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Added value of ultra-deep sequencing (UDS) approach for detection of genotypic antiviral resistance of herpes simplex virus (HSV)

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ABSTRACT

Classically, Sanger sequencing is considered the gold standard for detection of HSV drug resistance mutations (DRMs). As a complementary method, ultra-deep sequencing (UDS) has an improved ability to detect minor variants and mixed populations. The aim of this work was to apply UDS performed on MiSeq® Illumina platform to the detection of HSV DRMs and to the evaluation of the subpopulation diversity in clinical samples in comparison with Sanger sequencing. A total of 59 HSV-positive clinical samples (31 HSV-1 and 28 HSV-2) recovered from 50 patients mainly immunocompromised (70%) were retrospectively analyzed. Remarkably, UDS analysis revealed significant differences of relative abundance according to the type of DRMs within TK and Pol: natural polymorphisms and amino acid changes associated with resistance to antivirals were identified as high-abundant mutations (> 96%), whereas TK frameshifts conferring resistance to ACV were systematically detected at lower abundance (≈80%). This work also revealed that UDS can detect low-frequency DRMs and provides extensive information on viral population composition.

1. Introduction

Herpes simplex virus (HSV) is usually associated with self-limited orolabial (HSV-1) or genital (both HSV-1 and HSV-2) mucocutaneous manifestations. However, HSV may also be responsible for severe infections (e.g., encephalitis) or extensive and chronic lesions among immunocompromised individuals. After primary infection, HSV establishes lifelong latency in sensory ganglia leading to a risk of subsequent reactivation under particular conditions, such as stress, fever, and immunosuppression (Whitley et al., 2007).

Currently licensed drugs target the replication of HSV genome by DNA polymerase (Pol, UL30 gene). Acyclovir (ACV) and its prodrug, valacyclovir (VACV), are considered first-line therapy, whereas foscarnet (FOS) and cidofovir (CDV) constitute alternative options. ACV activity depends on an initial phosphorylation by the virus-encoded thymidine kinase (TK, UL23 gene) followed by two successive phosphorylations by cellular kinases. The ACV triphosphate form then inhibits the viral genome replication by a chain termination mechanism. According to this mechanism of action, viral mutations conferring resistance to ACV have been mapped both in TK and Pol. Mutations associated with FOS and CDV resistance are only detected in Pol since FOS directly inhibits Pol and CDV does not require TK activation. HSV resistance to ACV is mostly mediated by TK alterations (95% of cases), consisting in either nucleotidic insertion or deletion (indel) - leading to a translational frameshift, sometimes associated with premature stop codon - or nucleotidic substitution inducing an amino acid change. In the remaining 5% of cases of HSV resistance to ACV, nucleotidic substitutions are detected within Pol (Burrel et al., 2010; Piret and Boivin, 2014; Sauerbrei et al., 2016).

Classically, Sanger sequencing has been recognized as the gold standard for the detection of natural interstrain sequence variations (natural polymorphisms) and drug resistance mutations (DRMs) in HSV TK and Pol. However, this approach cannot detect minor DRMs in the viral population with a frequency below 20%. As a complementary method, ultra-deep sequencing (UDS) has an improved ability and enhanced sensitivity to detect minor variants and mixed populations with a detection of mutants with a frequency of 5%–10% of the viral population (Chin et al., 2013). Even though UDS is mainly dedicated to RNA viruses such as HIV for drug resistance testing, this technology has been recently demonstrated to be appropriate for DNA viruses implicated in human pathology such as HSV-1, varicella-zoster virus (VZV), and cytomegalovirus (CMV) (Benzi et al., 2012; Chou et al.,

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Table 1
Characteristics of the study population and the clinical samples.

Sample ID [a]	HSV type	Characteristics of the patient		Clinical context (HSV associated disease)	Antiviral therapy [b]	Clinical samples
		Age (years)	Gender			
1	HSV-1	34	F	IC (pneumopathy)	ACV	BAL
2	HSV-1	7	F	Lymhoma (orolabial herpes)	ACV and FOS	Mucocutaneous swab
3	HSV-1	30	F	HSCT (orolabial herpes)	ACV and FOS	Mucocutaneous swab
4	HSV-1	1.5	M	HSCT (orolabial herpes)	VACV	Mucocutaneous swab
5	HSV-1	47	M	Lymhoma (pneumopathy)	ACV	BAL
6	HSV-1	56	F	IC (herpetic keratitis)	VACV	Corneal swab
7	HSV-1	1	F	HSCT (orolabial herpes)	ACV and FOS	Mucocutaneous swab
8	HSV-1	64	M	IC (herpetic keratitis)	VACV	Tear specimen
9	HSV-1	73	F	IC (herpetic keratitis)	VACV	Tear specimen
10	HSV-1	60	F	IC (herpetic keratitis after corneal graft)	ACV and VACV	Corneal swab
11_A	HSV-1	61	M	HSCT (orolabial herpes)	VACV	Mucocutaneous swab
11_B	HSV-1		M	HSCT (orolabial herpes)	VACV	Mucocutaneous swab
12_A	HSV-1	49	F	HSCT (gingivostomatitis)	ACV	Mucocutaneous swab
12_B	HSV-1		F	HSCT (ear lesion)	VACV, ACV, and FOS	Mucocutaneous swab
13_A	HSV-1	11	F	Primary immunodeficiency (anal herpes)	VACV and FOS	Mucocutaneous swab
13_B	HSV-1		F	Primary immunodeficiency (buttock lesions)	VACV and FOS	Mucocutaneous swab
14	HSV-1	8	F	Primary immunodeficiency (orolabial herpes)	VACV	Mucocutaneous swab
15	HSV-1	58	M	HSCT (pneumopathy)	ACV and VACV	BAL
16	HSV-1	34	M	IC (herpetic keratitis)	VACV	Corneal swab
17	HSV-1	37	M	IC (herpetic keratitis)	VACV	Corneal swab
18	HSV-1	67	M	IC (herpetic keratitis)	ACV	Corneal swab
19	HSV-1	47	F	IC (orolabial herpes)	ACV and VACV	Mucocutaneous swab
20	HSV-1	7	F	Primary immunodeficiency (orolabial herpes)	ACV	Mucocutaneous swab
21	HSV-1	55	M	HSCT (orolabial herpes)	VACV	Mucocutaneous swab
22	HSV-1	64	F	IC (orolabial herpes)	ACV	Mucocutaneous swab
23	HSV-1	39	F	IC (orolabial herpes)	ACV and VACV	Mucocutaneous swab
24	HSV-1	44	F	IC (herpetic keratitis)	FCV	Corneal swab
25	HSV-1	64	M	Diabetes (corneal graft)	VACV	Corneal swab
26	HSV-1	68	M	Heart transplantation (oral herpes)	VACV	Mucocutaneous swab
27_A	HSV-1	66	F	Renal transplantation (hepatitis)	ACV and VACV	Whole blood
27_B	HSV-1		F	Renal transplantation (hepatitis)	ACV and VACV	Whole blood
28	HSV-2	64	M	HIV infection and renal transplantation (genital herpes)	ACV	Mucocutaneous swab
29	HSV-2	64	M	HIV infection (genital herpes)	ACV	Mucocutaneous swab
30	HSV-2	67	M	HIV infection (anal herpes)	ACV and FOS	Mucocutaneous swab
31	HSV-2	51	M	HIV infection (anal lesions)	VACV and imiquimod	Mucocutaneous swab
32	HSV-2	49	F	HIV infection (genital herpes)	VACV, ACV, and FOS	Mucocutaneous swab
33	HSV-2	86	M	Long-term corticosteroid therapy (genital herpes)	VACV	Mucocutaneous swab
34	HSV-2	67	M	HIV infection (genital herpes)	VACV	Mucocutaneous swab
35	HSV-2	51	M	HIV infection and renal transplantation (anal and genital herpes)	ACV and VACV	Mucocutaneous swab
36	HSV-2	52	M	HSCT (genital herpes)	VACV	Mucocutaneous swab
37	HSV-2	47	F	HIV infection (genital herpes)	VACV	Mucocutaneous swab
38	HSV-2	65	M	HIV infection (genital herpes)	VACV	Mucocutaneous swab
39_A	HSV-2	45	M	HIV infection (buttock lesions)	VACV	Mucocutaneous swab
39_B	HSV-2		M	HIV infection (buttock lesions)	VACV	Mucocutaneous swab
40_A	HSV-2	55	M	HIV infection (buttock lesions)	VACV and FOS	Mucocutaneous swab
40_B	HSV-2		M	HIV infection (buttock lesions)	VACV and FOS	Mucocutaneous swab
41_A	HSV-2	70	F	HIV infection (genital herpes)	VACV	Mucocutaneous swab
41_B	HSV-2		F	HIV infection (genital herpes)	VACV	Mucocutaneous swab
42	HSV-2	65	F	Chemotherapy treatment for cancer (buttock lesions)	VACV	Mucocutaneous swab
43	HSV-2	58	F	HIV infection (buttock lesions)	VACV	Mucocutaneous swab
44	HSV-2	51	F	HIV and HCV infection (thigh lesions)	VACV	Mucocutaneous swab
45	HSV-2	29	F	Crohn disease (genital herpes)	VACV	Mucocutaneous swab
46	HSV-2	59	M	HSCT (anal herpes and buttock lesions)	VACV	Mucocutaneous swab
47	HSV-2	55	F	IC (genital herpes)	VACV	Mucocutaneous swab
48	HSV-2	49	M	IC (genital herpes)	VACV	Mucocutaneous swab
49_A	HSV-2	59	F	Chronic lymphocytic leukemia (hepatitis)	ACV and FOS	Whole blood
49_B	HSV-2		F	Chronic lymphocytic leukemia (hepatitis)	ACV and FOS	Whole blood
50_A	HSV-2	22	M	IC (herpetic acute retinal necrosis)	ACV	Aqueous humor
50_B	HSV-2		M	IC (herpetic acute retinal necrosis)	ACV and FOS	Aqueous humor

ACV: acyclovir; BAL: bronchoalveolar lavage; F: female; FOS: foscarnet; HCV: hepatitis C virus; HIV: human immunodeficiency virus; HSCT: hematopoietic stem cells transplantation; HSV-1/-2: herpes simplex virus; IC: immunocompetent individual; M: male; VACV: valacyclovir.

[a] For some patients, sequential HSV-positive samples were tested for antiviral resistance in order to optimize antiviral treatment as exemplified by the following time-separated samples designed with A and B letters: 11 (2 months apart), 12 (1 month apart), 13 (4 months apart), 26 (1 month apart), 38 (1 week apart), 39 (2 months apart), 40 (3 months apart), 48 (1 month apart), and 49 (1 week apart).

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

2014; Garrigue et al., 2016; Kampmann et al., 2011; Karamitros et al., 2016; Sahoo et al., 2013; Mercier-Darty et al., 2018). However, to date, the impact of minor variants of all those herpesviruses remains unknown.

The aim of the work presented herein was to develop an UDS assay for genotypic detection of HSV-1 and HSV-2 DRMs in clinical samples using the MiSeq® Illumina platform. Accuracy, error rates and ability to assess the subpopulation diversity of this assay were evaluated to

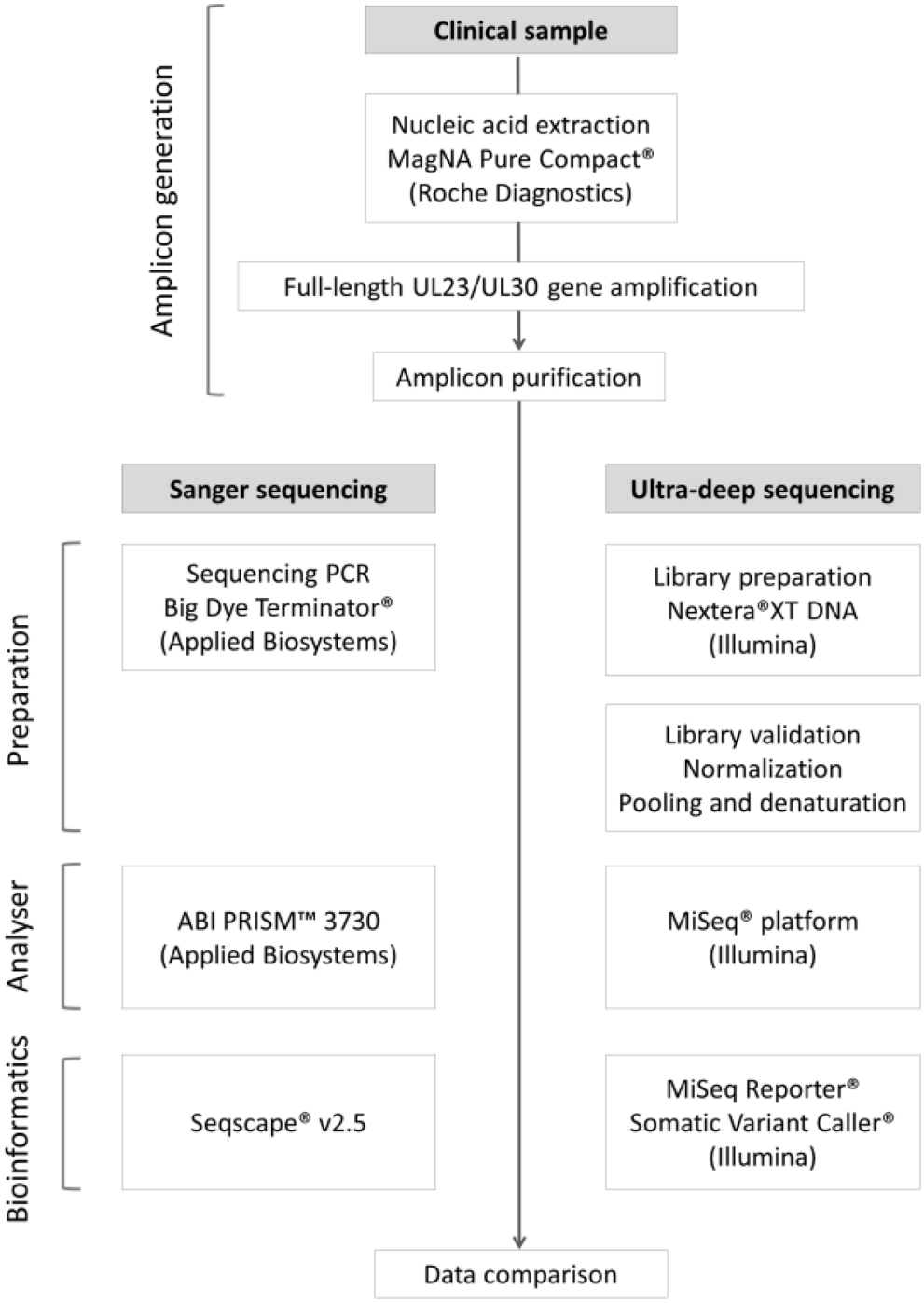

Fig. 1. Detailed workflows for both Sanger and UDS methods.

ensure high quality results.

2. Materials and methods

2.1. Patients and clinical samples

A total of 59 HSV-positive clinical samples (31 HSV-1 and 28 HSV-2) were recovered from 50 patients mainly immunocompromised and experiencing ACV and/or FOS treatment failure of HSV infection (median, 1 sample per patient; range, 1–2) (Table 1).

2.2. Sequencing analysis: comparison of Sanger and UDS methods

The detailed workflows for both Sanger and UDS methods are depicted in Fig. 1.

2.2.1. Sanger method

Samples collected for routine monitoring of HSV resistance to antivirals were tested by Sanger method, as previously described (Burrel et al., 2010). Briefly, viral DNA was extracted from clinical samples using MagNA Pure Compact Instrument® (Roche Diagnostics, Meylan, France). After viral DNA extraction from clinical samples, full-length TK and Pol genes were amplified with HSV type specific systems using a proofreading enzyme Expand High Fidelity® (Roche Diagnostics,

Meylan, France) dedicated to long-range PCR (~1.5 kbp for TK and ~4.0 kbp for Pol) and 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. For each gene, the first PCR was performed with 10 µL of DNA extract and a nested PCR with inner primers was carried out with 5 µL of the first PCR products if prerequisite. Cycling conditions for both first and nested PCRs included 40 cycles of amplification. Sanger sequencing data were analyzed using Seqscape® v2.5 software.

2.2.2. UDS assay

TK and Pol gene amplicons generated for initial Sanger analysis were thereafter sequenced by UDS after shotgun strategy on the MiSeq® platform (Illumina, San Diego, CA, USA), as previously described (Mercier-Darty et al., 2018). Briefly, 5 µL of amplicons at 0.2 ng/µL were used to prepare DNA library by means of Nextera® XT DNA (Illumina), according to the manufacturer's protocol. The quality and quantity of each library were evaluated by means of D1000 ScreenTape on TapeStation (Agilent, Santa Clara, CA, USA) and Quant-it dsDNA Assay kit (ThermoFischer, Waltham, MA, USA) on Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), respectively. All libraries were normalized at 4 nM, pooled and denatured before pair-end sequencing (v3, 2 × 300 bp) on a MiSeq® platform (Illumina). After UDS analysis, only nucleotidic sequences with mean Phred score Q30 (corresponding to an accuracy of 99.9%) were taken into account for analysis performed with Illumina on-board MiSeq Reporter® software. As quality filter for amino acid variant calling, only variant above Phred score Q20 (corresponding to an accuracy of 99%) with a depth of coverage of 10 or more were used to characterize the mutation frequencies with Illumina on-board Somatic Variant Caller® software. TK and Pol sequences obtained with Sanger and UDS methods were compared with those of viral references with GenBank accession numbers **X14112** for HSV-1 (strain 17) and **Z86099** for HSV-2 (strain HG52). In order to quantify errors due to amplification and sequencing, the UDS results for 2 plasmids containing either wild-type TK or wild-type Pol genes were compared to those obtained from Sanger sequencing. Plasmids were used at 10^5 cp/mL. Additionally, the well-defined PhiX® control (Illumina) was analyzed to evaluate the assay error rate. Statistical analyses were performed using MedCalc® software and $p < 0.05$ was considered to be statistically significant.

2.3. GenBank accession numbers

All TK and Pol Sanger sequences determined in this study have been deposited in the GenBank database under accessions numbers **MH697422** through **MH697533**.

2.4. Ethics

French Health Public Law does not require specific informed consent or ethics committee approval for such investigation. However, patients are truly informed that medical data could be used for research purposes according to a local ethic charter.

3. Results

3.1. Study population and sample characteristics

The study population included 25 (50%) males and 25 (50%) females. The median age was 55 years (range, 1 to 86). Fifteen (30%) individuals had no underlying medical condition, referred as immunocompetent individuals, and 35 (70%) patients exhibited immunosuppression consisting in human immunodeficiency virus (HIV)-infection (n = 14), hematopoietic stem cell transplantation (HSCT; n = 9), hemopathy (n = 3), immune disorder (n = 3), solid organ transplantation (SOT; n = 2), long-term immunosuppressive therapy or chemotherapy treatment for cancer (n = 2), Crohn disease (n = 1), and diabetes (n = 1) (Table 1). Clinical samples included 41 (69.5%) mucocutaneous swabs, 11 (18.6%) ocular specimens, 4 (6.8%) blood samples, and 3 (5.1%) bronchoalveolar lavages. Sequential clinical samples recovered from 9 patients with profound immunosuppression and long drug exposure were included in this study (patients 11, 12, 13, 27, 39, 40, 41, 49, 50) (Table 1).

3.2. Analytical characteristics of UDS method

Using our approach, we achieved reliable detection of mutations that were present in at least 5% of the population with about 46,000X and 55,000X mean coverage for TK and Pol, respectively (see Supplemental Table 1). Overall, the error rate was evaluated < 2%. Concerning the analysis of the clinical samples, UDS data analyzed with a 20% subpopulation cut-off were fully concordant with Sanger sequencing results for both HSV-1 and HSV-2. Overall, no significant differences were observed between replicates in experiments for inter-run and intra-run variability (see Supplemental Tables 2 and 3). We have also assessed the assay precision and its ability to detect viral subpopulations under our experimental conditions. To estimate the reliable percentage of minor variants detection, a wild-type amplicon and an amplicon containing single DRM were normalized at 1 ng/µL and were mixed according to different ratios, so that the mutated amplicon contributed to 5, 15, or 30% of the whole viral population. The overall precision was evaluated by running twice two libraries of the same sample, either in separate experiments for inter-run variability or within the same experiment for intra-run variability.

3.3. Natural polymorphisms

With UDS, TK natural polymorphisms consisting in amino acid changes were almost all detected as high-abundant mutations, with a frequency > 99% (exception: N376T ≈ 40% only within sample 8_TK1; see Supplemental Table 3). Interestingly, several HSV-2 clinical isolates harbored amino acid insertions or deletions within Pol previously associated with natural polymorphism (Burrel et al., 2010): Ins DGDE 683–686 in samples 39_A_Pol 2 and 39_B_Pol2 (estimated to represent 44.8% and 41.9% of the viral population, respectively), Del DDED 676–679 in sample 33_Pol2 (63.6%), and Del DGDE 683–686 in sample 47_Pol2 (76.7%) (data not shown).

3.4. Analysis of clinical samples with one single DRM detected by both Sanger and UDS methods (Table 2)

Remarkably, using UDS method, differences of abundance of DRMs in HSV TK conferring resistance to ACV were observed according to the type of mutation, nucleotidic substitutions generating an amino acid change *versus* indels generating a frameshift. All single amino acid changes were detected as high-abundant mutations with UDS, above 96% (mean 99.1%, range [96.6%–99.9%]). Otherwise, TK frameshifts were systematically detected at lower abundance with a frequency mean of 81.2% (79.4% for HSV-1 and 82.6% for HSV-2) ranging from 48.7 to 94.6% for both HSV-1 and HSV-2 (Table 1). Moreover, when sequential samples were available for chronically infected patients, proportions of TK frameshifts significantly varied over time whereas proportions of amino acid changes remained stable, as exemplified by samples 11_A/B_TK1 and samples 40_A/B_TK2, respectively. Indeed, C-nucleotidic deletion at position 548–553 (Del 1C nt 548–553, frameshift associated with a premature stop codon at amino acid residue 267 [V267Stop]) was raised from 48.7% to 83.2% within a 2-month period in samples 11_TK1, whereas amino acid change A157T was detected at 99.3% within the 3-month separated samples of 40_TK2. Regarding Pol,

Table 2
Analysis of clinical samples with DRMs within TK and Pol.

Sample ID number_gene	Mutations associated with antiviral resistance		Sequencing method	
	Nucleotidic change	Amino acid change	Sanger (detection of mutation)	UDS (frequency of mutations, %)
1_TK1	Substitution	Q342Stop	+	99.8
2_TK1	Indel	Frameshift (Del 1C nt C 548–553)	+	89.5
2_Pol 1	Substitution	R700G	+ (MP)	59.1
	Substitution	A719V	+ (MP)	32.4
3_TK1	Substitution	E83K	+	99.8
4_TK1	Indel	E225Stop (Ins 1G nt 430–436)	+	78.5
5_TK1	Substitution	P84S	+	96.6
6_TK1	Substitution	M130L	+	99.9
7_TK1	Indel	M182Stop (Del 1G nt 430–436)	+	88.7
8_TK1	Substitution	M128L	+	99.1
9_TK1	Indel	D228Stop (Ins 1G nt 548–553)	+	92.1
	Substitution	D55G[a]	–	6.0
10_TK1	Indel	D229Stop (Ins 1G nt 430–436)	+	79.7
11_A_TK1	Indel	V267Stop (Del 1C nt 548–553)	+ (MP)	48.7
11_B_TK1	Indel	V267Stop (Del 1C nt 548–553)	+	83.2
12_A_TK1	Substitution	L139P	+	98.2
12_B_TK1	Substitution	L139P	+	98.8
13_A_TK1	Indel	M182Stop (Del 1G nt 430–436)	+	87.3
13_B_TK1	Substitution	Y177C[a]	–	15.2
28_TK2	Indel	D229Stop (Ins 1G nt 433–439)	+	81.2
29_TK2	Indel	M183Stop (Del 1G nt 586–590)	+	70.8
30_TK2	Indel	L263Stop (Del 1G nt 551–556)	+	89.6
31_TK2	Substitution	G201D	+	99.6
32_TK2	Indel	L263Stop (Del 1G nt 779–782)	+	93.0
33_TK2	Substitution	K62N	+ (MP)	31.4
	Substitution	E84G[a]	–	16.9
	Substitution	T288M	+ (MP)	32.8
33_Pol 2	Substitution	A724V	–	7.1
34_TK2	Indel	I54Stop (Del 1G nt 105–109)	+	94.4
35_TK2	Indel	M348Stop (Del 1G nt G 837–840)	+	94.6
36_TK2	Indel	D229Stop (Ins 1G nt G 433–439)	+	80.8
37_TK2	Indel	D229Stop (Ins 1T nt 419–420)	+ (MP)	53.1
38_TK2	Substitution	R217C[a]	+	99.7
39_A_TK2	Indel	L263Stop (Del 1C nt 551–556)	+	86.0
39_B_TK2	Indel	L263Stop (Del 1C nt 551–556)	+	82.8
40_A_TK2	Substitution	A157T	+	99.3
40_B_TK2	Substitution	A157T	+	99.3

The names of TK alterations involving frameshifts are indicated as "M182Stop (Del 1G nt 430–436)" meaning that a part of viral population bears a G deletion in a homopolymer region containing 7 G's at nucleotides 430–436 leading to an ORF frameshift with apparition of premature stop codon at amino acid residue 182.

[a] Amino acid substitutions potentially conferring antiviral resistance. +: positive detection; -: non detection; Del: deletion; Indel: nucleotide insertion/deletion; Ins: insertion; MP: mixed population; nt: nucleotide.

the abundance of amino acid changes conferring resistance was similar to what was observed for TK. Indeed, two amino acid changes conferring ACV-resistance were detected within Pol: S775N (97.2%) and S779N (99.4%) within Pol 1 and Pol 2, respectively (see Supplemental Table 2).

3.5. Analysis of clinical samples with multiple DRMs and clinical samples with low-abundance DRMs detected only by UDS (Table 2)

In some cases, Sanger method revealed distinct DRMs in either TK or Pol, for which low-abundance frequencies were showed by UDS, supporting the idea of a heterogeneous viral population in those cases, as exemplified by samples 33_TK2 and 2_Pol 1. Indeed, for sample 33_TK2, T288M and K62N were estimated to represent 32.8% and 31.4% of the viral population, respectively. For sample 2_Pol1, R700G and A719V were estimated to represent 59.1% and 32.4% of the viral population, respectively. Interestingly, all those amino acid changes were initially detected as mixed population using Sanger method. Moreover, for few specimens including samples 9_TK1, 13_B_TK1, and 33_TK2, UDS also identified low-abundance mutations potentially associated with resistance (D55G [with 6% frequency] and Y177C [15.2%] in HSV-1 TK, and E84G [16.9%] in HSV-2 TK) or confirmed resistance (A724V [7.1%] in HSV-2 Pol) that had not been detected within TK or Pol by conventional Sanger sequencing. The potential role of these novel changes (D55G and Y177C in HSV-1 TK, and E84G and R217C in HSV-2 TK) in ACV resistance was analyzed according to their location within TK-1/-2 relatively to conserved regions and the existence of closely located mutations with previously assigned roles. Moreover, R217C change was identified within an isolate exhibiting an ACV-resistant/FOS-sensitive phenotype by plaque reduction assay (PRA) (data not shown).

4. Discussion

In a recent publication, we first reported the application of this technology for VZV (Mercier-Darty et al., 2018). Using this approach as a model, our work presented herein reports on the use of an UDS assay for HSV DRM detection in 59 HSV TK and Pol amplicons from clinical samples recovered from patients experiencing ineffective antiviral therapy.

Our UDS assay was validated with a cut off of 5%. As TK frameshifts occur mostly in homopolymeric regions of the TK gene, this could be a relevant technical problem for UDS. It is warranted to ensure that UDS using Illumina technology is not impacted. Previous comparative studies showed that Illumina technology does not generate significant artefacts on homopolymeric regions. Indeed, an evaluation of error rates showed that these regions have an error rate < 1% with Illumina technology on MiqSeq platform. Remarkably, UDS analysis revealed

significant differences of relative abundance according to the type of DRMs within TK and Pol. Natural polymorphisms and amino acid changes associated with resistance to antivirals were identified as high-abundant mutations, above 96%. In comparison, TK frameshifts conferring resistance to ACV were systematically detected at lower abundance for both HSV-1 and HSV-2 (~80%), even under maximal antiviral pressure. The percentage of TK frameshifts observed in our study is clearly above error rates observed in literature suggesting the results are not an artefact (Laehnemann et al., 2016). Those results are in agreement with previous data - obtained by cloning analyses, Sanger method, and other UDS technology - showing that heterogeneous populations (HSV TK-deficient and wild-type HSV mutants) may coexist within clinical isolates in variable proportions (Andrei et al., 2005; Piret and Boivin, 2011; Wang et al., 2007; Van velzen et al., 2012; Fujii et al., 2018; Duan et al., 2009; Seang et al., 2014). (). Moreover, HSV TK-deficient mutants have been reported to be significantly less virulent, to establish latency with a lower efficiency, and to reactivate poorly in comparison to wild-type viruses (Chen et al., 2006; Piret and Boivin, 2011; Wang et al., 2007).

Compiling our data and aforementioned studies, the differences of relative abundance may venture the idea that TK-deficient virus cannot stand alone without co-existing wild-type TK virus in clinical context conversely to virus harboring amino acid change accounting for resistance to antivirals. However, further studies are required to fully understand the clinical relevance of mutant subpopulations.

In conclusion, both Sanger method and UDS assay are validated for the detection of HSV DRMs with equivalent turnaround time for results (< 5 days). When the study was carried out, UDS cost slightly higher than Sanger sequencing (135 € vs. 100 €) when considering a limit of 5% for minor variants detection. However, UDS provides detailed and valuable information about the distribution of HSV DRMs. Moreover, UDS for HSV resistance testing allows the detection of minor variants, occasionally harboring well-characterized or potential DRMs, which are present below the sensitivity limit of Sanger method (< 20%). However, to date, the impact of DRM minor variants remains largely unknown. Larger studies, including sequential clinical samples from patients receiving antiviral treatment, are required to better characterize the clinical utility of UDS for HSV antiviral resistance genotyping over standardized Sanger methods in a real-life context. UDS technology has rapidly evolved in the past decade. In the future, we believe that targeted sequencing of viral genes implicated in HSV resistance to antivirals will likely move on whole genome sequencing providing full genetic data in the era of multiple antiviral therapies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.017.

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Transparency declaration

The authors declare no conflict of interest.

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