Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 79 (2008) 28–36
Antiviral Research
www.elsevier.com/locate/antiviral

Genotypic detection of acyclovir-resistant HSV-1: Characterization of 67 ACV-sensitive and 14 ACV-resistant viruses

Emilie Frobert [a,b,*], Jean-Claude Cortay [b], Tadamasa Ooka [b], Fatiha Najioullah [c], Danielle Thouvenot [a,b], Bruno Lina [a,b], Florence Morfin [a,b]

[a] *Laboratoire de Virologie, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, 59 boulevard Pinel, F-69677 Bron Cedex, Lyon, France*
[b] *FRE 3011 Virologie et Pathogénèse Humaine, CNRS, Université Lyon 1, rue Guillaume Paradin, F-69372 Lyon Cedex 08, France*
[c] *Laboratoire de Virologie-Immunologie, CHU Fort-de-France, Martinique*

Received 18 October 2007; accepted 28 January 2008

Abstract

Infections due to herpes simplex virus (HSV) resistant to acyclovir (ACV) represent an important clinical concern in immunocompromised patients. In order to switch promptly to an appropriate treatment, rapid viral susceptibility assays are required. We developed herein a genotyping analysis focusing on thymidine kinase gene (TK) mutations in order to detect acyclovir-resistant HSV in clinical specimens. A total of 85 HSV-1 positive specimens collected from 69 patients were analyzed. TK gene could be sequenced directly for 81 clinical specimens (95%) and 68 HSV-1 specimens could be characterized as sensitive or resistant by genotyping (84%). Genetic characterization of 67 susceptible HSV-1 specimens revealed 10 polymorphisms never previously described. Genetic characterization of 14 resistant HSV-1 revealed 12 HSV-1 with either TK gene additions/deletions (8 strains) or substitutions (4 strains) and 2 HSV-1 with no mutation in the TK gene. DNA polymerase gene was afterwards explored. With this rapid PCR-based assay, ACV-resistant HSV could be detected directly in clinical specimens within 24 h.

Keywords: Herpes simplex virus; ACV resistance; Thymidine kinase gene; Direct genotyping

1. Introduction

Acyclovir (ACV) is the first line treatment for herpes simplex virus (HSV) infection. ACV is a guanosine analogue that needs to be phosphorylated three times to become active. The first phosphorylation is performed by the viral thymidine kinase (TK), the two others being done by cellular kinases. The triphosphate form of ACV is then incorporated in nascent viral DNA and blocks HSV replication by inhibiting viral DNA polymerase (DNA pol). Despite the frequent use of ACV since its commercialisation in the 1980s, prevalence of ACV resistance does not seem to increase. In immunocompetent patients, prevalence has been estimated at 0.5% (Christophers et al., 1998; Danve-Szatanek et al., 2004; Nugier et al., 1992), and infections improve in most of the cases. HSV infections resistant to ACV remain an important clinical concern in immunocompromised patients. In these patients, the prevalence is around 5% but can reach 14–30% in allogenic bone marrow transplant patients (Danve-Szatanek et al., 2004; Morfin et al., 2004; Stránská et al., 2005). Lesions can become chronic and extensive with a risk of dissemination (Momméja-Marin et al., 2003; Sevilla et al., 2004). The switch to an appropriate treatment such as foscarnet (PFA) or cidofovir, which mechanisms of action do not depend on viral TK, needs to be done promptly.

ACV resistance can be due to mutations occuring either in TK gene or in DNA polymerase gene. TK gene mutations are involved in 95% of the cases of ACV resistance (Gaudreau et al., 1998; Hill et al., 1991; Morfin et al., 2000). TK is not necessary to viral replication in cell culture, but contributes to HSV neurovirulence and reactivation (Harris et al., 2003). TK is a 376 amino acid protein which presents six conserved regions including an ATP binding site (aa 51–63), a nucleoside binding site (aa 168–176) and a cysteine at codon 336 important to the functional conformation of the enzyme (Balasubramaniam et al., 1990; Darby et al., 1986). Since the crystal structure of the TK has been published, specific interactions involving also amino acids located ouside active or conserved sites have been shown

* Corresponding author at: Laboratoire de Virologie, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, 59 boulevard Pinel, F-69677 Bron Cedex, Lyon, France. Tel.: +33 4 72 12 96 17; fax: +33 4 72 12 95 00.
E-mail address: efrobert@sante.univ-lyon1.fr (E. Frobert).

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.01.153

to be necessary for its functionnality (Brown et al., 1995; Wild et al., 1997). Half of the case of ACV resistance are due to TK gene nucleotide additions or deletions, occuring frequently in G or C homopolymers which are considered as hot spots for point mutations (Gaudreau et al., 1998; Morfin et al., 2000). These additions or deletions can lead to a frameshift reading resulting in a truncated enzyme (Oram et al., 2000). Half of the other cases of ACV resistance are due to the presence of nucleotide substitutions, most of them occurring in active or conserved sites of the enzyme. Genotypic characterization of sensitive strains also reveals a high degree of polymorphism for TK gene; many mutations being not implied in ACV resistance.

DNA pol is a 1235 amino acid protein which includes eight conserved regions named I–VII and δ-C region (Hwang et al., 1992; Wong et al., 1988; Zhang et al., 1991). DNA pol mutations related to resistance are nucleotide substitutions mainly located in conserved regions II, III and VI. HSV resistance due to mutations in the DNA pol gene remains rare and most of the strains are derived from laboratory manipulations. Mutations located in the DNA pol gene can lead to PFA resistance (Chibo et al., 2002; Schmit and Boivin, 1999), ACV resistance (Larder and Darby, 1985) or resistance to both drugs (Hwang and Chen, 1995; Stránská et al., 2004). Mutation Ser724Asn (region II) has been frequently described and leads to ACV and PFA cross-resistance (Andrei et al., 2000; Bestman-Smith et al., 2001; Schmit and Boivin, 1999; Stránská et al., 2004). Till now, only one clinical case of HSV resistant to cidofovir has been reported, involving a strain harboring mutations truncating the DNA pol C′end (Wyles et al., 2005).

At the present time, phenotypic assays represent the gold standard for HSV susceptibility testing. Different tests do exist but they all require virus isolation and are thus time-consuming and laborious (Langlois et al., 1986; Pavić et al., 1997; Safrin et al., 1996; Swierkosz et al., 1987). Real-time PCR has also been expanded to HSV determination of antiviral drug susceptibility, but virus isolation is still required (Stránská et al., 2002; Thi et al., 2006). In case of ACV resistance, the switch to an appropriate treatment can thus be delayed. Moreover, in cerebrospinal fluids or ocular specimens, virus isolation is often not possible. Rapid methods for the detection of HSV resistance have to be developed, especially for immunocompromised patients for whom these infections may present high morbidity and mortality. A genotypic detection of resistance, as developed for HIV, HBV or CMV, represents an attractive approach (Vahey et al., 1999; Wen and Li, 2007; Yeo et al., 2005). Herein, we present a rapid genotyping assay, performed directly on clinical samples, based on the amplification of the entire TK gene followed by PCR products sequencing. This test allows the detection of all the mutations located in the TK gene that could be related to ACV resistance.

2. Materials and methods

2.1. Patients and specimens

Eighty-five HSV-1 positive specimens from 69 patients were analyzed. Thirty-four were collected in adults, including 9 immunocompetent, 10 bone-marrow-transplant patients, 2 lung-transplant patients and clinical information were not available for the 13 remaining. Thirty-five were collected from children. Nine were immunocompetent and 22 were immunocompromised including 13 bone marrow transplant patients, 3 with Kaposi–Juliusberg syndrome, 2 Burkitt's lymphoma, 2 kidney transplant patients, 1 lupus and 1 lymphoma. Clinical information were not available for the four remaining children. Samples were swabs taken from cutaneous sites (16), mouth (40), throat (17), nose (6), eye (6) or genital site (1) and 2 bronchoalveolar lavages (BAL). Swabs were discharged in transport medium (Eagle's Minimum Essential Medium supplemented with 2% fetal calf serum, Hepes buffer). All specimens were supplemented with penicillin/streptomycin mixture.

2.2. Virus susceptibility studies

A neutral red dye uptake assay was performed to determine sensitivity of HSV isolates to ACV (GlaxoWellcome, France) and PFA (Astra, France), as previously described (Langlois et al., 1986). ACV and PFA concentrations causing 50% inhibition of in vitro viral replication was determined as the inhibitory concentration-50 (IC_{50}). The HSV-1 IC_{50} cutoff values for ACV and PFA resistance were 6.5 and 350 μM, respectively.

2.3. TK gene analysis

2.3.1. Extraction

Total DNA extraction was performed directly from 200 μl of clinical specimens using the "High pure viral nucleic acid kit" (Roche, France). The purified nucleic acid was eluted in 100 μl of sterile water.

2.3.2. Amplification

TK gene was amplified from 10 μl of the purified nucleic acid using the proofreading enzyme Expand High Fidelity® (Roche, France) with a mix containing 1× buffer, 1.5 mM $MgCl_2$, 800 μM dNTP and 600 nM forward TKf and reverse TKr primers (Table 1). Amplification conditions included an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 2 min at 72 °C, with a final extension step of 5 min at 72 °C. Before sequencing, PCR products were purified with the GFX® Gel Band Purification kit (Amersham, France).

2.3.3. Sensitivity of TK PCR

The sensitivity of TK PCR was assessed by testing serial dilutions of a quantified HSV strain (MacIntyre strain, Advanced Biotechnologies, ATCC number VR-539).

2.3.4. Inhibitors testing

When TK gene amplification failed, the putative presence of inhibitors was tested by spiking the nucleic acid extract with 10^5 copies/ml of pGEM-TK KOS, a pGEM-T Easy vector (Promega) in which TK gene of ACV-sensitive HSV-1 reference strain KOS was previously cloned (Frobert et al., 2005). TK gene

Table 1
Primers and probes used for PCR, sequencing and real-time TaqMan PCR assays

Function	Target gene	Name	Sequence (5′ → 3′)
Amplification	TK	TKf	F: GATCTTGGTGGCGTGAAACTCC
		TKr	R: GGTTCCTTCCGGTATTGTCTCC
Sequencing	TK	TKA	F: TGACTTACTGGCAGGTGCTG
		TKC1 + TKf and TKr	R: GGGTCATGCTGCCCATAAGGTA
Real-time TaqMan PCR	Glycoprotein B	GB1	F: GCATCGTCGAGGAGGTGGAC
		GB2	R: TTGAAGCGGTCGGCGGCGTA
		HSP	Probe FAM: CGACCCCTCCCGGTAGCCGT
Amplification	DNA pol	LP6	F: GAGCACGTCCTCCTGTTTTC
		RP6	R CCGAGTTACACACGACCTTG
		LP7	F: GTATTAACATCACCCGCACC
		RP7	R: CGGAGACGGTATCGTCGTAA
Sequencing	DNA pol	RP8	F: ATGCAGTACTCGCCGATCAC
		LP8 + LP6, RP6, LP7 and RP7	R: TGTAACTCGGTGTACGGGTTC

F, forward primer; R, reverse primer.

amplification of the spiked specimen was carried out in the same conditions as described above.

2.3.5. Sequencing

Amplification products were sequenced (MWG-biotech, Germany). In order to get the 1214 bp of the TK gene, TKf, TKr, TKA and TKC1 primers were used for sequencing (Table 1). Sequence analysis and comparison were performed using SeqmanII software (DNAStar Inc.).

2.4. DNA polymerase gene analysis

2.4.1. Extraction

Nucleic acid extraction was performed from HSV culture isolates by using a standard phenol–chloroform protocol, as previously described (Frobert et al., 2005).

2.4.2. Amplification

DNA pol gene was amplified in two fragments using 1 μg of extracted DNA with LP6/RP6 and LP7/RP7 primers (Table 1). DNA pol gene amplification was carried out in the same conditions as described for TK gene amplification.

2.4.3. Sequencing

DNA polymerase gene was sequenced (MWG-biotech, Germany) using LP6/LP7 and RP6/RP7 primers (Table 1). SeqmanII software (DNAStar Inc.) was also used for sequence analysis and comparison.

2.5. Viral load in specimens

Viral load in specimens was determined by real-time PCR using the TaqMan® Universal PCR Master mix (Applied Biosystems, Roche). Each 50 μl of PCR reaction contained 10 μl of purified nucleic acid, 500 nM of GB1 and GB2 primers and 200 nM of HSP probe (sequences of primers and probes are described in Table 1). Each sample was analyzed in duplicate using the ABI Prism 7700 (Applied Biosystems) under the following conditions: incubation for 2 min at 50 °C, and then for 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.6. Sensitive and resistant virus mixtures

Two artificial mixtures were prepared from the ACV-resistant and -sensitive viruses isolated from the same patient. The patient TO mixture contained an ACV-resistant HSV-1 presenting the Gly240Glu substitution. The patient PR mixture contained a resistant HSV-1 harboring a + 1C addition in codon 184–185. Ratios 90:10, 80:20, 70:30, 60:40, 50:50, 40:60 and 20:80 represent the percentage of sensitive and resistant strains, respectively.

3. Results

3.1. Virus drug susceptibility

Eighty-five strains were first subjected to test ACV susceptibility according to the phenotypic assay. When a strain proved to be ACV-resistant, PFA susceptibility was then tested. Among the 85 samples, 71 strains were ACV-sensitive and 14 were ACV-resistant. The 14 ACV-resistant strains were isolated from 8 immunocompromised patients. Two ACV-resistant strains were also PFA-resistant and one had an intermediate susceptibility to this drug (Table 2).

3.2. Direct TK gene amplification from clinical specimens

After optimisation, the sensitivity of TK PCR was 5000 copies/ml. Nevertheless, the MacIntyre HSV strain quantified at 1000 copies/ml could be detected twice out of 3 assays.

Among the 85 culture positive HSV-1 isolates, 81 could be directly amplified and sequenced from the clinical samples (95%). Two samples that were not amplified had been exposed to inhibitors. The two others were at detection limit, with 1500 and 1100 copies/ml, respectively.

Table 2
Genotypic analyses of TK and DNA pol genes mutations of 18 clinical isolates from 8 immunocompromised patients

Patient	Age (year)	Clinical context	Isolates		Susceptibility		TK mutations (aa)			DNA pol mutations (aa)		
			Sample	Date	ACV IC_{50} (μM)	PFA IC_{50} (μM)	Polymorphism	Resistance	Not characterized	Polymorphism	Resistance	Not characterized
PR	12	BMT, mucite	Mouth	18 May 1999	0.8 (S)	ND		No mutation			Not performed[a]	
			Mouth	07 June 1999	20 (R)	125 (S)		+1C 184–185 = Stop 682		Val904Met		
			Mouth	20 December 1999	37 (R)	143 (S)						
CA	11	ID	Mouth	15 April 2002	26 (R)	80 (S)		+1C 184–185 = Stop 682		Val904Met		
DA	12	BMT	Lips	07 May 2001	20 (R)	96 (S)	Cys6Gly Leu42Pro	−1G 333 = frameshift		Val904Met		
			Mouth	09 June 2001	20 (R)	105 (S)	Gln89Arg Gly251Cys					
KO	32	Lymphoma, mucite	Throat	12 December 2000	65 (R)	173 (S)		−1G 145–146 = Stop 545		Val904Met		
			BAL	13 December 2000	34 (R)	146 (S)						Asp671Asn
BA	28	ID	Throat	02 July 2003	63 (R)	87 (S)	Glu36Lys Gln89Arg	−1C 184–185 = frameshift		Pro920Ser		Ala645Thr
VA	21	BMT	Lips	21 January 2000	38 (R)	95 (S)						
			Mouth	23 April 2000	32 (R)	84 (S)	Met85Ile	Gln103Stop		Val904Met		
			Cheek	08 September 2000	24 (R)	125 (S)						
TO	38	BMT	Throat	02 February 2005	1,9 (S)	179 (S)	Gly240Glu			No mutation		
			Throat	16 February 2005	35 (R)	334 (I)	Gly240Glu		Gly200Ser			Ala910Thr
RI	3	BMT	Mouth	19 October 2000	1.5 (S)	86 (S)	Cys6Gly			Val904Met		
			Tongue	25 October 2000	2.6 (S)	95 (S)	Gln89Arg			Val904Met	Ala719Val	
			Nose	10 November 2000	14 (R)	500 (R)	Ala192Val Gly251Cys Val267Leu			Val904Met	Ala719Val	Arg604Trp
			Chin	10 November 2000	16 (R)	500 (R)	Pro268Thr Asp286Glu Asn376His			Val904Met		Asp614Asn

BMT, bone marrow transplant patient; ID, immunodepressed patient; BAL, bronchoalveolar lavage; aa, amino acid; S, sensitive; R, resistant; I, intermediate; ND, not determined. The HSV-1 IC_{50} cutoff values for ACV and PFA resistance were 6.5 and 350 μM, respectively. TK sequence comparison was made with the sensitive KOS reference strain (Irmiere et al., 1989).

[a] DNA pol gene genotypic analyse was not performed as the strain could not be recultured.

Table 3
TK gene polymorphims detected from 67 ACV-sensitive strains and never previously described

Polymorphisms of the present report	Associated polymorphisms	Number of clinical isolates	References of associated polymorphisms
Asp14Tyr	Cys6Gly Leu42Pro Gln89Arg	1	Kudo et al. (1998)
Arg32Cys	0	1	Kudo et al., 1998
	Cys6Gly Leu42Pro Gln89Arg Gly240Glu	1	
His151Tyr	0	1	
Ala243Ser	Gly251Cys Val267Leu Pro268Thr Ala272Thr Asp286Glu	1	Morfin et al. (2000) and Kudo et al. (1998)
Ala272Thr	Gly251Cys Val267Leu Pro268Thr Asp286Glu Asp328Glu	1	Morfin et al. (2000) and Kudo et al. (1998)
	Ala243Ser Gly251Cys Val267Leu Pro268Thr Asp286Glu	1	
Pro274Thr	Cys6Gly Gly240Glu	1	Kudo et al. (1998)
Ala294Val	Gly240Glu	2	Kudo et al. (1998)
Leu327Met	Ser23Asn Glu36Lys Gln89Arg Gly240Glu	1	Kudo et al. (1998)
Asp328Glu	Gly251Cys Val267Leu Pro268Thr Ala272Thr Asp286Glu	1	Morfin et al. (2000) and Kudo et al. (1998)
Ser332Pro	Val348Ile	1	Chibo et al. (2004)

3.3. TK gene analysis

3.3.1. ACV-sensitive strains

Direct sequencing of the TK gene from 67 clinical samples with sensitive HSV-1 revealed 10 polymorphisms never previously described: Asp14Tyr, Arg32His, His151Tyr, Ala243Ser, Ala272Thr, Pro274Thr, Ala294Val, Leu327Met, Asp328Glu and Ser332Pro. Most of them were found in sensitive HSV-1 isolates harboring also other polymorphisms as compared to the KOS reference strain TK gene sequence (Irmiere et al., 1989). Up to six polymorphisms could be detected in the same virus (Table 3).

3.3.2. ACV-resistant strains

Eight patients excreted ACV-resistant viruses (Table 2). Five had a virus harboring a C or a G insertion or deletion either at codon 184–185 (patients PR, CA and BA), at codon 145–146 (patient KO) or at codon 333 (patient DA). These nucleotide additions or deletions created a frameshift and in three cases a premature stop codon. Two patients had strains presenting substitutions. Patient VA showed a virus presenting a substitution at codon 103 leading to a premature stop codon (Gln103Stop). Patient TO had an ACV-resistant HSV-1 harboring a Gly200Ser substitution and a Gly240Glu substitution which was also found in the ACV-sensitive HSV-1 previously isolated from the same patient. Patient RI showed an ACV- and PFA-resistant HSV-1 with no mutation in its TK gene, but mutations in its DNA pol gene could be detected (see Section 3.5).

3.3.3. Genotypic detection of ACV-resistant HSV-1

By genotyping applied directly on clinical samples, 57 out the 67 ACV-sensitive HSV-1 and 11 out of the 14 ACV-resistant HSV-1 could have been characterized as sensitive or resistant HSV-1 (68/81, 84%). Viruses that could not be genotypically

characterized harbored either substitutions not previously characterized in the literature (11 viruses) or mutations in their DNA pol gene (2 viruses).

3.4. Heterogeneous populations

Our genotyping assay allowed the detection of 20% of resistant virus in a mixture when a substitution was involved (patient TO mixture). In the PR patient mixture, composed of a resistant strain harboring a + 1C addition in an homopolymer, the heterogeneous population was only detectable at a ratio of 20:80, with the resistant strain being about 80% of the total viral population.

3.5. DNA polymerase gene analysis

DNA pol gene was analyzed from HSV culture isolates for all ACV-resistant strains and for sensitive strains previously isolated from the same patient (Table 2). Sequencing of DNA pol gene revealed a Val904Met substitution in 14 isolates. Two were ACV- and PFA-sensitive strains, 10 were ACV-resistant and PFA-sensitive strains and 2 others were ACV- and PFA-resistant strains. Associations of substitutions were also detected in PFA-sensitive strains (Asp671Asn with Val904Met and Ala645Thr with Pro920Ser). Associations Ala719Val/Val904Met and Arg604Trp/Asp614Asn/Val904Met were detected in ACV and PFA-resistant strains (patient RI).

4. Discussion

The aim of our study was to set up a rapid genotyping assay for the detection of ACV-resistant HSV directly in clinical samples. Such a molecular detection of HSV resistance should be performed in 24 h, allowing a rapid switch to an effective antiviral therapy using foscarnet or cidofovir. We focused our work on TK gene as it is involved in 95% of HSV ACV resistance. Our study revealed that TK gene could be amplified and sequenced directly from clinical samples in 95% of HSV-1 positive specimens according to the 85 samples retrospectively analyzed. In regard of the TK gene mutations involved in ACV resistance that have been previously published, TK sequences could have been interpretated in term of virus resistance or susceptibility to ACV in 84% of the cases.

PCR-based sequencing approach to detect resistance in clinical specimens had been developed in the past concerning HBV, HCV, HIV and CMV, as reviewed by Arens (2001). HSV TK sequencing related to ACV resistance has also been used previously but always after culture: see publications aimed to correlate phenotype and genotype, not to detect resistance directly from clinical samples (Chibo et al., 2004; Morfin et al., 2000; Saijo et al., 2002; Stránská et al., 2004).

HSV TK gene presents a large degree of polymorphism (Kudo et al., 1998; Morfin et al., 2000). According to the KOS reference strain TK gene sequence (Irmiere et al., 1989), the sequence of the 67 ACV-sensitive HSV-1 analyzed detected 10 TK polymorphisms never previously described. They are related to TK gene polymorphism as they do not affect TK activity as the strains were sensitive by phenotypic tests; all these mutations were located outside any active or conserved site of the enzyme.

TK gene sequencing of resistant strains showed nucleotide insertion or deletion for 8 out of the 14 ACV-resistant HSV-1, occurring most of the time in homopolymer repeats that are considered as hot spots for point mutations (Gaudreau et al., 1998). These nucleotide insertions or deletions in hot spots have been reported in about half of the cases of TK-related ACV resistance (Gaudreau et al., 1998; Morfin et al., 2000).

One patient showed an ACV-resistant and PFA-intermediate HSV-1 harboring two substitutions in its TK gene, Gly200Ser and Gly240Glu, and an Ala910Thr substitution in its DNA pol gene (patient TO). The TK Gly240Glu substitution might correspond to TK polymorphism as it was also detected in the ACV-sensitive HSV-1 isolated from the same patient. Moreover TK Gly240Glu polymorphism has been previously reported by Kudo et al. (1998). The role of the Gly200Ser substitution in ACV resistance remains uncertain. Even if this mutation is located outside any active or conserved site, it does not exclude its implication in ACV resistance and its role could be assessed by a site-directed mutagenesis study (Frobert et al., 2005, 2007). Concerning DNA pol gene, Saijo et al. (2005) previously described the Ala910Val substitution in a PFA-resistant laboratory strain which presented no mutation in its TK gene (Saijo et al., 2005). These substitutions at codon 910 could probably be related to PFA resistance, but their putative role in ACV resistance needs to be further explored. This could be performed by using a non-radioactive DNA polymerase assay as recently developed by Ducancelle et al. (2007).

Sequencing DNA pol gene of ACV-resistant strains and sensitive strains previously isolated from the same patient showed a Val904Met substitution that could surely account for DNA pol gene polymorphism. This substitution was detected in an ACV-resistant strains, but also in an ACV/PFA-sensitive strain (patient RI). This substitution has never been described before in a HSV-1 isolate, but three close substitutions (Ala904Gly, Ala905Glu and Gly906Ala) have been reported in ACV-sensitive HSV-2 (Chibo et al., 2004). Other substitutions are also probably related to DNA pol gene polymorphism even if it needs to be confirmed: Asp671Asn and Ala645Thr. Asp671Asn substitution was found in an ACV-resistant/PFA-sensitive strain (patient KO). ACV resistance was related to a −1G deletion at codon 145 in the TK gene. Nevertheless, the role of Asp671Asn in ACV resistance remains to be assessed. A double mutation (Ala645Thr + Pro920Ser) was detected in the DNA pol gene of an ACV-resistant and PFA-sensitive strain (patient BA). The TK gene of this strain also harbored −1C deletion that can explain the ACV-resistance phenotype. Ala645Thr has never been reported so far and needs to be investigated. By generating recombinant HSV using a system of overlapping cosmids and plasmids, Pro920Ser DNA pol substitution has been characterized as not involved in ACV and PFA resistance (Bestman-Smith and Boivin, 2003).

Clinical ACV and PFA cross-resistance associated with DNA pol gene mutations is rare but does exist (Collins et al., 1989; Sacks et al., 1989). These strains are as pathogenic as wild type strains and can be reactivated from latency, in contrast to most of TK-deficient strains (Field and Coen, 1986). Two strains with

ACV and PFA cross-resistance associated to DNA pol gene mutations were detected in patient RI. TK gene sequencing of these two viruses revealed eight already described TK polymorphisms. Strains isolated from the tongue and the nose showed two DNA pol substitutions: Val904Met and Ala719Val. The latter, located in region II of the DNA pol gene, was previously described by Larder et al. (1987) in an ACV and PFA cross-resistant laboratory strain (Larder et al., 1987). More recently, Saijo et al. (2002) isolated an ACV and PFA cross-resistant strain with a Val715Gly substitution; this mutation being located in region II of the DNA pol (Collins et al., 1989). This region is known to confer resistance to both ACV and PFA (Andrei et al., 2000; Schmit and Boivin, 1999; Stránská et al., 2004). In the chin isolate, substitutions Arg604Trp and Asp614Asn, localised in region A were detected. Their role is not known yet. A close substitution, Ala605Val, has been recently described by Saijo et al. (2005) in an ACV- and PFA-resistant laboratory strain (Saijo et al., 2005). The role of these two substitutions remains to be investigated.

In patient RI, sequencing of DNA pol gene revealed the development of a resistant population before its detection by phenotypic tests. The strain isolated from the tongue was PFA sensitive with an IC_{50} of 95 μM (cut off = 350 μM). Nevertheless, DNA pol sequencing revealed a double population with the Ala719Val substitution. This mutation is known to confer ACV and PFA resistance (Larder et al., 1987). Real-time investigation of resistance by genotypic methods would have detected the emergence of a resistant subclone before phenotypic assays.

Clinical specimens can contain a mix population of sensitive and resistant viruses (Larder and Darby, 1984; McLaren et al., 1983). Phenotypic tests can detect a subpopulation of 20% of ACV-resistant HSV (Langlois et al., 1986). In comparison, Baldanti et al. (1998) reported that sequencing analysis does not detect mutated viral CMV subpopulations when present in less than 25–30%. Our genotyping method allows the detection of an ACV-resistant subpopulation when this population reaches 20%, when a TK gene substitution is involved. However, in mixtures containing a mutated virus harboring an addition of one C in the homopolymer at codon 184–185, the resistant subpopulation can only be detected when it reaches 80% of the total population. Homopolymers additions or deletions in mixture could be easier to detect using pyrosequencing or using a real-time PCR with molecular beacons (Lindström et al., 2004; Yeo et al., 2005).

As to our results, detection of ACV resistance directly from clinical samples by genotyping can be confronted to some limits as TK sequences interpretation, detection of heterogeneous population or presence of DNA pol mutations. The interpretation of sequencing results can be successfully carried out thanks to a database of HSV TK gene mutations related either to ACV resistance or to gene polymorphism. To improve TK gene sequencing interpretation, this database should be completed as more and more mutations will be described. Nevertheless, sequencing interpretation is easy if an addition or a deletion is detected in the TK gene (half of the cases of resistance). The remaining half cases of resistance involve substitutions. For this latter mechanism, TK sequence can be interpretated if these mutations have been previously described and characterized. According to the literature, 88% of the substitutions described are characterized (Gilbert et al., 2002; Kudo et al., 1998; Morfin et al., 2000). However, if a substitution is located in an active or conserved site, it is most likely related to ACV resistance. In case of a substitution located outside active or conserved sites, TK gene polymorphism should be accounted, but exceptions do exist (Frobert et al., 2007). Three dimensional models and crystallographic studies would help to improve the understanding of specific interactions involved in TK functionality, particularly for amino acids located outside active sites (Vogt et al., 2000). Moreover, such studies could take in account the potentially compensatory effect of several TK mutations, even if it has not frequently been reported (Michael et al., 1995; Pilger et al., 1999).

In conclusion, genotyping testing represents an attractive approach as it could detect HSV resistance directly from clinical samples within 24 h. A rapid switch to an effective antiviral therapy using foscarnet or cidofovir would be possible, particularly in immunocompromised patients. Application of this genotyping test in routine diagnosis needs to be evaluated in a prospective study.

Conflict of interest

None.

References

Andrei, G., Snoeck, R., De Clercq, E., Esnouf, R., Fiten, P., Opdenakker, G., 2000. Resistance of herpes simplex virus type 1 against different phosphonylmethoxyalkyl derivatives of purines and pyrimidines due to specific mutations in the viral DNA polymerase gene. J. Gen. Virol. 81, 639–648.

Arens, M., 2001. Clinically relevant sequence-based genotyping of HBV, HCV, CMV, and HIV. J. Clin. Virol. 22, 11–29.

Balasubramaniam, N.K., Veerisetty, V., Gentry, G.A., 1990. Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation. J. Gen. Virol. 71, 2979–2987.

Baldanti, F., Biron, K.K., Gerna, G., 1998. Interpreting human cytomegalovirus antiviral drug susceptibility testing: the role of mixed virus populations. J. Infect. Dis. 177, 823–824.

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. J. Virol. 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. J. Virol. 75, 3105–3110.

Brown, D.G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P.J., Melitz, C., Summers, W.C., Sanderson, M.R., 1995. Crystal structures of the thymidine kinase from herpes simplex virus type-1 in complex with deoxythymidine and ganciclovir. Nat. Struct. Biol. 2, 876–881.

Chibo, D., Mijch, A., Doherty, R., Birch, C., 2002. Novel mutations in the thymidine kinase and DNA polymerase genes of acyclovir and foscarnet resistant herpes simplex viruses infecting an immunocompromised patient. J. Clin. Virol. 25, 165–170.

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

Christophers, J., Clayton, J., Craske, J., Ward, R., Collins, P., Trowbridge, M., Darby, G., 1998. Survey of resistance of herpes simplex virus to acyclovir in northwest England. Antimicrob. Agents Chemother. 42, 868–872.

Collins, P., Larder, B.A., Oliver, N.M., Kemp, S., Smith, I.W., Darby, G., 1989. Characterization of a DNA polymerase mutant of herpes simplex virus from a severely immunocompromised patient receiving acyclovir. J. Gen. Virol. 70, 375–382.

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. J. Clin. Microbiol. 42, 242–249.

Darby, G., Larder, B.A., Inglis, M.M., 1986. Evidence that the "active centre" of the herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide. J. Gen. Virol. 67, 753–758.

Ducancelle, A., Alain, S., Petit, F., Sanson Le Pors, M.J., Mazeron, M.C., 2007. Development and validation of a non-radioactive DNA polymerase assay for studying cytomegalovirus resistance to foscarnet. J. Virol. Methods 141, 212–215.

Field, H.J., Coen, D.M., 1986. Pathogenicity of herpes simplex virus mutants containing drug resistance mutations in the viral DNA polymerase gene. J. Virol. 60, 286–289.

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. Antimicrob. Agents Chemother. 49, 1055–1059.

Frobert, E., Ooka, T., Cortay, J.C., Lina, B., Thouvenot, D., Morfin, F., 2007. Resistance of herpes simplex virus type 1 to acyclovir: thymidine kinase gene mutagenesis study. Antiviral Res. 73, 147–150.

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. J. Infect. Dis. 178, 297–303.

Gilbert, C., Bestman-Smith, J., Boivin, G., 2002. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist. Updat. 5, 88–114.

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 acyclovir. J. Gen. Virol. 84, 1393–1401.

Hill, E.L., Hunter, G.A., Ellis, M.N., 1991. In vitro and in vivo characterization of herpes simplex virus clinical isolates recovered from patients infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 35, 2322–2328.

Hwang, C.B., Chen, H.J., 1995. An altered spectrum of herpes simplex virus mutations mediated by an antimutator DNA polymerase. Gene 152, 191–193.

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. J. Virol. 66, 1774–1776.

Irmiere, A.F., Manos, M.M., Jacobson, J.G., Gibbs, J.S., Coen, D.M., 1989. Effect of an amber mutation in the herpes simplex virus thymidine kinase gene on polypeptide synthesis and stability. Virology 168, 210–220.

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. J. Med. Virol. 56, 151–158.

Langlois, M., Allard, J.P., Nugier, F., Aymard, M., 1986. A rapid and automated colorimetric assay for evaluating the sensitivity of herpes simplex strains to antiviral drugs. J. Biol. Stand. 14, 201–211.

Larder, B.A., Darby, G., 1984. Virus drug-resistance: mechanisms and consequences. Antiviral Res. 4, 1–42.

Larder, B.A., Darby, G., 1985. Selection and characterisation of acyclovir-resistant herpes simplex virus type 1 mutants inducing altered DNA polymerase activities. Virology 146, 262–271.

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

Lindström, A., Odeberg, J., Albert, J., 2004. Pyrosequencing for detection of lamivudine-resistant hepatitis B virus. J. Clin. Microbiol. 42, 4788–4795.

McLaren, C., Corey, L., Dekket, C., Barry, D.W., 1983. In vitro sensitivity to acyclovir in genital herpes simplex viruses from acyclovir-treated patients. J. Infect. Dis. 148, 868–875.

Michael, M., Fetzer, J., Folkers, G., 1995. Site-directed mutagenesis of herpes simplex virus type 1 thymidine kinase opposes the importance of amino acid positions 251, 321 and 348 for selective recognition of substrate analogs. Biochem. Biophys. Res. Commun. 209, 966–973.

Momméja-Marin, H., Lafaurie, M., Scieux, C., Galicier, L., Oksenhendler, E., Molina, J.M., 2003. Herpes simplex virus type 2 as a cause of severe meningitis in immunocompromised adults. Clin. Infect. Dis. 37, 1527–1533.

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. J. Infect. Dis. 182, 290–293.

Morfin, F., Bilger, K., Boucher, A., Thiebaut, A., Najioullah, F., Bleyzac, N., Raus, N., Bosshard, S., Aymard, M., Michallet, M., Thouvenot, D., 2004. HSV excretion after bone marrow transplantation: a 4-year survey. J. Clin. Virol. 30, 341–345.

Nugier, F., Colin, J.N., Aymard, M., Langlois, M., 1992. Occurrence and characterization of acyclovir-resistant herpes simplex virus isolates: report on a two-year sensitivity screening survey. J. Med. Virol. 36, 1–12.

Oram, R.J., Marcellino, D., Strauss, D., Gustafson, E., Talarico, C.L., Root, A.K., Sharma, P.L., Thompson, K., Fingeroth, J.D., Crumpacker, C., Herold, B.C., 2000. Characterization of an acyclovir-resistant herpes simplex virus type 2 strain isolated from a premature neonate. J. Infect. Dis. 181, 1458–1461.

Pavić, I., Hartmann, A., Zimmermann, A., Michel, D., Hampl, W., Schleyer, I., Mertens, T., 1997. Flow cytometric analysis of herpes simplex virus type 1 susceptibility to acyclovir, ganciclovir, and foscarnet. Antimicrob. Agents Chemother. 41, 2686–2692.

Pilger, B.D., Perozzo, R., Alber, F., Wurth, C., Folkers, G., Scapozza, L., 1999. Substrate diversity of herpes simplex virus thymidine kinase. Impact of the kinematics of the enzyme. J. Biol. Chem. 274, 31967–31973.

Sacks, S.L., Wanklin, R.J., Reece, D.E., Hicks, K.A., Tyler, K.L., Coen, D.M., 1989. Progressive esophagitis from acyclovir-resistant herpes simplex. Clinical roles for DNA polymerase mutants and viral heterogeneity? Ann. Intern. Med. 111, 893–899.

Safrin, S., Palacios, E., Leahy, B.J., 1996. Comparative evaluation of microplate enzyme-linked immunosorbent assay versus plaque reduction assay for antiviral susceptibility testing of herpes simplex virus isolates. Antimicrob. Agents Chemother. 40, 1017–1019.

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 Res. 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. Antimicrob. Agents Chemother. 49, 606–611.

Schmit, I., Boivin, G., 1999. Characterization of the DNA polymerase and thymidine kinase genes of herpes simplex virus isolates from AIDS patients in whom acyclovir and foscarnet therapy sequentially failed. J. Infect. Dis. 180, 487–490.

Sevilla, J., Fernández-Plaza, S., González-Vicent, M., Colmenero, I., Echevarria, J.M., Pérez, A., Díaz, M.A., Madero, L., 2004. Fatal hepatic failure secondary to acute herpes simplex virus infection. J. Pediatr. Hematol. Oncol. 26, 686–688.

Stránská, R., van Loon, A.M., Polman, M., Schuurman, R., 2002. Application of real-time PCR for determination of antiviral drug susceptibility of herpes simplex virus. Antimicrob. Agents Chemother. 46, 2943–2947.

Stránská, R., van Loon, A.M., Polman, M., Beersma, M.F., Bredius, R.G., Lankester, A.C., Meijer, E., Schuurman, R., 2004. Genotypic and phenotypic characterization of acyclovir-resistant herpes simplex viruses isolated from haematopoietic stem cell transplant recipients. Antiviral Ther. 9, 565–575.

Stránská, 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. J. Clin. Virol. 32, 7–18.

Swierkosz, E.M., Scholl, D.R., Brown, J.L., Jollick, J.D., Gleaves, C.A., 1987. Improved DNA hybridization method for detection of acyclovir-resistant herpes simplex virus. Antimicrob. Agents Chemother. 31, 1465–1469.

Thi, T.N., Deback, C., Malet, I., Bonnafous, P., Ait-Arkoub, Z., Agut, H., 2006. Rapid determination of antiviral drug susceptibility of herpes sim-

plex virus types 1 and 2 by real-time PCR. Antiviral Res. 69, 152–157.

Vahey, M., Nau, M.E., Barrick, S., Cooley, J.D., Sawyer, R., Sleeker, A.A., Vickerman, P., Bloor, S., Larder, B., Michael, N.L., Wegner, S.A., 1999. Performance of the Affymetrix GeneChip HIV PRT 440 platform for antiretroviral drug resistance genotyping of human immunodeficiency virus type 1 clades and viral isolates with length polymorphisms. J. Clin. Microbiol. 37, 2533–2537.

Vogt, J., Perozzo, R., Pautsch, A., Prota, A., Schelling, P., Pilger, B., Folkers, G., Scapozza, L., Schulz, G.E., 2000. Nucleoside binding site of herpes simplex type 1 thymidine kinase analyzed by X-ray crystallography. Proteins 41, 545–553.

Wen, H., Li, Q., 2007. Real-time PCR detection of multiple lamivudine-resistant mutations with displacing probes in a single tube. J. Clin. Virol. 38, 334–340.

Wild, K., Bohner, T., Folkers, G., Schulz, G.E., 1997. The structures of thymidine kinase from herpes simplex virus type 1 in complex with substrates and a substrate analogue. Protein Sci. 6, 2097–2106.

Wong, S.W., Wahl, A.F., Yuan, P.M., Arai, N., Pearson, B.E., Arai, K., Korn, D., Hunkapiller, M.W., Wang, T.S., 1988. Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. 7, 37–47.

Wyles, D.L., Patel, A., Madinger, N., Bessesen, M., Krause, P.R., Weinberg, A., 2005. Development of herpes simplex virus disease in patients who are receiving cidofovir. Clin. Infect. Dis. 41, 676–680.

Yeo, A.C., Chan, K.P., Kumarasinghe, G., Yap, H.K., 2005. Rapid detection of codon 460 mutations in the UL97 gene of ganciclovir-resistant cytomegalovirus clinical isolates by real-time PCR using molecular beacons. Mol. Cell. Probes 19, 389–393.

Zhang, J., Chung, D.W., Tan, C.K., Downey, K.M., Davie, E.W., So, A.G., 1991. Primary structure of the catalytic subunit of calf thymus DNA polymerase delta: sequence similarities with other DNA polymerases. Biochemistry 30, 11742–11750.