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Surveillance of herpes simplex virus resistance to antivirals: A 4-year survey

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ABSTRACT

Herpes simplex virus (HSV) resistance to antivirals constitutes a therapeutic challenge, especially among immunocompromised patients. This observational survey on HSV resistance to antivirals was conducted retrospectively over a 4-year period (2008–2012). A total of 211 HSV-positive clinical samples (94 HSV-1 and 117 HSV-2) recovered from 139 patients (11 immunocompetent patients, 85 immunocompromised patients, and 43 patients with unknown immune status) with suspected HSV drug-resistance were analyzed for acyclovir and foscarnet susceptibility. Antiviral resistance testing consisted in a two-step procedure including a first-step genotypic assay, based on UL23 (thymidine kinase, TK) and UL30 (Pol) gene sequencing, and a second-step phenotypic assay (i.e., plaque reduction assay) performed when unpreviously described mutations were detected. As a whole, susceptibility and resistance to antivirals were evidenced for 58 (30.7%) and 86 (45.5%) HSV, respectively, whereas antiviral profile remained undetermined for 45 (23.8%) HSV. The prevalence of drug resistance was significantly higher among HSV-2 isolates than among HSV-1 isolates (53.8% *vs.* 34.9%; $p = 0.012$). The majority (i.e., 79.7%) of cases of ACV resistance conferred by TK mutations resulted from UL23 gene frameshift reading. Apart from the changes surely related to natural polymorphism or drug-resistance, 91 unpreviously reported mutations were identified in TK and Pol, including 51 potential natural polymorphisms, 22 mutations likely conferring resistance to antivirals, and 18 mutations of unclear significance.

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1. Introduction

The significant morbidity and mortality associated with herpes simplex virus (HSV) infections, requires antiviral treatments to prevent or to cure HSV-associated diseases. UL30 DNA polymerase (Pol) constitutes the viral target of the currently licensed drugs acyclovir (ACV) and penciclovir (PCV), with their respective prodrugs valacyclovir (VACV) and famciclovir (FCV), foscarnet (FOS), and cidofovir (CDV). Viral-encoded UL23 thymidine kinase (TK) is required for ACV and PCV phosphorylation (Piret and Boivin, 2011). Antiviral treatments may result in the emergence of HSV resistance. The prevalence of HSV resistance to antivirals among immunocompetent individuals is low (<1%) (Bacon et al., 2002; Collins and Ellis, 1993). Among immunocompromised patients, this prevalence reaches 3.5% to 10%, with profound immunosuppression and long-term drug exposure as associated risk factors (Danve-Szatanek et al., 2004; Piret and Boivin, 2011). The molecular mechanisms of HSV resistance rely on the presence of mutations within UL23 and UL30 genes. TK alterations, accounting for 95% of HSV resistance to ACV, consist in single-base insertions/deletions, leading to the shift of the translational reading frame of UL23 gene, or missense point mutations. HSV resistance to FOS and/or ACV may be related to mutations within conserved regions of Pol (Piret and Boivin, 2011). Conversely to time-consuming and cumbersome phenotypic antiviral resistance assays, genotypic assays, based on UL23 and UL30 gene sequencing, allow the determination of HSV resistance to antivirals in a clinically relevant time frame (Burrel et al., 2010). However, the involvement of numerous reported mutations in drug-resistance remains to be clearly established. This work reports the results from an observational and retrospective 4-year survey of HSV resistance to antivirals performed in a single center using a genotypic/phenotypic two-step procedure.

2. Materials and methods

2.1. Patients and clinical samples

Between June 2008 and May 2012, clinical samples collected from patients with HSV infection and experiencing incomplete

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virological response despite a well-conducted antiviral treatment (i.e., chronic mucocutaneous lesions and/or persistent viral load) were tested for HSV resistance to antivirals. Each sample was accompanied by a standardized form including: (i) identification of the patient and the hospital department, (ii) age and sex, (iii) immune status, (iv) antiviral treatment, (v) date and site of clinical sample. Samples were tested by real-time PCR assay (Qiagen, Courtaboeuf, France) in order to measure HSV load and to identify HSV type 1 or 2. Remaining sample was frozen at −80 °C for further investigations.

2.2. Antiviral resistance testing procedure

The analysis of HSV resistance to antivirals was performed using the two-step approach implemented in the laboratory. First, a genotypic assay, based on UL23 and UL30 gene sequencing from all HSV-positive samples, allowed the identification of mutations associated with antiviral resistance or natural polymorphism. When the results of the genotypic assay were not conclusive, due to unpreviously described or undefined mutations, a phenotypic assay was performed.

2.3. Genotypic resistance assay

Genotypic resistance assay was performed as previously described (Burrel et al., 2010). Briefly, full-length UL23 and UL30 genes were amplified using HSV type-specific PCR systems with the proofreading enzyme Expand High Fidelity (Roche Diagnostics, Meylan, France). Amplified products were sequenced using overlapping primer pairs with the Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Courtaboeuf, France) and analyzed with the automated sequencer ABI PRISM™ 3730 Genetic Analyser (Applied Biosystems). In order to rule out any PCR artefacts, all sequences were performed twice on both DNA strands. All nucleotide sequences were compared with that of reference strains 17 (HSV-1) and HG52 (HSV-2) (GenBank accession numbers X14112 and Z86099, respectively) using Seqscape v2.5 software (McGeoch et al., 1985; McGeoch et al., 1987).

2.4. Phenotypic resistance assay

HSV isolates were obtained from clinical samples by propagation in Vero cells or human fibroblasts (MRC5). A plaque reduction assay was performed in Vero cell culture for the measurement of the antiviral 50% effective concentration (EC_{50}) towards ACV (Merck, Lyon, France) and FOS (AstraZeneca, Rueil-Malmaison, France). HSV isolates were considered to be resistant at EC_{50} values $\geqslant 7$ μM and 330 μM for ACV and FOS, respectively, as previously reported (Burrel et al., 2010).

2.5. Statistical analyses

Statistical analyses were performed using MedCalc® software. Frequencies and means were compared by use of Fisher's exact test and Student's *t*-test, respectively. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of the study population

During the 4-year period, 211 HSV-positive clinical samples (94 HSV-1 and 117 HSV-2) recovered from 139 patients (median, 1 sample per patient; range, 1–7) were collected for antiviral resistance testing (Table 1). The study population included 73 (52.5%) males and 58 (41.7%) females. The median age was 47 years (range, 3–86) Gender and age were not specified for 8 (5.8%) and 4 (2.9%) patients, respectively. Eleven (7.9%) individuals had no underlying medical condition and 85 (61.2%) patients exhibited immunosuppression consisting in HIV-infection ($n = 39$), hematopoietic stem cell transplantation (HSCT; $n = 23$), solid organ transplantation (SOT; $n = 5$), hemopathy ($n = 10$), solid cancer ($n = 3$), and immune disorder ($n = 5$). Data concerning immune status were missing for 43 (30.9%) patients. Overall, 200 (94.8%) clinical samples were received from France (metropolitan France, France overseas departments and territories) and 11 (5.2%) from other Europe countries (Switzerland, Belgium, Czech Republic). Samples included 169 (80.1%) mucocutaneous lesions, with oro-facial ($n = 46$) and anogenital ($n = 76$) vesicles, skin swabs ($n = 30$), and keratitis lesions ($n = 17$), 8 (3.8%) cerebrospinal fluids, 5 (2.4%) bronchoalveolar lavage fluids, and 5 (2.4%) blood samples. Collection sites were not itemized for 24 (11.4%) of the clinical samples. Antiviral treatment information was unavailable for 39 (28.1%) patients for whom clinical samples were sent mainly by outside physicians. For the remaining patients, antiviral treatments consisted of intravenous ACV and/or oral VACV ($n = 97$), FOS ($n = 21$), ganciclovir (GCV) ($n = 3$), famciclovir (FCV) ($n = 1$) and cidofovir (CDV) ($n = 1$). Several patients were given successive-line regimens or bitherapy in case of antiviral resistance (Table 1).

Table 1
Characteristics of the study population.

Characteristics	Patients ($n = 139$)
Median age (years), [range]	47 [3–86]
Gender	
Male	73
Female	58
Non specified	8
Demographic data	
Paris and surroundings	93
Provincial town	36
French overseas	3
European countries	7
Immunosuppression	
HIV infection	39
HSCT	23
SOT	5
Hemopathy	10
Solid cancer	3
Immune disorder	5
None	11
Non specified	43
Samples[a]	
Mucocutaneous lesions	169
CSF	8
BAL	5
Blood	5
Non specified	24
Antiviral treatment[b]	
Acyclovir/valacyclovir	97
Foscarnet	21
Ganciclovir	3
Famciclovir	1
Cidofovir	1
Non specified	39

BAL: bronchoalveolar lavage fluid; CSF: cerebrospinal fluid; HIV: human immunodeficiency virus; HSCT: hematopoietic stem cells transplantation; SOT: solid organ transplantation.

[a] For some patients, sequential HSV-positive samples were tested by antiviral resistance testing assay in order to optimize antiviral treatment.

[b] Some patients received several lines of antiviral treatment or multitherapy during HSV infections.

3.2. Prevalence of HSV resistance to antivirals

HSV resistance to antivirals was investigated using a two-step procedure. Low HSV load in 22 clinical samples impaired the amplification of UL23 and UL30 genes. The first-line genotypic assay performed on the 189 remaining samples led to the unambiguous identification of 46 (24.3%) antiviral-sensitive HSV (22 HSV-1 and 24 HSV-2) and 75 (39.7%) antiviral-resistant HSV (27 HSV-1 and 48 HSV-2). HSV genotypic resistance concerned ACV alone ($n = 71$) or ACV and FOS ($n = 4$). The interpretation of viral sequences from 68 (36.0%) samples (34 HSV-1 and 34 HSV-2) deserved the performance a phenotypic assay, due to the detection of unpreviously reported or undefined mutations. HSV isolates could be recovered in cell culture from 23 (12.2%) clinical samples. The second-line phenotypic assay evidenced 12 antiviral-sensitive isolates (5 HSV-1 and 7 HSV-2) and 11 isolates (2 HSV-1 and 9 HSV-2) harboring resistance to ACV alone ($n = 8$) or ACV and FOS ($n = 3$). As a whole, this two-step procedure permitted to characterize 144 (76.2%) HSV-positive clinical samples with 58 (30.7%) HSV (27 HSV-1 and 31 HSV-2) exhibiting antiviral susceptibility and 86 (45.5%) HSV (29 HSV-1 and 57 HSV-2) exhibiting antiviral resistance. The genotypic antiviral profile remained undetermined for 45 (23.8%) HSV (27 HSV-1 and 18 HSV-2). The prevalence of drug resistance was significantly higher among HSV-2 isolates than among HSV-1 isolates (53.8% *vs.* 34.9%; $p = 0.012$). Clinical samples harboring HSV resistance were collected from 60 (43.2%) distinct patients (Table 2). For 27 (45%) of them, sequential HSV-positive samples were tested in order to optimize antiviral treatment. Among them, 12 patients exhibited successive excretions of ACV-resistant and ACV-sensitive HSV, 10 patients excreted HSV with different genotypic resistance profiles, and five patients excreted HSV with the same genotypic resistance profile. An antiviral treatment, based on (V)ACV, FCV, GCV and/or FOS, was reported for 46 (76.7%) of them. The highest rates of HSV resistance were observed among patients with hemopathy (70.0%) or HIV infection (61.5%). The prevalence of HSV resistance to antivirals did not differ significantly according to the age and gender of patients, or to the anatomic site of sampling ($p > 0.05$). Among transplant recipients, the prevalence of HSV resistance observed in HSCT patients tended to be higher that the one observed in SOT patients (70.0% *vs.* 25.0%; $p = 0.13$) (Table 2).

3.3. Genotypic characterization of HSV natural polymorphism and resistance to antivirals

During the 4-year period, the number of natural polymorphisms identified among clinical samples was 21 for HSV-1 and 9 for HSV-2 in TK, and 22 for HSV-1 and 23 for HSV-2 in Pol (Fig. 1 and Supplementary Table). Results of genotypic resistance analysis are summarized in Table 3. TK mutations conferring resistance to ACV were evidenced for 74 (39.2%) HSV (26 HSV-1 and 48 HSV-2). Those mutations consisted of 59 (79.7%) G/C insertions/deletions in UL23 gene (57 led to a premature stop codon), 14 (18.9%) previously described missense point mutations leading to amino acid changes, and one (1.4%) 248-amino-acid-long deletion in a HSV-2 isolate. TK amino acid changes conferring resistance to ACV were P84S (Saijo et al., 2002), D162N (van Velzen et al., 2013), L170P (Burrel et al., 2012; Burrel et al., 2010), R176W (Burrel et al., 2012; Burrel et al., 2010; Suzutani et al., 2003), S181N (Duan et al., 2008) and C336Y (Bestman-Smith et al., 2001; Burrel et al., 2010; Larder et al., 1987; Saijo et al., 2002; van Velzen et al., 2013) for HSV-1, G201D (Harris et al., 2003) and T288M (Gaudreau et al., 1998) for HSV-2. Two amino acid changes within Pol conferring drug-resistance were observed among 4 (6.6%) HSV-1: S724N (Burrel et al., 2010) ($n = 3$) and T821M (Andrei et al., 2007; Bestman-Smith and Boivin, 2003) ($n = 1$) (Table 3 and Fig. 1). As a whole, 95% of HSV isolates were resistant only to ACV.

Apart from the mutational changes described above and surely related to natural polymorphism or drug-resistance, 91 unpreviously reported mutations (10 in HSV-1 TK, 21 in HSV-2 TK, 28 in HSV-1 Pol and 32 in HSV-2 Pol) were identified in the present study (Table 4 and Fig. 1). The potential role of these novel changes was analyzed according to their location within TK or Pol relatively to conserved regions, the existence of closely located mutations with previously assigned roles, and the results of phenotypic assay. Considering TK, 7 changes (4 for HSV-1 and 3 for HSV-2), all of them located outside conserved regions (L29P, D303N, I358V and A375P for HSV-1, R76C, D274H and L341P for HSV-2) except one (R220T for HSV-2), were considered as potential natural polymorphisms, and eight changes (2 for HSV-1 and 6 for HSV-2) were considered to confer potential drug-resistance, with 6 (H58Y for HSV-1, G61W, E84G/K, S169P and R221C for HSV-2) and 2 (M121L for HSV-1 and A94V for HSV-2) of them located within and outside conserved domains, respectively. All 42 potential natural polymorphisms described in Pol (24 for HSV-1 and 18 for HSV-2) were located outside conserved domains with exception of I942L (domain VII in HSV-2) (Table 4 and Fig. 1). Furthermore, 2 amino acid insertions, InsA650-651 and InsDEDGDED684-685, located between domains δ-C and II were newly evidenced as potential natural polymorphisms in HSV-2 Pol (Table 4 and Fig. 1). The 14 novel changes in Pol potentially conferring resistance to antivirals (4 for HSV-1 and 10 for HSV-2), with exception of D307N and A915V (HSV-2), were located in conserved domains: S599L (HSV-1) and Q619R (HSV-2) in δ-C domain; F733C (HSV-1), A724V and Q732R (HSV-2) in domain II; S775N (HSV-1) in domain VI; N820S, Q829R, T843A, T844I and G846A (HSV-2) in domain III; R881C (HSV-1) in domain I (Fig. 1). Ultimately, 18 novel mutations remained of unclear significance: Y101C, D215N, T245K, and

Table 2
Characteristics of the patients according to HSV species and susceptibility to antivirals.

Characteristics	HSV-1		HSV-2	
	Drug-sensitive ($n = 27$)	Drug-resistant ($n = 29$)	Drug-sensitive ($n = 31$)	Drug-resistant ($n = 57$)
Median age (years), [range]	48.5 [8–79]	18 [3–81]	47 [7–70]	50 [7–76]
Gender				
Male	10	17	12	22
Female	10	4	11	14
Type of graft				
HSCT	5	13	1	1
SOT	2	0	1	1
Anatomic site				
Oro-facial	18	16	1	0
Ano-genital	1	2	20	31

HSCT: hematopoietic stem cells transplantation; SOT: solid organ transplantation.

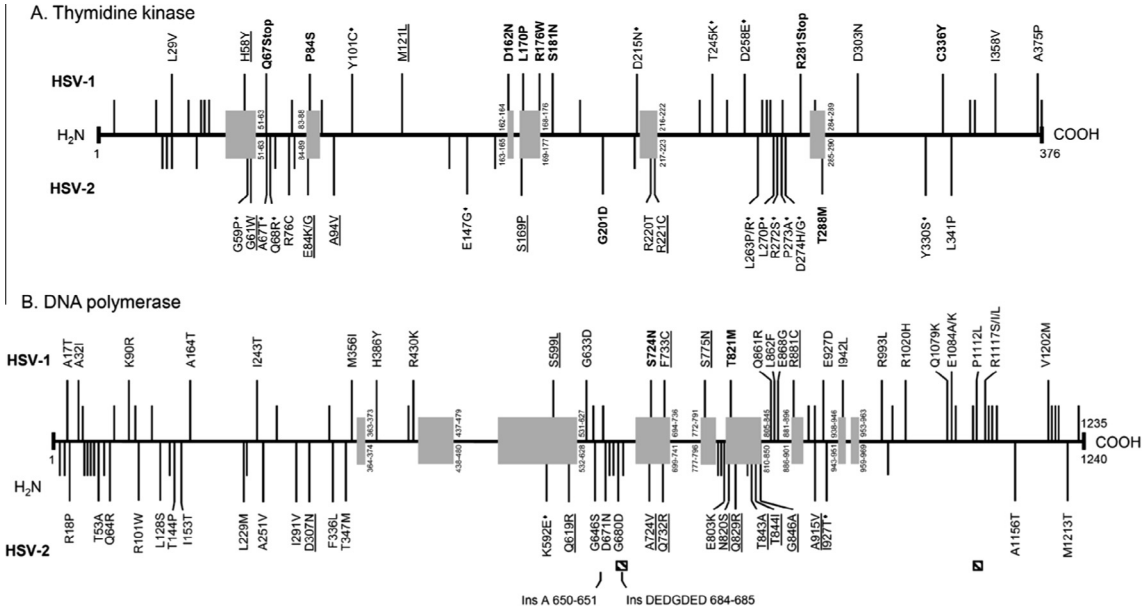

Fig. 1. Mutation map (to scale) of thymidine kinase (TK) (A) and DNA polymerase (B) among HSV-1 and HSV-2 isolates. For each viral enzyme, conserved regions and functional domains are indicated by the gray boxes, and mutations identified in the present study are represented separately for HSV-1 (above) and HSV-2 (below). HSV TK contains an ATP binding site (codons 51 to 63) and a nucleoside binding site (codons 168 to 176 for HSV-1 and 169 to 177 for HSV-2). DNA polymerase reveals a series of nine conserved domains represented along protein diagram and named as follows: Exo I, domain IV, δ-C region, domain II, domain VI, domain III, domain I, domain VII and domain V. Short vertical bars correspond to previously reported natural polymorphisms and long vertical bars with unmarked amino acid changes correspond to newly reported potential natural polymorphisms. Regarding HSV-2 DNA polymerase, hatched boxes indicate amino acid insertions/deletions related to natural polymorphism and previously reported. Amino acid changes in bold correspond to previously reported drug-resistance mutations, underlined changes correspond to newly described mutations potentially conferring resistance to antivirals. The role of amino acid changes marked with diamond remains unclear.

D258E in HSV-1 TK; G59P, A67T, Q68R, E147G, L263P/R, L270P, R272S, P273A, D274G/H and Y330S in HSV-2 TK; K592E and I927T in HSV-2 Pol (Table 4 and Fig. 1).

4. Discussion

Between June 2008 and May 2012, 211 clinical samples recovered from 139 patients suffering from persistent HSV disease despite a well-conducted antiviral treatment were investigated in our laboratory for HSV resistance to antivirals using a genotypic/phenotypic two-step procedure. Under these conditions, HSV antiviral susceptibility profile could be determined readily by genotypic assay for 64% of samples tested, and the phenotypic assay permitted the identification of antiviral susceptibility profile for 12.2% additional samples. As a whole, we were able to inform clinicians about HSV antiviral susceptibility for 76.2% of samples received during the study period. Surprisingly, the prolonged antiviral treatment received by most of the patients was not consistently associated with antiviral resistance, underlying the importance of HSV-specific immune response in HSV infections. However, one limitation of this study is that therapeutic data were not provided in the standardized form accompanying biological samples for 39 patients (28.1%).

When considering only the first-line genotypic assay, results could be interpreted for 59% of HSV-1-positive samples and 68% of HSV-2-positive samples. This rate raised almost 80% when considering only HSV-1 TK sequencing results, which is in accordance with a previous study reporting a success rate of genotypic assay interpretation of 84% (Frobert et al., 2008). Genotypic antiviral resistance assays constitute an attractive approach for the detection of drug-resistance in a clinically relevant frame in comparison with time-consuming and tedious phenotypic assays (Burrel et al., 2010). However, in this survey, the interpretation of genotypic resistance results remained impaired for 36% of clinical samples. Therefore, our knowledge concerning UL23 and UL30 gene alterations from both HSV-1 and HSV-2 has to be greatly enriched and deserves further multicentric studies with centers deeply involved in the field of HSV resistance to antivirals.

In the present study, the prevalence of HSV resistance to antivirals was significantly higher among HSV-2 isolates than among HSV-1 isolates, which is in contrast with the similar prevalence reported by a French surveillance network (Danve-Szatanek et al., 2004). This discrepancy may be linked to the following reason: conversely to this previous study for which HSV resistance to antivirals was investigated in all viral strains recovered from unselected patients, only clinical samples from patients receiving antiviral treatment without improvement of herpetic lesions were included in the present study. The prevalence of HSV resistance to antivirals tended to be higher in HSCT than in SOT patients, in accordance with previous results (Danve-Szatanek et al., 2004). Among patients with sequential clinical samples, different patterns of antiviral resistance were observed: alternating antiviral-resistance and antiviral-susceptibility, HSV resistance associated either with different genotypic resistance profiles or with always the same one. This phenomenon has been previously reported in sequential HSV-1 corneal isolates from patients with recurrent herpetic keratitis (Duan et al., 2009). Moreover, the recovery of different drug-resistant HSV isolates during successive episodes of HSV infection suggests that drug-resistant HSV can establish latency and reactivate intermittently (van Velzen et al., 2012).

Mutations located in HSV TK account for 95% of the cases of ACV-resistance (Piret and Boivin, 2011). Hitherto, it was reported that half of these mutations consisted in nucleotide insertions/deletions leading to the shift of the translational reading frame of UL23 gene, and half of them in amino acid substitutions (Gaudreau et al., 1998; Morfin et al., 2000). In our study, sequence analysis of UL23 gene revealed that 79.7% of ACV-resistance profiles consisted in a frameshift within open reading frame or in a premature stop codon, whereas only 18.9% resulted from a point mutation previously implicated in resistance to ACV (Table 3 and Fig. 1). Of note, this proportion of nucleotide insertions/deletions within UL23

Table 3
Genotypic resistance patterns of HSV TK and DNA polymerase.

HSV-1 (n = 83)		HSV-2 (n = 106)	
Mutation	No of samples (%)	Mutation	No of samples (%)
TK nucleotide mutation		*TK nucleotide mutation*	
Del nt C553 (Frameshift)	2 (2.4)	Del aa 1-248	1 (0.9)
Del nt G856 (Frameshift)	1 (1.2)	nt C664T (Q222Stop)	1 (0.9)
nt C199T (Q67Stop)	1 (1.2)	Del nt G222 (M86Stop)	1 (0.9)
Del nt A187 (M85Stop)	2 (2.4)	Del nt G280 (L98Stop)	4 (3.8)
Del nt G277 (V120Stop)	1 (1.2)	Del nt G439 (M183Stop)	1 (0.9)
nt C526T (R176Stop)	1 (1.2)	Ins nt G439 (D229Stop)	1 (0.9)
Del nt G436 (M182Stop)	1 (1.2)	Del nt C452 (M183Stop)	1 (0.9)
Ins nt G436-437 (T183Stop)	2 (2.4)	Del nt C467 (M183Stop)	10 (9.4)
Ins nt G436-437 (D228Stop)	1 (1.2)	Del nt C556 (L263Stop)	8 (7.5)
nt C841T (R281Stop)	2 (2.4)	Del nt C590 (L263Stop)	1 (0.9)
Del nt CG 753-754 (Y305Stop)	1 (1.2)	Del nt CCTG 651-654 (L263Stop)	1 (0.9)
Del nt G782 (L263Stop)	1 (1.2)	Del nt G782 (L263Stop)	2 (1.9)
		Ins nt G440-441 (D229Stop)	7 (6.6)
		Del nt G440 (D229Stop)	2 (1.9)
		Ins nt C556-557 (D229Stop)	1 (0.9)
		Del 13-nt 814-826 (M348Stop)	1 (0.9)
		Del 13-nt 821-833 (M348Stop)	1 (0.9)
Total	**16 (19.3)**	Total	**44 (41.5)**
TK aa substitution		*TK aa substitution*	
P84S	3 (3.6)	G201D	3 (2.8)
D162N	1 (1.2)	T288M	1 (0.9)
L170P	2 (2.4)		
R176W	2 (2.4)		
S181N	1 (1.2)		
C336Y	1 (1.2)		
Total	**10 (8.3)**	Total	**4 (3.8)**
DNA polymerase aa substitution[a]		*DNA polymerase aa substitution*	
S724N	3 (4.9)		
T821M	1 (1.6)		
Total	**4 (6.6)**	Total	**0 (0.0)**

Amino acid changes or premature codon stop associated with aciclovir-resistance have been previously described: P84S Saijo et al. (2002), D162N van Velzen et al. (2013), L170P Burrel et al. (2012, 2010), van Velzen et al. (2013), R176W (Burrel et al. (2012, 2010), Suzutani et al. (2003), van Velzen et al. (2013), S181N Duan et al. (2008), L263Stop Chibo et al. (2004), R281Stop Gaudreau et al. (1998) and C336Y Bestman-Smith et al. (2001), Burrel et al. (2010), Darby et al. (1981), Saijo et al. (2002), van Velzen et al. (2013) for HSV-1, M183Stop Burrel et al. (2010), G201D Harris et al. (2003), D229Stop Bestman-Smith et al. (2001), Burrel et al. (2010) and T288M Gaudreau et al. (1998) for HSV-2 within TK.
Amino acid changes within ADN polymerase conferring drug-resistance were observed among HSV-1: S724N Burrel et al. (2010) and T821M Andrei et al. (2007), Bestman-Smith and Boivin (2003).
All nucleotide sequences were compared with that of reference strains 17 (HSV-1) and HG52 (HSV-2) (GenBank accession numbers X14112 and Z86099, respectively) (McGeoch et al., 1985, 1987). aa: amino acid; Del: deletion; Ins: insertion; nt: nucleotide; TK: thymidine kinase.
[a] UL30 gene could not be amplified in 22 HSV-1-positive and 5 HSV-2-positive clinical samples because of low HSV load.

gene conferring resistance to ACV remained similar (i.e., 76.2%) when sequential drug-resistant viruses isolated from the same patients were omitted. This discrepancy with previous data may be due to the small numbers of HSV strains characterized in the two previous studies (30 and 11, respectively) in comparison with the present one (n = 189) (Gaudreau et al., 1998; Morfin et al., 2000). Regarding HSV Pol, S724N and T821M changes, conferring resistance to ACV/FOS and ACV, respectively, were identified in HSV-1 strains (Table 3 and Fig. 1) (Andrei et al., 2007; Bestman-Smith and Boivin, 2003).

To date, the genetic variability of HSV TK and Pol has been widely described. Numerous previously reported natural polymorphisms were detected in this study (Supplementary Table and Fig. 1) (Bohn et al., 2011; Burrel et al., 2010; Chibo et al., 2004; Frobert et al., 2005; Morfin et al., 2000; Sauerbrei et al., 2010; Schmit and Boivin, 1999; Stranska et al., 2005). A recent study raised doubt on R41H within HSV-1 TK as a natural polymorphism (van Velzen et al., 2013). Thus, this mutation was shown to impair ACV phosphorylation by recombinant HSV-1 TK in a functional mass spectrometry assay. However, R41H was previously reported to have no impact on BrdU phosphorylation activity by recombinant HSV-1 TK using an enzyme linked immunosorbent assay (Sauerbrei et al., 2012). Further studies using marker rescue experiments are warranted to assess the exact role of R41H within HSV-1 TK.

In addition, 51 novel potential natural polymorphisms, including two amino acid insertions, were also detected both in TK (4 for HSV-1 and 3 for HSV-2) and in Pol (24 for HSV-1 and 20 for HSV-2) (Table 4 and Fig. 1). The association of these mutations with resistance to antivirals was not likely since they were located outside conserved domains and/or close to previously characterized natural polymorphisms. In the present study, 22 unpreviously described changes in TK and Pol, situated in conserved domains or located nearby changes previously implicated in drug-resistance, were considered to confer potential resistance to antivirals (Fig. 1). Furthermore, the phenotypic characterization of corresponding HSV isolates recovered in cell culture evidenced resistance to ACV and/or FOS for some of them (Table 4). The role of some novel mutations detected in TK and Pol remained undetermined because the phenotypic characterization of HSV isolates could not be obtained in parallel of the genotypic assay (Fig. 1). However, all these novel mutations described in this study deserve now further investigations, including site-directed mutagenesis or marker transfer experiments, to assess their role as natural polymorphisms or in resistance to antivirals (Burrel et al., 2012; Piret and Boivin, 2011; Sauerbrei et al., 2012).

Despite the availability of phenotypic data, the interpretation of the role of some unpreviously reported mutations for HSV-2 isolates remained tricky (Table 4 and Fig. 1). The genotypic assay of isolate 11 (HSV-2), exhibiting phenotypic resistance to ACV and

Table 4
Previously described resistance mutations and novel mutations potentially associated with resistance to antivirals or natural polymorphism identified within HSV-1 and HSV-2 isolates.

No.	HSV species	Phenotypic susceptibility (EC_{50} [μM])[a]		Resistance mutations[b]	Unknown mutations[c]			
		ACV	FOS		Potential resistance mutations	Potential natural polymorphisms	Mutations of unclear significance	Comments[d]
1	HSV-1	**>50**	**>330**	–	TK: M121L Pol: S599L*	–	–	TK: M121R (ACV-R) Gibbs et al. (1988), Larder et al. (1987), Sauerbrei et al. (2011) Pol: E597 K/D (ACV-R; FOS-S/R) (Gibbs et al. (1988), Larder et al. (1987)
2	HSV-1	**14**	**>330**	Pol: S724N	–	Pol: E1084A	–	Pol: E1082K (NP) Burrel et al. (2010)
3	HSV-1	**24**	89.1	TK: C336Y	–	Pol: H386Y, Q1079K, E1084K	–	Pol: I352V (NP), V1077L (NP), E1082K (NP) Bohn et al. (2011), Burrel et al. (2010)
4	HSV-1	**11**	89.4	TK: D162N*	–	Pol: M356I	–	TK: D162A/H (ACV-R) Chibo et al. (2004), Stranska et al. (2005) Pol: I352V (NP) Burrel et al. (2010)
5	HSV-1	**>10**	107.3	TK: R281Stop	Pol: F733C*	Pol: E868G	–	Pol: S724 N (ACV-R; FOS-S/R), A870G (NP) Bestman-Smith and Boivin, (2003), Burrel et al. (2010), Gibbs et al. (1988), Larder et al. (1987)
6	HSV-1	**>10**	27	–	Pol: S775N*	–	–	Pol: E771Q (ACV-R; FOS-S), L774F (ACV-R; FOS-R) Chibo et al. (2004), Hwang et al. (2004)
7	HSV-1	1.7	<66	–	–	Pol: I942L*	–	Pol: Y941H (ACV-R; FOS-R/S) Bestman-Smith and Boivin (2003), Hwang et al. (1992), Pelosi et al. (1998)
8	HSV-1	<1	<66	–	–	Pol: A17T, A32I	–	Pol: A20V (NP); S33G (NP) Burrel et al. (2010), Chibo et al. (2004), Sauerbrei et al. (2010)
9	HSV-1	<1	<66	–	–	TK: A375P	–	TK: N376H/P/T (NP) Burrel et al. (2010), Chibo et al. (2004), Kudo et al. (1998), Sauerbrei et al. (2010)
10	HSV-1	<1	45.5	–	–	Pol: Q861R	–	Pol: A870G (NP) Burrel et al. (2010)
11	HSV-2	**>50**	**>330**	TK: M183Stop	–	Pol: Q64R, G680D	TK: A67T Pol: K592E*	TK: T65N (ACV-R, HSV-1) Saijo et al. (2002) Pol: L60P (NP), R628C (ACV-R), G680R (NP) Burrel et al. (2010), Chibo et al. (2004), Sauerbrei et al. (2010)
12	HSV-2	**>50**	**>330**	TK: L263Stop	Pol: T844I*	–	–	Pol: L850I (ACV-S, FOS-R) Schmit and Boivin (1999)
13	HSV-2	**>50**	**>330**	–	TK: A94V Pol: Q732R*		–	TK: Q105P (ACV-R) Chatis and Crumpacker (1991) Pol: S729N (ACV-R, FOS-R) Schmit and Boivin (1999)
14	HSV-2	**17.6**	**>330**	–	Pol: Q732R*	TK: D274H Pol: T53A, T144P, G646S	–	TK: T288M (ACV-R) Gaudreau et al. (1998) Pol: S729N (ACV-R, FOS-R) Schmit and Boivin (1999)
15	HSV-2	**>50**	66	TK: L263Stop	–	Pol: R101W, L128S	–	Pol: E139K (NP) Burrel et al. (2010), Chibo et al. (2004), Sauerbrei et al. (2010)
16	HSV-2	**>50**	<66	TK: L263Stop	–	Pol: A251V	–	Pol: N286Y (NP), A232T (NP) Burrel et al. (2010), Sauerbrei et al. (2010)
17	HSV-2	**>50**	<66	TK: G201D	–	Pol: A1156T	–	Pol: E1149G (NP) Burrel et al. (2010)
18	HSV-2	**>50**	103.3	–	TK: G61W*	–	–	TK: G61V (ACV-R, HSV-1), K62N (ACV-R, HSV-1) Gaudreau et al. (1998), Liu and Summers (1988)
19	HSV-2	**>50**	201	–	TK: S169P*	–	Pol: K592E*	TK: L170P, L158P (ACV-R) Burrel et al. (2012); Harris et al. (20030 Pol: R628C (ACV-R) Chibo et al. (2004)
20	HSV-2	**>50**	209	TK: L98stop	Pol: A724V*	–	–	Pol: A724T (ACV-S; FOS-R) Schmit and Boivin (1999), A719T (ACV-R; FOS-R, HSV-1) Larder et al. (1987)
21	HSV-2	**>50**	214.5	–	Pol: A915V	TK: L341P	–	TK: R363C (NP) Stranska et al. (2005) Pol: A910V (ACV-S; FOS-R, HSV-1) Saijo et al. (2005)
22	HSV-2	**>50**	256.7	TK: L98stop	Pol: T843A*	Pol: F336L	–	Pol: G324E (NP) Burrel et al. (2010), L850I (ACV-S, FOS-R) Schmit and Boivin (1999)
23	HSV-2	**47**	<66	TK: L263Stop	–	Pol: T53A, T144P, G646S, Ins A 650-651	–	Pol: H49Y (NP), E139K (NP), D672N (HSV-1; NP), Ins DGDE 686-687 (NP) Burrel et al. (2010), Chibo et al. (2004), Sauerbrei et al. (2010)
24	HSV-2	**43.1**	149.5	TK: D229Stop	–	–	Pol: I927T	Pol: D912V/A (ACV-R, FOS-R) Schmit and Boivin (1999)
25	HSV-2	**25**	<66	–	TK: A94V	–	–	TK: Q105P (ACV-R) Chatis and Crumpacker (1991)
26	HSV-2	**20**	66.6	–	TK: R221C*	–	–	TK: R221H (ACV-R) Sauerbrei et al. (2010), R220C (HSV-1; ACV-R) Chibo et al. (2004), Stranska et al. (2005)
27	HSV-2	**9.1**	71	–	Pol: Q829R*	–	–	
28	HSV-2	**9**	85.1	–	Pol: D307N	–	–	
29	HSV-2	6.8	145.2	–	–	TK: R220T*	–	TK: R220K (NP) Chibo et al. (2004)
30	HSV-2	3.1	<66	–	–	–	TK: G59P*	TK: G59P (ACV-R) Schmit and Boivin (1999)
31	HSV-2	5.1	<66	–	–	Pol: D671N	–	Pol: D676G/N (NP) Burrel et al. (2010)
32	HSV-2	5.8	82.8	–	–	Pol: M1213T	–	Pol: A1223V (NP) Burrel et al. (2010)

ACV, acyclovir; ACV-R/S, acyclovir resistance/susceptibility; FOS, foscarnet; FOS-R/S, foscarnet resistance/susceptibility; NA, not available; NP, natural polymorphism; Pol, DNA polymerase; TK, thymidine kinase.

[a] The EC_{50} cutoff values for resistance to ACV and FOS were 7 and 330 μM, respectively (Burrel et al., 2010). EC_{50} associated with resistance to antivirals are indicated in bold.

[b] Previously described resistance-associated mutations.

[c] Mutations unpreviously assigned either to natural polymorphism or to resistance to antivirals.

[d] Amino acid substitutions previously reported to constitute natural polymorphisms or resistance mutations and located either at the same codon or next to the codon of the novel mutation identified in this study.

* Mutations located in conserved regions or catalytic domains of TK or Pol.

FOS, revealed A67T change, located close to the ATP-binding site of TK, and K592E change within the δ-C region of Pol (Table 4). To date, these two amino acid changes have not been involved in HSV-2 resistance to antivirals. Since isolate 11 (HSV-2) isolate harbored a frameshift mutation leading to a premature stop codon in TK (M183Stop), it was difficult to interpret the role of A67T change regarding ACV-resistance. However, K592E change in Pol may confer resistance to FOS. Consistent with this, K592E change was also identified in Pol of isolate 19 (HSV-2) for which an elevation of FOS EC_{50} (201 μM) was evidenced. Isolate 24 (HSV-2), with ACV-resistant/FOS-sensitive phenotype, contained I927T change between the conserved regions I and VII of Pol. This change has never been reported so far. Nevertheless, the closely located D912A/N/V changes have been previously reported to be related to antiviral resistance phenotype in HSV-2 isolates (Chibo et al., 2004; Schmit and Boivin, 1999). The precise role of I927T change remained unclear and required to be assessed, especially as the premature stop codon in position 229 of TK was at least in part responsible for ACV-resistance. G59P change has been previously reported within TK from ACV-resistant strain (Bestman-Smith et al., 2001). In our study, this change was evidenced the ACV-sensitive H2-20 isolate (Table 4). Despite this change is located inside ATP-binding site, its role regarding TK activity remains to be clarified.

In conclusion, the results of this retrospective 4-year period survey support the need for a continuous surveillance of the emergence of HSV resistance to antivirals and the mutations associated with this phenomenon. This is essential to establish a robust and exhaustive database for HSV drug-resistance, as already performed for cytomegalovirus (CMV) (Chevillotte et al., 2010). The present observational survey provides numerous mutations both in TK and Pol for which the role in conferring HSV resistance to antivirals remains speculative. Further characterization of these mutations by marker transfer experiments using bacterial artificial chromosome (BAC) technology is now highly required. However, conversely to CMV (Chou, 2010; Martin et al., 2006), BAC technology for drug-resistance study is not yet available for HSV. Therefore, the evaluation of enzymatic activity of recombinant viral proteins using non-radioactive methods, as previously reported for TK, will be useful in that field (Burrel et al., 2012; Malartre et al., 2012; Sauerbrei et al., 2012).

Transparency declaration

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.09.012.

References

Andrei, G., Fiten, P., Froeyen, M., De Clercq, E., Opdenakker, G., Snoeck, R., 2007. DNA polymerase mutations in drug-resistant herpes simplex virus mutants determine in vivo neurovirulence and drug-enzyme interactions. Antiviral Therapy 12, 719–732.

Bacon, T.H., Boon, R.J., Schultz, M., Hodges-Savola, C., 2002. Surveillance for antiviral-agent-resistant herpes simplex virus in the general population with recurrent herpes labialis. Antimicrobial Agents and Chemotherapy 46, 3042–3044.

Bestman-Smith, J., Boivin, G., 2003. Drug resistance patterns of recombinant herpes simplex virus DNA polymerase mutants generated with a set of overlapping cosmids and plasmids. Journal of Virology 77, 7820–7829.

Bestman-Smith, J., Schmit, I., Papadopoulou, B., Boivin, G., 2001. Highly reliable heterologous system for evaluating resistance of clinical herpes simplex virus isolates to nucleoside analogues. Journal of Virology 75, 3105–3110.

Bohn, K., Zell, R., Schacke, M., Wutzler, P., Sauerbrei, A., 2011. Gene polymorphism of thymidine kinase and DNA polymerase in clinical strains of herpes simplex virus. Antiviral Therapy 16, 989–997.

Burrel, S., Deback, C., Agut, H., Boutolleau, D., 2010. Genotypic characterization of UL23 thymidine kinase and UL30 DNA polymerase of clinical isolates of herpes simplex virus: natural polymorphism and mutations associated with resistance to antivirals. Antimicrobial Agents and Chemotherapy 54, 4833–4842.

Burrel, S., Bonnafous, P., Hubacek, P., Agut, H., Boutolleau, D., 2012. Impact of novel mutations of herpes simplex virus 1 and 2 thymidine kinases on acyclovir phosphorylation activity. Antiviral Research 96, 386–390.

Chevillotte, M., von Einem, J., Meier, B.M., Lin, F.M., Kestler, H.A., Mertens, T., 2010. A new tool linking human cytomegalovirus drug resistance mutations to resistance phenotypes. Antiviral Research 85, 318–327.

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

Chou, S., 2010. Recombinant phenotyping of cytomegalovirus UL97 kinase sequence variants for ganciclovir resistance. Antimicrobial Agents and Chemotherapy 54, 2371–2378.

Collins, P., Ellis, M.N., 1993. Sensitivity monitoring of clinical isolates of herpes simplex virus to acyclovir. Journal of Medical Virology Suppl. 1, 58–66.

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

Darby, G., Field, H.J., Salisbury, S.A., 1981. Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature 289, 81–83.

Duan, R., de Vries, R.D., Osterhaus, A.D., Remeijer, L., Verjans, G.M., 2008. Acyclovir-resistant corneal HSV-1 isolates from patients with herpetic keratitis. The Journal of Infectious Diseases 198, 659–663.

Duan, R., de Vries, R.D., van Dun, J.M., van Loenen, F.B., Osterhaus, A.D., Remeijer, L., Verjans, G.M., 2009. Acyclovir susceptibility and genetic characteristics of sequential herpes simplex virus type 1 corneal isolates from patients with recurrent herpetic keratitis. The Journal of Infectious Diseases 200, 1402–1414.

Frobert, E., Ooka, T., Cortay, J.C., Lina, B., Thouvenot, D., Morfin, F., 2005. Herpes simplex virus thymidine kinase mutations associated with resistance to acyclovir: a site-directed mutagenesis study. Antimicrobial Agents and Chemotherapy 49, 1055–1059.

Frobert, E., Cortay, J.C., Ooka, T., Najioullah, F., Thouvenot, D., Lina, B., Morfin, F., 2008. Genotypic detection of acyclovir-resistant HSV-1: characterization of 67 ACV-sensitive and 14 ACV-resistant viruses. Antiviral Research 79, 28–36.

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

Gibbs, J.S., Chiou, H.C., Bastow, K.F., Cheng, Y.C., Coen, D.M., 1988. Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug recognition. Proceedings of the National Academy of Sciences of the United States of America 85, 6672–6676.

Harris, W., Collins, P., Fenton, R.J., Snowden, W., Sowa, M., Darby, G., 2003. Phenotypic and genotypic characterization of clinical isolates of herpes simplex virus resistant to aciclovir. The Journal of General Virology 84, 1393–1401.

Hwang, C.B., Ruffner, K.L., Coen, D.M., 1992. A point mutation within a distinct conserved region of the herpes simplex virus DNA polymerase gene confers drug resistance. Journal of Virology 66, 1774–1776.

Hwang, Y.T., Zuccola, H.J., Lu, Q., Hwang, C.B., 2004. A point mutation within conserved region VI of herpes simplex virus type 1 DNA polymerase confers altered drug sensitivity and enhances replication fidelity. Journal of Virology 78, 650–657.

Kudo, E., Shiota, H., Naito, T., Satake, K., Itakura, M., 1998. Polymorphisms of thymidine kinase gene in herpes simplex virus type 1: analysis of clinical isolates from herpetic keratitis patients and laboratory strains. Journal of Medical Virology 56, 151–158.

Larder, B.A., Kemp, S.D., Darby, G., 1987. Related functional domains in virus DNA polymerases. The EMBO Journal 6, 169–175.

Liu, Q.Y., Summers, W.C., 1988. Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. Virology 163, 638–642.

Malartre, N., Boulieu, R., Falah, N., Cortay, J.C., Lina, B., Morfin, F., Frobert, E., 2012. Effects of mutations on herpes simplex virus 1 thymidine kinase functionality: an in vitro assay based on detection of monophosphate forms of acyclovir and thymidine using HPLC/DAD. Antiviral Research 95, 224–228.

Martin, M., Gilbert, C., Covington, E., Boivin, G., 2006. Characterization of human cytomegalovirus (HCMV) UL97 mutations found in a valganciclovir/oral

ganciclovir prophylactic trial by use of a bacterial artificial chromosome containing the HCMV genome. The Journal of Infectious Diseases 194, 579–583.
McGeoch, D.J., Dolan, A., Donald, S., Rixon, F.J., 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. Journal of Molecular Biology 181, 1–13.
McGeoch, D.J., Moss, H.W., McNab, D., Frame, M.C., 1987. DNA sequence and genetic content of the HindIII l region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein G, and evolutionary comparisons. The Journal of General Virology 68 (Pt 1), 19–38.
Morfin, F., Souillet, G., Bilger, K., Ooka, T., Aymard, M., Thouvenot, D., 2000. Genetic characterization of thymidine kinase from acyclovir-resistant and -susceptible herpes simplex virus type 1 isolated from bone marrow transplant recipients. The Journal of Infectious Diseases 182, 290–293.
Pelosi, E., Rozenberg, F., Coen, D.M., Tyler, K.L., 1998. A herpes simplex virus DNA polymerase mutation that specifically attenuates neurovirulence in mice. Virology 252, 364–372.
Piret, J., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrobial Agents and Chemotherapy 55, 459–472.
Saijo, M., Suzutani, T., De Clercq, E., Niikura, M., Maeda, A., Morikawa, S., Kurane, I., 2002. Genotypic and phenotypic characterization of the thymidine kinase of ACV-resistant HSV-1 derived from an acyclovir-sensitive herpes simplex virus type 1 strain. Antiviral Research 56, 253–262.
Saijo, M., Suzutani, T., Morikawa, S., Kurane, I., 2005. Genotypic characterization of the DNA polymerase and sensitivity to antiviral compounds of foscarnet-resistant herpes simplex virus type 1 (HSV-1) derived from a foscarnet-sensitive HSV-1 strain. Antimicrobial Agents and Chemotherapy 49, 606–611.
Sauerbrei, A., Deinhardt, S., Zell, R., Wutzler, P., 2010. Phenotypic and genotypic characterization of acyclovir-resistant clinical isolates of herpes simplex virus. Antiviral Research 86, 246–252.
Sauerbrei, A., Bohn, K., Heim, A., Hofmann, J., Weissbrich, B., Schnitzler, P., Hoffmann, D., Zell, R., Jahn, G., Wutzler, P., Hamprecht, K., 2011. Novel resistance-associated mutations of thymidine kinase and DNA polymerase genes of herpes simplex virus type 1 and type 2. Antiviral Therapy 16, 1297–1308.
Sauerbrei, A., Liermann, K., Bohn, K., Henke, A., Zell, R., Gronowitz, S., Wutzler, P., 2012. Significance of amino acid substitutions in the thymidine kinase gene of herpes simplex virus type 1 for resistance. Antiviral Research 96, 105–107.
Schmit, I., Boivin, G., 1999. Characterization of the DNA polymerase and thymidine kinase genesof herpes simplex virus isolates from AIDS patients in whom acyclovirand foscarnet therapy sequentially failed. The Journal of Infectious Diseases 180, 487–490.
Stranska, R., Schuurman, R., Nienhuis, E., Goedegebuure, I.W., Polman, M., Weel, J.F., Wertheim-Van Dillen, P.M., Berkhout, R.J., van Loon, A.M., 2005. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. Journal of Clinical Virology 32, 7–18.
Suzutani, T., Ishioka, K., De Clercq, E., Ishibashi, K., Kaneko, H., Kira, T., Hashimoto, K., Ogasawara, M., Ohtani, K., Wakamiya, N., Saijo, M., 2003. Differential mutation patterns in thymidine kinase and DNA polymerase genes of herpes simplex virus type 1 clones passaged in the presence of acyclovir or penciclovir. Antimicrobial Agents and Chemotherapy 47, 1707–1713.
van Velzen, M., van Loenen, F.B., Meesters, R.J., de Graaf, M., Remeijer, L., Luider, T.M., Osterhaus, A.D., Verjans, G.M., 2012. Latent acyclovir-resistant herpes simplex virus type 1 in trigeminal ganglia of immunocompetent individuals. The Journal of Infectious Diseases 205, 1539–1543.
van Velzen, M., Missotten, T., van Loenen, F.B., Meesters, R.J., Luider, T.M., Baarsma, G.S., Osterhaus, A.D., Verjans, G.M., 2013. Acyclovir-resistant herpes simplex virus type 1 in intra-ocular fluid samples of herpetic uveitis patients. Journal of Clinical Virology 57, 215–221.