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

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Abstract

Background: Widespread and frequent use of acyclovir (ACV) for treatment, suppressive therapy and prophylaxis of herpes simplex virus (HSV) infections and its over the counter availability may be associated with emergence of HSV resistance. Objectives: To determine the prevalence of ACV-resistant HSV isolates in different patient groups between 1999 and 2002 in the Netherlands. Study design: 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. The genotypic analysis of the HSV thymidine kinase gene was performed to identify resistance-associated mutations. Results: 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 nine 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: four 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. Conclusions: 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.

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Keywords: Acyclovir-resistant herpes simplex virus; Acyclovir; HSV thymidine kinase gene

1. 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 (Whitley and Lakeman, 1995). Oral or intravenous ACV is frequently used for prophylaxis and treatment of HSV infections in immunocompromised patients. In the context of severe immunosuppression, as is 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 (Bergmann et al., 1995; Chen et al., 2001; Dignani et al., 2002; Shepp et al., 1987; Wood, 1998). 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) (Bestman-Smith et al., 2001) and, to a much lesser extent by mutations in the viral DNA polymerase (DNA pol) gene (Larder and Darby, 1985, 1986).

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 (Parris and Harrington, 1982; Sarsky et al., 2001). 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 (Chen et al., 2001). Studies reporting on the prevalence of ACV-resistant HSV have been recently reviewed (Bacon et al., 2003). Combined with the results from the most recent studies by Danve-Szatanek et al. (Danve-Szatanek et al., 2004) and Reyes et al. (Reyes et al., 2003) it can be concluded that the prevalence of HSV infections with reduced susceptibility to ACV in immunocompromised patients varies from 3.5% and 7.1% (Bacon et al., 2003; Christophers et al., 1998; Englund et al., 1990; Nugier et al., 1992). The highest prevalence rates are reported for recipients of HSCT, with a range from 4.1% to 10.9% (Chen et al., 2001; Danve-Szatanek et al., 2004; Wade et al., 1983; Williamson et al., 1999). A recent report described an even higher frequency (36%) of ACV resistance (Langston et al., 2002). Similarly, the prevalence in HIV-positive patients ranges from 3.5% to 7% (Englund et al., 1990; Danve-Szatanek et al., 2004; Reyes et al., 2003) and in solid organ transplant (SOT) recipients from 2.5% to 10% (Christophers et al., 1998; Danve-Szatanek et al., 2004). The numbers of patients in the SOT group, however, are usually low.

In contrast, ACV-resistant isolates have been reported infrequently in immunocompetent subjects (Bacon et al., 2003). 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 (Cox et al., 1989). 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 (Collins and Ellis, 1993). No increase in the prevalence of resistance has been observed since then (range from 0.1% to 0.7%) (Bacon et al., 2002; Bacon et al., 2003; Boon et al., 2000; Christophers et al., 1998; Danve-Szatanek et al., 2004; Reyes et al., 2003).

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.

2. Methods

2.1. 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.

2.2. 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 patients’ specimens 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.
2.3. Well-characterized strains and clinical isolates

The ACV-sensitive HSV-1 strain KOS, the KOS-derived mutants AraA8, PFA8 (both ACV- and PFA-resistant), and AraA8 (moderately resistant to ACV/resistant to PFA) were kindly provided by D.M. Coen (Harvard Medical School, Boston, MA) (Coen et al., 1982; Pelosi et al., 1998). 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 (Gueudreau et al., 1998) were a gift of N. Goyette (University of Laval, Que., 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 (Stranksa et al., 2004a, 2004b, 2004c).

2.4. 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 (Stranksa et al., 2004a). Selected stocks were subsequently subjected to detailed susceptibility testing using the latter method.

2.4.1. ELVIRA® HSV susceptibility assay

HFF cells were plated in the inner 60 wells of a 96-well microtiter plate (Costar) in a volume of 200 µl (6 x 10^5 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 (4-fold increments) and from 12.5 to 2000 µg/ml (2-fold increments), respectively. For ganciclovir (GCV), a range from 0.001 to 4 µg/ml (4-fold increments) was used. 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 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® culture 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-β-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 (OD560). 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 IC50 was calculated by linear regression analysis. Each virus isolate was tested at least in two independent experiments. Reference ACV- and PFA-sensitive and -resistant HSV-1 (KOS and AraA8) and HSV-2 (MS and 97.1218) strains were included as controls in each assay. IC50 values of 0.8 and 60 µg/ml were set as cut-off values for decreased susceptibility to ACV and PFA, respectively. 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-folds increase in IC50, compared to the IC50 of the pretherapy isolate, respectively (Andrei et al., 2000).

2.4.2. ELVIRA HSV screening assay

Confluent HFF monolayer plates were inoculated with an endpoint series of 5-fold dilutions of an un triturated 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 (Stranská et al., 2004a). 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 2SDs 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 (Stranská et al., 2004b). 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 by ELVIRA® HSV susceptibility assay for those isolates, which harbored so far unreported TK gene mutations (Table 4).

2.5. 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.

3. Results

3.1. 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}s) of a set of 40 ACV-sensitive and 30 ACV-resistant HSV-1 strains, consisting of laboratory strains and clinical isolates (see Section 2), 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} higher than 1 μg/ml (range, 1.22–58.6 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 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 × 10^4 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 × 10^5 PFU/ml with a range from 2.3 × 10^4 to 3.4 × 10^6 PFU/ml. Consequently, a 5-fold dilution series of virus stock starting at the dilution 1/5000 was tested in the screening assay.

3.2. 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-1 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 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-folds 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-folds).

3.3. 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.
### Table 1

**Results of EL VIRA HSV screening and susceptibility assays**

<table>
<thead>
<tr>
<th>Virus strain/isolate (code)</th>
<th>Isolate no</th>
<th>HSV type</th>
<th>EL VIRA screening (fold change)</th>
<th>EL VIRA susceptibility (ACV-IC₅₀)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-characterized strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KOS</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0.4 ± 0.20</td>
<td>–</td>
</tr>
<tr>
<td>AraA r 8</td>
<td>–</td>
<td>1</td>
<td>3.5</td>
<td>1.7 ± 0.51</td>
<td>DNA pol</td>
</tr>
<tr>
<td>PEV’S</td>
<td>–</td>
<td>1</td>
<td>5.4</td>
<td>2.1 ± 0.60</td>
<td>DNA pol</td>
</tr>
<tr>
<td>MS</td>
<td>2</td>
<td>14</td>
<td>1.7</td>
<td>0.5 ± 0.16</td>
<td>–</td>
</tr>
<tr>
<td>97.1218</td>
<td>–</td>
<td>2</td>
<td>7.5</td>
<td>78.1 ± 12.28</td>
<td>ND</td>
</tr>
<tr>
<td>A1</td>
<td>–</td>
<td>2</td>
<td>8.8</td>
<td>7.5 ± 3.50</td>
<td>TK</td>
</tr>
<tr>
<td>O24</td>
<td>2</td>
<td>1</td>
<td>7.8</td>
<td>11.7 ± 4.20</td>
<td>TK</td>
</tr>
<tr>
<td>McIntyre</td>
<td>1</td>
<td>1</td>
<td>0.4</td>
<td>0.3 ± 0.09</td>
<td>–</td>
</tr>
<tr>
<td>R39</td>
<td>1</td>
<td>1</td>
<td>0.3</td>
<td>0.4 ± 0.12</td>
<td>–</td>
</tr>
<tr>
<td>98.14742-PF1</td>
<td>–</td>
<td>1</td>
<td>7.5</td>
<td>19.1 ± 0.96</td>
<td>TK</td>
</tr>
<tr>
<td>Clinical isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>98.17628</td>
<td>1/1</td>
<td>1</td>
<td>0.4</td>
<td>0.2 ± 0.09</td>
<td>–</td>
</tr>
<tr>
<td>99.00820</td>
<td>1/2</td>
<td>1</td>
<td>13.7</td>
<td>42.9 ± 4.80</td>
<td>TK</td>
</tr>
<tr>
<td>99.20762</td>
<td>2/1</td>
<td>1</td>
<td>0.6</td>
<td>0.1 ± 0.06</td>
<td>–</td>
</tr>
<tr>
<td>99.22172</td>
<td>2/2</td>
<td>1</td>
<td>12.3</td>
<td>8.6 ± 0.83</td>
<td>TK</td>
</tr>
<tr>
<td>97.11896</td>
<td>3/1</td>
<td>1</td>
<td>0.5</td>
<td>0.2 ± 0.05</td>
<td>–</td>
</tr>
<tr>
<td>97.12736</td>
<td>3/2</td>
<td>1</td>
<td>8.0</td>
<td>5.8 ± 2.82</td>
<td>TK</td>
</tr>
<tr>
<td>01.14406</td>
<td>4/1</td>
<td>1</td>
<td>0.6</td>
<td>0.2 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>01.14630</td>
<td>4/2</td>
<td>1</td>
<td>0.7</td>
<td>0.3 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>01.19093</td>
<td>4/3</td>
<td>1</td>
<td>3.8</td>
<td>1.5 ± 0.33</td>
<td>DNA pol</td>
</tr>
<tr>
<td>01.22733</td>
<td>4/4</td>
<td>1</td>
<td>7.1</td>
<td>18±2 ± 3.10</td>
<td>TK</td>
</tr>
<tr>
<td>01.25004</td>
<td>4/5</td>
<td>1</td>
<td>4.2</td>
<td>1.5 ± 0.13</td>
<td>DNA pol</td>
</tr>
<tr>
<td>01.29319</td>
<td>4/6</td>
<td>1</td>
<td>4.4</td>
<td>2.3 ± 0.70</td>
<td>DNA pol</td>
</tr>
<tr>
<td>99.16237</td>
<td>5</td>
<td>1</td>
<td>18.5</td>
<td>45±2 ± 13.0</td>
<td>TK</td>
</tr>
</tbody>
</table>

**Note:** ND: Not determined.

- 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 two independent experiments.
- e Resistance-associated mutation in TK or DNA pol gene.

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 r 8, 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 EL VIRA 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 2

**Inter-assay variability of EL VIRA HSV screening assay**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance value, (%)</th>
<th>CV (%)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>12.1 ± 4.5 (13)</td>
<td>38.9</td>
<td>–</td>
</tr>
<tr>
<td>AraA r 8</td>
<td>66.2 ± 23.1 (13)</td>
<td>38.3</td>
<td>5.0 ± 1.3</td>
</tr>
<tr>
<td>MS</td>
<td>22.7 ± 3.2 (4)</td>
<td>13.9</td>
<td>2 –</td>
</tr>
<tr>
<td>97.1218</td>
<td>94.3 ± 20.2 (4)</td>
<td>21.4</td>
<td>4.3 ± 1.6</td>
</tr>
</tbody>
</table>

- a Virus yield at 1 µg/ml ACV as a percent 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.

### 3.4. 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%), and malignancy (21%) were the major groups (Table 3). Compared to the immunocompe-
Table 3

<table>
<thead>
<tr>
<th>Immune status of the patient</th>
<th>No. of patients</th>
<th>No. of patients with resistant HSV (%)</th>
<th>No. of isolates</th>
<th>No. of resistant HSV isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocompetent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>368</td>
<td>1 (0.27)</td>
<td>389</td>
<td>1</td>
</tr>
<tr>
<td>Immunocompromised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSCT</td>
<td>28</td>
<td>4 (14.3)</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>HIV</td>
<td>51</td>
<td>2 (3.92)</td>
<td>59</td>
<td>3</td>
</tr>
<tr>
<td>Malignancy</td>
<td>26</td>
<td>1 (3.85)</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>SOF</td>
<td>17</td>
<td>0 (0.00)</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>2 (na)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>9 (7.03)</td>
<td>153</td>
<td>12</td>
</tr>
<tr>
<td>Grand total</td>
<td>496</td>
<td>10 (2.02)</td>
<td>542</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not applicable.

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 ≥3-fold decrease in susceptibility to ACV (increase in resistance value) was found. Thirteen of these 38 isolates (eight HSV-1, five HSV-2; 10 patients) demonstrated a ≥5-fold decrease in susceptibility (increase in resistance value). Detailed IC<sub>50</sub> determination using the ELVIRA HSV susceptibility assaying and/or genotypic analysis demonstrated ACV resistance in all these 13 isolates. The mean ACV-IC<sub>50</sub> of these isolates ranged from 10.7 to 50.0 μg/ml. The remaining 25 isolates, which demonstrated a ≥3–5-fold increase in resistance value in the screening assay, displayed ACV-IC<sub>50</sub> in a range from 0.08 to 0.89 μg/ml. The distribution of the mean IC<sub>50</sub> 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). One out of 368 immunocompetent patients was identified by ACV susceptibility screening to be harbouring an ACV-resistant HSV (0.27%; 95% confidence interval [CI], 0.007%–1.5%). Nine out of 128 immunocompromised patients (7.0%) were found to carry resistant HSV (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.

3.5. Characterization of ACV-resistant isolates

3.5.1. 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
### Table 4
Summary of phenotypic, genotypic and clinical data for patients with ACV-resistant HSV

<table>
<thead>
<tr>
<th>Patient (age/gender)</th>
<th>Type of immunosupression</th>
<th>Isolate no(^a)</th>
<th>HSV type</th>
<th>Isolation site</th>
<th>Susceptibility: drug-IC(_{50}) (μg/ml)(^b)</th>
<th>Genotypic changes in TK gene(^c)</th>
<th>Antiviral treatment, day (d)(^d)</th>
<th>HSV disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (9/M) T-cell</td>
<td>1/1</td>
<td>Throat</td>
<td>0.16</td>
<td>ND</td>
<td>-</td>
<td>ACV PFA</td>
<td>nACV, d3–d22, HSV pneumonia.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lymphoma</td>
<td>1/2(^f)</td>
<td>Faeces</td>
<td>0.69(^g)</td>
<td>16.8</td>
<td></td>
<td></td>
<td>gastroenteritis;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
<td>50.0</td>
<td>8.5</td>
<td>FS185 (+C) stop 43 ds</td>
<td>PFA, d32–d36</td>
<td>5d16</td>
</tr>
<tr>
<td>2 (35/M) HIV- +</td>
<td>2/1(^f)</td>
<td>Anus</td>
<td>29</td>
<td>ND</td>
<td>-</td>
<td></td>
<td>ValACV</td>
<td>Recurrent genital herpes</td>
</tr>
<tr>
<td>3 (50/M) HIV- +</td>
<td>2</td>
<td>Scrotum</td>
<td>15.3</td>
<td>7.7</td>
<td>T288M</td>
<td></td>
<td>Previous ValACV and topical ACV</td>
<td>Recurrent genital herpes</td>
</tr>
<tr>
<td>4 (51/V) HSCT</td>
<td>2</td>
<td>Vagina</td>
<td>R(^g)</td>
<td>ND</td>
<td>E39G, G50E</td>
<td></td>
<td>GCV for CMV</td>
<td>Genital herpes</td>
</tr>
<tr>
<td>5 (68/M) High-dose prednisone in COPD</td>
<td>1</td>
<td>Oral cavity</td>
<td>R(^g)</td>
<td>ND</td>
<td>D162D/H</td>
<td></td>
<td>oACV, d2–d7</td>
<td>Oral lesions</td>
</tr>
<tr>
<td>6 (73/V) High-dose prednisone in COPD</td>
<td>None</td>
<td>Lung</td>
<td>10.7</td>
<td>16.4</td>
<td>R222H</td>
<td>nACV, d7–d11, oACV, d1–d17</td>
<td>HSV in lower respiratory tract</td>
<td></td>
</tr>
<tr>
<td>7 (38/M) None</td>
<td>2</td>
<td>Skin</td>
<td>35.3(^i)</td>
<td>11.5</td>
<td>T282A, R36/C</td>
<td></td>
<td>No therapy</td>
<td>Recurrent skin lesions</td>
</tr>
</tbody>
</table>

**Note:** FS: frameshift; ND: not determined; ds: downstream; o: oral.

\(^a\) 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).
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 seven 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 HSV isolates from three of the four HSCT recipients has been described previously (Stränská et al., 2004c).

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 five 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 prednisolone therapy 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 emphasisia, 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 2 weeks from recurrent HSV-2 skin infection resulting in blisters and lesions on his back without any previous antiviral therapy.

3.5.2. 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-IC50 of ACV-resistant isolates ranged from 10.7 to 50.0 μg/ml. De-
sistance (Boivin, 1998; Sarisky et al., 2002), 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 (Gilbert et al., 2002; Stranska et al., 2004b). 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 (Bestman-Smith and Boivin, 2003). 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 (Danne et al., 2002). 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 (Swierkosz, 2000). 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 h on isolates of unknown titer. The inter-assay variation was <50%, which is comparable to the reproducibility of other recently described susceptibility assays (Leahy et al., 1994; Rabella et al., 2002). 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 (Stranska et al., 2004a). 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 (Sprunck et al., 2003). 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 (Stranska et al., 2004b).

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 (Bacon et al., 2002; Christophers et al., 1998; Danve-Szatanek et al., 2004; Reyes et al., 2003). 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 (Christophers et al., 1998) also demonstrated a low prevalence of resistant HSV (0.5%). No resistant isolate was identified in our genital herpes patient group. Results from a recent large survey in a general population with genital herpes (Bacon et al., 2003) 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 (Bacon et al., 2002; Boon et al., 2000). In our study, a single immunocompetent patient shed ACV-resistant virus. This patient (87) 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 (Sarisky et al., 2000) 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 7.0% is in agreement with previous reports from other countries (Chen et al., 2001; Christophers et al., 1998; Englund et al., 1990; Morfin and Thouvenot, 2003; Nugier et al., 1992; Wade et al., 1983; Williamson et al., 1999). 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. (Chen et al., 2001) and Morfin and Thouvenot (Morfin and Thouvenot, 2003). 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 (Chen et al., 2001; Meyers et al., 1980). 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 (5–7 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 of 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 (Gaudreau et al., 1998; Morfin et al., 2000). 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 (Sarisky et al., 2000). 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 (Bestman-Smith et al., 1998; Kit et al., 1997; Sarisky et al., 2001). 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 (Balasubramaniam et al., 1990) responsible for coordination of Mg2+ ions, which are essential for catalysis (Black and Loeb, 1993; Brown et al., 1995). 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 phylogenetic 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 glycine-rich loop, GXXGXXGKT18 (residues 56–63), which is conserved in all herpesvirus TKs. The three glycins are playing an important role in binding of the β-phosphopheryl group of ATP, and replacement of any of them results in a loss of TK activity (Liu and Summers, 1988), 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 (Gaudreau et al., 1998). 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 (Brown et al., 1995; Schulz et al., 1990). 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 (Bestman-Smith et al., 2001; Sasadeusz et al., 1997), 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 (Halpern and Smiley, 1984; Vogt et al., 2000; Wild et al., 1995). Finally, additional and proven resistance-associated mutations were identified in the two reported resistant isolates containing Glu39Gly (Bestman-Smith et al., 2001; Sasadeusz et al., 1997).

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 (Palu et al., 1992; Sasadeusz et al., 1997).

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 (Gaudreau et al., 1998).

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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