Journal of Virological Methods 248 (2017) 181–186

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A high resolution melting (HRM) technology-based assay for cost-efficient clinical detection and genotyping of herpes simplex virus (HSV)-1 and HSV-2

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ARTICLE INFO

Keywords:
High resolution melting analysis
HSV-1
HSV-2
Genotyping
qPCR

ABSTRACT

Genital herpes can be caused by two very similar viruses, herpes simplex virus (HSV)-1 or HSV-2. These two HSV types cannot be distinguished clinically, but genotyping is recommended in the first-episodes of genital herpes to guide counselling and management. Quantitative polymerase chain reaction (qPCR) is the preferred diagnostic method for HSV typing. However, commercial qPCR methods use expensive fluorescent labeled probes for detection. Furthermore, most low-cost methods are not able to differentiate between HSV-1 and -2. The aim of this study was to develop a high resolution melting (HRM) technology-based assay for sensitive HSV-1 and HSV-2 detection and genotyping. Using a panel of 46 clinical specimens, the performance of the HRM assay was compared to two commercial HSV tests: the HRM assay detected HSV in all 23 positive samples, with no false positive results (100% concordance with HSV I/II Real-TM assay). Additionally, the HRM assay correctly genotyped both HSV types in a subset of these clinical samples, as determined by the Realstar HSV PCR Kit. The HSV HRM assay provides a cost-effective alternative method to conventional more expensive assays and can be used in routine clinical specimens, in cases where it is particularly necessary to detect and distinguish HSV-1 from -2.

1. Introduction

Clinical features of genital herpes, caused by herpes simplex virus (HSV)-1 or HSV-2, can be highly variable and may be confused with syphilis, candidiasis, herpes zoster, hand-foot-and-mouth disease, chancroid, granuloma inguinale or noninfectious conditions that can mimic genital herpes, including Reiter's syndrome, contact dermatitis, Crohn's disease, Behçet's syndrome, trauma, erythema multiforme, or lichen planus (Arias-Santiago et al., 2010; DiCarlo and Martin, 1997; Gnann and Whitley, 2016; Rosen and Brown, 1998). HSV-1 and HSV-2 infection occurs via inoculation of virus particles into susceptible mucosal surfaces. These neurotropic viruses can become latent in the local sensory ganglion, periodically reactivating to cause symptomatic lesions, or undergo asymptomatic viral release, with the potential for disease transmission and infection (Mitchell et al., 2003; Wakisaka et al., 2001).

Although it has been previously claimed that oral infections were most often caused by HSV-1 and genital infections by HSV-2 (Arduino and Porter, 2008; Wald and Corey, 2007), this paradigm changed in the last decades. Recent epidemiological data have shown that genital infections are more often caused by HSV-1 than by HSV-2 (Whitley, 2013). Infections with either HSV-1 and -2 can cause identical initial illness, though the actual clinical presentation may depend upon previous HSV-1 or HSV-2 infection, and previous sites of infection. Subsequent recurrence frequency is greater for HSV-2 than for HSV-1 disease when infection involves the genital area (Patel et al., 2015, 2011). As a consequence, although the same treatment is used for both HSV-1 and HSV-2 infections, the location of the lesions and the chronicity of the infection (primary or recurrent) determine dosage and frequency. It is therefore recommended to perform HSV-1 and HSV-2 genotyping in patients with first-episode genital herpes, to guide counselling and management (Gupta et al., 2007; Patel et al., 2015, 2011).

HSV-1 and HSV-2 share a high degree of genetic homology, but they do have specific regions with small nucleotide variations which may allow discrimination. Much emphasis has been placed on the HSV's ancient *UL30* gene and *RS1* gene because of their essential function in viral replication and virulence (Alazard-Dany et al., 2009; Muylaert et al., 2011; Pelosi et al., 1998; Weller and Coen, 2012). The *UL30* gene

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http://dx.doi.org/10.1016/j.jviromet.2017.07.005
Received 23 May 2016; Received in revised form 30 June 2017; Accepted 12 July 2017
Available online 19 July 2017

encodes a DNA polymerase catalytic subunit which constitutes the herpes virus simplex DNA polymerase (HSV pol) together with the processivity subunit, UL42 (Franz et al., 1999; Zuccola et al., 2000). The sequences of the HSV-1 and HSV-2 *UL30* genes are 99% identical (Blast alignment score). The *RS1* gene encodes the transcriptional regulator, ICP4. *In vitro* experiments showed that the ICP4 protein binds DNA and promotes transcription (Beard et al., 1986) of the ubiquitin gene during infection (Kemp and Latchman, 1988; Latchman et al., 1987).

HRM analysis is able to distinguish single nucleotide polymorphism very accurately (Taylor, 2009; Vossen et al., 2009) by increasing the temperature up to 95 °C to melt post-PCR amplicons in the presence of saturating DNA binding dyes. When the fluorescence signal is plotted against the temperature, the so-called melting curve is generated. The negative derivative (-d(RFU)/dt) of the melting curve gives rise to a melting peak plot and the area under the melting curve (AUC) reflects the amount of target in the analyzed sample (Al-Robaiy et al., 2001). Additionally, subtraction of the melting curves from a reference melting curve (baseline) gives rise to a difference plot (Erali et al., 2008). Due to these characteristics, HRM assays provide a candidate technology for sensitive, easy and cost-effective discrimination between HSV-1 and HSV-2 infections. The aim of the present study is to design a HRM-based test that can be used for HSV-1 and -2 genotyping in clinical samples.

2. Materials and methods

Swabs of 46 clinical specimens of variable origin (Table 1) were recovered from different patients and collected in viral transport medium (UTM, Copan, Brescia, Italy) (The retrospective study uses leftover specimens that would have been discarded if not used in the study. Therefore no ethical approval was necessary to conduct this study). For the HRM assay, automated DNA extraction in a 96-well format was performed in each sample, on the Cobas X 480 (Roche Diagnostics). The HSV I/II Real-TM (Sacace) was used as a reference test to assess the performance of the HSV HRM based method to detect HSV. This reference assay specifically targets the HSV glycoprotein D gene in the HSV-1 and -2 genome and although this test allows for qualitative detection of HSV-1 and -2, it does not enable differentiation of these virus genotypes. To verify the genotyping results of the HSV HRM assay and determine whether the AUC was indicative of viral load, HSV positive samples were analyzed with the RealStar® HSV PCR Kit 1.0 (Altona Diagnostics) according to the manufacturer's guidelines. Six samples could not be tested with the RealStar® HSV PCR Kit because DNA extraction of these samples failed. These samples were excluded for genotyping. A flowchart outlining the study design is displayed in Fig. 1.

Table 1
Clinical samples and corresponding site of origin.

Clinical Samples	Site of origin	Clinical Samples	Site of origin
1	Lip	24	Cervix
2	Finger	25	Cervix
3	Presumably oral infection	26	Lip
4	Pubic hair	27	Cervix
5	Glans penis	28	Upper lip
6	Vulva	29	Presumably genital infection
7	Chin	30	Vulva
8	Lower lip	31	Gingiva
9	Penis	32	Lower lip
10	Cervix	33	Vulva
11	Cervix-vagina	34	Vulva
12	Urethra	35	Presumably on facial skin
13	Eye lid	36	Vulva
14	Anus	37	Conjunctiva
15	Vagina	38	Urethra
16	Vagina	39	Urethra
17	Vagina	40	Gluteal
18	Presumably genital infection	41	Presumably genital infection
19	Urethra	42	Presumably genital infection
20	Vagina	43	Presumably genital infection
21	Glans penis	44	Presumably genital infection
22	Vulva	45	Presumably genital infection
23	Vulva	46	Vulva

For HRM, primer annealing regions were selected so that there was less than 99% sequence identity in the amplicons generated for HSV-1 and HSV-2 types and 100% sequence identity across the primer hybridization regions to avoid preferably binding to one HSV type. Sequences with GenBank accession number NC_001806.2 (Human herpesvirus 1 strain 17, complete genome) and NC_001798.2 (Human herpesvirus 2 strain HG52, complete genome) were used as reference for primer design of HSV-1 and HSV-2, respectively. Reference sequence (RefSeq) records for HSV-1 and -2 were derived from NCBI. Internal control (IC) primers specific for *GAPDH* gene (GenBank accession number NC_000012.12; Homo sapiens chromosome 12, GRCh38.p7 Primary Assembly) were also designed, to monitor input DNA quantity. All primer sets (Table 2) were aligned *in silico* to the sequences of a selected group of pathogens and to the human genome, to ensure primer specificity. Homogeneous primer hybridization sites to *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *cytomegalovirus, Epstein-Barr virus*, *Mycoplasma hominis*, *Candida albicans*, *Streptococcus pyogenus*, *Treponema pallidum*, *Listeria monocytogenes*, *Chlamydia trachomatis*, human papilloma virus (HPV) (types 11, 16, 18, 31, 33) and *Mycoplasma genitalium* whole genomes were inquired with BLAST (Basic Local Alignment Search Tool).

Primers were evaluated on 12 samples (sample 1–12, Table 1) and HSV-1 and HSV-2 ultramer sequences. Based on the discriminatory power for differentiating HSV-1 from -2, one primer pair was selected and included in the HRM assay. HSV-1 and -2 ultramers, encompassing the target *UL30* region, were included in each HRM run as a positive control (purchased at IDT). The ultramer sequences used as a positive control in each HRM run are summarized in Table 3.

HRM analysis was performed on the LightCycler® 480 (Roche Diagnostics). The reactions contained 4 µl Lightscanner® Master Mix (2.5 x) (Idaho Technology Inc.), 400 nM forward and reverse primer, 2 µl of molecular grade water and 2 µl DNA template. Cycling and post-PCR melting analysis were: (1) one cycle of 95 °C, 2 min and (2) 45 cycles of 95 °C, 5 min; 60 °C, 15 s; 72 °C, 15 s. Following PCR amplification, continuous, high-resolution melting was performed: amplicons were melted out in two separated programs. In melt out program 1, amplicons were melted from 58 to 78 °C. Subsequently, during melt out program 2, targets were melted from 78 °C to 97 °C. During melt out program 1, all primer dimers and most erroneous created amplicons are melted out. In melt out program 2 only generated amplicons are denaturated and measured.

After fluorescence data acquisition, the melting data was analyzed by Tm Calling analysis, melting curve genotyping analysis and Gene Scanning analysis (LightCycler480 software – Release 15.0). Tm Calling results include the melting temperature, the height and width of melting peak, and the AUC measured in each sample. The AUC reflects the amount of HSV viruses in each sample (Varga and James, 2006; Wilhelm and Pingoud, 2003). The genotyping module determines the genotypes of the tested samples by analyzing the shapes of the melting curves and by grouping curves with similar shapes. The median curve from each group is designated the “genotype standard” for that group. The melting curve and the position of the peak is a signature for each HSV type hence, infection with a particular HSV type gives rise to a

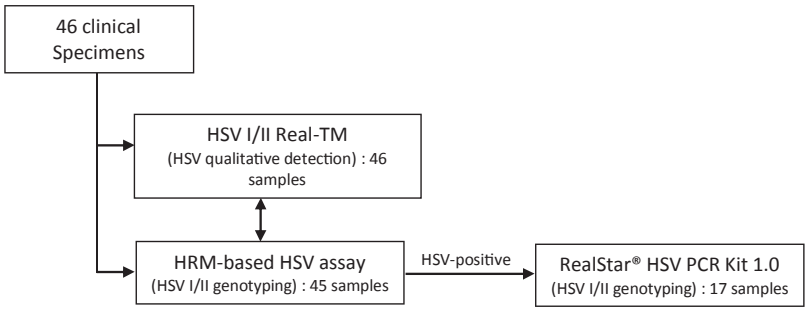

Fig. 1. Flowchart of the study. 46 swabs collected in viral transport medium (UTM, Copan, Brescia, Italy) were first subjected to HSV I/II Real-TM assay for a qualitative discrimination between HSV positive and negative samples, to analyze the qualitative performance of the in-house HRM-based HSV assay developed. Then, for the positive samples, a HSV-1 and HSV-2 genotyping comparison between the in-house HRM-based HSV assay and the commercially available RealStar® HSV PCR Kit 1.0 was performed.

particular melting curve. The gene scanning software was particularly used to analyze the differences in melting curve shape, by subtracting the curves from a reference curve. For each sample, a melting curve plot, a melting peak plot and difference plot was generated.

3. Results

Three pairs of flanking primers for the *UL30* HSV-1 and -2 gene (UL30-1, UL30-2 and UL30-3) were designed (Table 2). Attempts to design HRM primers in the *RS-1* gene region failed since no homogeneous primer annealing sites were found. The primer sets amplify regions of respectively, 127 bp, 130 bp and 112 bp within the HSV *UL30* gene. Alignment of the UL30-1 primers to the HSV-1 and -2 sequences is shown in Fig. 2. Evaluation with BLAST showed that none of the three primer pairs had homogeneous primer hybridization sites to the genomes of the investigated pathogens nor the human genome.

Discriminatory power of all three primer sets (Table 2) was evaluated on 11 HSV positive and 1 negative samples (sample 1–12, Table 1, previously tested with HSV I/II Real-TM). Primer pair 1 (UL30-1) clearly showed the greatest discriminatory power for differentiating HSV-1 from HSV-2 (supplementary data 2a, 2b and 2c). In order to remove background signals due to primer dimers and aspecific amplification, only melting out program 2 was used for data analysis. Based on the acquired data it was decided to adjust the temperatures for data acquisition in melting program 2: fluorescent data was collected starting at 86 °C to 99 °C, to avoid most of the background signals and facilitate interpretation.

A mean amplicon melting temperature difference of 1.0 °C (ranging from 0.63 °C to 1.57 °C, standard deviation of 0.1) between HSV-1 and -2 was observed (Fig. 3), resulting from an 8 base pair sequence variation between both genotypes (Fig. 2). The derivative melting plot displays a mean melting temperature of 90.8 °C for HSV-2 and of 91.9 °C for HSV-1 (Fig. 3). The temperature-shifted difference plot with HSV-2 positive control as baseline is shown in Fig. 4a. A shift in melting temperature between both genotypes could already be deduced from the melting curves (Fig. 4b). To check whether the observed melting profiles matched with each HSV genotype, the UL30-1 primer pair was tested on synthetic HSV-1 and -2 ultramers (300 copies/reaction), resembling HSV-1 or HSV-2 target regions. The melting peak of 91.9 °C and 90.8 °C for HSV-1 and -2, respectively, was confirmed (supplementary data 3).

Table 3
HSV-1 and HSV-2 UL30-1 ultramer sequences and length (in base pairs).

Annotation	Sequence	Length (base pairs)
UL30-1-HSV-1	AAT GTT GCA TAG AGC CTT TGA TAC TCT AGC ATG AGC CCC CCG TCG AAG CTG ATG TCC CTC ATT TTA CAA TAA ATG TCT GCG GCC GAC ACG GTC GGA ATC TCC GCG TCC GTG GGT TTC TCT GCG TTG CGC CGG ACC ACG AGC ACA AAC GTG CTC TGC CAC ACG TGG GCG ACG AAC CGG TAC	180
UL30-1-HSV-2	GAG CCT TTG ATA CTC TAG CAT GAG CCC CCC GTC GAA GCT GAT GTC CCG CAT CTT GCA ATA AAT GTC TGC GGC CGA CAC GGT CGG AAT TTC CGC GTC CGC TGG TTT CTC TGC GTT GCG TCT GAC CAC GAG CAC AAA CGT GCT CTG CCA CAC GTG GGC GGC GAA CCG GTA GCC GGG GCA CGC GGT	183

To ensure accurate genotyping for each experiment, HSV-1 and -2 positive controls were included in each PCR test plate. PCR efficiency and sample quantity were monitored by testing each sample using IC primers. The IC primers were designed to amplify a 107 bp region within the *GAPDH* gene. Amplification of the *GAPDH* gene with a cycle threshold (Ct) less than 35 cycles was interpreted as a valid PCR test. All tests were found valid under this criterion (data not shown).

The 46 clinical samples were first analyzed with a commercial kit HSV I/II Real-TM to test for presence or absence of HSV. 9 facial, 21 genital, 4 urine, 1 anal, 9 unknown and 2 samples of other origins were included (Table 1). In 23 of the 46 cases HSV was detected whereas other 23 samples were negative for HSV (Supplementary data 1). This assay cannot distinguish between HSV-1 and HSV-2. The HRM HSV technology-based genotyping assay identified HSV in all 23 samples that were found positive with the reference test. From the 23 HSV negative samples, 22 also tested negative with the *in house* HRM method. For 1 negative sample DNA extraction failed. A second attempt was performed to recover DNA from this sample, but also this effort failed probably due to insufficient sample quantity. This sample was then excluded from the study. The HRM-assay showed a perfect concordance with the HSV I/II Real-TM method (Supplementary data 1).

Of the 23 positive samples, the HRM HSV technology-based genotyping assay identified 12 HSV-1 positive samples and 11 HSV-2

Table 2
Primer sequences, hybridization position in the HSV-1/HSV-2 or GAPDH genome (GenBank accession number NC_001806.2/NC_001798.2 and NC_000012.12, respectively), melting temperature (Tm in °C) and amplicon length (base pairs) of HSV UL30 primer pairs evaluated for HRM. FP and RP stands for forward and reverse primer, respectively.

Annotation	Forward primer	Reverse primer	Genome position (NCBI)	Tm FP and RP (°C)	Amplicon length (base pairs)
UL30-1	CCCCCGTCGAAGCTGATGTCC	ACGTGTGGCAGAGCACGTTTGT	HSV-1: 66517–66643 HSV-2: 66990–67116	62.4–62.0	127
UL30-2	ACGCCAAGATCACCGAGAGTCTGT	GTTTCCTCCGCCGTAGCGCC	HSV-1: 66345–66474 HSV-2: 66818–66947	61.9–63.6	130
UL30-3	CGCGTTAGCCGAGCGCCCC	CTCCGGGTCGGTGATGCGCC	HSV-1: 65770–65881 HSV-2: 66243–66354	66.9–65.8	112
GAPDH	AGCGAGGCCTCCGTAAGATGCTT	ACGTCGCAGCTTGCCTACAACTGA	341–447	60.05–60.9	107

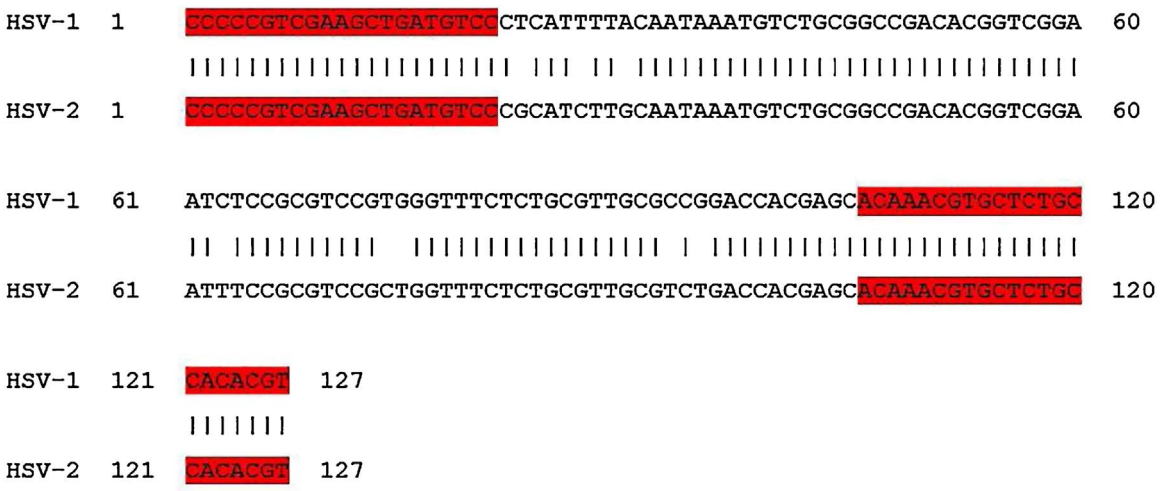

Fig. 2. HRM-HSV primers and sequence of HSV-1/HSV-2 PCR products: alignment of the selected set of UL30-1 PCR primers (red) used for the HRM-HSV assay in the sequence of HSV-1 and -2 amplicons. The selected primers amplify a 127 bp region of the gene that encodes a DNA polymerase catalytic subunit of the HSV pol, located between the nucleotide 66517 and 66643 of the HSV-1 genome (GenBank accession number: NC_001806.2) as well as the region located between nucleotides 66990 and 67116 of the HSV-2 genome (GenBank accession number: NC_001798.2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

positive samples. The melting profiles of the HSV-1 and HSV-2 samples allowed for accurate genotyping and grouping by the genotyping software (supplementary data 4 and 5). HRM genotyping results were validated and the viral load in each positive sample was determined with the RealStar® HSV PCR Kit (Altona Diagnostics) (supplementary data 6). All HSV types found with the HRM HSV assay were confirmed by the RealStar® HSV. However, in one of the samples, two HSV types were detected by the RealStar® HSV PCR test. Although the melting peak of this sample was positioned between HSV-1 and -2 ones (Fig. 3), the genotyping software grouped its melting curve as HSV-1 (Tm: 91.31 °C), probably as a consequence of higher viral load for HSV-1 over HSV-2.

The capacity of the AUC-value to estimate viral load in the HRM HSV technology-based genotyping assay was also evaluated. For that, the AUC of each sample was compared with the viral load quantified with the RealStar® HSV PCR kit (Supplementary data 6). In general, increasing amounts of HSV loads were correlated with an increase of the AUC, although this was not absolute (supplementary data 6).

With the presented in-house HRM-based HSV assay, a detection limit of at least 6.79 HSV-1 copies/µl and 10 HSV-2 copies/µl was reached for clinical samples. It was observed that even in case of a high absolute Ct-value (> 35), genotype specific post-PCR melting profiles can be generated, allowing for the detection of low HSV copy numbers (supplementary data 7).

4. Discussion

HSV can be detected in clinical samples by direct immunofluorescence assay, enzyme immunoassay, virus culture or DNA-based methods such as qPCR. HRM analysis is usually used for detection but is not routinely applied for diagnostic genotyping. With this study, it is shown that not only HRM analysis is a rapid and sensitive alternative detection method, but also that is able to clearly distinguish between HSV-1 and -2.

Up till now, HSV-1 and HSV-2 discrimination has only been performed using expensive labeled probes. Only one unlabeled probe assay based on HRM analysis for HSV genotyping has previously been described by Dames et al., 2007a. This unlabeled oligonucleotide probe (HSVP) was designed with 100% complementarity to HSV-2 and modified with a 3′ blocking modification to prevent DNA polymerase extension (Dames et al., 2007a). However, it was also reported that sometimes aberrant melting profiles were created with the described unlabeled probe assay. It was determined that for exon 10, *RET gene*, incomplete 3′ blocking of the unlabeled probe allowed polymerase-mediated probe extension, resulting in extension products that led to aberrant melting profiles. An evaluation of different blocking modifications was performed but none of the modifications tested had 100% effective blocking ability (Dames et al., 2007b). A concordance of 98% was reported for the HSV genotyping test with the *in-house* validated reference method.

The use of an intercalated dye instead of fluorescent-labeled oligonucleotides is the major advantage of the HRM –based assay, reducing

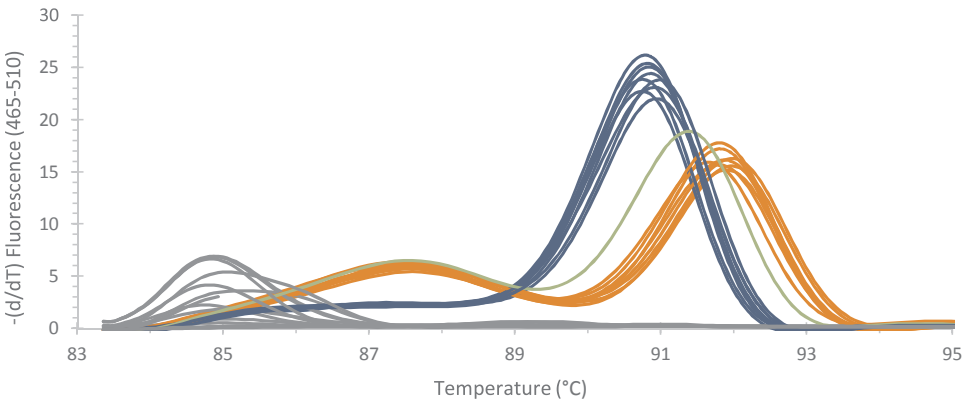

Fig. 3. The derivative melting plot of a set of samples. Melting curves of representative samples positive for HSV-1 (orange), HSV-2 (blue) and HSV negative samples (grey). Melting curves of only 18 of the 46 samples are displayed to reduce the complexity of the figure and to allow the evaluation of the individual melting curves. Sample 34, classified as positive for HSV-2 and HSV-1 with the RealStar HSV Altona test, has a melting peak between HSV-1 and 2 (green curve). HSV negative samples are showed in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

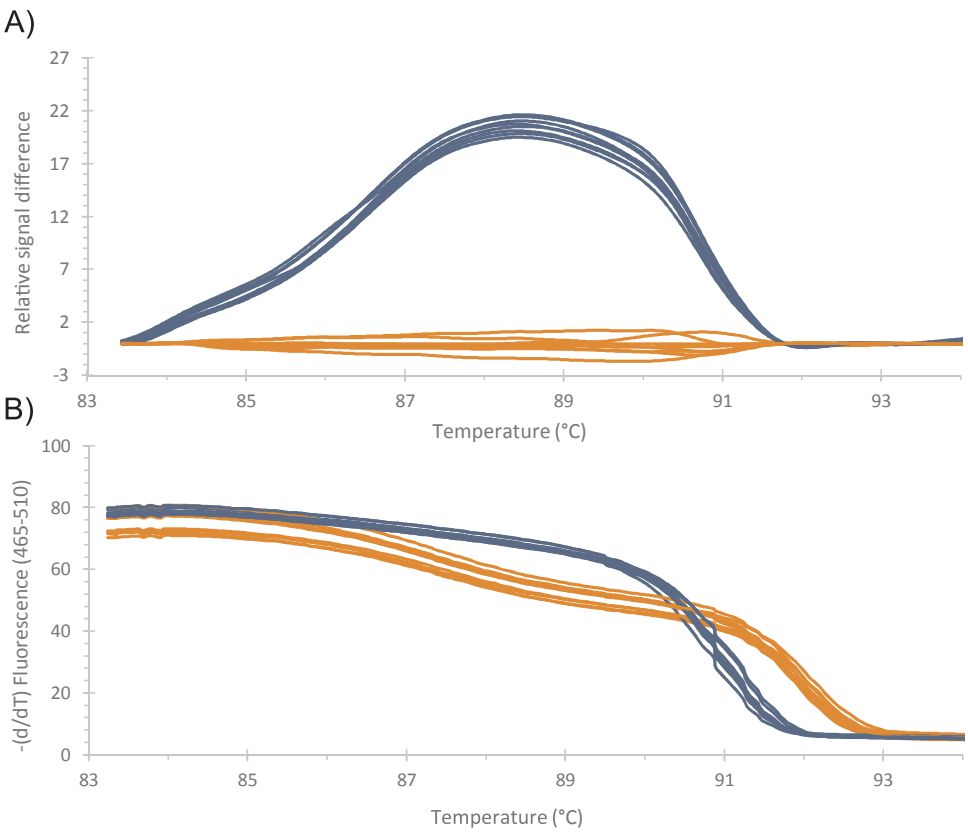

Fig. 4. Melting profiles of HSV positive clinical samples. HSV positive samples are indicated in blue (HSV-2) and orange (HSV-1). Melting curves (b) and difference plot (a) for a set of test samples (sample 7, 11, 15, 22, 31, 35, 39, 16, 21, 36, 40, 41, 42, 45). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the global costs of operation: the cost of HSV-HRM assay is 1.3 euro per sample which is significantly lower compared with the HSV I/II Real-TM assay and RealStar HSV PCR kit 1.0 assay, with 46 euro and 13 euro per sample, respectively. However, it also limits the assay to the differentiation of only a restricted number of genotypes in one test, as genotyping has to be based on melting curve analysis. Concurrently, the discriminatory power of HRM analysis largely depends on the efficiency and specificity of the primer set used, on the accessibility of the target region and the nature and amount of mismatches between the genotypes that should be differentiated.

Summing up, the HRM-based assay presented in this study seems to be a promising alternative for the genotyping of HSV-1 and HSV-2, although quantification is not possible as no correlation between viral load and the AUC was found. However, only a limited number of mucosal or dermal specimens were evaluated, which forms an important drawback. Further studies are needed to evaluate the HRM based HSV detection on a larger number of samples and in other specimens such as cerebrospinal fluid or blood.

5. Conclusions

Even targeting different genes of HSV, the HRM technology-based HSV assay had a performance comparable to that of commercially available PCR assays (HSV I/II Real-TM and RealStar HSV PCR kit 1.0), at a much lower cost, and is able to correctly distinguish between HSV-1 and -2. When applying the HSV HRM technology test with an appropriate HSV-1 and HSV-2 genotyping standard, as described in this experiment, the assay may be useful for research applications or routine clinical detection of HSV-1 and HSV-2.

Acknowledgement

We thank Pathlicon NV for assistance with the HRM technology-based HSV assay.

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.jviromet.2017.07.005.

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