Antiviral drug resistance of herpes simplex virus

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Antivirale resistentie van het herpes simplex virus

Rezistence viru herpes simplex vůči virostatikům

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No pessimist ever discovered
the secrets of the stars,
or sailed to uncharted land,
or opened a new doorway for the human spirit.

Helen Keller

Věnováno mým rodičům
Voor mijn ouders

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Chapter 1

Introduction

Herpesviruses

The family of *Herpesviridae* comprises a diverse group of large enveloped DNA viruses of vertebrates. The fundamental characteristic of this virus group is the ability to establish latent infection and periodically reactivate. Human herpesviruses (HHV) include herpes simplex virus (HSV) type 1 and 2, varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and HHV-6, HHV-7 and HHV-8. Based on the host range, duration of replication cycle, cytopathology and type of latent infection, herpesviruses are divided into 3 subfamilies: *Alpha-, Beta- and Gammaherpesvirinae.*

Alphaherpesviruses

Members of the *α-herpesvirinae* subfamily are characterized by a short replication cycle, wide host range and a rapid initial lytic infection of mucocutaneous cells followed by establishment of neuronal latency. This subfamily comprises 3 human herpesviruses: HSV-1, HSV-2 and VZV. All three are associated with primary and recurrent infections of mucocutaneous tissues, which may manifest as severe, persistent and disseminated disease among immunocompromised patients[108]. The seroprevalence varies between 50-95% for HSV-1 and 6-50% for HSV-2 depending on the age, sex, race, marital and socioeconomic status and geographical location[130]. About 95% of population above the age of 10 years is seropositive for VZV[108,157].

Pathogenesis of HSV infections

After primary lytic infection in peripheral mucocutaneous tissues, HSV establishes a lifelong latent ganglionic infection of sensory neurons that innervate the area of the primary infection. From its state of latency, when no virus replication takes place, the virus periodically reactivates and travels by axonal transport to the epithelial surface of the inervated region where it causes a recurrent infection[154]. Primary as well as recurrent infections may be associated with clinical symptoms or be asymptomatic, but in both cases virus shedding occurs and virus transmission is possible.

Primary HSV-1 infections of immunocompetent subjects are usually self limiting, and clinically manifest as gingivostomatitis, keratoconjuctivitis, cutaneous or genital herpes. Recurrences lead to the same symptoms but are of lesser severity and of shorter duration, with exception of HSV encephalitis. HSV-2 causes primary genital herpes, but is also an important cause of meningoencephalitis and neonatal herpes. In immunocompromised patients, the above mentioned HSV infections are often more severe and have a protracted course. In addition, HSV esophagitis, pneumonia, hepatitis or disseminated infection may occur in these patients.

VZV is the causative agent of chicken pox upon primary infection and can result in herpes zoster (shingles) in adults or in the disseminated infections in immunocompromised patients.

Laboratory diagnosis of HSV infections

Detection of HSV belongs to the frequent diagnostic procedures in the clinical virology laboratory. For years, virus isolation by cell culture or rapid shell vial culture combined with immunoflouorescence detection of virus antigen using type-specific monoclonal antibodies

have been the methods of choice[133]. HSV can be isolated from various specimens such as swabs from mucocutaneous lesions, vesicle fluid, less frequently sputum, bronchoalveolar lavage, conjuctival fluid or tissue biopsies. The highest isolation rates are obtained upon immediate inoculation on cell cultures. Although they are the gold standard, culture-based diagnostic tests are generally laborious and time-consuming, and may yield a high frequency of false-negative results due to the stage of the clinical lesion, inadequate collection of specimen, improper transport and storage conditions or inhibitory substances in the specimen[11,33,71].

Direct virus antigen detection in clinical specimen either by ELISA or immunofluorescence is rapid but has a sensitivity of 80-90% compared to culture[85,129]. Serological detection of virus specific antibodies can provide retrospective information on a primary or recurrent infection, but its role in rapid diagnosis is limited, as weeks have to elapse before a significant rise in antibodies is detectable[143]. For some time, the use of serological assays specific for glycoprotein G (gG) may distinguish between HSV-1 and HSV-2 infection[6]. The use of PCR techniques has been largely restricted to the diagnosis of HSV encephalitis[90,94]. Although numerous PCR assays have been established for detection of HSV and these assays have demonstrated to be significantly more rapid, sensitive and specific than virus culture and/or antigen assays, their implementation in routine diagnosis of HSV infections has been hindered by the high demands on laboratory logistics and personnel and the high contamination risk[6,117,129]. This situation has changed with the development of the real-time PCR. This new PCR format has overcome the need for post amplification manipulation of the sample, it has a rapid turn-around time and high capacity. Real-time PCR assays demonstrated to have sensitivity comparable to conventional PCR systems for detection of various viruses[1,89,109,144]. In addition, real-time PCR assays can be combined with automated nucleic acid extraction systems thereby providing rapid and highly standardized molecular diagnosis of HSV infections.

Current antiherpesvirus drugs and their mechanisms of action

The alphaherpesviruses were the first viruses for which effective, non-toxic antiviral drugs have been developed. The members of the first class of the present antiherpesvirus drugs, nucleoside analogues, have been developed in the past 25 years and remain the first line treatment of HSV infections. They exhibit potent and selective antiviral activity and very good safety. These drugs are actually prodrugs that require activation, which consists of three phosphorylation steps, the first one being exclusively performed by the viral thymidine kinase (TK) and the subsequent ones by cellular kinases. The triphosphate forms of the drugs then compete with the deoxynucleoside triphosphates (dNTPs) as a substrate for viral DNA polymerase (DNA pol). The incorporation of the nucleoside analogues into viral DNA results in termination of DNA elongation and functional inactivation of DNA pol[106] (Fig. 1). This class of drugs includes acyclovir (ACV) and penciclovir (PCV) and their respective oral prodrugs valacyclovir (ValACV) and famciclovir (FCV), and, in addition, ganciclovir (GCV), an anti-CMV drug, which has been also used to treat HSV and CMV co-infections[28,37,40,44,45,134,137,152].

The second class of antiherpesvirus drugs includes pyrophosphate analogues, such as foscarnet (PFA), which directly inhibit the DNA pol of HSV without requiring activation by viral TK. Pyrophosphate analogues bind to the pyrophosphate binding site of the viral DNA pol and prevent cleavage of the pyrophosphate (PP) from the dNTPs, thereby interfering with DNA chain elongation[111,148].

Cidofovir (HPMPC) is a member of the third class of antiherpesvirus drugs, acyclic nucleoside phosphonates, which are also direct DNA pol inhibitors with a broad spectrum anti-DNA virus activity. Similarly to PFA, cidofovir is also independent of viral TK activity. It is phosphorylated by cellular enzymes to its diphosphate form, which acts as a chain terminator upon its two sequential incorporations[8,27,41,131,132] (Fig. 1). All antiherpesvirus drugs mentioned above are summarized in Table 1.

The high specificity of nucleoside analogues for viral enzymes accounts for their low toxicity, except for GCV, which is myelosuppressive and nephrotoxic. The major side effect of the drugs of the second and third class is their nephrotoxicity[151]. The weak point of anti-HSV therapy is that the drugs inhibit only productive infection but are not effective against latent virus and thus can never cure HSV infection[69].

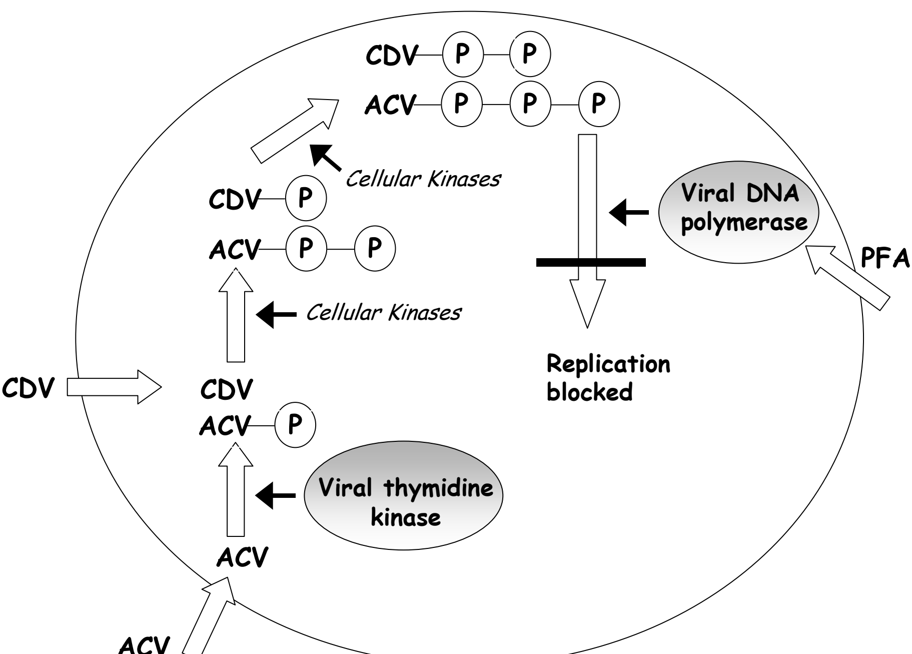

Fig. 1. Mechanims of action of the three major classes of current antiherpesvirus drugs: nucleoside analogues (acyclovir), pyrophosphate analogues (foscarnet) and acyclic nucleoside phosphonates (cidofovir). ACV, acyclovir; CDV, cidofovir; P, phosphate; PFA, foscarnet. Adapted from[58].

Table 1. Anti-HSV drugs targeted against viral DNA polymerase.

Class	Mechanism of action	Antiviral drug	Abbreviation	Market name	Specific information	Indication for use	Structure
Nucleoside analogues	TK dependent	Acyclovir	ACV	Zovirax		Prophylaxis and treatment of mucosal, cutaneous and systemic HSV infections (genital herpes, herpes labialis, keratoconjuctivitis, encephalitis, neonatal herpes)	
		Valacyclovir	ValACV	Valtrex Zelitrex	Oral prodrug of ACV	Same as oral ACV	
		Penciclovir	PCV	Denavir Vectavir	Longer half-life, greater activity than ACV in a short time scale	Herpes labialis	
		Famciclovir	FCV	Famvir	Oral prodrug of PCV	Same as oral ACV	
		Ganciclovir	GCV	Cymevene Cytovene	ACV-resistant HSV with mutations in the TK gene are cross-resistant to GCV	CMV infections	
Pyrophosphate analogues	TK independent, directly inhibits DNA pol	Foscarnet	PFA	Foscavir	Nephrotoxic	Treatment of ACV-resistant HSV and VZV infections in immunocompromised patients	
Acyclic nucleoside phosphonates	TK independent, directly inhibits DNA pol	Cidofovir	HPMPC CDV	Vistide Forvade	Nephrotoxic	Treatment of ACV- and PFA-resistant HSV infections, CMV retinitis	

[a] Adapted from[40] and[151].

HSV antiviral drug resistance: clinical incidence and significance

ACV was approved in 1982, and in the same year the first case of ACV resistance was reported[38]. In 1989, a report was published on 12 human immunodeficiency virus (HIV)-infected patients with ACV-resistant HSV isolates, predicting the increasing occurrence of resistant virus among immunocompromised individuals[49]. All early resistant isolates were found in severely immunocompromised patients [AIDS, hematopoietic stem cell transplant (HSCT) recipients, cancer patients, or patients on immunosuppressive therapy] and have been reported to cause meningitis, esophagitis and pneumonia, in addition to the usual persistent mucocutaneous manifestation[64,88,110,145]. The increased incidence of ACV-resistant HSV among clinical isolates was a direct consequence of the number of patients infected with HIV[51].

In immunocompromised patients, the viral load during infection is high, and viral replication may not be completely suppressed by the antiviral therapy. This subsequently increases the chance of drug-resistant mutants emergence. Furthermore, mixed populations of sensitive and naturally occurring resistant viruses can be present in clinical isolates[100,110] and the use of an antiviral drug may then favor the selection of the resistant strain. ACV resistance was observed almost exclusively in patients receiving therapeutic rather than prophylactic treatment[18]. Prophylactic treatment suppresses viral replication and is thought to minimize the likelihood of emergence of resistance[2].

The prevalence of HSV infections with reduced susceptibility to ACV in immunocompromised patients generally varies from 4.0 to 7.1%[24,26,48,96,99,107,147], with the majority of the studies focused on HSCT recipients and HIV-positive individuals. The highest prevalence rates have been reported for recipients of HSCT, with a range from 6 to 14%. The prevalence of resistant HSV ranges from 3.5 to 7% in HIV-positive individuals[48,84,107], and from 2.8 to 10% in solid organ transplant recipients[26,84].

In contrast, isolation of an ACV-resistant HSV strain from immunocompetent patients is rare and does not seem to correlate with a clinical outcome[153]. The immune system, especially the CD4+ and CD8+ T cells, plays a pivotal role in controlling HSV infection[32]. A low prevalence of resistant HSV in immunocompetent patients was reported by extensive screening surveys performed in the UK (0.7%) and the USA (0.3%) between 1980-1992[35]. No increase in the prevalence of resistance has been observed since then (range from 0.1 to 0.7%) even though these studies also included patients on chronic suppressive therapy for genital herpes as well as patients from the general population using ACV/PCV topical preparations for management of herpes labialis[7,9,19,26,84,107]. It has been also demonstrated that the long term antiviral treatment does not result in an increased emergence of resistant HSV in immunocompetent hosts[54]. Only a few cases of emergence of a clinical HSV resistance have been described in immunocompetent patients. These were found in patients on suppressive ACV therapy for genital herpes[46,70,98,138] and in patients with HSV keratitis[9,99,120].

Due to the increasing widespread episodic or chronic use of antiviral drugs for management of recurrent herpes labialis and genital herpes, resistance to these drugs may increase[70]. This issue has been addressed by mathematical modelling for both herpes labialis and genital herpes[16,86]. Although the level of acquired resistance and the transmission

potential of resistant viruses are uncertain parameters in these models, the effect of the increased drug use on resistance was predicted to be minimal.

Mechanisms of antiviral drug resistance

As can be anticipated from the mechanism of anti-HSV drug action, the resistance to these drugs is conferred by mutations in genes coding for the thymidine kinase and the DNA polymerase, which are enzymes directly involved in viral DNA replication. The DNA pol is essential for virus replication, as it is directly responsible for the viral DNA replication process. The viral TK catalyses the formation of deoxynucleoside triphosphate precursors. It is not essential for virus replication *in vitro*, but is required for virus replication in the peripheral and central nervous system, and has been demonstrated to play a role in the pathogenesis of *in vivo* infections[29].

There are three types of TK mutations that confer ACV-resistance. The simplest type, which confers the highest level of resistance, totally abrogates the TK activity (TK-negative, TK^-). The second type of mutations leads to reduced levels of TK activity (TK-partial, TK^p). The third type of mutations alters TK so that it can still phosphorylate natural substrates but is impaired for ACV phosphorylation (TK-altered, TK^a)[31]. The pathogenicity of these three types of mutants differs, depending on the level of the TK activity.

ACV-resistance can also be conferred by mutations in the DNA pol gene. DNA pol mutations also confer (cross)-resistance to the two other classes of anti-HSV drugs including foscarnet and cidofovir. Resistance mutations result in an enzyme that is less susceptible to drug inhibition. DNA pol mutants are much less frequent than TK mutants both *in vivo* and *in vitro*[25,78,79,125].

ACV-resistant TK mutants have been detected as minority populations (0.01-0.15%) within wild type clinical isolates[100,128]. Consequently, these mutants can be readily selected *in vitro*, and can also be isolated from immunocompromised patients, with the TK^- mutants being the most frequent (96%)[56,95,105].

Based on evidence from animal studies, it has been generally accepted that TK^- mutants have low virulence (poor replication in sensory ganglia, low neurovirulence, no zosteriform spread) and are unable to reactivate from latency[32,43]. However, this concept is confronted by several studies, showing that these mutants, despite their non-functional TK gene, can reactivate and may also cause severe disease[65,70,97,122]. It has been shown that HSV has developed mechanisms that allow the most common TK^- mutants to escape ACV therapy without loosing their pathogenicity[61]. These mechanisms include: replication errors (genetic instability) creating subpopulations of TK-positive virus, ribosomal frameshifting leading to expression of low levels of TK or compensation of TK activity by other gene products[60,61,65,67,122,123].

TK^p mutants are less attenuated than TK^- mutants, and are generally able to reactivate from latency. TK^a and DNA pol mutants are the most pathogenic of the ACV-resistant mutants and thus may be of a significant clinical concern[52,77,78,103], although reduced pathogenicity has been described for some DNA pol mutants[4,62]. Moreover, mixtures of different resistant mutants or mixtures of resistant and sensitive virus can retain high pathogenicity, and yet increase their resistance in animal models[29,47,53]. Finally, both the TK^a

and the DNA pol mutants may pose a risk for transmission of drug-resistant HSV[34]. A general characteristic of the ACV-resistant mutants is described in Table 2.

Table 2. General characteristics of ACV-resistant mutants[a].

Type of mutant	Thymidine phosphorylation (%)	Pathogenicity in mice (relative to wild type, %)[b]		
		Neurovirulence	Replication at periphery	Reactivation from latency
Wild type	100	100	100	100
TK-negative	<1	0.01-3	10-100	-(exceptions)
TK-partial	1-15	3-100	20-100	10-100
TK-altered	Mutation dependent	10	100	50-100
DNA pol	100	1-10	20-100	50-100

[a] Adapted from[9] and[30].
[b] The ranges reflect behaviour of different mutants due to molecular differences, different wild-type strains or differing routes of inoculation.

Resistance-associated mutations

Thymidine kinase: structure-function correlates of resistance

The HSV thymidine kinase is a deoxypyrimidine kinase with a broader range of substrate specificities compared to cellular kinases. Apart from catalysing the transfer of a γ-phosphate group from ATP to thymidine, it also phosphorylates other pyrimidine and purine nucleoside analogues including those carrying acyclic sugar moieties, such as ACV[50]. This laxity in HSV TK specificity already suggests the mutability of this enzyme, which is greater than that of the cellular TK.

HSV TK is a 376 amino acid (aa) protein functioning as a homodimer in complex with 4 water molecules. Its three dimensional structure is homologous to nucleoside monophosphate (NMP) kinases including a core region with 5 β-sheets and 12 α-helices, a NMP binding domain and a LID domain enclosing ATP-binding site (Fig. 2)[156].

The active site of TK has been generally described based on the preliminary model proposed by Darby *et al.*[59], and based on regions of high sequence homologies between herpesviral thymidine kinases identified by Balasubramaniam[10]. These studies have defined the location of 6 conserved regions (aa 56-63, 83-88, 162-164, 171-173, 216-222 and 284-289) comprising the ATP-binding site (aa 51-63) and the nucleoside-binding site (aa 168-176). In addition, based on the recently elucidated crystal structure of TK[20,146,155] and studies on the functional role of TK amino acid residues[104] it was demonstrated, that not only residues in the conserved regions play an important role in TK function, but also residues outside these regions can have major effect on TK activity. Thus, distant point mutations that alter relative orientations of the helices, which surround the nucleoside, can result in substantial variances in substrate specificity[50].

The nucleoside-binding domain is hidden in protein interior of TK and is composed of amino acid side chains of 3 parallel helices that interact with substrate through hydrogen bonds. The substrate binding itself is established by specific amino acids in close contact with nucleoside as well as subsidiary stabilizing factors (amino acids from several separate distant locations)[15,22,50,156].

The ATP binding site is composed of a conserved glycine-rich (G*XX*G*X*GKT) motif (aa 56-63), the so-called P-loop, which is common to all nucleotide kinases. This loop forms a large anion hole which accommodates the β-phosphate of ATP[87]. Together with the arginine-rich LID domain (aa 215-226) it holds ATP in the binding site[75].

The N-terminal 45 residues of TK does not seem to be essential for TK enzymatic activity[63].

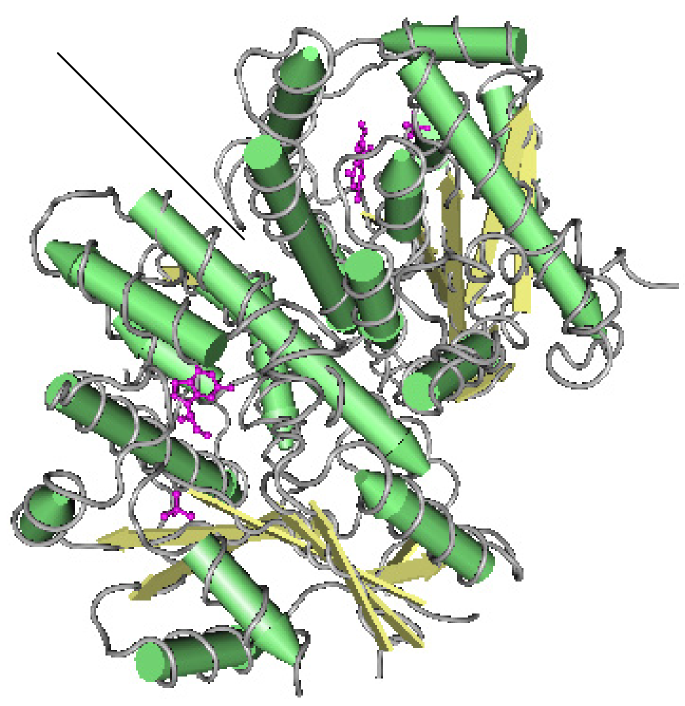

Fig. 2. HSV-1 TK dimer with bound ACV and phosphate (both in purple). Backbone of the protein is shown with five central β-sheets (yellow) surrounded by 12 α-helices (green). PDB number 2KI5 A.

Thymidine kinase: resistance-associated mutations

Single mutations in the TK gene may be associated with reduced susceptibility to ACV and/or other nucleoside analogues such as GCV and PCV. Two main types of mutations that occur at approximately equal frequencies have been identified. i) Frameshift (FS) mutations are caused by mostly single nucleotide additions/deletions in one of the homopolymeric repeats of cytosines (C) or guanines (G) that are frequently present throughout the TK gene[58] (Fig. 3a). These frameshifts result in a premature stop codon leading to a nonfunctional TK[14,95,123]. ii) The second type of mutations comprises single amino acid substitutions. These

are usually located in the substrate binding sites, in conserved regions or at highly conserved individual codons (as described above, Fig. 3a)[14,56,95]. A small number of resistance-associated substitutions has been reported outside the above mentioned regions[14,23,56,141]. These, however, have to be carefully differentiated from the relatively frequent mutations due to natural polymorphism[73]. Other type of mutations have been rarely reported; those include deletions of multiple to several hundred nucleotides which ultimately lead to a nonfunctional TK[25,123].

Mutations resulting in a nonfunctional TK confer a highest degree of resistance to ACV (>100-fold increase in the IC_{50}). The degree of resistance conferred by other mutations can range from marginal (2-fold increase in IC_{50}) to high resistance (100-fold increase)[31].

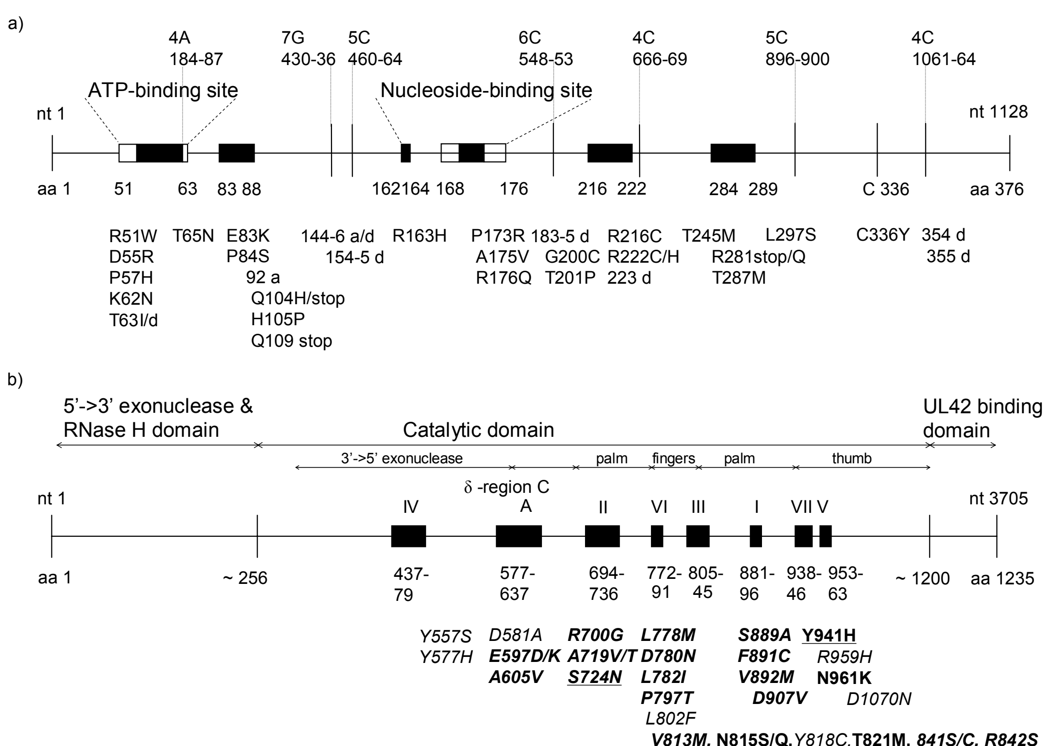

Fig. 3. Schematic representation of drug resistance-associated mutations in a) HSV-1 TK found in ACV-resistant clinical isolates. Conserved regions, substrate binding sites (conserved regions forming part of ATP and nucleoside-binding sites: aa 56-63, 171-173) and mutational homopolymeric runs are indicated; b) HSV-1 DNA pol gene found in laboratory mutants and clinical isolates (underlined). Mutations indicated in bold, italics and combination of both confer resistance to ACV, PFA and both drugs, respectively. Black boxes indicate conserved regions. a, addition; d, deletion; nt nucleotide.

DNA polymerase: structure and resistance-associated mutations

The HSV-1 DNA polymerase is a 1235 amino acid multifunctional enzyme with DNA-dependent DNA polymerase activity, 5'→3' exonuclease/RNaseH and 3'→5' exonuclease activity. It binds to the UL42 processivity factor that is essential for viral replication[42,80](Fig. 3b). Due to the tight linkage of catalytic functions in the DNA pol, single mutations can confer resistance to multiple antiviral drugs. However, compared to TK, these mutations are

restricted only to specific regions or residues, since they cannot interfere with the polymerase function[66]. It seems that no specific region is solely involved in recognition of a single class of anti-HSV drugs. Rather, the specific substrate recognition sites are formed through interactions of several non-sequential regions and amino acid residues upon folding[29,92].

The presumed catalytic domain of the DNA pol enzyme includes eight conserved regions, designated I to VII and the δ-C region (Fig. 3b). These regions share sequence homologies with other herpesvirus DNA polymerases. The structure of the HSV DNA polymerase domain resembles a hand, like that of other polymerases, with a thumb, finger subdomains and a palm subdomain. Catalytic center of HSV-1 DNA pol is located in conserved regions I and II of the palm subdomain and is formed by three aspartate residues at positions 717, 886 and 888[150].

Because DNA polymerase mutants are quite rare *in vivo*, most mutations affecting susceptibility to antiviral drugs have been identified in engineered virus mutants [reviewed in[58]] and have been broadly located between residues 500 and 1028[74]. Mutations conferring resistance to ACV and PFA cluster mainly in conserved regions II and III. Therefore, these regions seem to be most likely to interact with drugs and natural ligands. Residue Ser724 in region II is highly conserved among herpesvirus polymerases and seems to play a central role in the interaction with multiple classes of antiviral drugs. Mutation Ser724Asn confers resistance to ACV, pyrophosphate analogues and also to phosphonylmethoxyalkyl (PME) derivatives[4,57,80]. Recently, region VI has also been identified as a multidrug recognition site[13]. Region I is the most highly conserved region in the HSV DNA pol as well as in all α-like DNA polymerases, and is critical for catalytic activity. Consequently, no natural polymorphisms have been identified in this region and engineered mutations in this region are lethal or severely impair virus replication[91]. Region δ-C is a part of the 3’→5’ exonuclease domain[158]. Mutations conferring resistance to pyrophosphate analogues and less frequently to nucleoside analogues has been mapped in this region[81]. Some mutations in this region, especially in the ExoIII motif, significantly increase the mutation rate[68]. Only a few mutations associated with resistance to ACV have been described in regions V and VII. Mutations outside conserved regions, although less frequent, may also confer reduced drug susceptibility[4,57,125]. Most of these mutations were reported to confer unique resistance to cidofovir and not to other drugs[4]. However, some recent findings have undermined the exclusive nature of interaction of cidofovir with the enzyme, through identification of ACV and PFA resistance-associated mutations conferring cross-resistance to cidofovir[13]. However, as yet, resistance to cidofovir has only been reported in vitro[4]. Similarly to TK, natural polymorphisms in the DNA pol gene are frequent[13].

A recently developed assay, based on recombinant HSV mutants generated using a set of overlapping plasmids and cosmids, might contribute to a better understanding of the role of specific DNA pol mutations in drug susceptibility and thus to a better identification of the drug binding sites[13].

To date only a small number of clinical isolates with DNA pol mutations has been genotypically characterized[58]. Although evidence for cross-resistance to ACV and PFA, or even primary PFA resistance has been increasingly reported in HSCT recipients[21,24,131], no genotypic data on these mutants are available.

Mutations in both TK and DNA pol genes were rarely reported in clinical isolates, These mutations always demonstrated an additive effect on the ACV-resistant phenotype[12,118].

Antiviral drug susceptibility testing of HSV

Phenotypic assays

Phenotypic susceptibility testing has been an essential tool for identification and monitoring of resistant viruses, elucidation of the mechanisms of antiviral resistance, determination of cross-resistance and for discovery of new antiviral agents[139]. Safrin *et al.*[114] has clearly demonstrated the clinical relevance of antiviral susceptibility testing of HSV clinical isolates. So far, phenotypic tests remain the standard for susceptibility testing of HSV.

Since the replication cycle of HSV is rapid (18 hours), the results of susceptibility assays could be theoretically available in time for use in patient management. However, the clinical role of these assays is at present only marginal, mainly due to their laborious and time-consuming character and the lack of the standardization. There are indeed many variables that can influence the susceptibility result. These include: the cell line, the viral inoculum size, the incubation time, the range of drug concentrations, the reference strains, the assay type and the calculation and the interpretation of the endpoint.

Susceptibility assays can be grouped by the type of endpoint that is determined. This can be: i) the number of virus plaques (plaque reduction assay)[114], ii) the amount of infectious virus produced (yield reduction assay)[8], iii) the level of cytopathic effect (CPE) determined either microscopically (CPE reduction assay)[36,72,124] or colorimetrically (dye uptake assay)[76,93,124], iv) the production of virus antigen (ELISA, microplate in-situ ELISA)[82,115,149], v) the number of infected cells (flow cytometric analysis)[101], vi) the viral DNA production determined either by DNA hybridization[55,135,140] or real-time PCR[136] and vii) the induction of a transgene by HSV inducible reporter cells (ELVIRA HSV assay)[142].

The plaque reduction assay (PRA) has been a golden standard method, to which other assays have been compared and it is the only assay so far, for which susceptibility results have been shown to correlate with the clinical outcome[114]. However, due to its laborious and time-consuming nature (need for titrated virus stock, prolonged development of plaques, subjective endpoint), the results are often too late to play a role in therapeutic decisions.

Rapid screening assays for high throughput susceptibility testing of HSV isolates have also been developed, mostly for use in surveillance studies. These assays are often a modification of assays already established[39,113]. They are usually set up with one or two drug concentrations only and a range of virus stock dilutions.

Interpretation of susceptibility results

Cut-off (threshold) values for reduced susceptibility are usually set by researchers in the field based on the correlation between *in vitro* IC_{50} values and clinical treatment failure[119]. At the moment, however, there are no definitive standards for interpretation of results of HSV susceptibility testing[139]. Although for ACV, a threshold of ≥2 µg/ml in the plaque reduction assay has long been proposed as an absolute IC_{50} value indicating resistance of HSV[114], several investigators argue that the use of cut-offs could be misleading for the following

reasons. Firstly, the cut-off values defining sensitive and resistant virus can differ depending on the applied assay including different cell types and readout systems[116,139]. Secondly, for many drugs IC_{50} cut-offs are poorly defined. Moreover, determination of cut-offs based on the drug concentrations in the serum may not be adequate for some site-specific diseases. Consequently, several other criteria for decreased susceptibility were suggested and applied[3,4,13,17,19]. Recently, Sarisky *et al.* suggested to compare the IC_{50} of an isolate with that of a sensitive reference strain in the same assay to create an IC_{50} ratio[121]. A ratio greater than 10 was considered indicative of a significant decrease in susceptibility. This ratio may provide a better estimation of the true susceptibility of the virus, and may enable more reliable comparison of data generated with different assays. However, in order to adopt this cut-off value on a large scale, the use of specific reference strains needs to be standardized and the clinical significance of this value determined.

Genotypic assays

Genotypic determination of viral resistance-conferring mutations have been demonstrated to be of clinical relevance in the case of HIV[5]. However, genotypic assays have some specific limitations when applied to HSV. These limitations include the incomplete knowledge of consequences of specific genotypic changes on phenotypic susceptibility and the wide range of types of mutations and their disperse location within the HSV TK or DNA pol gene. On the other hand, interpretation of genotyping results might be relatively easy, as in most cases antiviral drug resistance of HSV is caused by a single mutation. Genotypic drug resistance tests allow detection of virus mutants directly in the clinical specimen. This prevents the potential selection of specific viruses during culture passage in the absence of the drug pressure. In addition, a sensitive detection of mutant virus in a mixture at a specific nucleotide (20-30%) may result in an early detection of emerging resistance. Genotypic assays may be more rapid and more efficient in detection of resistance (provided resistance is conferred by known mutations) compared to phenotypic assays. Should such assays be implemented, their utility in the management of HSV infections would need to be thoroughly evaluated and supplemented with adequate quality assurance monitoring[126].

The results of phenotypic and genotypic drug susceptibility assays cannot always accurately predict clinical outcome. *In-vitro* resistance is not always associated with severe or prolonged disease[83], and vice versa, not all treatment failures described in the past have been associated with ACV-resistant virus[112,127]. Clinical response of the patient also depends on host-specific factors, such as the severity of immunosuppression, graft versus host disease (GVHD), renal function, drug pharmacokinetics and drug penetration[147]. The viral heterogeneity in the clinical isolates is another variable that may influence the results of susceptibility testing. It has been long established that clinical isolates contain a mixture of sensitive and resistant virus variants[100,102]. A transition in a dominant strain in the isolate that is ongoing at the time of sampling may possibly lead to misleading susceptibility test results[100]. In addition, selection for wild type strain *in vitro* during culture in the absence of the drug may also lead to false results[127].

The objectives of this thesis

In this thesis, antiviral drug resistance of HSV was investigated from a virological and clinical perspective. Focus was directed at the detection, prevalence and characterization of drug-resistant HSV. The specific objectives of this thesis were:

1. to develop, evaluate and implement novel molecular and biological assays for rapid HSV detection and determination of susceptibility of HSV to antiviral drugs, which would improve diagnosis of HSV infections and identification of HSV drug resistance (chapters 2, 3 and 4)

2. to determine the prevalence of ACV-resistant HSV infections in the general population as well as in specific patient groups in The Netherlands, in order to establish baseline estimates for future assessments of changes in susceptibility (chapter 5)

3. to investigate molecular changes underlying the drug-resistant phenotypes of HSV clinical isolates, in order to define molecular correlates of HSV antiviral drug resistance (chapters 5, 6 and 7)

4. to investigate the evolution of drug-resistant HSV variants in immunocompromised patients and to determine their clinical significance (chapters 6 and 7)

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Chapter 2

Routine use of a highly automated and internally controlled real-time PCR assay for the diagnosis of herpes simplex and varicella-zoster virus infections

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ABSTRACT

Background: Detection of herpesviruses can be significantly improved by PCR. The development of real-time PCR, which has overcome several limitations of conventional PCR, improved the prospects for implementation of PCR-based assays in diagnostic laboratory. *Objectives*: To compare the diagnostic performance of an automated sample extraction procedure in combination with an internally controlled real-time PCR assay for detection of herpes simplex virus (HSV) and varicella-zoster virus (VZV) to conventional shell vial culture.

Study design: One hundred eighty-two consecutive specimens from patients suspected of HSV or VZV infection were examined by internally controlled PCR and shell vial culture. An internal control consisting of phocine herpesvirus was processed along with the specimens during the entire procedure and permitted to monitor extraction and amplification efficiency, including inhibition.

Results: A total of 48 (26.4%) specimens were positive for HSV or VZV by culture, and 77 (42.3%) by real-time PCR. Thus, overall sensitivity increased by 60.4%. All culture-positive specimens were detected and typed correctly by PCR, except for a single specimen that contained PCR inhibitors. Specifically, the real-time PCR assay increased the detection rate for HSV-1 and HSV-2 by 43.9% and 62.5%, respectively. In PCR-positive specimens, lower levels of viral DNA were found in culture-negative than in culture-positive specimens. The increase of HSV detection rates by PCR varied with the origin of specimen and was particularly significant for skin specimens (7/21 versus 4/21 detected by culture) and bronchoalveolar lavages (8/16 versus 1/16). In addition, real-time PCR significantly increased the detection rate for VZV.

Conclusions: Compared to shell vial culture, our real-time PCR assay demonstrated a superior sensitivity and an added value of using internal control for checking the quality of examination of each specimen. These results provide a solid basis for implementation of real-time PCR in the routine diagnosis of HSV and VZV infections in various clinical specimens.

INTRODUCTION

PCR techniques have been widely demonstrated to be significantly more rapid, sensitive and specific than virus culture and/or antigen assays for the diagnosis of HSV or VZV infections[16,17,19]. For the diagnosis of HSV infections of the central nervous system (CNS) in particular, PCR has shown to be a major improvement and is nowadays recognized as the diagnostic method of choice[11,13]. However, the implementation of the PCR-based assays for the routine diagnosis of HSV infections other than those of the CNS has not been very forthcoming until recently[2]. This was mainly due to the technically cumbersome and labor-intensive nature of the PCR assays, the high risk of contamination, and a still limited throughput[16,19]. Additional drawbacks of the conventional PCR assays were the high costs and the need for dedicated laboratory space (three separate areas) and for highly trained personnel.

The recent development of real-time PCR systems (e.g. ABI Prism™ systems by Applied Biosystems, LightCycler by Roche Diagnostics, Rotor-Gene by Corbett Research, iCycler by Bio-Rad, Smart Cycler by Cepheid and Mx4000™ Multiplex Quantitative PCR system by Stratagene) will greatly facilitate the application of the PCR assays in the routine diagnostic laboratory. Real-time PCR is to be preferred over conventional PCR for routine diagnostic applications because it is performed in a closed system and does not require post amplification manipulation of the sample. This significantly reduces the risk for carry-over contamination, and eliminates the time-consuming amplicon detection step. In addition, real-time PCR assays have demonstrated equivalent sensitivities to conventional PCR systems, a high degree of reproducibility and a superior turn-around time[1,10,15,20]. By combining real-time PCR detection with automated nucleic acid extraction (e.g. MagNA Pure by Roche Diagnostics, BioRobot 9604 by Quiagen) highly standardized and easy-to-perform diagnostic assays can be developed, which also have the potential for quantification of the pathogen. Finally, the quality of such diagnostic assays can be monitored by incorporation of internal controls during the entire laboratory procedure, which allows for detection of PCR inhibitors.

Here we report on a comparison of a highly automated, internally controlled real-time PCR assay based on a combination of the nucleic acid extraction using MagNA Pure extractor (Roche Diagnostics) and a real-time TaqMan PCR (Applied Biosystems), for detection of HSV-1, HSV-2 and VZV in clinical specimens with conventional shell vial culture in a routine diagnostic setting.

MATERIALS AND METHODS

Clinical specimens. Consecutive specimens (n=182) were obtained from patients with clinical signs and symptoms suggestive of HSV or VZV infection between January and September 2001. These specimens were sent to our clinical laboratory with a request for HSV/VZV diagnosis. The far majority of specimens were swabs from skin, oro-facial or ano-genital vesicles or lesions suspected to be due to HSV or VZV infection. In addition, a few specimens were from patients suffering from conjunctivitis, keratitis or uveitis; throat swabs and bronchoalveolar lavage (BAL) specimens were from immunocompromised patients. All specimens except for BAL were collected in 2 ml of serum free virus transport medium (VTM) and centrifuged at 2000 × *g* for 15 min. Approximately 1 ml of supernatant was used directly for virus culture and the remainder was stored at -70°C for PCR analysis.

Shell vial culture and typing. Human diploid embryonic lung fibroblasts were cultured in shell vials in Eagle's MEM (BioWhittaker, Verviers, Belgium) supplemented with 5% fetal bovine serum, amphotericin B and antibiotics. Cells were inoculated with approximately 0.2 ml of clinical specimen in VTM and centrifuged at 700 × *g* for 75 min. Thereafter, 1 ml of culture medium was added to the shell vials and the cultures were subsequently incubated in a stationary phase at 37°C. Virus antigen detection and typing was performed on day 2 after inoculation for HSV and on days 3 and 7 for VZV by using commercial monoclonal antibodies specific for HSV-1, HSV-2 or VZV (Argene Biosoft, Varilhes, France).

Molecular detection of HSV/VZV. The molecular detection of HSV-1, HSV-2 and VZV was performed by using an automated nucleic acid extraction in combination with an internally controlled real-time TaqMan PCR assay.

Nucleic acid extraction. Total nucleic acid was extracted from 0.2 ml of clinical specimen in VTM with the MagNA Pure LC automated extractor (Roche Diagnostics, Penzberg, Germany) and the MagNA Pure LC total nucleic acid isolation kit. The purified nucleic acid was eluted in 100 µl of elution buffer.

A phocine herpesvirus (PhHV-1) culture supernatant, kindly provided by Dr. H. G. Niesters (Erasmus Medical Center Rotterdam, The Netherlands), was used as an internal control. A fixed, low concentration of PhHV-1 (mean cycle threshold (Ct) value, 34.3; standard deviation, 1.18) was added into each sample before extraction to monitor the PCR inhibition and extraction efficiency[14].

Real-time TaqMan PCR amplification and detection of HSV-1, HSV-2 and VZV DNA. Specific primers and TaqMan fluorescent probes for detection of HSV-1, HSV-2 and VZV were used as described earlier[8,15]. Additional primers and probes for amplification of PhHV-1 DNA (internal control) were generously provided by Dr. H. G. Niesters (Erasmus Medical Center Rotterdam, The Netherlands)[14]. Each 25 µl PCR reaction mix contained 5 µl of extracted DNA. Each extracted sample was analyzed in duplicate. The sequences and concentrations of primers and probes for each virus specific PCR are shown in Table 1. All four different PCRs were performed separately but in the same run using the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) under the following conditions: incubation for 2 min at 50ºC, and then for 10 min at 95ºC followed by 45 cycles of 15 s at 95ºC and 1 min at 60ºC. During the annealing-extension step, the ABI Prism sequence detector monitored the amplification by quantitatively analyzing fluorescence emissions. The reporter dye (FAM, VIC or TET) signal was measured relative to the internal reference dye (ROX) signal to normalize for non-PCR related fluorescence fluctuations occurring from well to well. The threshold was set at 10 times the standard deviation of the mean baseline emission calculated for cycles 3-15. The threshold cycle number (Ct value) represented the refraction cycle number at which a positive amplification reaction was measured.

The sensitivity of the real-time TaqMan PCR assays was determined by using serial dilutions of electron microscopy (EM) counted virus stocks of HSV-1, McIntyre strain, and HSV-2, G strain or a quantified VZV genomic DNA, ROD strain (Advanced Biotechnologies Inc., Columbia, Md.). In case of HSV, sensitivity and specificity were also evaluated using samples from the 4th HSV proficiency panel distributed by Quality Control of Molecular Diagnostics (QCMD), Glasgow, Scotland.

Assay controls. Positive controls derived from the EM counted stocks of HSV-1, HSV-2, and quantified VZV DNA were included in each assay. These controls were used at concentration yielding following Ct values (mean ± SD): 25.7 ± 1.1 for HSV-1, 26.6 ± 1.2 for HSV-2 and 30.4 ± 1.6 for VZV. Negative controls were routinely included in the whole extraction-amplification procedure to control for carry-over contamination.

Table 1. Primers and probes used for the real-time TaqMan PCR assays.

Virus	Target gene	Final PCR concentration (nM)	Primer/probe sequence (5'→3')
HSV-1[a]	Glycoprotein G	900	Forward: TCC TG/CG TTC CTA/C ACG/T GCC TCC C
		900	Reverse: GCA GIC AC/TA CGT AAC GCA CGC T
		150	Probe FAM: CGT CTG GAC CAA CCG CCA CAC AGG T
HSV-2	Glycoprotein D	50	Forward: CGC CAA ATA CGC CTT AGC A
		900	Reverse: GAG GTT CTT CCC GCG AAA T
		175	Probe VIC: CTC GCT TAA GAT GGC CGA TCC CAA T
VZV[b]	Gene 38	300	Forward: AAG TTC CCC CCG TTC GC
		300	Reverse: TGG ACT TGA AGA TGA ACT TAA TGA AGC
		50	Probe FAM: CCG CAA CAA CTG CAG TAT ATA TCG TC
PhHV-1[c]	Glycoprotein B	50	Forward: GGG CGA ATC ACA GAT TGA ATC
		50	Reverse: GCG GTT CCA AAC GTA CCA A
		200	Probe TET: TTT TTA TGT GTC CGC CAC CAT CTG GAT C

a 15
b 8
c 14

Criteria for validation of assay results. For each virus-specific positive control a threshold Ct range (mean Ct ± 2 SD) was set for run validation. The positive controls were evaluated in each run and the entire run was repeated if the set conditions were not met. For each specimen, the amplification of the internal control was analyzed. The threshold for amplification of the internal control was set 2 SD above the mean Ct value (mean 34.3, SD 1.18). If the Ct value for the internal control was higher than 36.66, the amplification of the specimen was considered inhibited. In those cases, the entire assay procedure should be repeated for that specimen.

Statistical analysis. The Mann-Whitney test was used for the analysis of the real-time PCR data in comparison to the shell vial assay.

RESULTS

Analytical sensitivity. The sensitivity of the assay procedure described here was evaluated by using serial dilution of EM counted HSV stocks or quantified VZV DNA. All three assays showed a detection sensitivity as low as at least 6 DNA copies per reaction, which translates into 600 DNA copies per ml of clinical specimen in VTM prior to extraction.

The real-time PCR assays were additionally validated by using QCMD's 4th HSV proficiency panel. The HSV-1 real-time PCR assay detected all samples correctly, from 1-3 × 10^3 to 3-9 × 10^7 genome equivalents (GE)/ml, except for the "weak-positive" HSV-1 sample containing 3-9 × 10^2 GE/ml. The real-time PCR for HSV-2 was able to detect samples containing 1-3 × 10^3 to 1-3 × 10^7 GE/ml correctly, including the HSV-2 sample containing 1-3 × 10^3 GE/ml. The one sample containing VZV was detected correctly by VZV real-time PCR. No false positive results were obtained in this proficiency evaluation.

Validation of the assay on clinical specimens. Out of 182 specimens submitted to our laboratory for detection of HSV and/or VZV, 41 (22.5%) were positive for HSV-1, 8 (4.4%) for HSV-2 and 1 (0.6%) for VZV by shell vial culture. With the exception of the one culture-positive specimen that demonstrated PCR inhibition, all specimens were also positive in the respective real-time PCR assay. Furthermore, 18, 5, and 6 additional specimens were found positive by PCR for HSV-1, HSV-2 or VZV, respectively. Thus, the real-time PCR assay increased the detection rate for each of these three viruses by 43.9, 62.5 and 600%, respectively (Table 2). The PCR typing results showed 100% concordance with the results obtained by shell vial culture-based typing, confirming the specificity of the three real-time PCR assays.

One double positive specimen, genital swab with HSV-1 and HSV-2, was detected by culture and it also tested double positive by real-time PCR.

Table 2. Detection of HSV-1, HSV-2 and VZV by real-time PCR and shell vial culture.

PCR	Culture	HSV1	HSV2	VZV	–	Total
+	+	40	8	1		49[b]
+	–	18	5	6		29
–	+	1[a]	0	0		1
–	–				104	104
Total		59	13	7	104	183[b]

[a] HSV-1 culture-positive specimen (genital swab) contained PCR inhibitors.
[b] One specimen was PCR- and culture-positive for HSV-1 and HSV-2.

The Ct values of real-time PCR results demonstrated a significant difference in the amount of HSV-1 or HSV-2 DNA between culture-positive and culture-negative specimens (Fig. 1). The median Ct value for culture-negative HSV specimens was 34.0 (range, 18.5 to 39.1), whereas the median Ct value for culture-positive specimens was 24.0 (range, 14.9 to 36.1) ($P<0.0001$). More specifically, median Ct values for culture-positives and -negatives were 24.6 and 34.5 for HSV-1, and 21.5 and 27.6 for HSV-2.

All HSV culture-positive specimens had a Ct value of less than 36, whereas an additional 7 out of 23 culture-negative specimens became PCR-positive at Ct values between 36 and 40. By a Ct value of 30, 90% of all culture-positives were detected, whereas only 35% of culture-negatives were detected at this Ct value.

The relationship between the origin of the specimen and positivity by either culture or real-time Taqman PCR was determined for the HSV-positive specimens (Table 3). The detection rate for specimens collected from genital or anal areas increased by 33% by PCR (32/56 versus 24/56 detected by culture) and a similar increase, 37.5% (11/31 versus 8/31) in the detection rate, was observed for oro-facial specimens. However, HSV was detected by culture and PCR equally well in throat specimens. The virus detection rate for other skin specimens and for BAL demonstrated an 75% (7/21 versus 4/21 detected by culture) and 700% increase (8/16 versus 1/16), respectively.

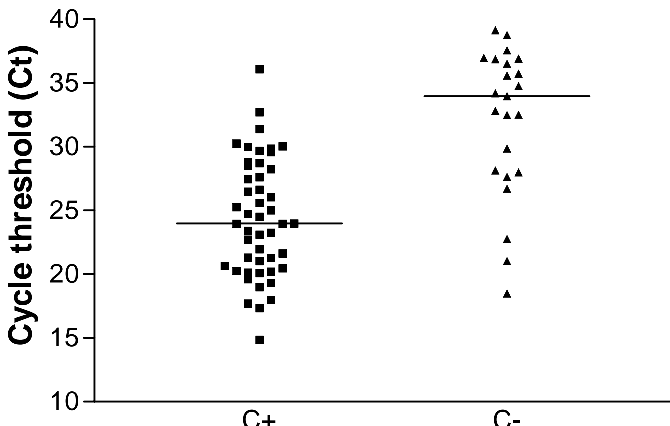

Fig. 1. Ct values detected in the HSV real-time PCR assays in culture-positive (C+) and culture-negative (C-) specimens. The values shown are means of duplicate amplifications analyzed in a single run. Bars indicate median value.

Reproducibility. The reproducibility of duplicate Ct values was assessed on all PCR-positive clinical specimens. The mean intra-assay coefficient of variation calculated from duplicate Ct values was 0.8% (range, 0.0% to 3.6%) over the range of viral DNA levels in the clinical specimens, indicating a high level of reproducibility.

Table 3. Numbers of real-time PCR and shell vial culture results for HSV, by origin of specimen.

PCR	Culture	Origin of specimen					
		Skin	Ano-genital	Oro-facial	Eye	Throat	BAL
+	+	4 (21.1)	24 (23.4)	8 (26.1)	0	5 (26.4)	1 (32.7)
+	–	3 (38.3)	8 (29.6)	3 (34.2)	1 (34.8)	0	7 (31.2)
–	–	14	24	20	8	12	8
Total		21	56	31	9	17	16

The median Ct values are indicated in parenthesis.

DISCUSSION

Examination of patient specimens for the detection of herpesvirus infections is an important part of the routine diagnostic testing in the virological laboratory. So far, virus culture is the method of choice for laboratory diagnosis of HSV infections, especially for mucocutaneous HSV infections. However, culture is not always successful, which may be due to the stage of the clinical lesion, inadequate collection of specimen or improper transport and storage conditions[9]. For VZV, the traditional cell culture is even less successful, and has

previously been reported to be inferior to direct antigen detection or PCR[3,4]. While accepted as the method of choice for laboratory diagnosis of herpesvirus infection in CNS, PCR has so far succeeded only marginally in replacing traditional or shell vial culture for the detection of HSV or VZV infection. The limited diagnostic implementation of conventional PCR assays is due to several limitations such as the high demand on laboratory logistics and personnel and the contamination risk[16,19]. The development of real-time PCR, however, has improved considerably the prospects of implementation of molecular diagnostics in the routine laboratory.

We evaluated an automated, internally controlled, real-time PCR assay for the detection of three herpesviruses, HSV-1, HSV-2 and VZV. The real-time PCR assay demonstrated an increased sensitivity for all three virus types examined. Out of the 182 specimens from patients with clinical signs suggestive of HSV or VZV infection, 48 were positive by both shell vial culture and PCR, and an additional 29 were detected exclusively by PCR. Only one specimen positive by culture could not be evaluated by PCR, because of inhibition. Thus, PCR increased the combined overall detection rate by 60.4%. Similar results have been described using the LightCycler PCR[5,7,18] or by LightCycler Syber Green PCR[7] for detection of HSV. The detection of VZV was also improved remarkably using real-time TaqMan PCR, even though the number of positive specimens was small. This also corresponds to the previously reported results obtained by using LightCycler PCR[6].

As expected, the HSV specimens tested positive by real-time PCR assay but not culture, contained significantly lower virus DNA levels than the specimens that were culture-positive. Their median Ct value (34.0) was significantly higher than that of culture-positives (24.0; $P<0.0001$). This difference of 10 cycles translates into an approximately 1024 (2^{10})-fold difference in median virus DNA levels between the culture positives and negatives.

As a result of its high sensitivity, real-time PCR increased the overall detection rate for all types of specimens collected from different body sites, except for specimens collected from the throat (Table 3). The 33% increase in detection rate in ano-genital specimens is comparable to the 24% reported in a large study using the LightCycler PCR platform[18]. A seven-fold increase in detection rate was even found in BAL specimens. All these specimens were HSV-1 positive and had been obtained from immunocompromised patients. For the immunocompromised patients in particular, where rapid diagnosis is needed for timely initiation of antiviral therapy, real-time PCR examination would be a considerable improvement. However, the clinical significance of these findings is not always completely clear. A distinction has to be made between a true infection of the lower respiratory tract and possible contamination from the oral cavity. The possibility of quantification of the virus could help to resolve this issue.

Culture-based diagnostic tests are laborious and time-consuming, potentially highly variable and difficult to standardize and control. In the absence of an internal control in culture-based assays, the frequency of false-negative results due to culture inhibition or other circumstances cannot be determined. Our real-time PCR assay set-up does monitor for PCR inhibition and false-negative results. Furthermore, the automated extractor allows processing of clinical specimens immediately upon arrival and the universal amplification format used in the ABI Prism 7700 enables detection of different pathogens in a single run. Thus, the

capacity of the instrument is used efficiently, and can be also used easily for urgent determinations. Replacement of time- and personnel-consuming cell culture detection by real-time PCR has been already reported to be cost-effective[12,18].

Based on the results of this study, the real-time PCR assays described here were implemented in our routine diagnostic virology laboratory for diagnosis of HSV-1/2 and VZV.

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Chapter 3

Application of real-time PCR for antiviral drug susceptibility determination of herpes simplex virus

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ABSTRACT

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-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 read-out 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 (PRA). 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.

INTRODUCTION

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 relatively frequently especially in immunocompromised patients such as those undergoing bone marrow (6-12%) or solid organ transplantation (~4%), or AIDS patients (~6%) and can be associated with serious disease[4,17]. 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[1,21].

Safrin *et al.*[26] 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 emphasized 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[26]. The majority of alternative susceptibility assays is based on reduction in cytopathic effect (CPE), which is either microscopically evaluated or colorimetrically detected[7,13,15,20,25,31]. Assays based on enzyme-linked immunosorbent assay (ELISA) include the sandwich ELISA[35] and the microplate in-situ ELISA (MISE-test)[16,23,27]. The latter has been shown to correlate well with PRA. Other currently used antiviral susceptibility assays involve the use of DNA hybridization[9,32,33], flow cytometric analysis[22] and transgenic HSV inducible reporter cells[34].

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 (AraA^{r}7, AraA^{r}8, AraA^{r}13, F891C, PFA^{r}5 and PAA^{r}5)[5,6,10,18] 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 (21) 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 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.*[30].

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.*[24]. 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 of probe. Amplification was performed using the Applied Biosystems Sequence Detector 7700 under the following conditions: incubation for 2 min at 50ºC, and then for 10 min at 95ºC followed by 45 cycles of 15 s at 95ºC and 1 min at 60ºC. Each PCR run contained two negative controls and a dilution series of HSV-1 DNA (6×10^2 to 6×10^8 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 IC_{50}s was determined in parallel experiments in which cell cultures were infected with a half-$\log_{10}$ 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, 72 hours post infection, and the 50% inhibitory concentrations (IC_{50}s) 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 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 (6×10^5 cells/ml). Serial twofold dilutions of ACV (Sigma, Zwijndrecht, The Netherlands) ranging from 0.06 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 culture supernatants were collected, cleared by centrifugation (1,100 × *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 IC_{50} 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 plaque-forming units (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 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 real-time PCR assay. The IC_{50} 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 IC_{50} 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 10^7 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 a 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 $\log_{10}$ 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).

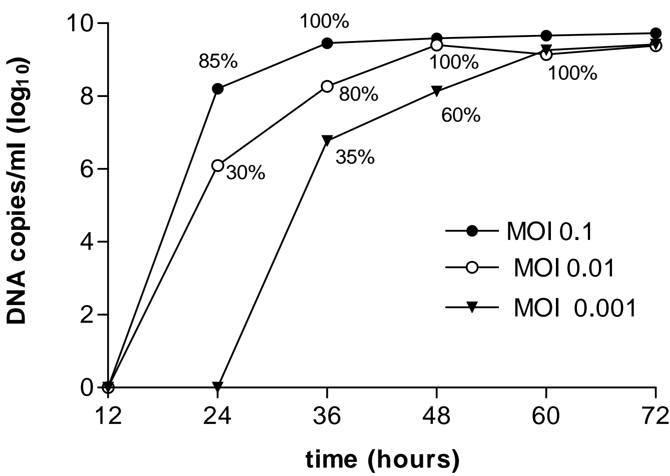

Fig. 1. Time course of changes in the yield of HSV-1 DNA after infection at different MOIs (PFU/cell). The DNA levels were measured in culture supernatants by real-time PCR. The percent values represent the percent CPE observed in the wells and are indicated in the graph until the first time point that a 100% CPE was reached for each MOI.

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 reaction. Particularly the presence of ACV, which is a DNA polymerase inhibitor, in culture supernatants could inhibit the PCR[19]. 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 IC_{50}. The IC_{50}s of ACV in the TaqMan PCR assay at different MOIs and incubation times varied between 0.16 and 0.61 µg/ml (Table 1). Only a slight increase in IC_{50}s 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 IC_{50} was observed among the cultures infected with a large range of MOIs from MOI of 0.001 to 0.5 PFU/cell. For each MOI an increase in IC_{50} with incubation time was observed (Table 1).

Table 1. Effect of MOI and incubation time on ACV IC_{50}s in Vero cells measured by real-time PCR assay (HSV-1 McIntyre).

Incubation time (h)	MOI (PFU/cell)	Mean ACV $IC_{50} \pm$ SD (µg/ml)[a]	% CPE virus control
24	0.001	ND[b]	
	0.005	0.17 ± 0.09	10
	0.01	0.16 ± 0.06	30
	0.05	0.18 ± 0.04	50
	0.1	0.24 ± 0.03	80
	0.5	0.30 ± 0.05	100
48	0.001	0.19 ± 0.03	50
	0.005	0.28 ± 0.04	70
	0.01	0.29 ± 0.03	80
	0.05	0.34 ± 0.06	100
	0.1	0.35 ± 0.05	100
	0.5	0.61 ± 0.09	100
72	0.001	0.23 ± 0.04	90
	0.005	0.45 ± 0.05	100
	0.01	0.41 ± 0.02	100
	0.05	0.45 ± 0.06	100
	0.1	0.42 ± 0.08	100
	0.5	0.49 ± 0.10	100

[a] Mean from two separate experiments.
[b] ND, no data because of insufficient CPE and undetectable levels of HSV DNA in the supernatant (Fig. 1).

The IC_{50}s detected 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 6 clinical isolates (Table 2). ACV IC_{50}s determined by real-time PCR assay correlated well with those from PRA (r=0.86, 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}s 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}s was 7.7-fold (range 4.1 to 15.0) between the two assays.

Table 2. ACV IC_{50}s for HSV-1 strains determined by real-time PCR assay and plaque reduction assay.

Virus strain	Mean ACV $IC_{50} \pm$ SD (μg/ml)[a] as determined by:	
	Real-time PCR assay	Plaque reduction assay
ACV-sensitive		
KOS	0.15 ± 0.03	1.30 ± 0.35
McIntyre	0.26 ± 0.05	1.91 ± 0.56
R39	0.06 ± 0.01	0.46 ± 0.04
ACV-resistant		
PAA^r5	1.38 ± 0.07	20.75 ± 6.61
PFA^r5	1.19 ± 0.23	8.69 ± 0.14
$AraA^r7$	1.23 ± 0.16	18.38 ± 3.56
$AraA^r8$	0.40 ± 0.09	4.50 ± 1.65
$AraA^r13$	1.03 ± 0.21	6.10 ± 2.06
F891C	2.24 ± 0.07	10.04 ± 3.13
Clinical isolates		
97.12961	3.92 ± 0.13	25.10 ± 2.51
98.15779-VA/2	2.33 ± 0.37	9.47 ± 2.48
98.14742-PE/1	1.37 ± 0.10	8.65 ± 0.96
98.25733-MA/3	2.45 ± 0.41	10.21 ± 4.26
99.16237	1.60 ± 0.13	11.53 ± 0.86
00.29392	0.30 ± 0.05	1.63 ± 0.63

[a] Mean from two separate experiments.

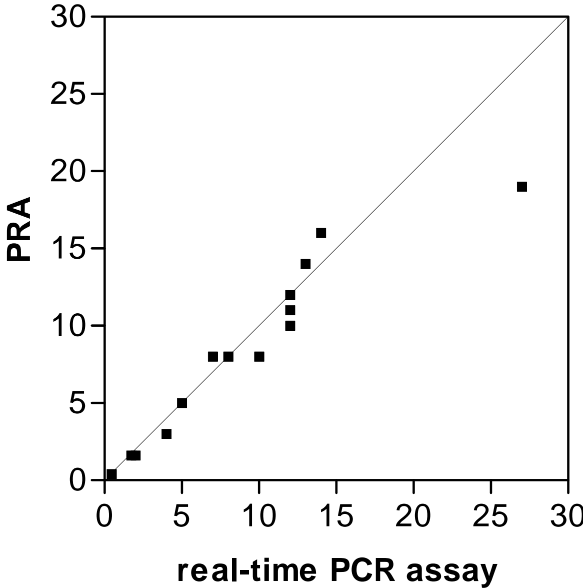

Fig. 2. Fold differences in susceptibility (IC_{50}) between the reference strain KOS and tested strains as determined by real-time PCR assay and PRA (*r*=0.99). The trend line is aligned with the correlation coefficient 1.0.

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 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; 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 readout parameters. The determination of these different parameters may explain the differences in absolute IC_{50}s between the two assays. Moreover, PRA does 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 plaques in drug-treated wells consist of lower numbers of virus-infected cells but are counted equally to

plaques 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 IC_{50} 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 hours 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 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 IC_{50}s and also the fold differences in susceptibility between the reference sensitive and tested strains highly correlated.

Based on the range of IC_{50}s obtained for sensitive HSV strains and clinical isolates, a cutoff value of 0.3 μg/ml of ACV was considered as a discriminative concentration for sensitive and resistant strains in the real-time TaqMan assay. For a better and more accurate definition 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[28], 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[29].

The real-time PCR assay described here allows a 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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Chapter 4

ELVIRA® HSV - a yield reduction assay for rapid antiviral susceptibility testing of herpes simplex virus

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ABSTRACT

A novel colorimetric assay ELVIRA® HSV (Enzyme-Linked Virus Inhibitor Reporter Assay) was developed to determine the susceptibility of herpes simplex virus (HSV) to antiviral drugs. It utilizes an HSV inducible reporter cell line (BHKICP6LacZ-5), which expresses β-galactosidase upon HSV infection, and thus enables to detect and quantify HSV. ELVIRA® HSV (ELVIRA) is a yield reduction assay, which uses two different cell lines. Initial infection of human diploid fibroblasts in the presence of an antiviral drug is followed by a secondary infection of the reporter cells, which triggers the production of β-galactosidase. The β-galactosidase activity is measured colorimetrically in cell lysates. The assay conditions were optimized for use in microtiter plates with a viral inoculum of 70 to 300 plaque-forming units (PFU)/ml and a turn around time of 2.5 days. The assay was evaluated on well-characterized drug-sensitive and -resistant strains and clinical isolates. Results demonstrated to be comparable with those obtained with gold standard plaque reduction assay (PRA). The total inter-assay variability was less than 50%. The assay was further validated for susceptibility testing to acyclovir and foscarnet of 30 and 33 therapy-naive HSV-1 clinical isolates, respectively. ELVIRA is a simple and rapid drug susceptibility assay with good reproducibility, objective readout and low inoculum size, which may be amenable for HSV susceptibility testing in the diagnostic virology laboratory.

INTRODUCTION

Antiviral drug resistance of herpes simplex virus (HSV) has emerged shortly after the introduction of acyclovir (ACV) in the early 1980s[6]. The increased number of immunocompromised patients due to the HIV infection, immunosuppressive treatment or immunodeficiencies has resulted in an increased frequency of HSV infections. As antiviral treatment is usually administered for prolonged periods, drug-resistant HSV are frequently isolated from these patients. Recent data demonstrated that the prevalence of drug-resistant HSV in immunocompromised patients is approximately 2.8 to 14% depending on the type of immunosupression[4,12], and even higher prevalence of resistance was reported especially in allogeneic stem cell transplant recipients[8]. In immunocompetent patients, drug resistance is considered to be rare, with a prevalence ranging from 0.1 to 0.7 %[1,7,17]. Given the increased use of antiviral treatment for HSV and the rise in the prevalence of drug-resistant HSV, HSV drug susceptibility determinations become of importance for routine clinical use and patient care. The availability of a diagnostically applicable, simple and rapid drug susceptibility assay is an essential prerequisite for adequate and effective clinical use of antiviral drugs.

The standard *in vitro* assay for determination of HSV susceptibility to antiviral drugs, the plaque reduction assay (PRA), is laborious and time-consuming, has a subjective endpoint and results are often available too late to play a role in therapeutic decisions[14]. There has been a considerable effort to develop less laborious and more rapid assays (reviewed in[18]). One of the strategies described earlier was a modified plaque reduction assay using transgenic cell lines (Vero or CV1) expressing β-galactosidase upon infection with HSV[19,20]. The β-

galactosidase activity in infected cells was detected histochemically 48 h post infection. The readout of the test was microscopic counting of blue plaques. This test demonstrated to correlate well with PRA.

Here we describe a quantitative colorimetric antiviral drug susceptibility assay for HSV, ELVIRA® HSV (Enzyme-Linked Virus Inhibitor Reporter Assay), which is based on a previously described HSV inducible reporter cell line BHKICP6LacZ-5[15]. The induction of β-galactosidase activity upon infection with HSV-1 or HSV-2 is conferred by the *E. coli lacZ* gene under the control of the HSV-1 early promoter ICP6 stably transformed in the cell genome. The assay protocol consists of initial infection of human fibroblasts in the presence of the antiviral drug and a subsequent second round of infection of the reporter cells, which are used as an overlay readout cell line. The β-galactosidase activity is measured in cell lysates and reflects the number of infected transgenic cells and thereby the yield of infectious virus upon the drug action. We demonstrated the usefulness of the ELVIRA® HSV assay (ELVIRA) for drug susceptibility testing of HSV. The assay evaluation on well-characterized strains and clinical isolates showed a good correlation of the IC_{50} data with those of the PRA.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFF) and African green monkey kidney cells (Vero) were cultured in Iscove's modified Dulbecco's medium (IMDM, Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum and antibiotics. The HFF cells were used until passage 20. The BHKICP6LacZ-5 cells (ELVIRA reporter cells) were obtained as frozen aliquots of 7.5×10^5 cells/vial from Diagnostic Hybrids, Inc., Athens, Ohio.

The HSV-1 strain KOS and the KOS-derived mutant $AraA^r8$ (moderately resistant to ACV/resistant to PFA) were kindly provided by D. M. Coen (Harvard Medical School, Boston, Mass.)[5]. The HSV-1 ACV-sensitive strains McIntyre and R39 and the ACV-sensitive HSV-2 strain MS and ACV/PFA-resistant clinical isolate HSV-2 97.1218 were generously provided by A. Linde (Swedish Institute for Infectious Disease Control, Solna, Sweden). The ACV-resistant clinical isolate HSV-1 98.14742-PE/1 was a gift of M. Aymard (Université Claude Bernard, Lyon, France). Propagation and stock preparation of these virus strains was performed on Vero cells. Clinical isolates of HSV type 1 and 2 were used from our own collection. These isolates were obtained from clinical specimens of immunocompromised as well as immunocompetent patients. In addition, a set of 10 HSV-1 clinical isolates was obtained from bone marrow transplant recipients suffering from severe HSV infections despite ACV therapy. Viruses were isolated on human diploid embryonic lung fibroblasts and virus stocks were propagated in Vero cells. The infectious virus titer was determined using the ELVIS™ HSV Test Kit (Diagnostic Hybrids, Inc., Athens, Ohio) according to the manufacturer's instructions[13].

Antiviral drugs. ACV and foscarnet (PFA) (Sigma, Zwijndrecht, The Netherlands) were dissolved in water and stored as 0.5 mg/ml and 10 mg/ml stocks, respectively.

ELVIRA. HFF cells were plated in the inner 60 wells of a culture microtiter plate (Costar) in a volume of 200 µl (6×10^4 cell/ml) and cultured for 2 days. Confluent monolayers were inoculated with 100 µl of virus suspension containing 70 to 300 plaque-forming units (PFU)/ml. Subsequently, 100 µl of antiviral drug diluted in culture medium were added. The concentrations of ACV and PFA ranged from 0.25 to 64 µg/ml (in fourfold increments) and from 12.5 to 200 µg/ml (twofold increments), respectively. Triplicate wells were used for each drug concentration. Virus adsorption was enhanced by centrifugation of the plates for 1 h at 700 × g. After 22-24 h of incubation, a suspension of ELVIRA reporter cells was prepared from frozen stocks using IMDM (final concentration 29,000 cells/ml). The culture supernatant was aspirated and a 200 µl of the ELVIRA cell suspension was added to each well and allowed to settle onto the HFF monolayer. After overnight incubation at 37°C, the culture supernatant was aspirated, 150 µl of 0.03% sodium desoxycholate was added and cell cultures were lysed for 30 min. Subsequently, β-galactosidase activity in the lysates was determined by incubating the plates at 37°C for 15-90 min in the presence of 100 µl of substrate solution (chlorophenol red-beta-D-galactopyranoside monosodium salt; CPRG [Roche Diagnostics, Almere, The Netherlands] 3 mg/ml, 4.35 mM magnesium chloride in PBS). Beta-galactosidase activity was determined spectrophotometrically (OD_{570}). For each drug concentration mock-infected OD values were subtracted from the average OD value of triplicate wells and converted to indicate a percentage of that of no-drug virus control. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of antiviral drug that reduced the OD by 50% relative to the virus control. Each virus was tested at least in two independent experiments. Reference ACV- and PFA-sensitive and -resistant HSV-1 and HSV-2 strains were included as controls in each assay. A schematic description of ELVIRA is shown in Fig. 1.

PRA. The PRA was performed as previously described[16]. 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 antiviral drug and 0.5% agar. The same drug concentrations were used as in ELVIRA. Each drug concentration was tested in quadruplicate. The plates were incubated at 37°C for 2 to 4 days until plaques were observed in the no-drug control wells. Subsequently, the monolayers were stained using a second overlay medium containing 0.08% neutral red in 0.8% agar. The IC_{50} was defined as the drug concentration that reduced the number of plaques by 50% compared to the untreated control wells. Isolates were considered resistant to ACV and PFA at an IC_{50} of ≥ 2 µg/ml and >100 µg/ml, respectively[18].

Statistical analysis. The intra-assay variability of ELVIRA was assessed using ANOVA and F-test. Results of the ELVIRA assay and the PRA were analyzed and compared using Wilcoxon's signed rank test and Spearman's correlation coefficient.

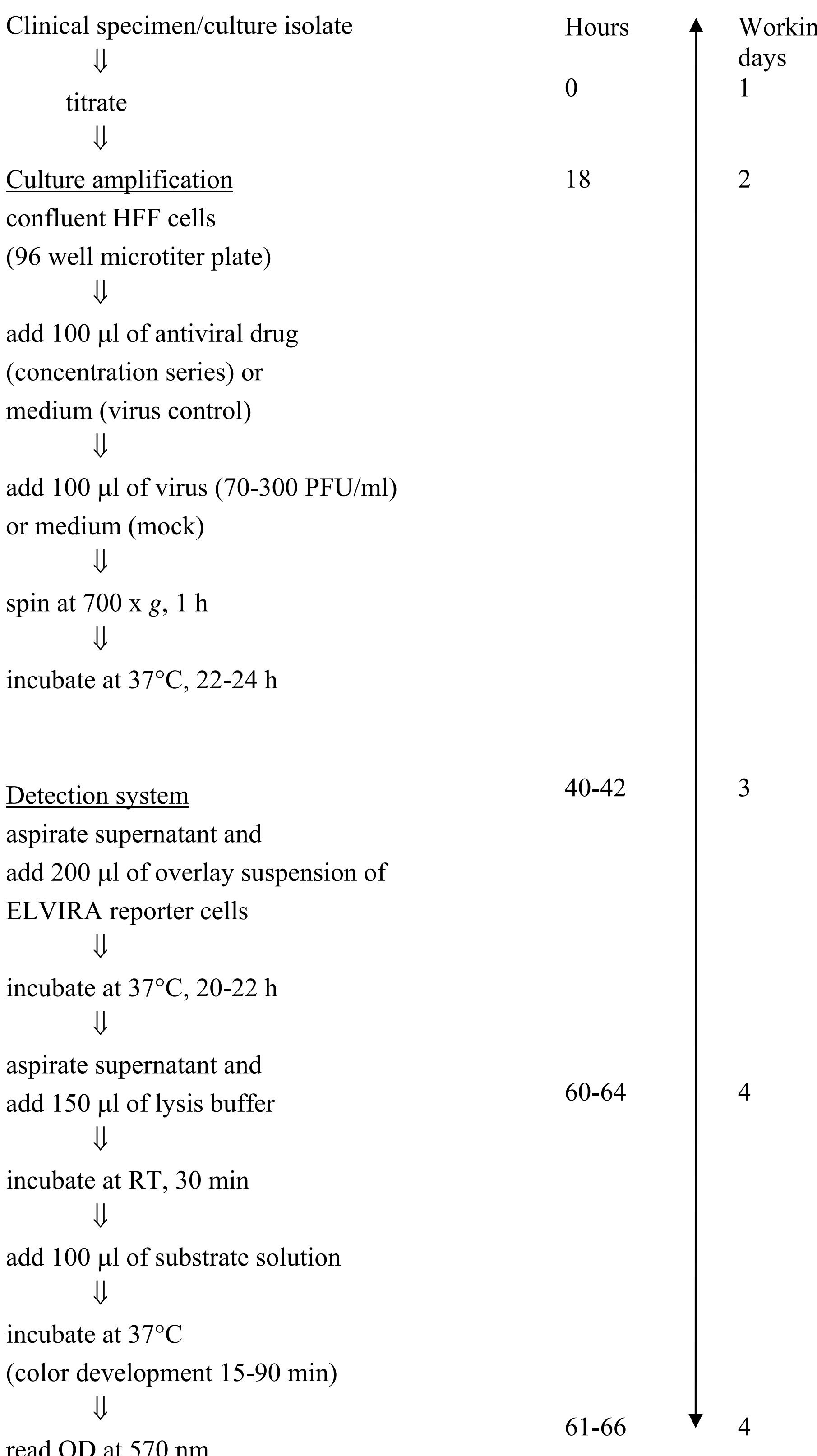

Fig. 1. Schematic overview of ELVIRA® HSV assay procedure.

RESULTS

We focused on the development of a rapid drug susceptibility assay for HSV using HSV inducible reporter cells. Our objective was to replace the laborious so-called "modified PRA"[20] by a rapid, semi-automated microtiter plate-based assay with colorimetric detection of enzymatic activity in culture lysates. A yield reduction assay was set up, in which virus infects human cell line in the presence of antiviral drug and subsequently the non-human reporter cell line (BHKICP6LacZ-5) is used for virus detection. After this initial setup was chosen, several parameters of the assay were tested and optimized. Thereafter, the assay was evaluated on a panel of well-characterized laboratory strains and clinical isolates and finally compared with PRA.

Assay optimization. In order to determine the optimal duration of infection (culture amplification step), confluent HFF monolayers were inoculated with a range of virus inocula from 37.5 to 600 PFU/ml (MOI 0.00013 to 0.00200 PFU/cell) and incubated at 37°C. After 12, 16, 20 and 24 h ELVIRA reporter cells were added and the assay was carried out as described in Materials and Methods. The results are shown on Fig. 2. An increased incubation time post infection resulted in an increased sensitivity of the assay, with a detection of inocula as small as 37.5 PFU/ml. The enzymatic activity showed a plateau at the highest inoculum of 600 PFU/ml after 20 h. Similar data were obtained for HSV-2 (data not shown). Thus, an incubation of 22-24 h was chosen for further use.

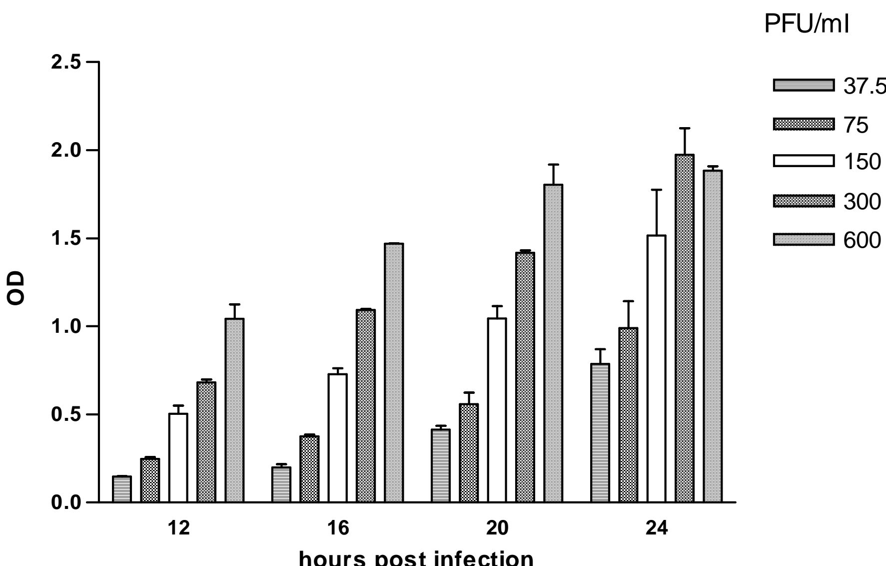

Fig. 2. The effect of the duration of infection of HFF cells on the sensitivity of ELVIRA. HFF monolayers were infected with a range of HSV-1 virus inocula (see Results). After 12, 16, 20 and 24 h, ELVIRA reporter cells were added and the assay was carried out as described in Materials and Methods. Results are representative (HSV-1) of four independent experiments in triplicates; error bars indicate SD.

Detection of viral replication was performed upon infection of overlay reporter cells for 20-22 h. This incubation time was based on previously determined expression kinetics of β-galactosidase by the reporter cells upon HSV infection[15,19].

Linear range. The linear range of the assay was determined by inoculation of HFF cells with a reference strains KOS (HSV-1) and MS (HSV-2) with inocula ranging from 1 to 1 × 10^6 PFU/ml (~ MOI 3 ×10^{-6} to 3 PFU/cell). The assay demonstrated to be linear within an inoculum size ranging from 70 to 300 PFU/ml (~ MOI 0.0002 to 0.0010 PFU/cell). A plateau was observed with inocula higher than 300 PFU/ml, and a decrease in β-galactosidase activity was observed with inocula higher than 1,500 PFU/ml (Fig. 3). Low inocula ranging from 10 to 70 PFU/ml resulted in decreased reproducibility of OD values. The mean coefficient of variation (CV) of triplicate OD values from five random determinations was 74% compared to a mean CV of 17% (P=0.002) for inocula higher then 70 PFU/ml. An inoculum range between >70 and 300 PFU/ml was selected for routine use.

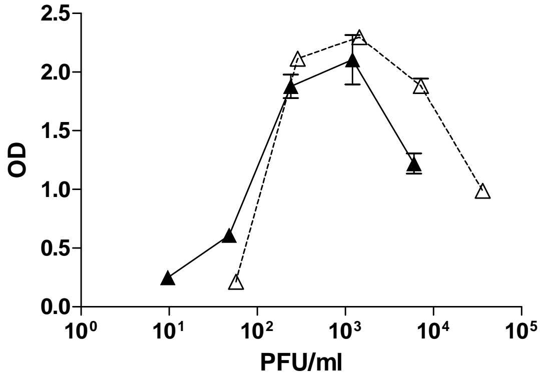

Fig. 3. Linear range of ELVIRA. Different serial dilutions of reference strains (HSV-1 strain KOS and HSV-2 strain MS) were inoculated on HFF cells (see Results) and the assay was carried out as described in Materials and Methods. Results are representative of four independent experiments in duplicates; error bars indicate SD. Triangles indicate HSV-1 strain KOS (▲) and HSV-2 strain MS (△).

Susceptibility testing

Susceptibility of reference strains. The utility of the assay for susceptibility testing was evaluated on two pairs of well-characterized ACV/PFA-sensitive and -resistant HSV-1 and HSV-2 strains: i) HSV-1 strain KOS and a KOS-derived mutant AraA^{r}8, and ii) HSV-2 strain MS and a mutant 97.1218. In addition, two HSV-1 sensitive reference strains R39 and McIntyre were included. A PRA was performed in parallel. Mean IC_{50}s of at least three independent determinations for these strains are shown in Table 1. There was a good agreement in susceptibility results between ELVIRA and PRA for all 6 strains examined. The antiviral effect of ACV and PFA measured in ELVIRA corresponded to the results of PRA. The data in Table 1 clearly demonstrate the capacity of the ELVIRA to discriminate between sensitive and resistant HSV strains.

Table 1. Results of ACV and PFA susceptibility testing using ELVIRA and PRA of well-characterized reference strains, mutants and clinical isolates.

Virus strain[a]	HSV type	ACV-IC_{50} (μg/ml)[b]		PFA-IC_{50} (μg/ml)	
		ELVIRA	PRA	ELVIRA	PRA
ACV^s					
KOS	1	0.52 ± 0.17	1.26 ± 0.37	33.2 ± 8.9	50.3 ± 1.8
McIntyre	1	0.33 ± 0.09	1.91 ± 0.56	32.9 ± 6.8	ND[c]
R39	1	0.38 ± 0.12	0.46 ± 0.04	41.5 ± 5.3	ND
MS	2	0.51 ± 0.16	1.63 ± 0.63	21.5 ± 8.3	50.6 ± 7.2
ACV^r/PFA^r					
AraA^{r}8	1	1.70 ± 0.51	4.50 ± 1.65	122.3 ± 15.5	133.0 ± 49.8
97.1218	2	78.10 ± 12.28	70.50 ± 15.3	190.0 ± 10.1	189.4 ± 20.3
ACV^s/PFA^s clinical isolates					
97.11896	1	0.19 ± 0.05	0.24 ± 0.19	22.6 ± 5.6	14.5 ± 2.1
97.09227	1	0.24 ± 0.01	0.87 ± 0.19	26.9 ± 5.6	14.9 ± 3.6
97.06348	1	0.22 ± 0.01	1.05 ± 0.28	21.5 ± 1.9	36.8 ± 4.1
97.07631	1	0.07 ± 0.02	0.76 ± 0.49	14.3 ± 2.4	27.3 ± 2.3
01.14606	1	0.23 ± 0.01	0.37 ± 0.14	24.0 ± 5.5	24.4 ± 3.1
ACV^r/PFA^s clinical isolates					
96.05940	1	44.18 ± 6.96	10.74 ± 6.89	24.9 ± 8.9	30.5 ± 3.3
96.07922	1	43.14 ± 6.76	9.93 ± 4.17	31.3 ± 3.3	28.3 ± 4.5
97.10788	1	35.18 ± 3.56	14.15 ± 2.26	33.9 ± 6.2	32.7 ± 5.3
97.07632	1	8.60 ± 2.35	4.21 ± 1.88	18.4 ± 0.2	27.6 ± 1.6
01.22388	1	9.38 ± 2.96	6.23 ± 2.30	14.7 ± 3.6	31.5 ± 7.0
01.22733	1	18.21 ± 3.10	2.31 ± 1.10	38.4 ± 10.6	20.4 ± 4.8
98.14742-PE/1	1	19.12 ± 0.96	8.65 ± 1.37	ND	ND
99.16237	1	45.15 ± 13.0	11.52 ± 0.86	ND	ND
99.17213	1	20.61 ± 6.73	5.73 ± 1.76	9.2 ± 3.1	10.3 ± 2.8
01.28565	2	33.56 ± 3.00	14.97 ± 3.10	11.5 ± 1.4	16.4 ± 2.3
ACV^r/PFA^r clinical isolates					
01.24080	1	7.68 ± 2.31	4.31 ± 1.60	131.7 ± 4.1	111.6 ± 7.8

[a] The superscript ^{s} and ^{r} indicate sensitive and resistant, respectively.
[b] The IC_{50} values shown are expressed as mean ± SD from at least 2 independent experiments.
[c] ND, not determined.

Evaluation of ELVIRA for susceptibility testing. Upon the initial testing with reference strains, ELVIRA was further evaluated on a set of clinical isolates (n=16) with different susceptibilities to ACV and PFA. All isolates were tested by ELVIRA and PRA in parallel (Table 1). Compared to the results of PRA, ELVIRA identified all isolates correctly as

sensitive or resistant. In addition, the ACV-IC_{50}s determined by ELVIRA showed a good correlation with the results of PRA (r_{ACV}=0.94). The IC_{50} values for ACV-sensitive strains and isolates determined by ELVIRA were on average five times lower compared to those of PRA (*P*=0.002). For ACV-resistant clinical isolates ELVIRA showed significantly higher mean IC_{50}s than PRA (*P*=0.0005). For PFA, IC_{50}s by ELVIRA did not differ significantly from those of PRA for sensitive isolates (*P*=0.21). There were only 3 PFA-resistant isolates available for testing and their IC_{50}s were also similar to those determined by PRA (*P*=0.75) (Table 1). Given the small number of isolates tested, a further research into the correlation of the PFA-IC_{50}s with PRA is warranted.

In addition, the fold changes in IC_{50}s between the reference strain KOS and each of the tested strains (relative susceptibility) between ELVIRA and PRA were determined (Fig. 4). For ACV-sensitive strains, the fold changes in ACV-IC_{50}s were not significantly different between the two assays (*P*=0.07). For ACV-resistant strains, however, ELVIRA results demonstrated significantly higher fold changes in ACV-IC_{50}s than PRA (*P*=0.0005).

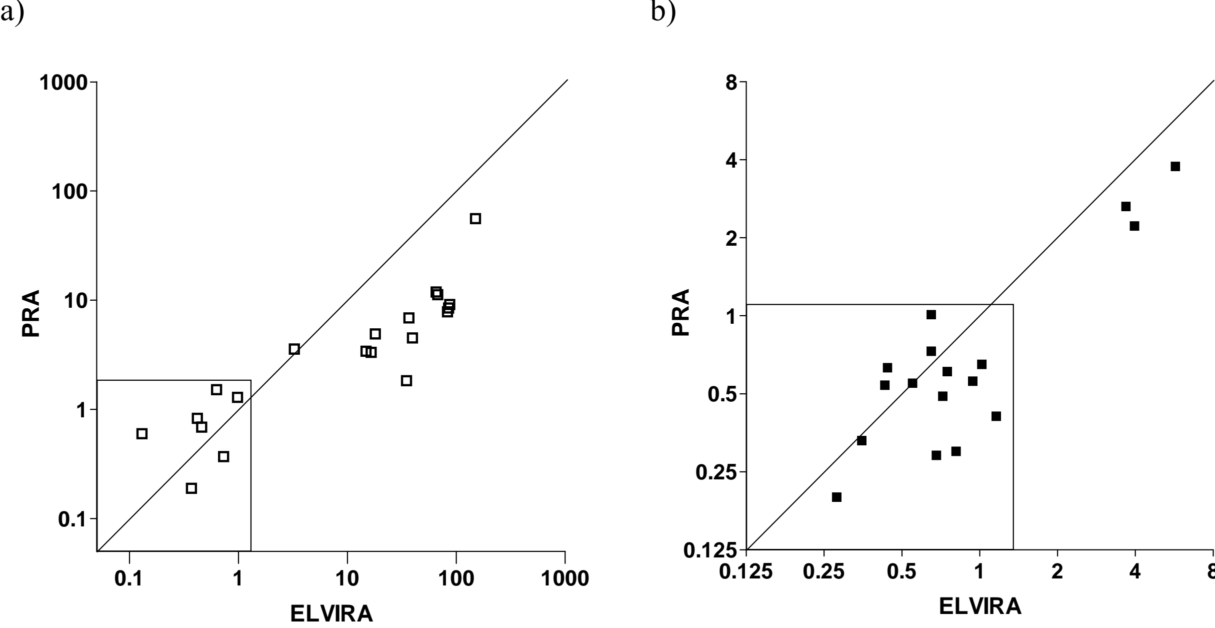

Fig. 4. Mean fold changes in IC_{50} between reference strain KOS and tested strains determined by ELVIRA and PRA. a) ACV; axes in log_{10} scale; b) PFA; axes in log_2 scale. Square area in the graphs indicates drug-sensitive isolates.

For PFA, the fold changes between the two assays were comparable for PFA-sensitive strains (*P*=0.08). For PFA-resistant strains, the fold changes in PFA-IC_{50}s determined by ELVIRA were somewhat higher than those of PRA (*P*=0.029). However the number of PFA-resistant isolates tested was small (n=3).

Reproducibility. To assess the inter-assay variability, the IC_{50} of the sensitive HSV-1 strain KOS, HSV-2 strain MS and the resistant HSV-1 strain $AraA^r8$ to ACV and PFA were determined in seven repeated experiments (Table 2). The data showed a good reproducibility indicated by a CV ranging from 12 to 33% for each of both serotypes.

In addition, data were collected on the variation in susceptibility results of HSV-1 reference strains, which were included as controls in each assay. The 28 and 24 consecutive ACV-IC_{50} determinations for the reference strain KOS and the mutant $AraA^r8$ demonstrated a mean ± standard deviation (SD) of 0.60 ± 0.17 µg/ml and 1.7 ± 0.61 µg/ml and a variation of 28.3 and 35.9%, respectively.

The reproducibility of IC_{50} values of sensitive and resistant isolates was also examined. The mean variation was calculated from CVs from replicate susceptibility assays of clinical isolates shown in Table 1 and additional 10 clinical isolates used for assay validation (CV, 32.3%).

Table 2. Inter-assay variability of ELVIRA

Drug	Strain	Mean IC_{50} ± SD (µg/ml)[a]	CV (%)
ACV	KOS	0.52 ± 0.17	32.6
	MS	0.58 ± 0.15	26.5
	$AraA^r8$	1.75 ± 0.59	33.3
PFA	KOS	36.1 ± 8.1	22.4
	MS	24.5 ± 7.9	32.0
	$AraA^r8$	122.7 ± 15.6	12.7

[a] Mean and SD from seven independent experiments

Evaluation of ELVIRA with clinical isolates. ELVIRA was used to test the susceptibility of 30 and 33 HSV-1 clinical isolates to ACV and PFA, respectively, from specimens from untreated patients sent for routine HSV diagnostics in the past. The median ACV-IC_{50} was 0.17 µg/ml (range, 0.01 to 0.57 µg/ml SD: 0.12) and median PFA-IC_{50} was 20.98 µg/ml (range 9 to 47.1 µg/ml; SD: 8.5) (Fig. 5).

Clinical utility. Determination of the susceptibility profile of 10 HSV-1 clinical isolates from bone marrow transplant recipients suffering from HSV infection despite antiviral therapy was performed by ELVIRA and PRA. The results of ELVIRA correlated in all cases with the results of PRA, to the similar extent as shown for reference strains and clinical isolates in Table 1 (data not shown). Furthermore, results of genetic analysis of the resistance-associated thymidine kinase (TK) gene confirmed presence of sensitive or resistant virus as indicated by ELVIRA. Specifically, ELVIRA detected a decrease in susceptibility to ACV in isolates, which carried well-known ACV resistance-associated mutations in the TK gene. At the same time no decrease in susceptibility was noted for wild type or pretherapy isolates, which did not carry any mutation in TK gene.

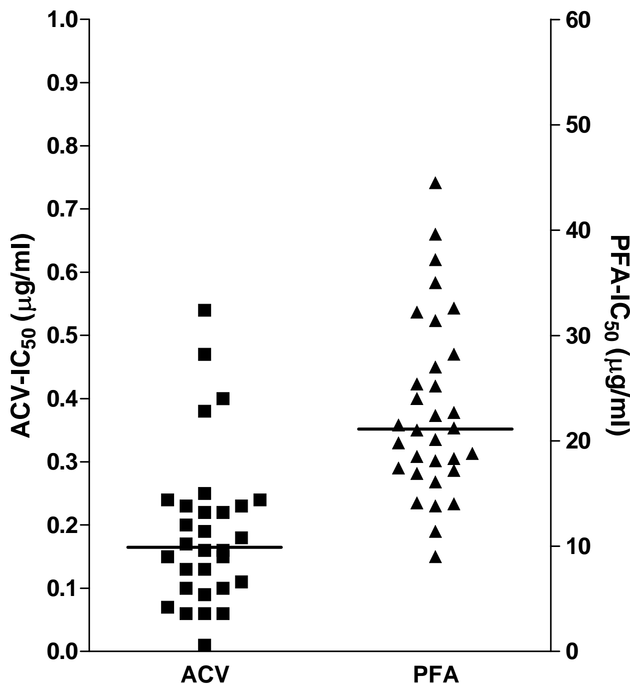

Fig. 5. Susceptibilities of HSV-1 clinical isolates to ACV (n=30) and PFA (n=33) determined by ELVIRA. The bars indicate median.

DISCUSSION

Here we describe a colorimetric dual-cell yield reduction assay, ELVIRA® HSV, which uses the transgenic HSV inducible cell line BHKICPLacZ-5 as a readout cell line for determination of susceptibility of HSV-1 and HSV-2 to antiviral drugs.

ELVIRA demonstrated a 100% correlation with PRA on identification of ACV- and PFA-sensitive and -resistant isolates of both HSV serotypes (n=22). In addition, the relative changes in susceptibility to ACV (fold changes in IC_{50}) correlated well between the two assays (r_{ACV}=0.95) (Fig. 4). Because of the lower frequency of PFA resistance in clinical isolates, there were only three PFA-resistant clinical isolates available for assay evaluation in our study. Thus only limited data for comparison of PFA-IC_{50}s and fold changes in susceptibility between the two assays could be obtained (r_{PFA}=0.54) (Fig. 4). Further experiments with additional PFA-resistant clinical isolates are warranted for more solid conclusions on the correlation of ELVIRA PFA-IC_{50}s with PRA. Our data indicate that ELVIRA® HSV could be used for drug susceptibility determination for each of the antiviral drugs currently applied in the clinic.

The reproducibility of the assay was adequate, with an inter-assay coefficient of variation ranging from 4 to 43%, which is within the acceptable range for biological assays[9].

A set of 30 and 33 HSV-1 clinical isolates was tested by ELVIRA in order to determine the natural variation in ACV and PFA drug susceptibility, respectively. Based on the range in

drug susceptibility of these clinical isolates, the drug susceptibility of reference strains and the reproducibility of the assay, an IC_{50} value of 0.8 μg/ml was set as a cut-off value for decreased susceptibility to ACV. Similarly, a cut-off value for decreased susceptibility to PFA was set at 60 μg/ml. However, additional testing of several clinical isolates and well-characterized strains is required for better and more accurate definition of these cut-offs.

The amount of virus needed to perform ELVIRA appeared to be low. Testing of clinical isolates containing as little as 70 PFU/ml could be performed. This high sensitivity was ensured, apart from the reporter cell detection system, by the total infection period of 44 to 46 h, providing sufficient time for multiple cycles of virus replication. In addition, the use of HFF cells also contributes to the high sensitivity of the assay. These cells are more susceptible to HSV infection requiring a smaller inoculum and less time to reach complete CPE than Vero cells[11]. This might enable direct susceptibility testing of HSV clinical specimens, without the need for prior virus isolation. In a clinical laboratory setting, the titration of the clinical specimen, using the ELVIS™ HSV Test Kit, can be performed the same day as seeding of HFF cells for ELVIRA. Within two days, which are routinely used for HFF cells to reach confluence, the titer of clinical specimen can be determined. Expression of β-galactosidase in ELVIRA reporter cells is driven by an immediate-early promoter of HSV-1 and occurs within hours after infection[15].

As a result, the virus detection part of ELVIRA is very rapid and can be completed even before CPE develops. The whole assay is thus more rapid than the CPE-based assays and assays based on detection of viral DNA, where more replication cycles are needed. Thus, a susceptibility result can be available within 5 days from sample arrival at the laboratory. The rapid availability of results as well as the good concordance of ELVIRA with PRA might stimulate the application of susceptibility testing in clinical routine and the use of results for decisions on subsequent treatment.

ELVIRA was set up as a yield reduction assay (YRA). The YRA determines the production of infectious virus rather than the formation of plaques[3]. The endpoint of an YRA presumably more closely reflects the in-vivo ability of the drug to inhibit virus shedding and thus may also be of greater relevance in the evaluation of new antiviral agents[10]. Moreover, in PRA only plaque numbers and not their size are measured, even though a decrease in plaque size also reflects the antiviral effect of the drug[2]. In ELVIRA, however, the true number of virus infected cells is determined, which reflects the amount of infectious virus. Another characteristic of ELVIRA is the use of liquid medium compared to solid/semisolid medium in PRA. The liquid medium allows drug-resistant virus to “amplify” and spread, consequently resulting in a more sensitive detection of small amounts of drug-resistant virus[18].

These above mentioned differences between ELVIRA and PRA in the assay setup and readout may probably explain the higher relative changes in IC_{50} between the ACV-sensitive and -resistant viruses observed for ELVIRA compared to PRA (Fig. 4). This indicates that ELVIRA might be more sensitive for discrimination between ACV-sensitive and -resistant isolates than PRA.

In conclusion, we describe the development and evaluation of a novel colorimetric, transgenic cell-based assay, ELVIRA® HSV, which can be used for rapid and reliable determination of HSV drug susceptibility and also for evaluation of new antiviral

compounds. The assay is able to determine differences in susceptibility to ACV and PFA for both HSV serotypes even in low titer specimens. The short turn-around time, easy-to-use format, objective endpoint, good reproducibility, and low inoculum size make ELVIRA a rapid susceptibility assay amenable for use in the routine diagnostic virology laboratory. The testing of larger number of sensitive and resistant clinical isolates is warranted for further validation of the assay.

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Chapter 5

Survey of acyclovir-resistant herpes simplex virus in The Netherlands: prevalence and characterization

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ABSTRACT

A survey of resistance to acyclovir (ACV) was performed on herpes simplex virus (HSV) strains isolated in The Netherlands between 1999 and 2002. A total of 542 isolates, 410 HSV-1 and 132 HSV-2, from 496 patients were screened for reduced susceptibility to ACV. A newly developed ELVIRA HSV screening assay was used that allowed a high throughput screening. Thirteen isolates, 8 HSV-1 and 5 HSV-2, from 10 patients (2%) were found resistant to ACV. A single ACV-resistant strain was identified among isolates from 368 immunocompetent patients (0.27%; 95% confidence interval [CI], 0.007%-1.5%), whereas in 9 isolates from 128 immunocompromised patients resistant HSV was identified (7%; 95% CI, 3.26%-12.93%). The highest frequency of ACV-resistant HSV was associated with bone marrow transplantation: 4 patients out of 28 (14.3%) shed resistant virus. In addition, resistant virus was obtained from two HIV-positive patients, one patient with a hematological malignancy and two patients on immunosuppressive drugs. Further testing showed that none of the isolates was resistant to foscarnet. Several new mutations were identified in the thymidine kinase gene of these resistant isolates, and their effect on ACV-resistance is discussed.

Our study shows that the prevalence of ACV resistance is low in immunocompetent patients (0.27%), whereas ACV-resistant HSV infections occur relatively frequently in immunocompromised patients (7%; $P<0.0001$). This emphasizes the need for drug susceptibility monitoring of HSV infections in immunocompromised patients with persisting infections despite antiviral therapy.

INTRODUCTION

For more than 20 years, acyclovir (ACV) has been the drug of choice for prophylaxis and treatment of herpes simplex virus (HSV) infections. Its use is indicated for treatment of primary and recurrent genital HSV infection as well as for chronic suppressive treatment of genital herpes, and as a first line treatment for HSV encephalitis, where timely administration of ACV is needed to prevent often fatal outcome[51]. Oral or intravenous ACV is frequently used for prophylaxis and treatment of HSV infections in immunocompromised patients. In the context of severe immunosuppression, as established for example during hematopoietic stem cell transplantation (HSCT), ACV prophylaxis dramatically decreases HSV reactivation in seropositive HSCT recipients, in whom the risk of chronic, severe and sometimes fatal HSV infections is high[5,12,18,43,54]. Finally, topical ACV and penciclovir (PCV) formulations are available in most countries as an over the counter drug for management of recurrent herpes labialis.

ACV, a nucleoside analogue, requires three phosphorylation steps to achieve an antiviral effect by competitive inhibition of viral DNA polymerase activity. The initial phosphorylation step is carried out by the viral thymidine kinase (TK), and the two subsequent ones by cellular kinases. Antiviral resistance is mostly conferred by mutations in

the TK gene (nucleotide additions, deletions or substitutions)[7] and, to a much lesser extent by mutations in the viral DNA polymerase (DNA pol) gene[25,26].

Since the introduction of ACV, concerns have been raised, that long-term treatment, prophylaxis and suppressive use may result in the development of resistance. ACV-resistant viruses have been found in clinical isolates, which were never exposed to ACV, and ACV-resistant TK mutants of HSV can be readily selected *in vitro*[34,40]. These *in vitro* observations were confirmed by an increased frequency of isolation of drug-resistant HSV viruses from ACV-treated immunocompromised patients since the early 1990s[12]. Studies reporting on the prevalence of ACV-resistant HSV have been recently reviewed[3]. Combined with the results from the most recent studies by Morfin *et al.*[31] and Reyes *et al.*[37] it can be concluded that the prevalence of HSV infections with reduced susceptibility to ACV in immunocompromised patients varies from 4.0 and 7.1%[3,13,19,32]. The highest prevalence rates are reported for recipients of HSCT, with a range from 6 to 14%[12,31,50,53]. A recent report described an even higher frequency (36%) of ACV resistance[24]. Similarly, the prevalence in HIV-positive patients ranges from 3.5 to 7%[19,31,37] and in solid organ transplant (SOT) recipients from 2.8 to 10%[13,31]. The numbers of patients in the SOT group, however, are usually low.

In contrast, ACV-resistant isolates have been reported infrequently in immunocompetent subjects (for review see[3]). This is probably due to the low pathogenic potential of the resistant virus variants and the presence of effective immune response, which results in rapid clearance of the virus[15]. A low prevalence of resistant HSV in immunocompetent patients was reported in extensive screening surveys performed in the UK (0.7%) and the USA (0.3%) between 1980 and 1992[16]. No increase in the prevalence of resistance has been observed since then (range from 0.1 to 0.7%)[2,3,10,13,31,37]. These studies, performed in the UK, USA and France, also included patients on chronic suppressive therapy for genital herpes as well as the general population using ACV/PCV topical preparations for herpes labialis.

Our study aimed at obtaining data on the prevalence of ACV-resistant HSV among isolates collected from different patient groups between 1999 and 2002 in The Netherlands, with a focus on determination of the prevalence rates in both the immunocompetent and immunocompromised patient populations. For both the general population as well as for specific patient groups, this study set baseline prevalence estimates for future national surveillance studies. In addition, our study also included a detailed phenotypic and genotypic characterization of resistant clinical isolates identified in the survey.

METHODS

Study participants. HSV isolates were obtained from major clinical virological laboratories that provide routine diagnostic service for university and regional hospitals, STD clinics as well as general practitioners' practices in The Netherlands.

Clinical specimens. HSV-positive specimens, either culture isolates or original materials, were obtained from the participating laboratories. The laboratories were asked to provide a random collection of HSV-positive specimens/isolates obtained between 1999 and 2002. No other selection criteria were applied. Isolates were coded and susceptibility testing was

performed without prior clinical information on the patient's specimen in order to avoid bias in the susceptibility test results. Clinical data were coupled to the results of susceptibility testing only when a final test result was obtained. Efforts were made to obtain information on the patients' diagnosis, immunostatus, clinical manifestations of HSV disease and previous use of ACV. Therefore, participating laboratories were requested to complete a basic questionnaire. Additional clinical information was collected from the medical records of specific patients of interest (i.e. patients with resistant HSV isolates). However, in several cases, complete and detailed information could not be obtained.

HSV isolation and serotyping were performed in the collaborating laboratories using their routine diagnostic procedures. Specimens included skin, oro-facial and ano-genital specimens obtained from vesicles or ulcerative lesions, as well as ocular, throat and lower respiratory tract specimens and biopsies. For susceptibility screening, small scale virus stocks were prepared on human foreskin fibroblasts (HFF) from all obtained specimens and stored at -70°C. The number of *in-vitro* passages for individual HSV-positive specimens was limited to two.

Well-characterized strains and clinical isolates. The ACV-sensitive HSV-1 strain KOS, the KOS-derived mutants AraA^{r}7, PFA^{r}5 (both ACV- and PFA-resistant), and AraA^{r}8 (moderately resistant to ACV/resistant to PFA) were kindly provided by D. M. Coen (Harvard Medical School, Boston, Mass.)[14,35]. The HSV-1 ACV-sensitive strains McIntyre and R39, and ACV-sensitive HSV-2 strain MS and ACV- and PFA-resistant HSV-2 strain 97.1218 were generously provided by A. Linde (Swedish Institute for Infectious Disease Control, Solna, Sweden). The ACV-resistant clinical isolate HSV-1 98.14742-PE/1 was a gift of M. Aymard (Université Claude Bernard, Lyon, France) and ACV-resistant HSV-2 isolates A1 and O24[20] were a gift of N. Goyette (Univeristy of Laval, Quebec, Canada).

Clinical isolates of HSV type 1 and 2 were obtained from our own collection of specimens received for routine HSV diagnostics as well as for antiviral drug susceptibility testing in the past. These clinical isolates originated from immunocompetent as well as immunocompromised patients and were all tested for susceptibility to ACV using ELVIRA® HSV susceptibility assay[45-47].

Susceptibility testing. Virus stocks were primarily screened for susceptibility to ACV by the ELVIRA HSV screening assay (described below), a modification of the ELVIRA® HSV susceptibility assay described previously[45]. Selected stocks were subsequently subjected to detailed susceptibility testing using the latter method.

ELVIRA® HSV susceptibility assay. This assay was performed as previously described[45]. HFF cells were plated in the inner 60 wells of a 96-well microtiter plate (Costar) in a volume of 200 µl (6×10^4 cell/ml) and cultured for 2 days. Confluent monolayers were inoculated with 100 µl of virus suspension containing 70 to 300 plaque-forming units (PFU)/ml. Subsequently, 100 µl of antiviral drug diluted in culture medium was added. The concentrations of ACV and foscarnet (PFA) ranged from 0.25 to 64 µg/ml (fourfold increments) and from 12.5 to 200 µg/ml (twofold increments), respectively. For ganciclovir (GCV), a range from 0.001 to 4 µg/ml (fourfold increments) was used. Triplicate wells were used for each drug concentration. Virus adsorption was enhanced by centrifugation of the plates for 1 hour at 700 × *g*. After 22-24 h of incubation, a suspension of ELVIRA reporter

cells in culture medium was prepared from frozen stocks (final concentration 29,000 cells/ml). The culture supernatant was aspirated and 200 µl of the ELVIRA cell suspension was added to each well and allowed to settle onto the HFF monolayer. After overnight incubation at 37°C, the culture supernatant was aspirated, 150 µl of a 0.03% sodium desoxycholate solution was added, and cell cultures were lysed for 30 min. Subsequently, β-galactosidase activity in the lysates was determined by incubating the plates at 37°C for 15-90 min in the presence of 100 µl of substrate solution (chlorophenol red-beta-D-galactopyranoside monosodium salt; CPRG [Roche Diagnostics, Almere, The Netherlands] 3 mg/ml, 4.35 mM magnesium chloride in PBS). The β-galactosidase activity was determined spectrophotometrically (OD_{570}). For each drug concentration average OD values of mock-infected wells were subtracted from the average OD value of HSV-infected wells. Subsequently, the percentage virus replication as a function of a drug concentration was calculated relative to the no drug control and the IC_{50} was determined. Each virus isolate was tested at least in two independent experiments. Reference ACV- and PFA-sensitive and -resistant HSV-1 (KOS and $AraA^r8$) and HSV-2 (MS and 97.1218) strains were included as controls in each assay. IC_{50} values of 0.8 µg/ml and 60 µg/ml were set as cut-off values for decreased susceptibility to ACV and PFA, respectively[45]. When determining the susceptibility of sequential isolates from a single patient, all changes in drug susceptibility were related to the susceptibility of the sensitive pretherapy or early therapy isolate if available. Decreased susceptibility to ACV and PFA was defined as an at least 5- and 3-fold increase in IC_{50}, compared to the IC_{50} of the pretherapy isolate, respectively[1].

ELVIRA HSV screening assay. Confluent HFF monolayers in microtiter plates were inoculated with an endpoint series of 5-fold dilutions of an untitered virus stock in a volume of 100 µl. Four dilutions of each virus stock were usually tested starting at the dilution 1/5000. A single concentration of ACV of 1 µg/ml was used for the primary screening. Each virus stock dilution was tested in duplicate in the drug and the no drug control wells. A schematic drawing of the assay setup is shown in Fig. 1. Subsequent culture amplification and detection steps of the assay were performed as described above for the ELVIRA® HSV susceptibility assay.

To express the susceptibility of the isolates a “resistance value” was defined, which represented virus yield (replication of the virus) in the presence of 1 µg/ml ACV expressed as a percentage of the no-drug virus control. This value was calculated for each virus stock dilution. The susceptibility of the isolate was determined from the stock dilution for which OD values of virus control were within the linear range of the assay[45]. Usually one (less often two) dilutions fulfilled these criteria. In case of two evaluable virus dilutions the mean resistance value was calculated. Standard endpoint dilutions of reference ACV-sensitive and -resistant HSV-1 and HSV-2 strains were included as controls in each assay as described above. The mean resistance values for each of the reference drug-sensitive and -resistant strains were determined in multiple experiments and further used in routine testing as reference values (see Results). A test result was accepted when the resistance values of the reference strains were within 2 SDs of the mean. The resistance value of each tested isolate was compared with that of the sensitive reference strain KOS (HSV-1) or MS (HSV-2), obtained in the same assay. The susceptibility was expressed as a fold change in resistance

value compared with the reference strain. The resistance value of sequential isolates was compared to that of the pretherapy or early therapy isolate.

For isolates that demonstrated decreased susceptibility to ACV in the screening assay, detailed susceptibility testing to ACV was performed, as described above. In case of confirmed ACV resistance, susceptibility to PFA was determined and genotypic analysis of resistance-associated TK gene was performed as described previously[46]. The entire TK gene sequence was compared to those of the pretherapy or drug-sensitive isolate if available, or with the HSV-1 or HSV-2 reference strains KOS and 333, respectively. Any mutations identified this way were compared with a database of published TK gene mutations. Susceptibility to GCV was determined for those isolates which harbored so far unreported TK gene mutations.

Statistical methods. Results of the ELVIRA® HSV susceptibility assay and the screening assay were analyzed and compared using Spearman's correlation coefficient. Data on prevalence of ACV resistance were analyzed and compared using 95% confidence intervals and Fischer's exact test.

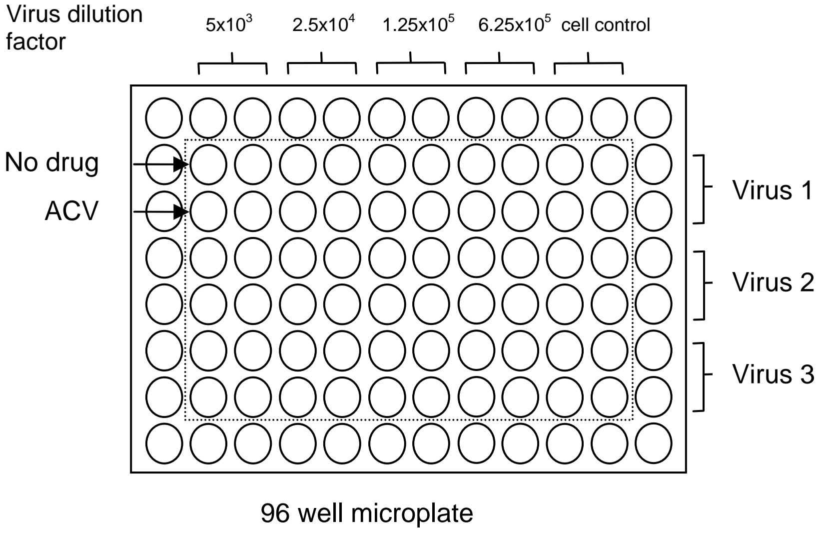

Fig. 1. Schematical drawing of the ELVIRA HSV screening assay.

RESULTS

ELVIRA HSV screening assay setup. The screening assay was developed using a single ACV concentration of 1 µg/ml. This concentration was chosen on the basis of ACV susceptibilities (IC_{50}) of a set of 40 ACV-sensitive and 30 ACV-resistant HSV-1 strains, consisting of laboratory strains and clinical isolates (see Materials and Methods), determined by the ELVIRA HSV susceptibility assay. The IC_{50}s of sensitive strains ranged from 0.01 to 0.76 µg/ml, while all resistant strains had IC_{50}s higher than 1 µg/ml (range, 1.22 to 58.6 µg/ml). A smaller set of HSV-2 strains and clinical isolates (6 sensitive and 6 resistant)

showed an IC_{50} range for sensitive isolates from 0.08 to 0.67 µg/ml and from 6.5 to 90 µg/ml for resistant isolates.

The titer of the virus in the clinical specimens varied considerably. Upon testing of 81 specimens, a median titer of 3.4×10^3 PFU/ml was obtained with a wide titer range from 10 to 1×10^6 PFU/ml. For susceptibility screening, small scale virus stocks were generated on HFF cells from all of the obtained specimens, which resulted in narrowing the virus titer range. Titration of 10 of such stocks showed a median titer of approximately 1.6×10^7 PFU/ml with a range from 2.3×10^6 to 3.4×10^7 PFU/ml. Consequently, a 5-fold dilution series of virus stock starting at the dilution 1/5000 was tested in the screening assay.

Susceptibility testing using ELVIRA HSV screening assay. The screening assay was evaluated in parallel to ELVIRA HSV susceptibility assay on a set of 10 well-characterized HSV strains and 13 clinical isolates of both serotypes, which included 9 ACV-sensitive and 14 ACV-resistant viruses, covering TK as well as DNA pol mutants (Table 1). Results of the screening assay (expressed as fold changes in resistance value compared with the reference strain KOS) were in a total agreement with the ELVIRA HSV susceptibility assay (discrimination of sensitive and resistant phenotype) and correlated well with the IC_{50} values (r=0.89, P<0.0001). All resistant isolates demonstrated decreased susceptibility (increase in resistance value) in a range from 3.5- to 13.7-fold compared to the reference strain KOS. Furthermore, screening assay results were compared with the virus genotype determined by sequencing. The screening assay detected a decrease in susceptibility to ACV in isolates, which carried ACV resistance-associated mutations in TK or DNA pol gene, while no significant decrease in susceptibility was noted for wild type or pretherapy isolates, which did not carry any mutation in any of the two genes (Table 1). The decrease in susceptibility was generally lower for viruses with DNA pol mutations (3.5- to 5.4-fold) compared to those with mutations in the TK gene (7.1- to 13.7-fold).

Reproducibility. The variation between the replicates was assessed for 10 clinical isolates in the presence and the absence of ACV for all virus dilutions within the linear range of the assay. The mean coefficient of variation (CV) was 6.8% with a range from 0.99 to 13.8%. If the variation between the replicates was >25%, the isolate was retested.

To assess the inter-assay variability, the resistance values of the two pairs of well-characterized ACV/PFA-sensitive and -resistant HSV-1 and HSV-2 strains: i) HSV-1 strains KOS and $AraA^r8$, and ii) HSV-2 strains MS and 97.1218 were determined in 13 and 4 repeated experiments, respectively. The data showed a CV ranging from 14 to 38% (Table 2).

The data described above clearly demonstrate that the ELVIRA HSV screening assay can be used to discriminate between ACV-sensitive and -resistant HSV strains. Based on this evaluation a ≥3-fold increase in resistance value compared with the reference strain was chosen as a breakpoint for decreased susceptibility for both HSV-1 and HSV-2 strains.

Table 1. Results of ELVIRA HSV screening and susceptibility assays.

Virus strain/isolate (code) [a]	Isolate no.[b]	HSV type	ELVIRA screening (Fold change)[c]	ELVIRA susceptibility (ACV-IC_{50})[d]	Genotype[e]
Well-characterized strains					
KOS	-	1	1	0.4 ± 0.20	-
AraA^{r}8	-	1	**3.5**	**1.7 ± 0.51**	DNA pol
PFA^{r}5	-	1	**5.4**	**2.1 ± 0.60**	DNA pol
MS	-	2	1.7	0.5 ± 0.16	-
97.1218	-	2	**7.5**	**78.1 ± 12.28**	ND
A1	-	2	**8.8**	**7.5 ± 3.50**	TK
O24	-	2	**7.8**	**11.7 ± 4.20**	TK
McIntyre	-	1	0.4	0.3 ± 0.09	-
R39	-	1	0.3	0.4 ± 0.12	-
98.14742-PE/1	-	1	**7.5**	**19.1 ± 0.96**	TK
Clinical isolates					
98.17628	1/1	1	0.4	0.2 ± 0.09	-
99.08230	1/2	1	**13.7**	**42.9 ± 4.80**	TK
99.20762	2/1	1	0.6	0.1 ± 0.06	-
99.22172	2/2	1	**12.3**	**8.6 ± 0.63**	TK
97.11896	3/1	1	0.5	0.2 ± 0.05	-
97.12576	3/2	1	**8.0**	**5.8 ± 2.82**	TK
01.14606	4/1	1	0.6	0.2 ± 0.01	-
01.14830	4/2	1	0.7	0.3 ± 0.01	-
01.19093	4/3	1	**3.8**	**1.5 ± 0.33**	DNA pol
01.22733	4/4	1	**7.1**	**18.2 ± 3.10**	TK
01.25504	4/5	1	**4.2**	**1.5 ± 0.13**	DNA pol
01.29319	4/6	1	**4.4**	**2.3 ± 0.70**	DNA pol
99.16237	5	1	**10.5**	**45.2 ± 13.0**	TK

[a] In the upper part of the table data from the evaluation of the assay on well-characterized strains are shown, the lower part represents evaluation on the clinical isolates.
[b] Sequential isolates from a single patient are numbered.
[c] Fold change in resistance value compared to the sensitive strain KOS. Numbers in boldface indicate resistance.
[d] Mean ± SD from at least 2 independent experiments.
[e] Resistance-associated mutation in TK or DNA pol gene.
Note: ND, not determined.

Table 2. Inter-assay variability of ELVIRA HSV screening assay.

Virus strain	Resistance value (%) [a] mean ± SD (n)	CV (%)	Fold change[b]
KOS	12.1 ± 4.5 (13)	36.9	-
AraA[r]8	60.2 ± 23.1 (13)	38.3	5.0 ± 1.3
MS	22.7 ± 3.2 (4)	13.9	-
97.1218	94.3 ± 20.2 (4)	21.4	4.3 ± 1.6

[a] Virus yield at 1 µg/ml ACV as a % of virus control. Mean ± SD from n-independent experiments.
[b] Mean ± SD of a fold change in the resistance values within the pair of resistant and sensitive reference strains of HSV-1 and HSV-2 (see Results).

Screening of ACV susceptibility of HSV clinical isolates from the Dutch patients. A total of 542 HSV isolates (410 of HSV-1, 132 of HSV-2) originating from 496 patients were available for susceptibility analysis.

The immunocompetent patient group consisted of 368 patients who had genital infections (n=131, 36%), oro-facial (n=115, 31%) or skin infections (n=28, 7.6%). Infections of the lower respiratory tract or the digestive tract were less common (6.5%). The immunocompromised patient group consisted of 128 patients with different underlying causes of immunosuppression; HIV (40%), HSCT (22%), solid organ transplantation (13%), malignancy (21%) were the major groups (Table 3). Compared to the immunocompetent group, these patients mostly suffered from oro-facial or throat infections (n=67, 52%). Genital herpes was identified in 31 patients (24%), and was associated with HIV seropositivity in 27 (87%) of these patients.

All HSV isolates were tested using the ELVIRA HSV screening assay. In 38 isolates obtained from 35 patients a decreased susceptibility to ACV (≥3-fold increase in resistance value) was found. Thirteen of these 38 isolates (8 HSV-1, 5 HSV-2; 10 patients) demonstrated a ≥5-fold decrease in susceptibility (increase in resistance value). Detailed susceptibility determination (IC_{50}) using the ELVIRA HSV susceptibility assay and/or genotypic analysis demonstrated ACV resistance in all these 13 isolates. The mean ACV-IC_{50}s of these isolates ranged from 10.7 to 50.0 µg/ml. The remaining 25 isolates, which demonstrated a ≥3- to 5-fold increase in resistance value in the screening assay, displayed ACV-IC_{50}s in a range from 0.08 to 0.89 µg/ml. The distribution of the mean IC_{50}s for these isolates is shown in Fig. 2. It has demonstrated that all isolates were ACV-sensitive. Isolate #22 repeatedly demonstrated an atypical response to increasing drug concentrations. The dose response curve formed a second replication peak at the high drug concentrations, suggestive of a presence of a subpopulation of resistant virus. Genotypic characterization revealed a mixture of wild type and resistant virus in this isolate (isolate 1/8, Table 4).

As a result of ACV susceptibility screening, one out of 368 immunocompetent patients carried an ACV-resistant HSV (0.27%; 95% confidence interval [CI], 0.007%-1.5%), whereas in nine out of 128 immunocompromised patients (7.0%) resistant HSV was identified (95% CI, 3.26%-12.93%) (Table 3). The prevalence of resistant HSV in

immunocompromised patients was significantly higher than that in the immunocompetent ones ($P<0.0001$). The distribution of these ACV-resistant isolates among major groups of immunocompromised patients is shown in Table 3. The highest rate of resistant isolates was found among HSCT recipients.

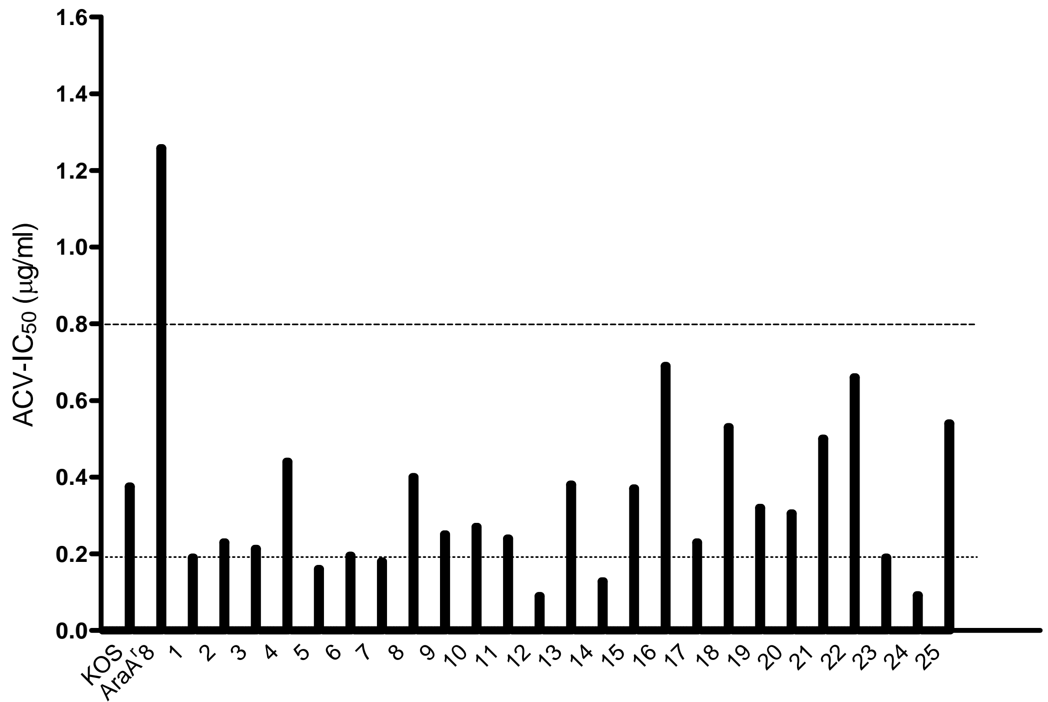

Fig. 2. Mean ACV-IC_{50}s of 25 isolates, which demonstrated a ≥3- to 5-fold increase in resistance value in the ELVIRA HSV screening assay, including HSV-1 controls. A cut-off IC_{50} for decreased susceptibility to ACV in the ELVIRA HSV susceptibility assay is shown as a dashed line. A median ACV-IC_{50} of 40 sensitive strains (0.19 µg/ml) is shown as dotted line.

Table 3. Frequency of ACV-resistant HSV in the population of immunocompetent and immunocompromised subjects.

Immune status of the patient	No. of patients	No. of patients with resistant HSV (%)	No. of isolates	No. of resistant HSV isolates
Immunocompetent				
Total	**368**	**1 (0.27)**	**389**	**1**
Immunocompromised				
HSCT	28	4 (14.3)	34	5
HIV	51	2 (3.92)	59	3
Malignancy	26	1 (3.85)	36	2
SOT	17	0 (0.00)	17	0
Other	6	2 (na)[a]	7	2
Total	**128**	**9 (7.03)**	**153**	**12**
Grand total	**496**	**10 (2.02)**	**542**	**13**

[a] Not applicable

Characterization of ACV-resistant isolates.

Virus isolates. The thirteen ACV-resistant HSV isolates from 10 patients were studied in detail. Nine of these patients were immunocompromised, either due to an HIV infection (n=2), a HSCT (n=4), cancer (n=1) or immunosuppressive drug treatment (n=2), and one patient had no signs of impaired immune response. Results of detailed phenotypic and genotypic analysis of ACV-resistant HSV isolates from 7 of these 10 patients as well as the summary of available clinical information with respect to the HSV infection is given in Table 4. The characterization of HSV isolates from 3 of the 4 HSCT recipients has been described previously [47].

From two patients (#1 and 2), sequential isolates were obtained. For patient #1, apart from resistant isolates, additional two isolates (1/1, a sensitive pretherapy isolate and a 1/8, therapy isolate with somewhat decreased susceptibility, see above) were available, and for patient #2 sequential resistant isolates were obtained. For the other 5 patients only a single resistant isolate was available for investigation.

Only limited information was available on the antiviral treatment and clinical response to therapy for these patients. Patient #1 had a T cell lymphoma and suffered from severe disseminated visceral HSV infections demonstrated as pneumonia and gastroenteritis. Antiviral therapy was switched to PFA when no response to long-term intravenous (iv) ACV was seen. However, the patient died shortly after the switch due to multiorgan failure. Patients #2 and 3, both HIV-positive, suffered from recurrent genital herpes. Isolate from patient #2 was obtained during ValACV therapy. Patient #3 experienced previous recurrences 1 and 3 years ago, which were treated with ValACV or topical ACV, but no ACV treatment had been given shortly before the isolation of the resistant isolate described here. Patient #4 received HSCT for treatment of chronic myeloid leukemia 18 month before development of genital herpes. Her posttransplantation period was complicated by cytomegalovirus (CMV) reactivation, for which she was treated with GCV. Two patients (#5 and 6) suffered from chronic obstructive pulmonary disease (COPD). Patient #5 was on high dose prednisone and had oral HSV lesions. Resistant HSV was isolated on the third day of oral ACV therapy. Patient #6 was admitted at the intensive care unit with emphysema, and treated with high dose prednisolone. The patient had also been treated in the past with inhaled corticosteroids. Seven days after start of prednisolone therapy, HSV was isolated from the lungs and treatment with iv ACV was initiated. A single immunocompetent patient (#7) suffered every two weeks from recurrent HSV-2 skin infection resulting in blisters and lesions on his back without any previous antiviral therapy.

Susceptibility to antiviral drugs and molecular analysis of the TK gene. Susceptibilities to ACV were determined for all isolates if sufficient virus stock was available (Table 4). The mean ACV-IC_{50}s of ACV-resistant isolates ranged from 10.7 to 50.0 µg/ml. The mean ACV-IC_{50} of 0.66 µg/ml was obtained for isolate 1/8, which consisted of a mixture of wild type and resistant virus. ACV-resistant isolates from all patients were subjected to PFA susceptibility testing. None of the isolates showed a PFA-resistant phenotype. The susceptibility to GCV of isolate from patient #7 was determined in order to further elucidate the role of the mutations identified in the TK gene. Cross-resistance to GCV was observed for this ACV-resistant isolate (Table 4).

Table 4. Summary of phenotypic, genotypic and clinical data for patients with ACV-resistant HSV.

Patient (age/ gender)	Type of immunosupression	Isolate no.[a]	HSV type	Isolation site	Susceptibility: drug-IC_{50} (µg/ml)[b]		Genotypic changes in TK gene[c]	Antiviral treatment, day (d)[d]	HSV disease
					ACV	PFA			
1 (9/M)	T-cell lymphoma	1/1	1	Throat	0.16	ND	-	ivACV, d3-d22, d26-d32 ; PFA, d32-d36	HSV pneumonia, gastroenteritis; †d36
		1/8		Faeces	0.66[e]	16.8	**R176R/Q**		
		1/24[f]		Lung	**50.0**	8.3	**FS185 (+C) stop 43 ds**		
2 (35/M)	HIV+	2/1[f]	2	Anus	**29.0**	ND	*A27T, S29A, G36E,* ***D229H***	On ValACV	Recurrent genital herpes
3 (50/M)	HIV+		2	Scrotum	**15.3**	7.7	**T288M**	Previous ValACV and topical ACV	Recurrent genital herpes
4 (51/V)	HSCT		2	Vagina	**R**[g]	ND	E39G**,** ***G56E***	On GCV for CMV	Genital herpes
5 (68/M)	High-dose prednisone in COPD		1	Oral cavity	**R**[g]	ND	***D162D/H***	oACV, d2-d?	Oral lesions
6 (73/V)	High-dose prednisolone in COPD		1	Lung	**10.7**	16.4	**R222H**	ivACV, d7-d?, oACV, d?-d27	HSV in lower respiratory tract
7 (38/M)	None		2	Skin	**35.3**[h]	11.5	***T202A,*** *R363C*	No therapy	Recurrent skin lesions
KOS			1		0.52	32.8	-		
98.14742			1		**23.2**	20.7	FS146 (+G)		
MS			2		0.45	17.7	-		
97.1218			2		**95.5**	**160.0**	ND		

[a] Number indicates the patient number and the day of collection relative to the first isolate.
[b] Mean IC_{50} values were calculated from at least two independent experiments. Inter-experimental variability for all IC_{50} values was <50%. Bold numbers: drug-resistant phenotype
[c] Bold text: TK mutations for which the (therapy) isolates differ from the pretherapy or earlier sensitive isolate or reference ACV-sensitive strains and which are presumably associated with ACV resistance. Italics: substitutions not reported before (shown for all isolates where found).
[d] Day of treatment relative to the day of collection of the first isolate.
[e] Isolate scored as intermediate in the screening assay.
[f] Patient 1 still shed resistant virus at day 30 and patient 2 still shed resistant virus at day 9.
[g] R, resistant in the screening assay. Isolate excluded from further phenotypic testing due to low virus stock titer and lack of original specimen.
[h] Strain showed decreased susceptibility to GCV (IC_{50} =2.23 µg/ml).
Note: FS, frameshift; ND, not determined; ds, downstream; o, oral.

All ACV-resistant isolates had mutations in the TK gene. Except for a single resistant isolate with a frameshift mutation, these mutations all involved amino acid substitutions (Table 4). Each of the HSV-1 isolates from 3 patients (#1, 5 and 6) contained a single amino acid substitution either in the active site (Asp162His, Arg176Gln) or in conserved regions of the TK gene (Arg222His). In addition, in patient #1, a frameshift mutation at codon 185 resulting from an insertion of cytosine was found (isolate 1/24). The isolate from patient #5 displayed a mixture of two genotypes at position 162, where both the wild type Asp as well as a mutation to His were identified. Because of the insufficient amount of stock specimen the correlations of this mixed genotype with IC_{50} could not be studied (Table 4).

A single isolate from patient #1, recovered prior to ACV therapy, was sensitive to ACV. The subsequent isolate recovered on day 8 from faeces demonstrated borderline resistance to ACV with a mean 4-fold increase in ACV-IC_{50} compared to the first ACV-sensitive isolate. Genotyping of this isolate identified a substitution Arg176Gln in a mixture with a wild type genotype. Subsequently, a fully resistant virus was recovered from the lungs of this patient (within 24 days after a sensitive isolate) with a frameshift mutation at codon 185. Alignments of TK gene sequences of these sequential virus isolates demonstrated a complete sequence homology except for the resistance conferring mutations.

Multiple amino acid substitutions, relative to the reference virus sequence, were usually observed in ACV-resistant HSV-2 isolates from 4 patients (#2, 3, 4, 7). All these mutations are shown in Table 4. In each of the isolates either known resistance-associated substitutions (Thr288Met) or new substitutions (Gly56Glu, Thr202Ala and Asp229His), which could affect ACV resistance, were identified. The remaining substitutions found in the isolates (Ala27Thr, Ser29Ala, Gly36Glu, Glu39Gly, Asn78Asp, Leu140Phe and Arg363Cys) were presumably natural polymorphisms.

DISCUSSION

In order to allow screening of large numbers of clinical isolates for decreased susceptibility to ACV, rapid screening assay was developed based on modification of the ELVIRA HSV susceptibility assay[45]. The susceptibility testing was performed on a dilution series of a virus stock with a single ACV concentration of 1 μg/ml. Determination of ACV susceptibility was based on a comparison with the sensitive reference strain KOS/MS. A 3-fold increase in resistance value was considered the threshold for decreased susceptibility. Although others suggested using a higher increase (5- or 10-fold) as a criterion for resistance[9,38], the use of the 3-fold criterion in our study ensured detection of low level (borderline) ACV resistance (Table 1). Indeed, isolates with borderline resistance causing ACV unresponsive infections have been encountered, albeit less frequently, in the clinic[21,46]. In addition, certain DNA pol mutations confer only low level of ACV resistance compared to the usually high level of ACV resistance for TK-negative mutants[6]. The 3-fold criterion used in our screening assay also demonstrated its usefulness in detecting emerging resistance (mixture of wild type and resistant virus) in a patient, who shed resistant virus later on (patient #1, Table 4). Others have also described clinical isolates with intermediate

(borderline) resistance that preceded isolation of ACV-resistant strain[17]. The magnitude of the resistance criterion used in our assay largely depended on the reference strain used. Indeed, a wide natural variation in susceptibilities exists among ACV-sensitive reference strains and clinical isolates[48]. Thus, in order to find consensus on the significant increase in the level of the resistance, the use of drug-sensitive reference strains needs to be standardized and the clinical significance of this value has to be evaluated.

Our screening test was completed within 43-48 hours on isolates of unknown titer. The inter-assay variation was less than 50%, which is comparable to the reproducibility of other recently described susceptibility assays[27,36]. Since the screening assay includes determination of the virus titer, it allows subsequent direct full susceptibility testing. Although in our survey virus stocks containing high virus titer were evaluated, testing of specimens containing as little as 70 PFU/ml of infectious virus could be performed[45]. This might enable direct susceptibility screening of HSV clinical specimens, without the need for prior virus culture. The range of dilutions of the clinical specimens tested would, however, need to be adjusted, because the titer of virus in clinical specimens may vary considerably[44]. The screening assay provided a reliable susceptibility determination, as its results have consistently been confirmed by the ELVIRA HSV susceptibility assay and by the results of genotyping. Thus, the ELVIRA HSV screening assay was suitable for rapid susceptibility testing of large numbers of isolates in our survey but it could as well be used in the routine clinical setting for rapid initial susceptibility evaluation of HSV isolates suspected of resistance. Regular drug susceptibility monitoring of HSV isolates causing persisting infections in immunocompromised patients on antiviral therapy is essential for optimal patient management[46]. The ELVIRA HSV screening assay can be applied for this purpose.

In this survey, data on the prevalence of ACV-resistant HSV in the general immunocompetent population and in immunocompromised patients in The Netherlands were generated. The susceptibility of HSV isolates of both serotypes (76% HSV-1, 24% HSV-2) from 496 patients was determined. Resistant HSV infections were identified in 0.27% of immunocompetent and in 7% of immunocompromised patients. These prevalence estimates are consistent with results from other recent surveys in other countries[2,13,31,37]. The fact that the majority of isolates included in the survey were collected by university hospital laboratories that receive specimens from regional hospitals spread throughout the country indicates, that data obtained may be sufficiently representative for the general patient population in The Netherlands. Indeed, the patient population showed diversity in age, backgrounds and geographic distribution within The Netherlands, and attended general practices, outpatient or STD clinics, or were hospitalized.

Among our group of specimens from immunocompetent patients, genital herpes specimens were the most frequent (36%), followed by oro-facial (31%) and skin specimens (7.6%). Although most cases of HSV resistance in immunocompetent patients reported so far have been identified in patients with genital herpes[3], no resistant isolate was identified in our genital herpes patient group. Results from a recent large survey in a general population with genital herpes[13] also demonstrated a low prevalence of resistant HSV (0.5%). No resistant isolate was recovered from patients with herpes labialis in our study, despite the frequent use of topical ACV or PCV for treatment of herpes labialis in the general population. Similarly,

low prevalence rates of 0.2% and 0.1%, were reported from two recent large studies in the general population with recurrent herpes labialis performed in USA and UK, respectively[2,10]. In our study, a single immunocompetent patient shed ACV-resistant virus. This patient (#7) suffered from recurrent HSV-2 skin infection despite lack of any previous ACV therapy. Two possible explanations exist for the unusual emergence of primary HSV resistance in this patient. Either this case reflects the low incidence of resistant virus variants, as a result of natural mutation rate of HSV-2[39] or a resistant virus was transmitted during primary infection. Our study in accordance with other large surveys indicates that the prevalence of resistance in the immunocompetent population remains low despite increased ACV usage.

The immunocompromised patient group consisted of 128 patients with different underlying causes of immunosuppression. In contrast to the immunocompetent group, these patients mostly suffered from oro-facial or throat infections (52%) while genital herpes was observed less frequently (24%). Although the total number of patients evaluated was limited, the overall prevalence of resistance of 7.1% is in agreement with previous reports from other countries[12,13,19,31,32,50,53]. In addition, the prevalence was significantly higher than that for the immunocompetent patient group. Considering the very small number of resistant isolates in various groups of immunocompromised patients and also the small numbers of subjects per group, the prevalence values per patient group have to be considered less reliable. The highest frequency of HSV resistance was found in HSCT recipients, which is in accordance with results of the recent large surveys in HSCT recipients performed by Chen *et al.*[12] and Morfin *et al.*[31]. Indeed, HSCT recipients generally demonstrate a higher prevalence of HSV resistance than other immunocompromised patients, which is probably due to the more severe and prolonged suppression of CD4+ and CD8+ T cell responses. Adequate T cell immunity is essential for protection from HSV reactivation and clearance of HSV[12,29]. Interestingly, resistant HSV isolates were identified in two patients with COPD, who received treatment with corticosteroids. Patients suffering from COPD usually receive daily doses of inhaled corticosteroids (based on the severity of the disease). For the treatment of acute exacerbations, a short (5 to 7 days) course of oral corticosteroids is often beneficial and thus administered frequently. Thus, the immunosuppression induced by corticosteroids in these patients might have led to HSV reactivation and subsequent emergence of resistant HSV variants. In addition, inhaled corticosteroids might also play a role in induction a local immunosuppression favoring emergence of resistant viruses.

All ACV-resistant infections identified in the 10 patients in our study were caused by viruses with mutations in the TK gene. Indeed, ACV-resistant TK mutants are most frequently isolated *in vivo*[20,30]. Interestingly, HSV-2 isolates in our study displayed more substitutions within the TK gene than HSV-1 isolates. This probably reflects the higher incidence of spontaneous mutations in HSV-2[39]. Four ACV-resistant isolates from three patients (#1, 3, 6) contained mutations previously shown to be associated with ACV-resistance: Arg176Gln, Arg222His and a frameshift at codon 185 (+C) in HSV-1 isolates and Thr288Met in a HSV-2 isolate[7,20,23,40].

Isolates from the four other patients expressed mutations that had not been previously associated with ACV resistance. An Asp162His mutation was identified in an isolate from patient #5. Asp162 in HSV-1 TK is a highly conserved residue[4] responsible for co-ordination

of Mg^{2+} ions, which are essential for catalysis[8,11]. Thus, it is likely that replacement of Asp162 by His could have led to abrogation of TK catalytic activity resulting in ACV resistance. Unfortunately, it was not possible to perform detailed phenotypic analysis of this isolate due to insufficient amount of specimen.

In patient #4 a Gly56Glu mutation was identified. Gly56 is a part of the ATP-binding glycin-rich loop, GXXGXGKT/S (residues 56-63), which is conserved in all herpesvirus TKs. The three glycins are playing an important role in binding of the β-phosphoryl group of ATP, and replacement of any of them results in a loss of TK activity[28], which presumably resulted in ACV resistance.

In patient #7, a Thr202Ala mutation was identified. Thr202 in HSV-2 corresponds to Thr201 in the HSV-1 TK. Substitution Thr201Pro has been previously identified in an ACV-resistant HSV-1 isolate[20]. Therefore, it is likely that the substitution Thr202Ala might be responsible for ACV-resistance. In addition, cross-resistance to GCV supports the role of Thr202Ala in ACV-resistance and excludes the involvement of DNA pol mutations.

From the set of four mutations detected in the TK of patient #2, the Asp229His is most likely to be associated with resistance. The location of Asp229 in the so-called "insertion loop" (residues 216-230, equivalent to 215-229 in HSV-1) of the TK gene that interacts with ATP-binding site might indicate its role in TK activity[11,42]. Other mutations identified in this isolate are located at nonconserved regions/codons.

Although current structural and functional data support the significance of the mutated residues identified in this study, no definitive conclusion can be made for these new mutations on their role in resistance without confirmatory testing by site directed mutagenesis.

The role of substitution Glu39Gly in HSV-2 TK, was elucidated in our study. Although this mutation was previously identified in two ACV-resistant isolates[7,41], it presumably does not confer ACV resistance. Firstly, in our study, this mutation was found in a sensitive isolate as well as in a resistant isolate, in which other mutation conferring resistance was present (#4). Secondly, Glu39 is located within the N-terminal 45 amino acids of TK gene, a region previously described to be dispensable for the TK activity[22,49,52]. Finally, additional and proven resistance-associated mutations were identified in the two reported resistant isolates containing Glu39Gly[7,41].

Similarly to Glu39, mutations at residues Ala27, Ser29 and Gly36 located within the N-terminal region of the HSV-2 TK presumably reflect natural polymorphisms. Residue Arg363 is not located in the conserved region of TK and thus mutation Arg363Cys may also reflect natural polymorphism not previously reported. The remaining mutations Asn78Asp and Leu140Phe are natural polymorphisms described before[33,41].

The results of genotyping showed that new mutations can still be identified in ACV-resistant isolates, especially in HSV-2, which complicates the use of genotypic assays for rapid susceptibility determination. TK gene mutations conferring reduced susceptibility to ACV might be more diverse than previously thought[20].

In conclusion, with a prevalence of 0.27% in immunocompetent and 7% in immunocompromised patients, the prevalence of HSV drug resistance in The Netherlands does not differ from that reported in recent years for other countries for both patient groups.

A rapid screening assay for routine surveillance of ACV-resistant HSV was established and used to obtain prevalence estimates for future assessments of changes in susceptibility. Several new mutations have been identified in resistant isolates, indicating that current use of genotypic drug resistance assays requires careful examination of the identified mutations and should always be accompanied by phenotypic drug susceptibility determination.

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Chapter 6

Genotypic and phenotypic characterization of acyclovir-resistant herpes simplex viruses isolated from hematopoietic stem cell transplant recipients

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ABSTRACT

Thirty-one herpes simplex virus type one (HSV-1) isolates from 12 hematopoietic stem cell transplant recipients with persistent HSV infections despite acyclovir (ACV) prophylaxis or treatment, were genotypically and phenotypically characterized. The relationship between drug susceptibility of the isolates and mutations in thymidine kinase (TK) and DNA polymerase (DNA pol) genes was examined. In all 12 patients, HSV infections were due to ACV-resistant, foscarnet-sensitive viruses. Out of 31 isolates examined, 23 were resistant and 8 were sensitive to ACV. Eight patients carried viruses with frameshift mutations in the TK gene (due to addition or deletion of single nucleotides in homopolymeric repeats). These mutations were found at codon 61 (G deletion, 1 patient), 146 (G addition, 5 patients) and 153 or 185 (C deletion, both 1 patient). In four patients viruses were selected during ACV therapy that contained novel amino acid substitutions in the TK gene (H58R, G129D, A189V, R216H, R220C). Their possible role in ACV resistance was further confirmed phenotypically and by the absence of any resistance-associated mutations in the DNA pol gene. These substitutions were located in ATP- or dNTP-binding sites or in conserved regions of the TK gene. In addition, a single mutation, Q570R, in the δ-C region of the DNA pol gene, was identified in an isolate from a single patient with resistance to ACV. Our study confirms and expands previous data on genotypic changes associated with ACV resistance of HSV-1 clinical isolates.

INTRODUCTION

Chronic, severe and sometimes fatal mucocutaneous herpes simplex virus (HSV) infections may occur upon immunosuppressive conditioning regimens used for patients undergoing hematopoietic stem cell transplantation (HSCT)[3,8]. Without prophylaxis, HSV seropositivity before HSCT results in virus reactivation in 70-80% of patients[60]. Current prophylactic strategies with oral valacyclovir (ValACV) or intravenous acyclovir (ACV) are quite effective in preventing recurrent HSV disease in both autologous and allogeneic HSCT recipients[14,49]. However, emergence of virus resistant to therapy still occurs in 6-12% of cases[8,10,16]. Recent data by Morfin *et al*. (14%) and Langston *et al*. (5/14, 36%) indicate an increased prevalence of drug resistant HSV in this patient group[29,36].

For treatment of severe HSV infections refractory to ACV or ValACV, alternative antiviral drugs include foscarnet (PFA) and cidofovir (HPMPC, CDV)[20]. Although CDV is licensed for treatment of CMV, it has also been used for successful treatment of HSV infections resistant to all other available antivirals[43,52]. Majority of HSV isolates resistant to ACV exhibit cross-resistance to ganciclovir (GCV)[41]. Each of the HSV antiviral drugs targets the viral DNA polymerase (DNA pol) though using different mechanisms. Nucleoside analogues, such as ACV, require a prior phosphorylation by the viral thymidine kinase (TK) and two subsequent phosphorylation steps by cellular kinases in order to exert an antiviral effect by competitive inhibition of viral DNA pol activity. On the other hand, PFA is a direct DNA pol inhibitor. Binding of PFA to the pyrophosphate binding site of DNA pol prevents

the cleavage of pyrophosphate from deoxynucleotide triphosphates, which then results in a blockage of chain elongation. CDV requires two phosphorylation steps by cellular enzymes to generate the biologically active diphosphate form that acts as a DNA chain terminator[13].

As a result of the mechanism of action, resistance to ACV results predominantly from mutations in the TK gene and, to a much lesser extent, from mutations in the DNA pol gene[30,31]. Several mutations have been described to be associated with reduced susceptibility of HSV to ACV. However, the spectrum of TK gene mutations conferring ACV resistance is still incomplete[36]. The mutations usually consist of single amino acid substitutions or nucleotide additions/deletions in homopolymeric repeats of cytosines (C) or guanines (G) that are present throughout the TK gene. The latter types of mutations occur most frequently. They result in a frameshift and consequently in a premature stop codon leading to a nonfunctional TK[5,35,46]. Single amino acid (aa) substitutions conferring ACV resistance are mostly located in the nucleoside-binding site (aa 162-176), in the ATP-binding site (aa 51-63)[21], in conserved regions of the gene (aa 56-63, 83-88, 162-164, 171-173, 216-222 and 284-289)[2], or at highly conserved individual codons. In addition, a small number of resistance-associated substitutions has been reported outside the above mentioned regions[5,18,28]. Resistance to PFA and CDV is limited to single amino acid substitutions in the conserved regions of the DNA pol gene[20]. As yet, resistance to CDV has only been reported in vitro[1].

Since the introduction of ACV, several resistant mutants have been generated in the laboratory and characterized in detail[44,55]. The numbers of genotypically and phenotypically characterized resistant viruses derived from well defined clinical isolates are much smaller and often limited to single cases[25,37,38,42,48]. The largest study of this kind was performed by Gadreau *et al*. with 30 resistant isolates collected predominantly from HIV-infected subjects between 1989 and 1996[18].

Here we report on the genotypic and phenotypic characterization of 31 HSV-1 isolates from 12 HSCT recipients suffering from clinically severe HSV infections despite ACV prophylaxis or treatment. The relationship between drug susceptibility and mutations in TK and DNA pol genes was examined in the context of the clinical history of the patients.

MATERIALS AND METHODS

Patients. A total of 12 patients that underwent an allogeneic stem cell transplantation between 1996 and 2001 at the Leiden University Medical Center or the University Medical Center Utrecht, The Netherlands were examined. The patients received HSCT for treatment of hematological malignancies, leukodystrophy or severe aplastic anemia. All patients had undergone conditioning regimens with cyclophosphamide, busulfan or total body irradiation, and additional antithymocyte globulin in cases of matched unrelated transplantations. Graft versus host disease (GVHD) was prevented by partial T cell depletion of donor marrow and cyclosporine. All patients had tested positive for HSV IgG prior to transplantation and

suffered from severe HSV infections. Patients received ACV prophylaxis and/or ACV treatment in accordance to the local protocol.

In Utrecht, prophylaxis consisted of intravenous (iv) ACV (5 mg/kg) three times daily for recipients of transplants from matched unrelated donors. The iv ACV prophylaxis started at the day of transplantation and continued until 3 weeks thereafter. Subsequently, oral prophylaxis was initiated with either ACV (200 mg four times daily; until 1998), or ValACV (500 mg twice daily, since 1998) until 1 year post transplantation.

Recipients of transplants from matched related donors received ValACV 500 mg twice daily as prophylaxis until 1 year post transplantation. Breakthrough HSV infections were treated with high dose iv ACV (10 mg/kg/day) or with PFA until resolution of symptoms.

In Leiden, no prophylactic treatment was given. Patients transplanted in this center received iv ACV therapy from the moment of laboratory-confirmed HSV infection (patients # 1, 2, 3, 6, 7). The pediatric patients received iv ACV 10 mg/kg three times daily, followed by ValACV 500 mg/m^2 three times daily while adults received iv ACV 500 mg three times daily. Treatment continued until resolution of symptoms or until recovery of cellular immunity. The patient characteristics are summarized in Table 1.

Table 1. Characteristics of 12 HSCT patients with drug-resistant HSV-1 infections.

Characteristics	Number
Age, median (years range)	27 (8-48)
Gender, male/female	7/5
Underlying disease	
Chronic myeloid leukemia	1
Acute myeloid leukemia	5
Myelodysplastic syndrome	3
X-linked adrenoleukodystrophy	1
Severe aplastic anemia	2
Type of transplant	
Identical sibling	1
Haploidentical family donor	5
Matched unrelated	6
Mortality	5
Antiviral management	
ACV, prophylaxis/therapy	7/5
PFA, therapy/response	6/5
GCV	3
CDV	1

Virus isolation. Clinical material was collected from the nose, mouth, throat, esophagus or the lungs. Human diploid embryonic lung fibroblasts were cultured in shell vials in Eagle's MEM (BioWhittaker, Verviers, Belgium) supplemented with 5% fetal bovine serum, amphotericin B and antibiotics. Duplicate shell vials with confluent cells were inoculated with approximately 0.2 ml of clinical specimen in virus transport medium (VTM) and centrifuged at 700 × *g* for 75 min. Thereafter, 1 ml of culture medium was added and the cultures were subsequently incubated at 37°C. Virus antigen detection and typing was performed two days after inoculation by using commercial monoclonal type-specific antibodies (Argene Biosoft, Varilhes, France). The second shell vial was maintained for the development of cytopathic effect (CPE) for a maximum of 14 days.

Viral stocks were generated for all HSV-positive specimens and the infectious virus titers were determined by plaque assay in Vero cells, as previously described by Schaffer *et al.*[47], or by the ELVIS™ HSV Test Kit (Diagnostic Hybrids, Inc., Athens, OH)[39].

Susceptibility testing. Susceptibility to ACV, PFA and GCV (Sigma, Zwijndrecht, The Netherlands) was determined by the ELVIRA® HSV assay as described previously[54]. Briefly, confluent monolayers of human foreskin fibroblasts cultured in 96-well culture plates were inoculated with 7 to 30 plaque-forming units (PFU) of virus; subsequently antiviral drug was added in various dilutions. The concentrations of ACV ranged from 0.25 to 64 μg/ml (fourfold increments); the concentrations of GCV and PFA ranged from 0.016 to 4 μg/ml (fourfold increments) and from 12.5 to 200 μg/ml (twofold increments), respectively. Each drug concentration was tested in triplicate wells. Virus adsorption was enhanced by centrifugation of the plates for 1 hour at 700 × *g*. After overnight incubation at 37°C, the culture supernatant was aspirated and 200 μl of the ELVIRA cell suspension (final concentration 29 000 cells/ml) was added to each well. After overnight incubation at 37°C, 150 μl of 0.03% sodium desoxycholate was added and cell cultures were lysed for 30 minutes. Subsequently, the β-galactosidase activity in the lysates was determined by incubating the plates at 37°C for 15-90 min in the presence of 100 μl of substrate solution based on CPRG (chlorophenol red-beta-D- polymerase galactopyranoside monosodium salt; Roche Diagnostics, Almere, The Netherlands). Beta-galactosidase activity was determined spectrophotometrically (OD_{570}). When sequential isolates from the same patient were available, changes in drug susceptibility were related to the susceptibility of the pretherapy isolate or the first available sensitive isolate around the start of the treatment. Decreased susceptibility to ACV/GCV and PFA was defined as an at least 5- and 3-fold increase in IC_{50}, compared to the IC_{50} of the pretherapy/the first sensitive isolate, respectively[1]. Non-sequential isolates were considered resistant to ACV at IC_{50}s of ≥ 0.8 μg/ml and to PFA at IC_{50}s of ≥ 60 μg/ml. HSV-1 strain KOS and the KOS-derived DNA pol mutant $AraA^{r}8$ (moderately resistant to ACV, resistant to PFA), kindly provided by D. M. Coen (Harvard Medical School, Boston, Mass.) and an ACV-resistant HSV-1 clinical isolate 98.14742, a TK-deficient mutant, a gift of M. Aymard (Université Claude Bernard, Lyon, France), were used as reference strains for susceptibility testing.

Genotypic analysis. Total nucleic acid was extracted from 0.1 ml of virus stock using the MagNA Pure LC automated nucleic acid extractor (Roche Diagnostics, Penzberg, Germany)

and the MagNA Pure LC total nucleic acid isolation kit. Purified nucleic acid was eluted in 100 µl of elution buffer.

TK gene. Amplification of the entire TK gene with flanking regions (nt -126 to nt 1149) was performed by PCR using the Gene Amp® XL PCR Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and the primers 5'-CTGTCTTTTTATTGCCGTCA-3' (forward) and 5'-TCCACTTCGCATATTAAGGT-3' (reverse) as described previously[53]. Each 50 µl PCR mixture consisted of 0.2 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1.7 mM magnesium acetate, XL Buffer II, 1.5 U of r*Tth* DNA polymerase, XL and 10 µl of extracted DNA. The cycling conditions were as follows: 1 min at 94ºC, 16 cycles of 15 s at 94°C, 30 s at 55°C and 2 min at 68°C, and an additional 19 cycles in which the length of the extension step at 68°C was extended by 5 s with every cycle. Finally, amplification was completed by incubation for 10 min at 72°C.

DNA polymerase gene. A 2.3 kb fragment (nt 948 to nt 3219) of the DNA polymerase gene representing the conserved regions I to VII and the σ-C region was amplified by a hot start PCR as described previously[53], using the Gene Amp® XL PCR Kit in combination with wax beads AmpliWax® PCR Gems (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Each 12.5 µl of lower reaction mix consisted of 0.4 µM of each primer, 0.2 mM of each dNTP, 0.8 mM magnesium acetate, and each 32.5 µl of upper mix consisted of XL Buffer II and 1.5 U of r*Tth* DNA polymerase, XL. The lower mix was sealed upon addition of a wax bead by incubation of the mixture for 5 minutes at 80°C followed by 5 minutes at room temperature. Thereafter the upper mix and 5 µl of extracted DNA was added. The cycling conditions were as follows: 1 min at 94ºC, followed by 16 cycles of 15 s at 94°C, 30 s at 57°C and 2 min 30 s at 68°C and additional 19 cycles with prolongation of the extension step, and a final extension as described above. The sequences of the HSV-1 specific primers used were: 5'-TCGTCACCTTCGGCTGGTA-3' (forward) and 5'-GTCTGGGCCACGATCACGTA-3' (reverse).

The PCR products were purified by using the QIAquick Gel Extraction Kit or the QIAquick PCR Purification kit (Westburg, Leusden, The Netherlands) and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) on the ABI PRISM 377A automated DNA Sequencer. The sequence of both strands of the entire TK gene was determined by using a set of 6 sequencing primers. Ten primers were used to determine the sequence of both strands of a 1.6 kb fragment of DNA pol gene (codon 499-1031) containing the conserved regions of the gene. The sequences of TK and DNA pol genes were compared to those of the pretherapy or drug-sensitive isolate if available, or with the HSV-1 reference strain KOS. Sequencing protocols used ensured detection of the presence of approximately 25% of mutant virus in a mixture, by comparison of the peak heights of two nucleotides at a single locus in a chromatogram.

Phylogenetic comparison of the TK gene sequences of clinical isolates from all 12 patients was conducted using MEGA version 2.1.[27]. The DNA sequences of the entire TK gene were used in the analysis. A phylogenetic tree was constructed by using the neighbor-joining method and bootstrap analysis of 1000 iterations.

RESULTS

Virus isolates. Thirty-one HSV clinical isolates were studied from 12 HSCT recipients. The isolates originated from the throat (17), mouth (9), nose (1), the lower respiratory tract (3) and the esophagus (1). Isolates were obtained prior to or shortly after the start of therapy, and upon therapeutic failure. All isolates were typed as HSV-1. For 10 out of 12 patients, sequential isolates were obtained, and for 7 of these 10, sensitive pre- or early-therapy isolates were available. For 3 patients sequential resistant isolates were available, and for 2 patients only a single resistant isolate was available for investigation. Clinical information with respect to the HSV infection, as well as the results of phenotypic and genotypic characterization of the isolates are shown in Table 2.

The first clinical manifestation of HSV reactivation occurred in the patients at a median of 6 days after transplantation (range, -1 to 64 days).

Seven out of 12 patients (#4, 5, 8-12) received oral or iv ACV prophylaxis. Patient #4 had been on long-term ValACV prophylaxis from 17 months until day 35 prior to HSCT and developed resistant HSV infection already before HSCT. Five patients (#1, 2, 3, 6, 7) were treated with iv ACV upon the occurrence of HSV lesions. From this group, patient #2 had already been treated with oral and iv ACV for recurrent HSV infection of the throat 7 and 2 months prior to HSCT. In 3 patients (#1, 2, 7), infections resolved under ACV pressure at median 70 days post HSCT. In patient #2, discontinuation of ACV and GCV was followed by the appearance of an ACV-sensitive virus. Active HSV infection remained in two patients (#4 and 10), while on ACV until death due to GVHD.

PFA was used to treat ACV unresponsive infections in 6 of the 12 patients (#3, 6, 8, 9, 11, 12); therapy was successful in all except one, as demonstrated by partial or complete resolution of the lesions. The remaining patient (#11) did not respond to PFA and died due to HSV pneumonia shortly after an unsuccessful second HSCT (Table 2).

HPMPC was administered to a single patient (#5) to suppress CMV reactivation. The patient was also receiving parallel treatment with ValACV for his HSV infection, which resolved on this double therapy.

GCV was used in 3 patients (#2, 5, 7) for treatment of CMV; in patient #5, GCV was given in parallel to ACV.

Susceptibility of clinical isolates to antiviral drugs. Susceptibilities to ACV and PFA were determined for all 31 isolates. For a selected number of isolates GCV susceptibilities were also determined. All of the drug-sensitive isolates (n = 8) were obtained shortly before or within 1 to 7 days after initiation of ACV therapy. All drug resistant isolates (n = 23) were obtained during or after antiviral treatment.

Resistant virus was recovered at a median of 25 days after transplantation (range, 2 to 64 days) or at a median of 21 days after initiation of ACV prophylaxis or treatment (range, 1 to 74 days). Patient #2 and 4 were excluded from this analysis because of their long term treatment for recurrent HSV prior to HSCT.

The ACV-IC_{50} values of ACV-sensitive and -resistant isolates ranged from 0.1 to 0.24 µg/ml and from 1.5 to 43.1 µg/ml, respectively. The fold changes in ACV-IC_{50} between pairs of sensitive and resistant isolates (7 patients) ranged from 26 to 335.

None of the 31 HSV isolates tested demonstrated resistance to PFA.

Susceptibility to GCV was determined for 9 isolates from patients # 1, 5, 6 and 11, in order to further elucidate the role of the mutations identified in the TK and DNA pol gene of these isolates. For isolates from patient #1, 6 and 11, GCV susceptibility data correlated to those of ACV. Cross-resistance to GCV was observed for all ACV-resistant isolates while ACV-sensitive isolates were also sensitive to GCV (Table 2). The ACV-resistant isolate from patient #5 was still sensitive to GCV.

Molecular analysis of clinical isolates. Alignments of TK and DNA pol gene sequences were performed for each patient where sequential virus isolates were available. Complete sequence homology, except for the resistance-associated mutations, was observed for sequential isolates from each individual patient. This suggests that the initial infection as well as the recurrences were caused by the same virus in each individual patient.

In addition, a phylogenetic analysis of TK gene sequences was performed to determine the intra- and inter-patient genetic distance between strains. In all cases sequential isolates from an individual patient clustered together and demonstrated to be genetically distant from any of the strains in each of the other patients (Fig. 1). This analysis demonstrated that every patient harbored a unique HSV strain.

Analysis of thymidine kinase mutations. In all but one isolate, reduced susceptibility to ACV could be confirmed by genotypic changes in the TK gene. Additions or deletions of a single G or C in homopolymeric repeats of Gs or Cs were found in 8 patients. Specifically, an addition of a G in a run of seven Gs (nt 430-436), resulted in a frameshift at codon 146 and a stop codon 82 aa downstream (3 patients: #2, 8, 9) or 79 aa downstream (2 patients: #3, 12). Another frameshift was detected at codon 61 in a single patient (#7) due to a deletion of one G in a run of four Gs (nt 180-183) resulting in a stop codon 24 aa downstream. A frameshift mutation at codon 153 due to a deletion of a single C in a run of four Cs (455-458) resulting in a stop codon 27 aa downstream, was found in patient #4. Patient #10 had a deletion of a single C in a run of six Cs (nt 548-553), which resulted in a frameshift mutations at codon 185 and a stop codon 82 aa downstream.

Single amino acid substitutions were observed in isolates from 4 patients (#1, 6, 10, 11). The mutations included His58Arg (G173A), Gly129Asp (G386A), Ala189Val (T641C), Arg216His (G647A) and Arg220Cys (C658T). The location of these mutations in the TK gene is shown in Fig. 2.

For 5 patients (#1, 3, 6, 7, 8), all resistant isolates obtained over time within one patient stably harbored the same TK mutation. In two additional patients (#10 and 11), however, two different TK mutants were identified. In patient #10, two isolates obtained on two consecutive days from different body sites showed different TK mutations; a deletion of C553 (10/25; throat) and an Arg216His substitution (10/26; BAL), respectively. In patient #11, isolates from the oral cavity and trachea harbored a His58Arg substitution (11/57,

11/62), while an earlier isolate from oral cavity (11/24) harbored a Gly129Asp substitution (Table 2).

Apart from mutational changes conferring drug resistance, several mutations associated with natural TK gene polymorphism were found in the patient isolates. These included Cys6Gly, Arg41His, Leu42Pro, Ala192Val, Ile214Thr, Gly251Cys, Val267Leu, Pro268Thr, Asp286Glu, Val348Ile and Asn376His.

Analysis of DNA polymerase mutations. In one patient (#5), the substitution Gln570Arg in the conserved σ-C region of DNA pol gene was found in a single isolate with low level resistance to ACV. No ACV resistance-associated mutation in the TK gene was identified in this isolate.

No evidence was found for any resistance-associated mutations in the DNA pol gene in isolates from the other patients.

DISCUSSION

In profoundly immunocompromised patients, such as HSCT recipients, HSV infections with a drug-resistant virus can be quite severe and sometimes even fatal[7]. Although the virulence of drug resistant viruses can be reduced[12], the severe mucocutaneous and visceral infections due to drug-resistant HSV in immunocompromised patients reported by several groups underline their pathogenic potential at least in these patients[33,51]. The increasing occurrence of HSV drug resistance in HSCT recipients, reported since the late 1980s, is probably caused by a higher number of matched unrelated or mismatched related (haplo-identical) allogeneic transplantations, which require stringent lymphocyte depletion of the graft and more aggressive immunosuppressive regimens[8]. Patients undergoing matched unrelated or mismatched allogeneic transplantations seem to be more at risk for breakthrough HSV infections than recipients of matched related allogeneic stem cell transplants[56]. Recently, Langston *et al.* have shown an unusually high frequency of ACV- and PFA-resistant HSV infections in recipients of haplo-identical HSCT. Out of 14 transplant recipients, 5 (36%) developed ACV-resistant lesions while on iv ACV prophylaxis[29].

The relatively high incidence and severe clinical consequences of resistant HSV infections in HSCT patients underline the need for adequate monitoring of the antiviral drug susceptibility of these viruses. *In vitro* tests for drug susceptibility determination are time-consuming, laborious and are poorly applicable for rapid identification of resistant virus in the clinical laboratory. The development of genotypic assays identifying the major resistance-associated mutations in a shorter time-frame, could result in increased testing and use of susceptibility results in clinical practice. This might greatly improve the efficacy of antiviral treatment of patients with persistent HSV infections. However, a detailed knowledge on the role of TK and DNA pol gene mutations in drug susceptibility is required before such tests can be implemented.

Table 2. Summary of phenotypic, genotypic and clinical data for HSV isolates from 12 HSCT recipients with drug resistant HSV-1 infections.

Patient (age/ gender)	Isolate no[a]	Source	Drug susceptibility[b] IC$_{50}$ (µg/ml)			Genotypic changes[c]		Antiviral treatment, day (d)	HSV disease and therapy outcome
			ACV	PFA	GCV	TK gene	DNA pol gene		
1 (8/M)	1/4	Throat	0.13	16.9	0.001	*I214T*	*A646T*	ivACV, d11-d32	Mucositis, persistent on ACV,
	1/24	Throat	**8.56**	22.7	**0.12**	***A189V***, *I214T*	*A646T*	ValACV, d32-d41	resolved by ~d90
	1/73	Throat	**3.40**	17.4	ND	***A189V***, *I214T*	*A646T*	ivACV, d41-d48	
								oACV, d48-d180	
2 (39/F)	2/-1	Throat	0.18	32.2	ND	*V348I*	ND[d]	ivACV, d0-d5	Mucositis, persistent on ACV,
	2/26[e]	Throat	**42.9**	44.5	ND	**FS146 (+G), stop 82 ds,** *V348I*	ND	ivGCV, d14-d27	resolved by ~d60; GCV for CMV
3 (35/F)	3/13	Throat	0.16	18.3	ND	-	ND	ivACV, d13-d18,	Stomatitis, no response to ACV,
	3/26	Mouth	0.40	22.5	ND	-	ND	d32-d46	resolved on PFA by ~d62
	3/39[f]	Throat	**8.70**	16.1	ND	**FS146 (+G), stop 79 ds**	ND	PFA, d46-d53	
								ValACV, d54-d62	
4 (16/F)	4/-78	Throat	**34.8**	17.3	ND	**FS153 (ΔC), stop 27 ds**	ND	ValACV, d(-515)-d(-35) ivACV, d(-15)-d52	Recurrent gingivitis pre- and post HSCT, no response to ACV †, d52: VOD, GVHD
	4/11[g]	Throat	ND	ND	ND	ND	ND		
5 (25/M)	5/64	Throat	**1.60**	8.54	0.02	*V348I*	***Q570R***	ivACV, d(-10)-d31 ValACV, d31-d59, d71-d142 ivGCV, d24-d37 CDV, d59-d81 (3 infusions)	Mucositis after ACV, resolved on CDV; GCV and CDV for CMV; †, d239: acute pancreatitis at VOD
6 (9/M)	6/2	Nose	**4.50**	21.5	**0.21**	***R220C***[h]	-	ivACV, d1-d11	Severe mucositis, progress on
	6/19	Throat	**6.00**	10.4	**0.47**	***R220C***	ND	ivACV, d19-d34	ACV to pharyngitis, response to
	6/26	Throat	**22.0**	19.3	**0.29**	***R220C***	-	PFA, d34-d49	PFA, resolved by ~d71
	6/33	Throat	**19.8**	11.1	**0.49**	***R220C***	ND	ValACV, d50-d71	
7 (11/F)	7/10	Throat	0.10	11.4	ND	*V348I*	-	ivACV, d10-d23	Mucositis, improved temporarily
	7/26	Throat	**21.2**	18.8	ND	***FS61 (ΔG), stop 24 ds***,*V348I*	ND	ValACV d36-d45	on initial ivACV; d40 severe
	7/40	Throat	**24.3**	20.1	ND	***FS61 (ΔG), stop 24 ds***,*V348I*	ND	ivACV, d45-d57	ulcerative esophagitis, resolved on
	7/54	Throat	**33.5**	13.8	ND	***FS61 (ΔG), stop 24 ds***,*V348I*	-	ivGCV, d58-d63	ACV and GCV by ~70;
								ivACV d63-d70	GCV for CMV
								oACV, d70-d79	

8 (47/M)	8/28	Mouth	**45.2**	28.5	ND	**FS146 (+G), stop 82 ds**	ND	ValACV, d1-d39	Esophagitis on ValACV, response to PFA
	8/39	Esophagus	**20.6**	9.2	ND	**FS146 (+G), stop 82 ds**	ND	PFA, d39-d59	
9 (22/M)	9/3	Mouth	0.19	22.6	ND	-	ND	ivACV, d1-d11	Mucositis on ACV, response to PFA
	9/11	Mouth	**5.81**	5.4	ND	**FS146 (+G), stop 82 ds**	ND	PFA, d12-d23	
10 (48/F)	10/6	Mouth	0.24	26.9	ND	-	*E1005K*	ivACV, d1-d24	Persistent lip and oral lesions on ACV, HSV pneumonia on ACV
	10/25	Throat	**35.2**	33.5	ND	**FS185 (ΔC), stop 82 ds**	ND	oACV, d24-d32	†, d50: GVHD
	10/26	BAL	**26.3**	22.5	ND	***R216H***	*E1005K*	ivACV, d32-d46	
	10/49	Throat	**42.6**	23.5	ND	**FS185 (ΔC), stop 82 ds**	ND	oACV, d47-d50	
11 (28/M)	11/24	Mouth	**44.2**	24.9	**0.71**	***G129D***	-	ivACV, d1-d31	Stomatitis on ACV, no response to ACV nor to PFA; 2nd HSCT d33, no engraftment;
	11/57	Mouth	**19.2**	20.1	**1.1**	***H58R***	-	PFA, d31-d40	†, d62: HSV pneumonia
	11/62	Trachea	**43.1**	31.3	ND	***H58R***	-	ivACV, d40-d62	
12 (40/M)	12/6	Mouth	0.22	15.5	ND	-	ND	ivACV, d1-d24	Mucositis on ACV, response to PFA;
	12/20	Mouth	**9.00**	18.4	ND	**FS146 (+G), stop 79 ds**	ND	PFA, d24-d39	†, d90: EBV NHL
								oACV, d40-d90	
KOS			0.43	36.5	0.001				
AraA^{r}8			**1.68**	**122.3**	ND				
98.14742			**19.12**	ND	**0.64**				

[a] Number indicates a patient number and a day of collection post first HSCT. Minus sign indicates before HSCT.
[b] Mean IC_{50} values were calculated from at least two independent experiments. Inter-experimental variability for all IC_{50} values was <50%. Bold numbers: drug-resistant phenotype
[c] Bold text: mutations for which the therapy isolates differ from the pretherapy or earlier sensitive isolate and which are presumably associated with ACV resistance. Italics: substitutions not reported before (shown for all isolates where found).
[d] ND, not determined.
[e] On day 53, an ACV/PFA-sensitive isolate was recovered from the throat.
[f] On day 44, an ACV-resistant/PFA-sensitive isolate was still recovered from the throat.
[g] Stock culture negative.
[h] Mixture of both wild type (R220) and mutant (C220) TK genotypes was detected in the isolate.
Note: ds, downstream; VOD, veno-occlusive disease; EBV, Epstein-Barr virus; NHL, non-Hodgkin lymphoma.

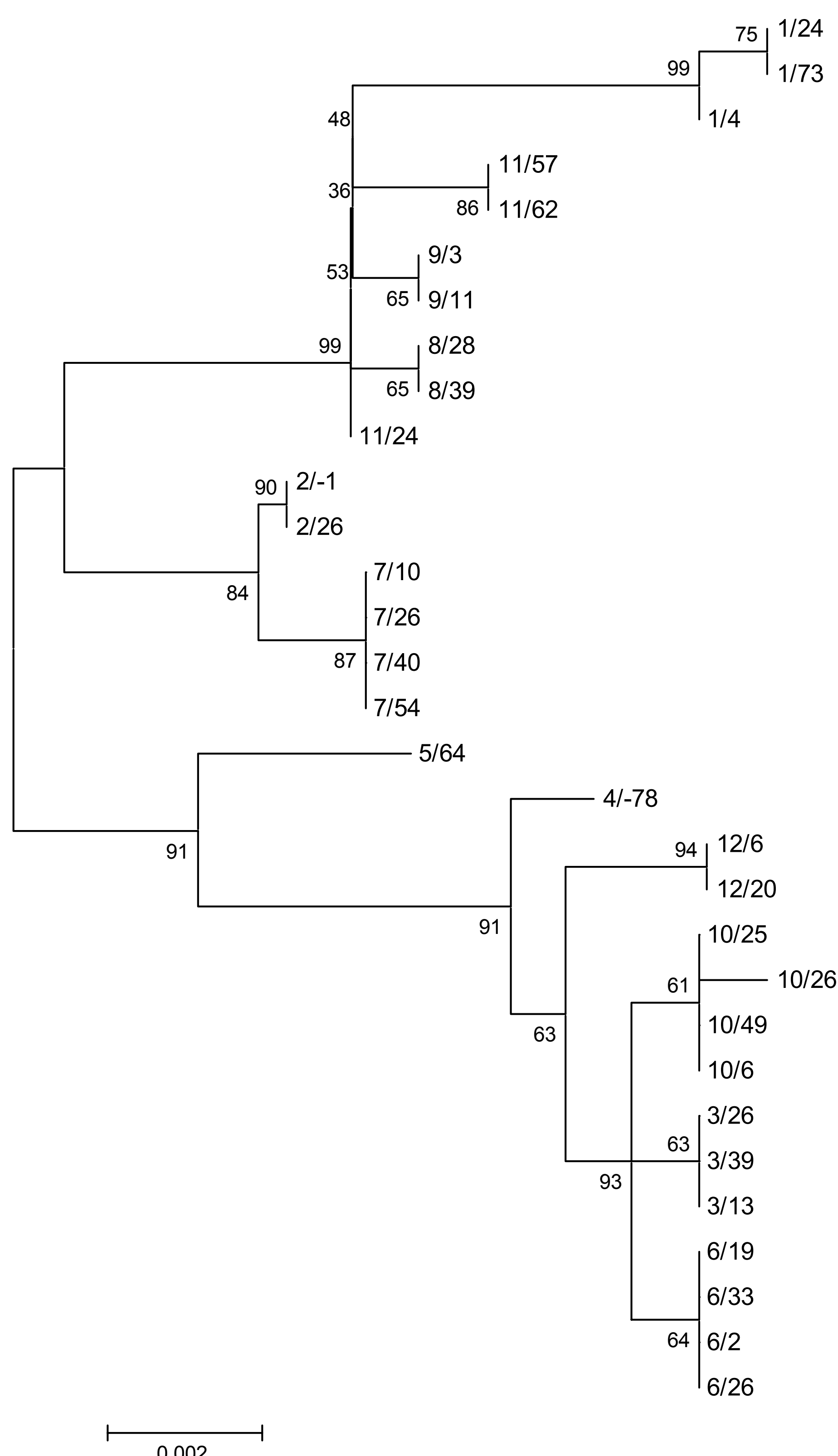

Fig. 1. Phylogenetic tree analysis comparing nucleotide sequences of the whole TK gene of HSV-1 isolates from all 12 patients. The unrooted phylogenetic tree was constructed by using the neighbor-joining method and gap-stripped aligned TK gene sequences. Selected bootstrap values are given.

Patients selected for our study suffered from severe and/or persistent HSV infections despite antiviral prophylaxis or treatment with ACV. These infections occurred following HSCT or during the immunosuppressive regimen preceding transplantation. Three of the 12 patients receiving ACV prophylaxis or treatment (#1, 2, 7) were able to clear the resistant HSV infections without a change in therapy within a median of 70 days after HSCT. The resolution of HSV infection in these patients was presumably due to the gradual immune recovery, as was confirmed by an increase of absolute leucocyte counts over 500/mm^3 (data available for patient #1 and 7). Emergence of a sensitive wild type virus in one of these three patients (#2) after early discontinuation of ACV and GCV therapy was presumably due to incomplete immune recovery at the moment of discontinuation of the therapy. Indeed, HSCT recipients usually demonstrate suppression of specific antiviral immunity for 60 days or more after transplantation[34]. CD4+ and CD8+ T cells are essential for protection from HSV reactivation and clearance of HSV. Healing of HSV lesions was shown to correlate with recovery of these effector T cells in patients after HSCT[29]. In addition, recovery of CD4+ T cells is significantly prolonged in the recipients of T-cell depleted grafts, who demonstrated frequent occurrence of life threatening viral infections[50]. T cell depletion might also be a risk factor for development of resistance[7]. Our patients received an aggressive pre-transplantation immunosuppressive treatment and T cell depleted grafts, which both could have contributed to the protracted immune recovery. However, no data were available either on total white blood cell counts or lymphocyte subpopulations for majority of our patients to make solid correlations between immune status and clinical outcome of HSV disease.

Two patients (#4 and 10) on ACV therapy with severe GVHD did not clear their ACV-resistant HSV infections. The presence of GVHD is known to be strongly associated with development of resistant HSV infections[7]. Patient #5 cleared his HSV infection upon combined therapy with ValACV and HPMPC. At the same time, the patient also experienced immune recovery, thus the role of both antiviral drugs in the clearance of the HSV infection is difficult to evaluate.

Five of six patients, who were treated with PFA after ACV treatment failure, demonstrated clearance of HSV lesions upon PFA therapy. Clinical response correlated with the results of PFA susceptibility testing and genotyping; all isolates carried mutations in TK gene. However, the contribution of recovering immune system to the clearance of the infections cannot be excluded. Failure of a single patient #11 to respond to PFA, despite the in-vitro PFA-sensitive HSV infection, was likely due to the rejection of the second transplant.

Eleven of twelve patients (91.7%) in our study suffered from resistant HSV infections conferred by TK mutants. Indeed, since TK is not essential for virus replication in most tissues, ACV-resistant TK mutants can be readily selected *in vitro*, and are also most frequently isolated *in vivo* from immunocompromised patients[18,35]. Drug-resistant viruses harboring mutations in the DNA pol gene have been found less frequently both *in vivo* and *in vitro*[30,31]. In our study, a DNA pol mutant was found in only one of the 12 patients (8.3%).

The resistant HSV strains from 7 patients harbored mutations previously reported to be associated with ACV resistance. Viruses from 5 additional patients harbored TK gene mutations that had not been described to be associated with clinical ACV resistance before. A high prevalence of frameshift mutations in the repeats of Cs or Gs, the mutational hotspots of

the TK gene, was reported previously[20], and was confirmed in our study. These were found in 67% (8/12) of the patients. Mutations of this type are the most frequent cause of ACV resistance and they result in a premature stop codon and thus in a nonfunctional TK. In our study frameshifts at codons 61, 146, 153 and 185 were detected. The deletion of a G at codon 61 is a novel mutation. The deletion of a C at codon 153 was previously reported only upon *in vitro* selection[44] (Fig. 1). These TK-deficient mutants have been reported to be less pathogenic in animal studies and to be unable to reactivate from latency[11,15]. However, recent reports revealed that these mutants, despite their non-functional TK gene, can reactivate and may also cause severe disease[37,45]. It has been shown that HSV has developed mechanisms that allow these most common TK-deficient frameshift mutants to escape from ACV therapy but still retain pathogenicity[22]. These mechanisms include: ribosomal frameshifting, which leads to expression of low levels of TK, errors during replication of homopolymeric repeats, which create subpopulations of TK^+ virus or compensatory function of other genes[22-24,45,46].

Amino acid substitutions due to single nucleotide mutations represent the second large group of TK mutations, with a reported frequency comparable to frameshifts[5]. Apart from resistance-associated mutations located in the six conserved regions of TK, some mutations localized outside these conserved regions have also been reported to be associated with resistance[2].

Novel substitutions presumably associated with ACV resistance found in our study include His58Arg, Gly129Asp, Ala189Val, Arg216His and Arg220Cys (Fig. 2).

His58 is part of a conserved glycine-rich (G*XX*G*X*GKT) motif (aa 56-63), the so-called P-loop, of the active site, which is common to all nucleotide kinases. This loop forms a giant anion hole which accommodates the beta-phosphate group of ATP[32]. His58 interacts with the substrate via hydrophobic interactions. Changes at this position remarkably affect the substrate affinity of the enzyme[40]. Replacement of His58 by leucine resulted in the loss of affinity to ACV and thus ACV resistance. The association of H58R with ACV resistance, observed in patient #11, is also supported by the fact that no resistance-associated mutation was found in the DNA pol gene[9].

Ala189 belongs to a larger area of high sequence conservation in herpetic thymidine kinases. This area is supposed to play a role in nucleoside binding. It is part of helix 7, which interacts hydrophobically with helices 5 and 6[6]. As suggested by Evans *et al.*, mutations that alter relative orientations of the helices that surround the nucleoside can result in substantial differences in substrate specificity, molecular integrity and enzymatic parameters[17]. The role of Ala189Val in ACV resistance is further supported by the absence of this mutation in the ACV-sensitive isolate obtained earlier from the same patient.

Arg216 and Arg220 are located within the LID domain, a region rich in arginines and lysines (aa 212-226) that forms a flap enclosing the active site of the TK. These strongly conserved arginine residues form a cluster of positive charge which interacts with residues of the ATP-binding loop. Arg216 helps to sandwich the adenine ring of the ATP[17]. Substitution of Arg216 with cystein and serine resulted in an ACV-resistant virus with altered and deficient TK, respectively[18,55]. The absence of the mutation Arg216His in the sensitive pretherapy isolate of the patient #10 as well the lack of resistance-associated mutations in the DNA pol gene support an association of this mutation with ACV resistance.

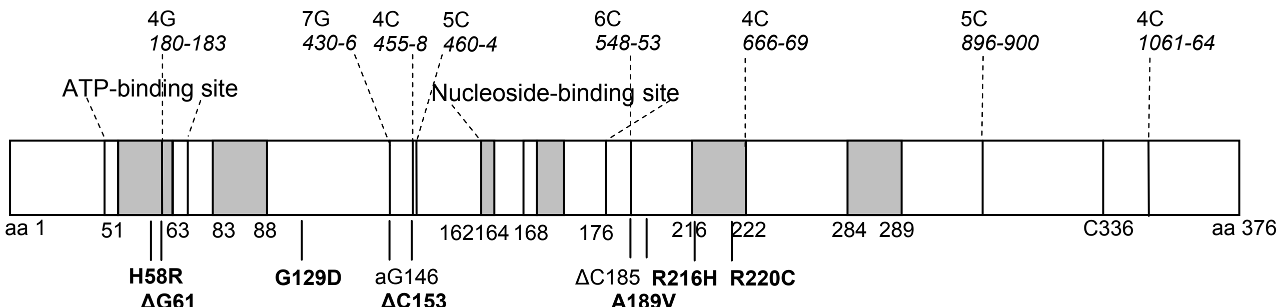

Fig. 2. Diagram of HSV-1 TK with conserved regions (grey; conserved regions aa 56-63 and aa 171-173 are not numbered), substrate binding sites and homopolymeric runs. Mutations identified in this study are shown: new mutations and mutations identified for the first time in the clinical isolate are in bold. Regular numbers indicate codons, numbers in italics indicate nucleotides. a, addition; Δ, deletion.

Arg220 is a strongly conserved residue in the active site of the enzyme. It interacts with the alpha-phosphate of ADP. In the presence of ADP-dTMP this complex associates with Glu225, thereby playing a central role in catalysis[17]. An ACV-resistant mutant with Arg220His substitution has been generated *in vitro* and it showed reduced susceptibility to ACV[55].

The role of the substitution Gly129Asp remains unclear so far. Gly129 lies within helix 3 (aa 114-130), one of the three parallel helices that contact natural substrate, thymidine, through amino acid side chains and thus establish a local environment for nucleoside substrate binding[17]. The adjacent Met128 is conserved and plays a role in the interaction of TK with the substrate. Replacement of Gly129 with a bulkier aspartate might change the local interaction of helix 3 with the substrate or hinder the interaction of the substrate with the adjacent Met128. Because of the high level of ACV resistance and the lack of a resistance-associated mutation in the DNA pol gene, we speculate that this substitution belongs to the group of ACV resistance-associated mutations in nonconserved residues outside the active site of TK.

The role of mutation Val348Ile, previously unclear, is elucidated in our study. In patients #2 and #7, it was detected in ACV-sensitive isolates as well as in the resistant ones, in which an additional frameshift mutation was clearly involved in the emergence of resistance. In addition, Val348 does not lie in the active or conserved sites of the TK gene. Our results indicate that this mutation is a natural polymorphism rather than associated with ACV resistance, as was suggested by Venard *et al.*[57]. Mutation Ile214Thr is another novel polymorphism found both in sensitive and resistant isolates. The remaining mutations detected in our study (Cys6Gly, Arg41His, Leu42Pro, Ala192Val, Gly251Cys, Val267Leu, Pro268Thr, Asp286Glu and Asn376His) are natural polymorphisms and have been described previously[5,35,57].

A novel DNA pol mutation Gln570Arg was found in a single isolate recovered from patient #5 after prolonged treatment with ACV. It is located in the σ-C region of the 3'→5'exonuclease domain of DNA pol. Mutations in the σ-C region have been associated with decreased exonuclease and polymerase activities and altered drug susceptibilities[19,26]. A mutation in a nearby residue V573M also resulted in a slightly decreased ACV susceptibility[4]. The isolate demonstrated low level resistance to ACV and sensitivity to PFA and GCV, and absence of any resistance-associated mutations in the TK gene. These data have led us to speculate on the possible role of this mutation in ACV resistance. However, since no pretherapy isolate from this patient was available for comparison, further investigation of the effect of this mutation on ACV resistance is required.

Two additional novel mutations Ala646Thr and Glu1005Lys in the DNA pol gene were found in isolates from patients #1 and #10. These mutations have presumably no effect on resistance as they are located outside of all conserved regions and they were found in both sensitive as well as resistant isolates. Resistance in these isolates was conferred by mutations in the TK gene.

Viruses expressing ACV resistance conferred by mutations in the TK gene demonstrated a median 82-fold reduction in drug susceptibility (range, 26 to 335), upon comparison of IC_{50}s of resistant isolates with those of the sensitive ones for the same patient. In concordance with the data obtained by Gadreau *et al.*[18], no clear relationship was found between levels of ACV-resistance and the type of TK mutation (frameshift or substitution), however the number of paired isolates for both types of mutations was small. Although frameshift mutations induced a higher median relative ACV-resistance (156-fold) than substitutions (67-fold), this difference was not statistically significant ($P = 0.21$).

Each individual patient in our study carried a unique HSV strain, as was demonstrated by genotypic and phylogenetic analysis. In the majority of patients, persistence of the same resistant virus was observed. However, coexistence of different ACV-resistant variants in different body sites, as well as temporal switching of different ACV-resistant variants, in the same patient was detected as well. These results might reflect the heterogeneity of the virus population[18]. However, they have to be interpreted carefully since selection of a predominant isolate by culture can occur.

Data obtained from the TK crystal structure[6,58,59] as well as the results of studies on the functional role of TK amino acid residues[40] support the importance of the mutated residues found in this study and thus provide additional evidence for association of these mutations with resistance. In addition, identification of ACV-resistant clinical isolates with mutations at positions 58, 153 and 220 in our study corroborates the results previously obtained with laboratory mutants[40,44,55].

The genotypic profile of drug-resistant HSV is more complex than previously thought. An extensive natural polymorphism in the TK gene, the still growing spectrum of novel mutations and the recently uncovered different functional roles of adjacent residues in the DNA pol gene emphasize the importance of larger surveys in immunocompromised patients[4]. Our study contributes with new information to the current knowledge of HSV drug resistance. In the end, this will help to get a more complete picture of genotypic changes and their consequences for drug susceptibility, which may lead to a better understanding of HSV

drug resistance and ultimately to a more rapid diagnosis and more effective treatment of drug-resistant HSV.

ACKNOWLEDGEMENTS

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Chapter 7

Sequential switching of DNA polymerase and thymidine kinase mediated HSV-1 drug resistance in an immunocompromised child

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ABSTRACT

Sequential herpes simplex virus type 1 (HSV-1) isolates were obtained from a pediatric hematopoietic stem cell transplant (HSCT) patient who received prolonged therapy with acyclovir (ACV) followed by foscarnet (PFA) and topical cidofovir (HPMPC) for severe persistent mucocutaneous HSV-1 infection. The isolates were retrospectively studied for drug resistance. The first resistant isolate associated with clinical failure of antiviral therapy emerged 44 days post initiation of ACV treatment. Susceptibility testing revealed an ACV resistant HSV strain that demonstrated cross-resistance to PFA in the absence of any previous PFA treatment. The observed cross-resistance was conferred by a single amino acid substitution Ser724Asn in the HSV DNA polymerase (DNA pol) gene. During the subsequent course of ACV therapy, the ACV/PFA cross-resistant isolates were replaced by ACV resistant, PFA sensitive isolates. These isolates carried no DNA pol mutations, but a substitution Arg163His in the thymidine kinase (TK) gene. Upon subsequent switching of antiviral therapy from ACV to PFA the original ACV/PFA cross-resistant DNA pol mutant re-appeared. Our study shows emergence of different drug resistant HSV variants during ongoing and unchanged ACV therapy. Furthermore a rapid re-selection of the original resistant variant was observed after switch. For optimal antiviral management of HSV infections in HSCT recipients, therapeutic decisions should be guided by drug susceptibility results whenever therapeutic failure is observed and/or when changes in antiviral treatment are considered.

INTRODUCTION

Resistant herpes simplex virus (HSV) infections occur relatively frequently in immunocompromised patients, and are associated with persistent, severe, sometimes fatal disease[10]. The highest prevalence of drug-resistant HSV (6-12%) has been reported in hematopoietic stem cell transplant (HSCT) recipients[12,17,30] and the percentage of chronic or recurrent HSV infections that no longer respond to antiviral therapy seems to be increasing in these patients[25]. This is probably due to the relative increase in the numbers of matched unrelated or mismatched related allogeneic HSC transplantations that require lymphocyte depletion of the graft and more severe immunosuppressive regimens[43]. Severe HSV infections in HSCT recipients that fail to respond to antiviral therapy require prompt and accurate determination of the antiviral drug susceptibility of the virus, to guide and optimize subsequent treatment. Although highly recommended, susceptibility testing still has not yet become common practice[2,32].

Acyclovir (ACV), or its prodrug valacyclovir (ValACV), is the primary drug of choice for prophylaxis and treatment of HSV infections. Resistance is mostly conferred by mutations in the thymidine kinase (TK) gene (nucleotide additions, deletions or substitutions)[5], which result in TK deficiency or in an altered TK substrate specificity. ACV-resistant TK mutants of HSV can be readily selected *in vitro*, and are also frequently isolated from immunocompromised patients[20,31]. Fortunately, TK-deficient mutants fail to reactivate from

latency and show significantly decreased virulence[13,16]. ACV-resistance can also be conferred by mutations in the DNA polymerase (DNA pol) gene. However, such mutants are much less frequent both *in vivo* and *in vitro*[27,28]. The same is true for DNA pol mutants resistant to foscarnet (PFA), which is used as salvage therapy for HSV infections with a failing response to ACV[11,39]. So far, mutations conferring cross-resistance to both ACV and PFA have only been described in 2 clinical isolates of HSV-1[15,23,35]. DNA pol mutants may be of a more significant clinical concern than the TK-deficient mutants because their pathogenicity is similar to wild type virus, which is also reflected in their ability to reactivate from latency[19,26,27]. Finally, given their wild type pathogenicity, the DNA pol mutants may pose a risk for transmission of drug-resistant HSV[14].

We report on the genotypic and phenotypic characterization of sequential HSV-1 isolates from a pediatric HSCT recipient suffering from chronic, persistent mucocutaneous HSV-1 infection, which did not respond to both ACV and PFA. An unusual drug resistance profile conferred by a DNA pol mutation in response to ACV resulted in an immediate cross-resistance to PFA, which was used in a later salvage therapy. Although the DNA pol mutants were replaced by the TK mutants, which arose during ongoing ACV therapy, the original DNA pol mutants re-emerged after administration of PFA. Our findings suggest that switching between different drug-resistant phenotypes during the antiviral therapy of persistent HSV infection can occur. Implications of our findings for the current antiviral therapy strategies in HSCT recipients are discussed.

CASE REPORT

In May 2001 a 7-year-old boy underwent an HLA haploidentical peripheral blood stem cell transplantation (PBSCT) from a parental donor, for treatment of the adult form of chronic myeloid leukaemia, Philadelphia chromosome-negative. The conditioning regimen consisted of cyclophosphamide (120 mg/kg), anti-thymocyte globulin (ATG, 10 mg/kg) and a single dose of total body irradiation (TBI, 7 Gy). Graft manipulation was performed by CD34+ stem cell selection. The patient was seropositive for HSV prior to transplantation. The clinical course of the patient and virological results are summarized in Figure 1.

On day 6 after transplantation (day +6) a lesion on the upper lip developed and HSV-1 (D6) was isolated by virus culture. Intravenous (iv) ACV therapy (10 mg/kg, three times a day) was initiated from day +7 onwards, which resulted in a slow regression and eventual disappearance of the lesions. During the first month after transplantation, adenovirus (ADV) type 1 was isolated from faeces; a PCR for ADV in plasma remained negative[38]. The ADV infection was cleared without clinical consequences.

Because of graft rejection a second haploidentical PBSCT was performed on day +36 using the other parent as donor. Conditioning consisted of fludarabine (150 mg/m^2), methylprednisolon (2 mg/kg) and an anti-T-cell monoclonal antibody (OKT3, 0.1 mg/kg/day, 14 days). The HSV lesion on the lip reappeared on day +51, and HSV-1 was isolated from the oropharynx. From day +45 onwards leukocytes increased steadily and donor engraftment was documented by chimerism analysis. On day +55 the patient was discharged. During the

period from day +51 to day +83 the HSV antiviral regimen was switched from iv ACV to oral ValACV (250 mg, three times a day) combined with topical treatment of lesions with ACV. Although the lip lesions regressed, HSV was isolated from the oropharynx on day +71 and +76. The patient was readmitted on day +72 because of signs of fever, rash and severe leukopenia, which proved to be a second rejection based on chimerism and bone marrow analysis. Because the HSV lesions on the lip progressed, ValACV treatment was discontinued on day +83 and switched back to iv ACV (10 mg/kg, three times a day). However, the lesions spread to his nose and mouth, increased in size and bleeded spontaneously. Therefore, ACV therapy was switched to PFA (60 mg/kg, three times a day) therapy on day +98.

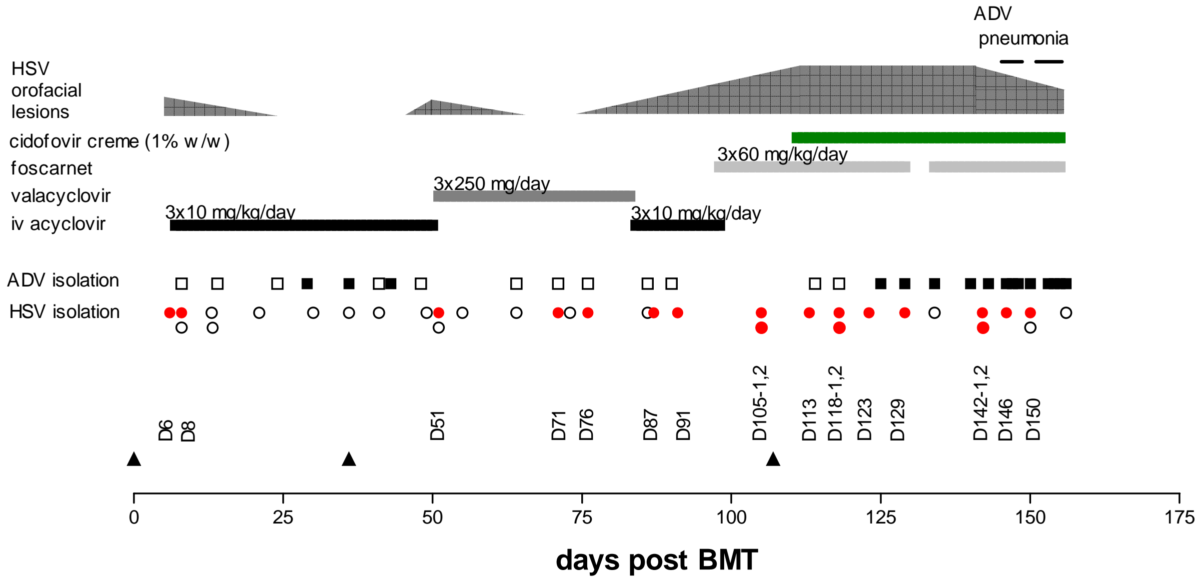

Fig. 1. Clinical course of HSV disease in a HSCT recipient. Circles indicate positive (●) and negative (○) HSV cultures. Squares indicate positive (■) and negative (□) ADV cultures. Triangles (▲) indicate HSCT.

On day +107 the patient received a third transplantation with T-cell depleted bone marrow from an HLA class I (HLA-A) and class II (homozygous) mismatched unrelated donor. The conditioning regimen consisted of thiotepa (5 mg/kg), fludarabine (150 mg/m^2) and ATG (6 mg/kg). Cyclosporin was administered to prevent GVHD. The severe HSV lesions disseminated to his nose, mouth and throat, and showed no signs of response to PFA; cultures obtained from these sites remained HSV positive. Additional topical therapy with cidofovir creme (1% w/w)[41] was started on day +111, leading to partial healing of the facial lesions. At the same time the patient showed signs of leukocyte engraftment of his third graft.

One week after his third transplantation, patient showed a poor cardiac and kidney function and severe fluid retention, which was treated with diuretics. From day +125 on, ADV type 1 was isolated from faeces, and from day +147 on, ADV was detected in blood by

PCR indicating systemic ADV infection[38]. The day after, the patient experienced a respiratory failure. Despite antiviral therapy with ribavirin the plasma ADV load in blood continued to increase. Patient died on the day +156 due to multiple organ failure and progressive respiratory acidosis. Postmortem viral cultures from lung biopsy were negative and PCR revealed only ADV.

Between day 1 and 156, 18 HSV-1 isolates were collected from different body sites.

MATERIALS AND METHODS

Virus isolation. Clinical material was collected from lip, nose, mouth, throat or lungs, and HSV was isolated by conventional virus culture using human embryonic lung fibroblasts. For typing type-specific monoclonal antibodies (Kallestadt Laboratories, Diessen, Germany) were used. Virus stocks were generated and the infectious titer was determined by plaque assay in Vero cells as previously described by Schaffer *et al.*[37].

Susceptibility testing. Susceptibility to ACV, PFA (Sigma, Zwijndrecht, The Netherlands) and cidofovir (HPMPC; gift of Gilead Sciences, Foster City, CA) was determined by plaque reduction assay as described previously[42]. For ACV and HPMPC the concentrations used ranged from 0.25 to 64 µg/ml (fourfold dilutions), for PFA concentrations ranged from 12.5 to 200 µg/ml (twofold dilutions). All changes in drug susceptibility were related to the susceptibility of the pretherapy isolate. Decreased susceptibility to ACV and PFA was defined as an at least 5- and 3-fold increase in IC_{50}, compared to the IC_{50} of the pretherapy isolate, respectively. For HPMPC, a 10-fold increase in IC_{50} was considered a significant decrease in susceptibility[1].

HSV-1 strain KOS and the KOS-derived DNA pol mutant $AraA^r8$ (moderately resistant to ACV, resistant to PFA), kindly provided by D. M. Coen (Harvard Medical School, Boston, Mass.) and an ACV-resistant HSV-1 clinical isolate 98.25733-MA/3, a TK-deficient mutant, a gift of M. Aymard (Université Claude Bernard, Lyon, France), were used as reference strains for susceptibility testing.

Genotypic analysis. Total nucleic acid was extracted from 0.1 ml of virus stock using the MagNA Pure LC automated nucleic acid extractor (Roche Diagnostics, Penzberg, Germany) and the MagNA Pure LC total nucleic acid isolation kit. The purified nucleic acid was eluted in 100 µl of elution buffer. Amplification of the entire TK gene with flanking regions (nt -126 to nt 1149) was performed by PCR using the Gene Amp® XL PCR Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and primers 5'-CTGTCTTTTATTGCCGTCA-3' (forward) and 5'-TCCACTTCGCATATTAAGGT-3' (reverse). Each 50 µl PCR mixture consisted of 0.2 µM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1.7 mM magnesium acetate, 3.3x XL Buffer II, 1.5 U of r*Tth* DNA polymerase, XL and 10 µl of extracted DNA. The cycling conditions were as follows: 1 min at 94ºC, 16 cycles of 15 s at 94°C, 30 s at 55°C and 2 min at 68°C, and an additional 19 cycles in which the length of the extension step at 68°C was extended by 5 s with every cycle. Finally, the reaction was incubated for 10 min at 72°C.

A 2.3 kb fragment of the DNA polymerase gene containing conserved regions (nt 948 to nt 3219) was amplified by a hot start PCR using the previously mentioned Gene Amp® XL PCR Kit in combination with wax beads AmpliWax® PCR Gems (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Each 12.5 µl of lower reaction mix consisted of 0.4 µM each primer, 0.2 mM each dNTP, 0.8 mM magnesium acetate, and each 32.5 µl of upper mix consisted of 3.3x XL Buffer II and 1.5 U of r*Tth* DNA polymerase, XL. After addition of the lower mix, a wax bead was added and the tubes were incubated 5 minutes at 80°C to melt the wax. Thereafter the upper mix and 5 µl of extracted DNA was added. The cycling conditions were as follows: 1 min at 94ºC, followed by 16 cycles of 15 s at 94°C, 30 s at 57°C and 2 min 30 s at 68°C and additional 19 cycles with prolongation of the extension step, and a final extension as described above. The sequences of the HSV-1 specific primers used were: 5'-TCGTCACCTTCGGCTGGTA-3' (forward) and 5'-GTCTGGGCCACGATCACGTA-3' (reverse). The PCR products were purified by using QIAquick Gel Extraction Kit or QIAquick PCR Purification kit (Westburg, Leusden, The Netherlands). PCR products were sequenced by using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and ABI PRISM 377A DNA Sequencer. A set of 6 sequencing primers for the TK gene and 10 sequencing primers for the DNA pol gene was used to sequence both strands. The sequences of TK and DNA pol genes were compared with that of the pretherapy isolate and also with the reference HSV-1 strain KOS. Our sequencing protocols ensured detection of the presence of approximately 25% of mutant virus in a mixture, by comparison of the peak heights of two nucleotides at a single locus in a chromatogram.

Phylogenetic comparison of the case patient TK gene sequences with TK gene sequences of 12 HSV-1 clinical isolates from Dutch patients collected at our department between 2000 and 2002 was conducted using MEGA version 2.1[24]. The DNA sequences of the whole TK gene were used in the analysis. A phylogenetic tree was constructed by using the neighbor-joining method and bootstrap analysis of 1000 iterations.

RESULTS

Phenotypic characterization. A total of 18 HSV-1 isolates were obtained from different specimens collected during the whole post-transplantation period (Fig. 1, Table 1). The first two isolates obtained before initiation (D6) and at the first day of ACV therapy (D8), were sensitive to both ACV and PFA. Intravenous ACV therapy resulted in a complete regression of lesions and no virus was isolated from 8 different specimens taken during a period of six weeks. After 44 days of iv ACV therapy (day +51) lesions re-emerged, and an ACV and PFA double-resistant virus was isolated (D51). It exhibited a 6.6- and a 3.7-fold reduced susceptibility to ACV and PFA, respectively, compared to the pretherapy isolate. During subsequent ValACV treatment the lip lesions partially regressed, although two ACV and PFA cross-resistant isolates (D71, D76) were obtained from the oropharynx during this period. These isolates showed a similar susceptibility profile with an average 25.9- and a 5.3-fold

reduction in ACV and PFA susceptibility. Shortly after the second switch to iv ACV, viral isolates resistant to ACV but sensitive to PFA were obtained (D87 and D91) showing a 16.8- and a 6.2-fold reduction in ACV susceptibility. Upon switching to PFA therapy, all isolates obtained until the end of antiviral therapy exhibited resistance to both ACV and PFA, with a 5.1- to 20.0-fold reduction in susceptibility to ACV and a 4.3- to 5.6-fold reduction in susceptibility to PFA compared to the pretherapy isolate. All 18 isolates were susceptible to HPMPC (data not shown).

Genotypic characterization. Sequencing of the TK gene of the selected isolates showed two distinct genotypes (Table 1). No resistance-associated mutations were observed in the TK gene of the pretherapy isolate D6 and the sensitive isolate D8. In addition, in the ACV and PFA cross-resistant isolates D51, D71 and D76, no mutations in the TK gene were observed. Similarly, no mutations in the TK gene were observed in any of the isolates obtained during PFA therapy. In the ACV-resistant, PFA-sensitive isolates D87 and D91 a single amino acid substitution was identified at position 163 in the TK gene, where arginine was replaced by histidine (R163H) due to a single nucleotide change, CGC into CAC (Table 1). Other mutations found in the TK gene relative to the KOS reference strain (C6G, R41H, Q89R, G251C, V267L, P268T and D286E) were also present in both pretherapy and resistant isolates. Each of these mutations had previously been reported to be natural polymorphisms [5].

Analysis of the DNA pol gene also revealed two distinct genotypes. No resistance-associated mutations were observed in the pretherapy isolate D6, sensitive isolate D8 and in the ACV-resistant, PFA-sensitive isolates D87 and D91. In all sequenced cross-resistant isolates (D51-D76, D105 and D150) a single amino acid substitution was identified of serine at position 724 to asparagine (S724N) due to a single nucleotide change, AGC into AAC (Table 1). No other mutations were identified in the DNA pol gene.

Multiple alignment of all TK gene sequences from all sequenced isolates revealed identical DNA patterns (except for the resistance mutations) in all sensitive as well as resistant isolates. This indicated that the initial infection as well as the recurrences were caused by the same virus. In addition, a phylogenetic tree analysis was performed to compare the TK gene sequences of isolates from our patient with sequences of 12 clinical isolates from our own database (Fig. 2). The analysis showed that the case patient isolates formed a single cluster clearly separate).ed from any of the other clinical strains. This cluster was supported by the bootstrap value of 94%. This indicates that all the case patient isolates belonged to the single unique virus strain.

Table 1. Phenotypic and genotypic characterization of sequential HSV isolates.

Isolate number[a]	Source	Antiviral therapy	Phenotypic drug susceptibility: ACV IC$_{50}$ (µg/ml)[c]	ACV Fold change[d]	ACV S/R[e]	PFA IC$_{50}$ (µg/ml)[c]	PFA Fold change[d]	PFA S/R[e]	Genotypic changes[b]: TK[f]	DNA pol
D6	vesicle	-	0.4 ± 0.14	-	S	24.4 ± 3.1	-	S		
D8	throat	ACV	0.3 ± 0.01	-	S	13.1 ± 2.1	-	S	-	-
D51	throat	ACV	2.4 ± 0.18	6.6	R	90.1 ± 0.1	3.7	R	-	S724N
D71, D76	throat	ValACV	9.6 ± 0.33	25.9	R	128.8 ± 21.4	5.3	R	-	S724N
D87	vesicle	ACV	6.2 ± 0.98	16.8	R	31.5 ± 7.0	1.3	S	R163H	-
D91	throat	ACV	2.3 ± 0.56	6.2	R	20.4 ± 4.8	0.8	S	R163H	-
D105	throat	PFA	4.8 ± 2.18	13.0	R	124.5 ± 19.0	5.1	R	-	S724N
D113-D146	throat mouth nose	PFA HPMPC	4.3 ± 1.60	11.6	R	111.6 ± 7.8	4.6	R	-[g]	ND[h]
D150	sputum	PFA HPMPC	1.9 ± 0.54	5.1	R	106.9 ± 15.2	4.4	R	-	S724N
KOS			0.8 ± 0.33		S	47.2 ± 5.3		S		
AraA^{r}8			3.2 ± 1.22		R	124.8 ± 27.7		R		
98.25733			10.2 ± 4.26		R	32.7 ± 5.3		S		

[a] Number indicates a day of collection post first HSCT.
[b] Amino acid substitutions in which the therapy isolates differ from the pretherapy isolate D6 are indicated.
[c] Mean IC$_{50}$ ± SD for each isolate was calculated based on at least two independent experiments. For groups of isolates means of these data are shown. Inter-assay variability of the PRA was 40%, based on multiple susceptibility determinations of KOS and AraA^{r}8 strains.
[d] Fold change in IC$_{50}$ compared to pretherapy isolate D6.
[e] S, sensitive; R, resistant.
[f] GenBank accession numbers for TK gene sequences of isolates D6 and D87 are AY426827 and AY426828, respectively.
[g] TK gene sequence of isolate D118 and D146
[h] ND, not determined.

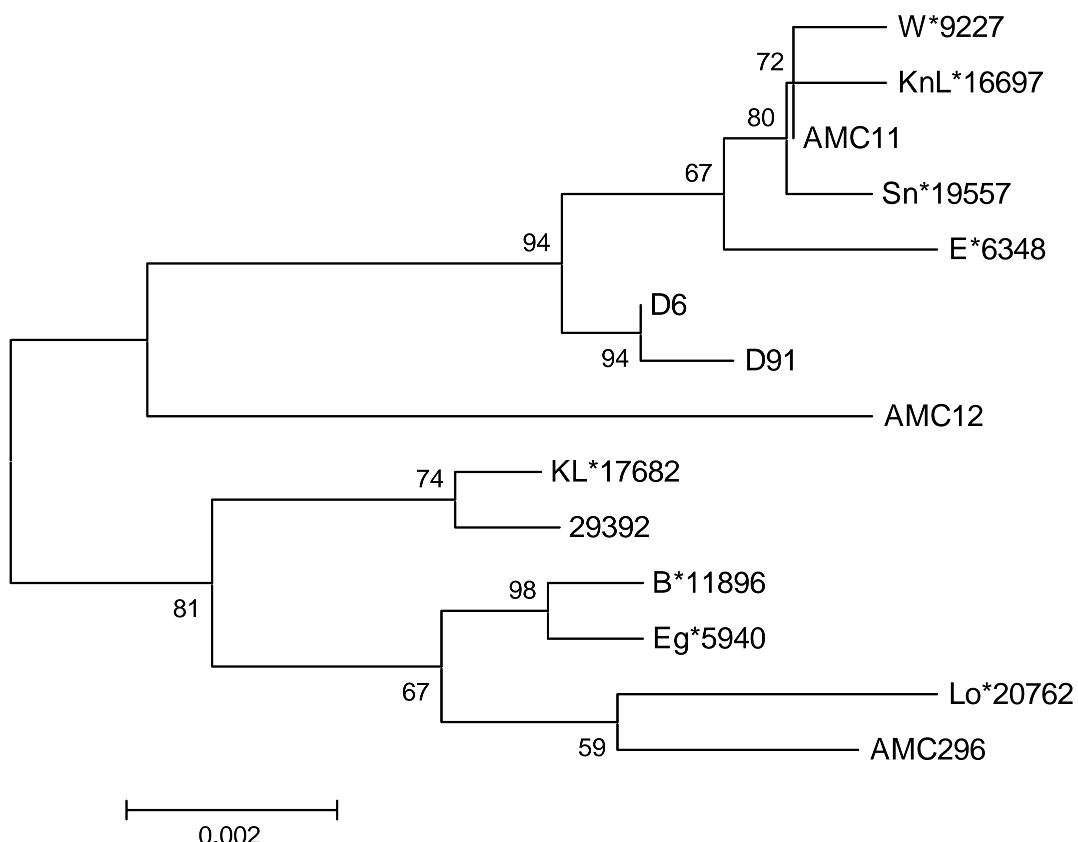

Fig. 2. Phylogenetic tree analysis comparing nucleotide sequences of the whole HSV-1 TK gene of wild type isolate D6, mutant isolate D91 of the case patient and 12 HSV-1 clinical isolates from Dutch patients. The unrooted phylogenetic tree was constructed by using the neighbor-joining method. Selected bootstrap values are given.

DISCUSSION

In this study sequential virus isolates of a HSCT recipient with severe HSV infection, refractory to antiviral treatment were examined. Drug resistance analysis at several time points demonstrated the emergence of virus variants with different resistance profiles during the ACV as well as during PFA treatment. Initially, a virus cross-resistant to ACV and PFA was selected, which was subsequently replaced by a virus that only exhibited ACV resistance. Upon a therapy switch to PFA, the virus variant cross-resistant to both ACV and PFA re-emerged.

Recently, cross-resistance to ACV and PFA, which mostly arises due to the mutations in the DNA pol gene, has been increasingly reported in matched unrelated HSCT recipients[8,40]. In addition, Chen *et al*. described three cases of unusual primary PFA resistance associated with ACV resistance which emerged without previous PFA treatment[10]. However, no data on the genotypic alterations of these mutants were available. In our study the genotyping of the HSV isolates, which exhibited primary resistance to PFA combined with resistance to ACV,

revealed a single mutation Ser724Asn in the DNA polymerase gene. The majority of mutations conferring resistance to nucleoside and pyrophosphate analogues have been mapped to the conserved regions δ-C, II, VI, III, I, VII and V (aa 557-961) of the catalytic domain of the DNA pol gene[29]. Region II is one of the most conserved regions and accordingly Ser724 is conserved in all herpesvirus DNA polymerases. Our results are in agreement with the phenotypes reported for laboratory-derived DNA pol mutants carrying a Ser724Asn substitution[21,29]. Conversely, HSV-1 clinical isolates with this mutation isolated previously from a single patient failing sequential ACV and PFA therapy, were PFA resistant but remained sensitive to ACV or showed only borderline resistance[3,39]. However, recent in vitro evaluation of Ser724Asn substitution using a novel system of overlapping cosmids and plasmids demonstrated its role in both ACV and PFA resistance[4]. This is the first report on the association of mutation Ser724Asn with clinical resistance to ACV.

The substitution Arg163His in the TK gene was the only mutation in which the ACV-resistant isolates D87 and D91 differed from the ACV-sensitive pretherapy isolate. Examination of the location of the Arg163 in the HSV-1 TK structure provides evidence for its importance for catalytic function of TK as Arg163 is a highly conserved hydrophilic residue mapped within the nucleoside-binding site[18]. It is involved in phosphorylation of natural substrates and of ACV[9], and its absence or substitution has been shown to be associated with very low levels of TK activity[6]. A VZV clinical isolate with a mutation at this codon also demonstrated to be highly resistant to ACV and this mutant also showed drastically reduced TK function[34,36].

The genotyping and phylogenetic tree analysis demonstrated that the two different mutant variants as well as the pretherapy wild type virus originated from the same HSV-1 strain. This indicates that probably no reactivation of a second virus population or an exogenous reinfection took place.

In our patient, the manifestation of the HSV infection shortly after first transplantation was presumably due to reactivation of the sensitive wild type virus. ACV treatment resulted in an initial disappearance of the lesions, as indicated by several negative culture results. We hypothesize that despite these negative cultures, some virus replication continued in the patient resulting in an induction and/or selection of the DNA pol mutant, which was cross-resistant to ACV and PFA. At present, it is still unclear why this DNA pol mutant emerged as the first resistant virus in the case patient. Primary emergence of DNA pol mutants during ACV treatment is rather uncommon. It is hypothesized that a replicative advantage of this mutant over other ACV-resistant mutants, i.e. TK mutants, might have been the reason. Prolonged ACV treatment subsequently led to the emergence of an ACV-resistant variant sensitive to PFA harboring a mutation in the TK gene (R163H). Presumably, the better replication capacity and thus better fitness of this TK mutant compared to that of the DNA pol mutant under the ACV pressure might have led to the selection of TK mutant as a majority population. The rapid recurrence of the original double-resistant DNA pol mutant after initiation of PFA therapy probably resulted from selection and outgrowth of the DNA pol mutant, which was present as a minority population in the patient. This suggests a heterogeneous nature of the virus pool in the patient. Previous studies demonstrated the preexistence of drug resistant variants in natural populations, even in the absence of prior

drug exposure[22,33]. The association of heterogeneous virus mixtures containing DNA pol mutants with a progressive HSV infection was previously reported by Sacks *et al.*[35]. In order to elucidate whether selection from naturally occurring mutants or de novo selection of resistant viruses occurred, further characterization of the isolates at the clonal level is warranted.

In our patient, persistent HSV infection was treated in the absence of data on virus susceptibility due to the lack of routine availability of drug susceptibility tests. Long-term ACV (98 days) and subsequent PFA therapy (57 days) were applied to treat the HSV infection without prolonged success. Therapeutic failure of the ACV regimen was caused by a DNA pol mutant that expressed cross-resistance to PFA. Therefore the attempt to manage the infection by subsequent switching to PFA therapy was also unsuccessful. Knowledge of the resistance profile of the virus failing ACV could have been helpful in making therapeutic decisions for this patient. Knowing the resistance profile at this stage might have led to a decision not to treat the patient with PFA but to switch to HPMPC.

HPMPC is the only drug available, which is effective against ACV and PFA resistant infections. HPMPC-resistant HSV clinical isolates have not been described yet[1] and were not obtained from our patient neither prior nor after introduction of topical HPMPC therapy. HPMPC therapy has been demonstrated to be effective in treatment of ACV-resistant HSV infections[40]. This strategy might have been useful in our patient. Although the efficacy and toxicity of HPMPC in children has not been evaluated yet, a dramatic improvement of HSV mucocutaneous infection in a child treated with iv HPMPC has been reported[7].

In conclusion, this study demonstrates that ACV treatment may result in the emergence of various resistant HSV-1 variants that can replace each other in time, without changes in the used therapeutics. Furthermore, the initial mutant virus re-emerged rapidly after switching to PFA therapy, indicating that this mutant was still present in the patient. Optimal use of the limited antiviral drugs available in immunocompromised patients therefore warrants availability of antiviral drug susceptibility information obtained at the initial moment of therapy failure as well as at the moment of switch.

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Chapter 8

General discussion

The problem of HSV drug resistance raises several important issues for clinicians as well as virologists. The work described in this thesis addresses the detection and prevalence of drug-resistant HSV strains, and their phenotypic and genotypic characterization. These are all fundamental for better understanding and optimal management of drug-resistant HSV infections at the patient as well as at the population level.

New approaches for detection of HSV and HSV drug susceptibility determination

The frequent severe and sometimes life-threatening HSV infections in immunocompromised patients and the relatively high incidence and serious clinical consequences of drug-resistant HSV infections in these patients underline the need for easy and well-standardized assays for rapid HSV diagnosis and determination of antiviral drug susceptibility. Application of real-time PCR technology appeared to be particularly attractive for these purposes. Various real-time PCR assays have demonstrated at least equal sensitivity to conventional PCRs, with a rapid turn-around time, high throughput, decreased risk of carry-over contaminations and potential for quantification of the pathogen. These advantages and the suitability of the real-time PCR for implementation into routine practice allow molecular assays to become a major tool in diagnostic virology. Consequently, there is a tendency to replace the traditional cell culture by real-time PCR-based assays for routine diagnosis of viral infections. In chapter 2, the diagnostic performance of an internally controlled real-time PCR (TaqMan) assay for detection of HSV-1, HSV-2 and VZV was evaluated. The results clearly demonstrated the superior sensitivity of the real-time PCR assay over shell vial culture in various clinical specimens. The virus detection rate using real-time PCR was 42.3% compared to 26.4% for culture. This is in agreement with several recent reports where similar detection rates and improvements over culture were obtained for molecular assays[16,18,19]. In addition, a combination of the real-time TaqMan PCR with an automated nucleic acid extraction resulted in a highly standardized and easy-to-perform diagnostic assay, the quality of which was monitored by incorporation of internal control. Such a sensitive and rapid assay represents a considerable diagnostic improvement and a clinical benefit especially for the immunocompromised patients, where rapid diagnosis is essential for timely initiation of antiviral therapy.

Although molecular detection of virus infections is gaining a place in routine clinical virology, the need for virus culture will stay. The isolation of the virus remains essential for antiviral drug susceptibility testing, for evaluation of new antiviral drugs or for the discovery of new viruses[59].

Currently, phenotypic tests are the golden standard for susceptibility testing of HSV. Several phenotypic assays have been described so far, which are based on different endpoint measurements (see Introduction). However, these assays are time-consuming, laborious, lack standardization and objective endpoints and are poorly applicable for rapid identification of resistant viruses in the clinical laboratory. In chapters 3 and 4 we describe the development of novel phenotypic assays for HSV susceptibility determination. These assays simplify and speed up susceptibility testing, generate objective and reproducible results and are amenable for implementation in the diagnostic virology laboratory. In chapter 3, we set out to use quantitative real-time PCR as a readout for determination of antiviral drug susceptibility of

HSV. The real-time PCR-based susceptibility assay was designed as a time- and labor-saving alternative for PRA for modern diagnostic virology laboratory, where real-time PCR assays are carried out on daily basis. In chapter 4, on the other hand, we describe the ELVIRA HSV assay, a quantitative yield reduction assay for determination of antiviral drug susceptibility of HSV, based on an HSV inducible reporter cell line. The ELVIRA is a result of a different design approach. Here the main idea has been to improve the cell culture part of the antiviral susceptibility assay and to develop an assay that can be used for rapid screening of clinical specimens for antiviral drug resistance. Both of these approaches seem to be useful for antiviral drug susceptibility testing. This has been demonstrated by recently developed real-time PCR-based drug susceptibility assays for HHV-6 and HHV-8[35,51] as well as by several reports on application of the reporter cells in the design of antiviral drug susceptibility assays[25,32,39,60,61]. Both of our assays demonstrated a good discrimination between ACV-sensitive and -resistant viruses and their results correlated well with the currently used golden standard test, the plaque reduction assay (PRA). Moreover, it seems that both may give a more accurate estimation of the effect of the drug on viral replication than the subjective and imprecise plaque counting as done for PRA. Both assays have an objective endpoint, are easy to perform and relatively rapid, with a total turn-around time of approximately 4-5 days, including the rapid virus titration. Compared to a fixed 3 day duration of susceptibility testing by ELVIRA, the cell culture part of real-time PCR-based assay might require longer incubation for slowly growing HSV clinical isolates. Real-time PCR-based assay seems to be less sensitive to variation in the amount of input virus as reflected by its relatively broad MOI range. This was also observed in a similar assay for HHV-6[35]. Our real-time PCR-based susceptibility assay combines a simple virus culture and a standard real-time PCR, which generally fits well with modern routine laboratory procedures. As such, this assay format could be easily implemented in diagnostic laboratories. The ELVIRA assay, on the other hand, due to its inherent high sensitivity but narrow MOI range, appears to be more useful in its modified version for rapid resistance screening using original clinical specimens or HSV isolates. Laboratories performing large scale surveys could benefit from the ELVIRA HSV screening assay. Furthermore, the yield reduction assay format of ELVIRA might be relevant in the evaluation of new antiviral agents[29].

An important observation from our work on susceptibility assays is that the use of a single drug concentration as a cut-off for defining resistance is unreliable. Firstly, the cut-off values can vary greatly with applied assay and its endpoint, as demonstrated in this thesis as well as in other studies[45,46,55]. For our assays we proposed preliminarily cut-offs for resistance, however, to achieve more accurate definition of these cut-offs, a thorough evaluation with substantial number of clinical isolates and well-characterized sensitive and resistant strains is required. Secondly, one has to consider the general inherent variability in day to day assay performance and the inter-laboratory variability in the drug susceptibility results. For these reasons it appears that the use of an internal standard in the form of a well-characterized sensitive reference strain would improve the quality of drug susceptibility testing[50]. The incorporation of an internal standard in every assay and a definition of resistance relative to this standard might allow detection of borderline resistance and might be a straightforward approach to compare results between different assays and different laboratories. This

approach has been successfully used in our ELVIRA HSV screening assay. However the use of sensitive reference strains needs to be standardized before criteria for decreased susceptibility can be defined. In addition the clinical relevance of these criteria should be evaluated[1,50].

The prevalence of drug-resistant HSV

Immunocompetent population

The prevalence of drug-resistant HSV in untreated individuals prior to the introduction of ACV was reported to be 0.3%. This was similar to the 0.5% reported for the treated population[12]. Recent surveys revealed that the prevalence of ACV-resistant HSV has remained low and virtually unchanged in the general population (0.1% to 0.7%) with no apparent differences between the ACV-treated and untreated groups[6,7,27]. Similar prevalence rates were reported for penciclovir and famciclovir (0.22%)[48]. The sporadic recovery of drug-resistant HSV from immunocompetent hosts only rarely correlates with clinical resistance[2]. Our survey of immunocompetent population consisting of 368 subjects identified a single case of ACV-resistant HSV infection resulting in a prevalence of 0.3% (CI: 0.007%-1.5%), which is in agreement with recent reports. This single resistant isolate was obtained from a recurrent skin lesion of a treatment-naive patient and could represent the background level of naturally occurring resistant viruses in the population[7,47]. However, the transmission of a drug-resistant HSV cannot be excluded. Our survey comprised a cross-sectional analysis of the Dutch general population over the last 4 years. An obvious extension of this study would be to determine the susceptibility of serial virus isolates collected before, during and after treatment, and thus gain insight into the level of acquired resistance upon episodic treatment, which is the current standard of care. Acquired resistance is an important parameter in predicting epidemiological consequences of increased drug use[5]. The few studies on episodic treatment that have been performed so far demonstrated no change in the antiviral drug susceptibility of the virus isolated upon either a single treatment course or several treatment episodes[27,47,52].

The relatively low prevalence of drug resistance for HSV compared to other drug-treated infections can be explained by i) the key role of the immune system in the control of HSV infection[36], ii) the decreased pathogenicity of the resistant mutants[8] and iii) the impaired capacity of resistant mutants to reactivate from latency[10,30]. These factors presumably also influence the transmissibility of the drug-resistant HSV[30], which is predicted to be low[27,54]. Indeed, so far there has been only one documented case of primary drug resistance due to possible transmission of a TK-altered mutant from an immunocompromised to a healthy individual[28]. Primary drug resistance in an immunocompetent patient in our study could have resulted from transmission of a resistant virus, however, as mentioned before, it could also reflect the very low natural prevalence of resistant HSV in the immunocompetent population.

The extensive use of ACV since its introduction in 1981 did not result in an increase in the prevalence of drug-resistant HSV in the general population nor in the treated immunocompetent population[2]. However, one should realize that the use of antiviral drugs is still low compared to the number of infected cases (especially genital herpes). Although recent mathematical models predict a low prevalence of resistance even with high antiviral

drug use[22,23], several important parameters in these models are unknown. Therefore, with the increased use of anti-HSV drugs predicted for the next ten years, continuing surveillance of drug susceptibility is warranted even among immunocompetent individuals[42].

It is evident from findings mentioned above that the development and transmission of HSV resistance in the immunocompetent population is not a major concern nowadays. In the immunocompromised patients, however, drug-resistant HSV infections are of much higher clinical importance.

Immunocompromised patients

The absence or impairment of virus-specific immunity in immunocompromised patients enables the virus to replicate extensively. Incomplete suppression of the extensive viral replication even in the presence of antiviral drug pressure creates a perfect setting for emergence of resistant viruses. Although the pathogenicity of drug-resistant viruses may be reduced[11], these viruses can still cause severe mucocutaneous and visceral infections in immunocompromised patients[31,53]. Indeed, as described in chapter 5 and 6, TK mutants, even with a TK-negative phenotype, can produce severe clinical disease in these patients.

Comparison of the results from recent surveys with those from the late 1980s reveals that the prevalence of ACV-resistant HSV has largely remained stable in immunocompromised patient population with a range from 4.0 to 7.1%. The majority of these studies focused on HSCT recipients and HIV-positive individuals. Considerably smaller number of studies with limited number of patients were performed in patients with malignancies, recipients of solid organ transplantations (SOT) or patients on high-dose steroid therapy. This implies that prevalence estimates are less reliable for these patient groups[7,17]. Our susceptibility data from a group of 128 immunocompromised patients with an HSV infection showed an overall prevalence of HSV resistance of 7%. We found a relatively high frequency of resistant infections in HSCT recipients (14%), with a recovery of resistant isolates only during or after ACV/GCV treatment. Similar prevalence rates in a much larger group of patients have been recently reported by Morfin *et al.*[38], indicating that drug-resistant HSV infections are a major concern in HSCT recipients. In our group of HIV-positive subjects, resistant HSV was identified in 3.9% of cases and was associated with genital HSV infection and previous ACV use. These data are in accordance with the prevalence of 3.5% reported by Morfin *et al.* in a European survey[38] and the 5.3% reported by Reyes *et al.* in a USA survey[42]. Interestingly, drug-resistant HSV was recovered from 2 patients diagnosed with COPD, who had received systemic prednisone treatment. In these patients, the prednisone-induced immunosuppression might result in the development of clinical HSV disease, and consequently, in an increased risk for emergence of ACV-resistant variants, as described for other groups of immunocompromised patients. In addition, COPD patients usually receive a maintenance therapy with inhaled corticosteroids, which may also contribute to local immunosuppresion favourable for virus reactivation and subsequent emergence of resistant viruses. It is of note that resistant viruses in the COPD patients were isolated from the throat and lungs.

The availability of sequential isolates from some patients, allowed us to investigate the evolution of HSV-1 infection and the emergence of resistance such as in the case of a pediatric HSCT recipient, who was treated over the clinical course of oral HSV infection with

all three available antiherpetic drugs, ACV, PFA and topical cidofovir (chapter 7). We demonstrated a sequential switching of two distinct resistant genotypes, an initial DNA pol mutant and a subsequent TK-altered mutant, during ongoing ACV treatment. Furthermore, the original DNA pol mutant reemerged after switching to PFA. Although DNA pol mutants are generally not impaired for replication at peripheral sites, the switch of the virus population to the TK-altered genotype might suggest a better fitness of this TK mutant, which could have led to its selection under the ACV pressure. Indeed, TK-altered mutants generally behave similar to wild type virus in terms of virulence and pathogenicity[15,43]. Data from animal models have also indicated that fitness advantages might influence the emergence and disappearance of resistant HSV mutants[49]. The subsequent reemergence of the original DNA pol mutant upon administration of PFA suggested the presence of both mutants in the virus pool of the patient, which was confirmed upon subsequent clonal characterization of the virus isolates (unpublished observation). These findings parallel previous observations describing the presence of heterogeneous virus populations[40]. The consequence of the presence of both viruses on viral pathogenicity needs further investigation[44].

The unusual finding in this patient was that the initial resistance to ACV was conferred by a DNA pol mutation, rather than by a mutation in the TK gene. Primary emergence of DNA pol mutants during ACV treatment is very uncommon[13,44]. Characterization of the pretherapy sensitive isolate at the clonal level suggested that *de novo* selection of resistant viruses occurred, since no resistant clones were identified (unpublished observation). It seems likely that a replicative advantage of this DNA pol mutant over other mutants resulted in its primary emergence, although reactivation of the DNA pol mutant from latency cannot be excluded.

Our survey on resistant HSV in immumocompromised patients as well as the study in HCST recipients confirmed that HSV antiviral drug resistance is relatively common. With the increasing number of immunocompromised patients HSV infections caused by drug-resistant viruses will remain to be found relatively frequently. Therefore, regular surveillance for drug-resistant HSV is necessary in this patient group. In addition, our studies in HSCT recipients demonstrated that the drug susceptibility determination upon therapeutic failure should be included in the clinical management of the patients.

Genotypic correlates of HSV antiviral drug resistance. Any role for genotypic assays?

Molecular assays may allow a rapid and efficient identification of resistant viruses, as they facilitate detection of resistant virus directly in a clinical specimen. With the advances in molecular assays it is likely that genotypic tests will achieve a more widespread diagnostic application. Assays based on either probes for detection of highly frequent mutations, probes targeting the conserved regions of the TK gene or assays based on RFLP analysis of PCR products (analogous to those used for CMV genotyping) can be envisaged[34]. However, a detailed knowledge on the role of individual TK and DNA pol gene mutations in drug susceptibility is a prerequisite for diagnostic application of such tests.

During our study on HSV antiviral drug resistance we characterized 26 clinical isolates (21 HSV-1 and 5 HSV-2) from 22 patients that expressed *in vitro* resistance to ACV (chapters 5, 6, and 7 and unpublished data). This is the second largest collection of drug-resistant HSV clinical isolates that were genotypically and phenotypically characterized[21].

HSV infections conferred by TK mutants prevailed in our study (20 out of 22 patients), while only single cases were identified with a DNA pol mutant or a mixed infection with both TK and DNA pol mutant, which is in agreement with previous studies[21,37]. Genotypic analysis of TK mutants revealed that 9 out of 24 mutants (38%) had frameshift mutations in the repeats of Cs or Gs (mutational hotspots), which presumably lead to a truncated TK polypeptide and thus TK-deficient phenotype[26]. It is believed that this type of mutation is relatively easy to create by the virus by slippage of the DNA polymerase while replicating the repeats of a single nucleotide[14]. The frameshift mutation at codon 146 (a repeat of 7 Gs) was found in 20% of isolates, which is in accordance with the fact that this is the most common mutation described to date. Thus, codon 146, as a mutational hotspot, could be one of the targets for molecular screening of ACV resistance. However, in agreeement with others, we also frequently identified frameshift mutations at other codons, spread throughout the TK gene, which preclude a straightforward genotypic approach. Instead, a phenotypic test, which screens for the size of the TK polypeptide and its enzymatic characteristics, developed by Suzutani *et al.*, might be useful for rapid detection of any type of frameshift[58].

Single amino acid substitutions in conserved or nonconserved regions of the TK gene were identified in 15 isolates (63%). In contrast to other studies reporting approximately equal frequencies of both mutation types, we found a higher, but not significant, overall frequency of substitutions compared to the frameshift mutations[4,21,37]. Substitutions were located in the ATP-binding site or regions involved in ATP binding, in the nucleoside-binding site or at residues interacting with the nucleoside substrate, and at conserved or nonconserved residues of the TK gene. Some of these genotypes were previously identified in the laboratory-selected ACV-resistant mutants[24,41,47,57].

Except for a frameshift at codons 146 and 185 identified in 5 and 2 isolates, respectively, unique and in several cases new mutations were detected. Although some of the mutations clustered in the active site, a substantial number of mutations was distributed throughout the gene. This correlates with TK structural data, which demonstrate that multiple residues, including those distant from active site, are involved in catalytic activity of TK[20,56]. The fact that of 24 TK mutants, 19 had a unique genotype demonstrates the high number of different mutations that can confer resistance, in contrast to what has been described for CMV[33]. In addition, identification of 10 presumably novel resistance-conferring mutations in the TK gene indicates that our knowledge of the resistance mutations is far from complete.

All new TK mutations identified in our studies were carefully examined based on comparison with sequences of pretherapy or drug-sensitive isolates from the same patient and with available sequences of ACV-sensitive reference strains. In addition, mutations were analysed in relation to TK structural and functional data, and/or additional drug susceptibility profiling was performed. Genotypic data still lack biochemical determination of TK activity of the resistant strains. Moreover, the exact assignment of the resistant phenotype to new mutations identified in our study requires their functional analysis using site directed mutagenesis.

DNA pol mutants could not be studied extensively in this thesis due to their rare occurrence in the clinic. Nevertheless, the spectrum of DNA pol gene mutations conferring resistance to ACV and other antiherpetic drugs is growing. The structure-functional

relationship of the catalytic domain of DNA pol is more complex than expected on the basis of studies on the HCMV DNA polymerase. It appears that the region of DNA pol that contains the drug binding sites is very large (codon 500-1030)[3,9]. In addition, mutations conferring resistance to a single drug have been identified in several conserved as well as nonconserved regions.

The high numbers of resistance-conferring mutations in drug-resistant HSV isolates identified in our study as well as in those of others suggest that development of rapid and simple genotypic assays for detection of resistant HSV mutants is at this stage practically impossible. For the moment, phenotypic assays will thus remain the first line approach for HSV resistance testing. The phenotypic assays described in this thesis have demonstrated to be easy-to-use and rapid alternatives of PRA. Phenotypic testing should however be paralleled by genotyping whenever possible in order to improve our understanding of resistance-associated mutations and their consequences for drug susceptibility and viral pathogenicity. With the ongoing improvements in the sequencing technology and microarray analysis, the easier and more rapid sequence analysis of large genes such as TK and DNA pol might soon be expected. Analysis of serial isolates and the recently developed recombinant assays might further speed up the identification of new resistance-associated mutations[3,4].

The studies described in this thesis contribute to a better understanding of clinical problem of HSV drug resistance, its mechanisms, prevalence and routine detection. Additional studies will be needed to further evaluate the clinical applicability of the newly developed approaches for adequate monitoring of the emergence and spread of drug-resistant HSV.

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Nederlandse samenvatting

Infecties met het herpes simplex virus (HSV) komen vaak voor en hebben meestal een asymptomatisch of mild verloop. De symptomen van blaasjes, zweren en wondjes die worden veroorzaakt door een HSV infectie kunnen voorkomen op de lip, neus en mond in geval van koortsuitslag, of in de anogenitale streek in het geval van een genitale herpes infectie. Ernstig verlopende HSV infecties komen met name voor bij HIV/AIDS patiënten of bij mensen die een orgaan transplantatie hebben ondergaan. Als er in deze situatie geen adequate antivirale therapie wordt toegepast, kan een infectie bij deze patiënten levensbedreigend zijn.

Sinds de introductie van acyclovir (ACV) in de jaren tachtig, wordt dit middel veelvoudig toegepast bij de behandeling van (ernstige) HSV infecties. Daarnaast wordt ACV in toenemende mate gebruikt als suppressieve therapie en als profylaxe voor HSV infecties met een recidiverend en/of ernstig beloop. Tegenwoordig is het gebruik van ACV wijdverbreid sinds het middel ook zonder recept verkrijgbaar is.

Kort na de introductie van ACV werd resistentie van HSV tegen het middel beschreven. In de laatste twee decennia is duidelijk geworden dat resistentie van HSV tegen ACV regelmatig voorkomt in immuunogecompromiteerde patiënten, maar dat resistentie nauwelijks voorkomt in de normale populatie vanwege een adequate controle van de infectie door het immuunsysteem van de gastheer in deze laatste groep. De aandacht voor resistentie van HSV neemt toe door het stijgende aantal immuungecompromiteerde patiënten en de ernstige klinische gevolgen van resistente HSV infecties. Dit onderstreept de behoefte aan ontwikkeling van makkelijke, snelle en betrouwbare fenotypische methoden voor bepaling van HSV resistentie en aan surveillance naar het voorkomen van antivirale resistentie in de normale populatie en in specifieke patiënten groepen. Een beter inzicht in de genetische veranderingen in het virus die verantwoordelijk zijn voor het ontstaan van HSV resistentie is nodig voor ontwikkeling van moleculair-biologisch methodes voor detectie van HSV resistentie. In dit proefschrift wordt de detectie, prevalentie en karakterisering van resistent HSV beschreven.

Er werden nieuwe methoden ontwikkeld voor de snelle en gevoelige detectie van HSV en bepaling van antivirale resistentie. In **hoofdstuk 2** wordt een methode, gebaseerd op kwantitatieve ‘real-time’ PCR, beschreven voor een snelle detectie van infecties door HSV en varicella-zoster virus in verschillende klinische monsters. Dit is een aanzienlijke diagnostische en klinische vooruitgang voor, met name, immuunogecompromiteerde patiënten, aangezien een snelle diagnose voor deze patiënten van belang is voor een tijdige start van antivirale therapie. Deze PCR methode werd ook toegepast als ‘uitleessysteem’ van een fenotypische bepaling van de antivirale gevoeligheid van HSV, zoals beschreven in **hoofdstuk 3**. Deze gevoeligheidstest, die gebruik maakt van combinatie van virus kweek en real-time PCR, is eenvoudig te implementeren in de dagelijkse routine van een klinisch virologisch laboratorium. In **hoofdstuk 4** wordt een snelle fenotypische test beschreven die is ontwikkeld voor screening van de antivirale gevoeligheid van HSV isolaten voor ACV. Deze test, genaamd ELVIRA HSV, is gebaseerd op een door HSV-induceerbare reporter cellijn en kan de gevoeligheid van HSV voor antivirale middelen in monsters met een lage virustiter binnen 48 uur vaststellen. Deze test werd toegepast in een grootschalig onderzoek naar de

prevalentie van ACV resistentie van HSV, zoals beschreven in **hoofdstuk 5.** Ter bepaling van de prevalentie van ACV resistentie van HSV in de Nederlandse populatie werden in totaal 542 HSV isolaten van 496 patiënten onderzocht. Bij 128 van hen was sprake van verminderde afweer. De prevalentie van ACV resistentie in de populatie van immuncompetente personen was 0.27% en week daarin niet af van de prevalentie van virussen met een verminderde gevoeligheid voor ACV die werd waargenomen in de periode voor de introductie van ACV. De prevalentie van ACV resistente HSV die werd waargenomen in immuungecompromiteerde patiënten was 7%. Uit deze resultaten blijkt dat na ruim 20 jaar gebruik van ACV, inclusief de beschikbaarheid zonder recept, er geen aanwijzingen zijn voor een toename van antivirale resistentie van HSV in de normale populatie. Echter, zoals blijkt uit de prevalentie van 7% in immuungecompromiteerde patiënten, is antivirale resistentie een groot probleem geworden in deze groep. De ACV-resistente stammen uit dit onderzoek werden verder onderzocht op de moleculaire veranderingen die ten grondslag liggen aan deze antivirale resistentie.

In patiënten die een beenmergtransplantatie hebben ondergaan, komen resistente HSV stammen relatief vaak voor. Dit kan resulteren in ernstige en soms fatale infecties. In **hoofdstuk 6** werden 31 HSV isolaten gekarakteriseerd van 12 beenmergtransplantatie patiënten met een ernstige HSV infectie die niet reageerde op ACV therapie. De relatie tussen de gevoeligheid van de isolaten voor de medicijnen en de moleculaire veranderingen in het genoom van het virus werd onderzocht. Er werden "nieuwe", niet eerder gerapporteerde mutaties geïdentificeerd in het thymidine kinase en DNA polymerase genen van het virus die mogelijk een rol spelen bij de resistentie tegen ACV. Deze informatie is van groot belang voor het inzicht in het ontstaan van antivirale resistentie, bij het opzetten van snelle, gevoelige genotypische bepalingen van antivirale resistentie direct in patiëntenmateriaal, en voor het ontwikkelen van nieuwe antivirale middelen. **In hoofdstuk 7** wordt een casus beschreven van een ernstige HSV infectie bij een kind dat na beenmergtransplantatie niet reageerde op langdurige antivirale therapie. Resistentie tegen ACV bleek de verklaring voor het therapiefalen. Bovendien bleek het ACV resistente virus tevens kruisresistent te zijn voor foscarnet. Deze casus laat zien dat in dergelijke gevallen van ernstige HSV infecties, de therapeutische keuzen gebaseerd zouden moeten worden op het resultaat van de bepaling van de antivirale gevoeligheid van het virus.

Shrnutí v českém jazyce

Infekce virem herpes simplex (HSV) jsou velmi časté, avšak většinou jsou zcela bez příznaků, nebo jde jen o lehké onemocnění. Po primární infekci virus navozuje latentní infekci centrálního nervového systému, během které může docházet k reaktivacím viru při dočasné imunosupresi organismu, či v důsledku získané imunodeficience. Nejčastější klinickou manifestací infekce či reaktivace virem HSV jsou kožní a slizniční leze na rtech, v dutině ústní nebo nosohltanu, známé jako opary, či v krajině ano-genitální, známé jako herpes genitalis. U imunosuprimovaných pacientů, jako jsou osoby infikované virem HIV, pacienti po transplantacích nebo protinádorové terapii, HSV však může způsobit velmi závažná onemocnění vyžadující protivirovou léčbu. Acyklovir (ACV), který se používá už od počátku 80. let, zaujímá výsadní místo mezi antivirotiky. Používá se nejen pro léčbu HSV infekcí s velmi vážným průběhem, ale i pro supresivní terapii a profylaxi, zejména u pacientů s rizikem častých nebo vážných recidiv. K širokému použití ACV v současné době přispívá i jeho volná dostupnost bez lékařského předpisu.

Velmi brzy po uvedení ACV do klinické praxe však byly popsány první případy HSV rezistence. Studie posledních dvaceti let ukázaly, že výskyt HSV mutant rezistentních na ACV je poměrně častý především u imunosuprimovaných osob, zatímco tyto viry byly izolovány jen velmi zřídka v běžné (imunokompetentní) populaci, kde dochází k eliminaci viru imunitním systémem. Rostoucí počet imunosuprimovaných pacientů, vážný klinický průběh HSV infekcí a riziko selekce rezistentních HSV u těchto pacientů, vyvolaly zvýšený zájem o problematiku HSV rezistence. Ukázalo se, že největší pozornost by měla být věnována vývoji jednoduchých, rychlých a spolehlivých metod pro detekci HSV rezistence (fenotypu rezistence). Tyto metody by pak umožnily monitorování rezistence HSV k antivirotikům, jak v běžné populaci, tak u specifických skupin pacientů. Neméně důležité je zmapování mutací způsobujících rezistenci na ACV a další antivirotika, což by mohlo vést k vývoji molekulárně-biologických metod pro detekci rezistentních HSV. Tato disertační práce se zabývá detekcí, prevalencí a charakterizací fenotypu a genotypu rezistentních HSV.

Byly vyvinuty nové metody pro rychlou a citlivou diagnostiku HSV infekcí a pro vyšetření HSV rezistence. V **kapitole 2** je popsána metoda, založená na principu kvantitativní polymerázové řetězové reakce (PCR, amplifikační test), která je určena pro rychlou diagnostiku infekcí virem HSV a virem varicella-zoster (VZV; původce pásového oparu) v různých typech klinických materiálů. Tato metoda je citlivější než klasická izolace viru na buněčných kulturách a znamená tak výrazný krok vpřed v diagnostice HSV a VZV. Má klinický význam především pro imunosuprimované pacienty, u kterých je rychlá diagnostika důležitá pro včasné nasazení protivirové léčby. Tato metoda díky kvantitativní PCR poskytuje objektivní výstupní data a proto byla využita i při vývoji diagnostického testu pro vyšetření citlivosti viru k různým antivirotikům, který je popsán v **kapitole 3.** Tento test, který je kombinací kultivace viru v přítomnosti antivirotika a kvantitativní PCR, je možné jednoduše začlenit mezi diagnostické testy běžně prováděné v klinické virologické laboratoři. V **kapitole 4** je popsán test, který byl navržen pro rychlý screening rezistence HSV izolátů k ACV. Tento test s názvem ELVIRA HSV je založen na buněčné linii s HSV inducibilním

reporterovým genem a s jeho pomocí je možné testovat rezistenci HSV během 48 hodin i ze vzorku s malým množstvím viru. Byl použit ve velké studii, která byla zaměřena na zjištění prevalence rezistence k ACV u izolátů HSV v Nizozemí, a ktera je popsána v **kapitole 5**. V této studii bylo testováno celkem 542 HSV izolátů ze 496 pacientů, z nichž 128 bylo imunosuprimovaných. Prevalence HSV rezistence k ACV v běžné populaci se ukázala nízká, 0,27%, což je procento srovnatelné s frekvencí přirozeně se vyskytujících rezistentních HSV v době před zavedením ACV. Naopak prevalence ACV rezistentních HSV u imunosuprimovaných pacientů byla 7%. Tyto výsledky ukazují, že více než 20 let užívání ACV, včetně volného prodeje, nevedlo k výraznému zvýšení prevalence ACV rezistentních HSV v populaci Nizozemska. Avšak prevalence 7% u imunosuprimovaných pacientů ukazuje, že HSV rezistence se stala v této skupině pacientů závažným problémem. HSV izoláty rezistentní k ACV, které byly identifikovány v této studii, byly dále zkoumány po molekulární stránce s cílem lokalizovat genetické změny související s rezistencí.

Zvláště u pacientů po transplantaci kostní dřeně se infekce rezistentními HSV objevují poměrně často a mohou mít chronický až fatální průběh. **Kapitola 6** popisuje charakterizaci 31 HSV izolátů z dvanácti pacientů po transplantaci kostní dřeně, u kterých byla diagnostikována HSV infekce rezistentní k léčbě ACV. Naším cílem bylo korelovat rezistenci izolátů vůči ACV s mutacemi ve virovém genomu, což vedlo k identifikaci nových mutací v genu pro virovou thymidinkinázu a DNA-polymerázu, které s velkou pravděpodobností způsobují rezistenci. V **kapitole 7** je popsán případ pacienta po transplantaci kostní dřeně, u kterého došlo k reaktivaci HSV v dutině ústní. Tato infekce byla rezistentní nejen na ACV, ale i na alternativní HSV inhibitor foskarnet, což vedlo k masivnímu rozšíření lézí po celém obličeji, v oblasti dutiny ústní, nosohltanu a dolních cest dýchacích. Vyšetření fenotypu rezistence vůči běžně používaným antivirotikům a genetická analýza ukázaly, že u tohoto pacienta došlo v průběhu dlouhodobé léčby ACV ke střídání rezistentních HSV mutant s různými fenotypy rezistence. Tento případ demonstruje důležitost výsledků testování fenotypu rezistence pro úspěšnou a efektivní antivirovou léčbu.

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…sorry, I have to stop here….this has to go to the printer today!…

Růža

Curriculum vitae

The author was born on 24 September 1975 in Brandýs nad Labem, Czech Republic. After finishing her secondary school education at the Grammar School of J. S. Machar in Brandýs nad Labem she entered the study of Biology at the Charles University in Prague and completed her BSc degree in 1997. During her study she worked at the Department of Experimental Virology of the Institute of Hematology and Blood Transfusion in Prague, under the supervision of Dr. Š. Němečková and Prof. V. Vonka. In autumn 1998 she followed a three-month traineeship in the clinical virology laboratory of the University Medical Center Utrecht under the supervision of Dr. Anton van Loon. In June 1999 she completed her MSc degree in Virology. From August 1999 until December 2003 she was working on her PhD research project at the Department of Virology of the University Medical Center Utrecht under the supervision of Dr. Anton van Loon and Dr. Rob Schuurman. During this period she has also completed the graduate course program at the Eijkman Graduate School for Immunology and Infectious Diseases.

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