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Longitudinal Study on Oral Shedding of Herpes Simplex Virus 1 and Varicella-Zoster Virus in Individuals Infected with HIV

Monique van Velzen¹, Werner J.D. Ouwendijk¹, Stacy Selke², Suzan D. Pas¹, Freek B. van Loenen^{1,3}, Albert D.M.E. Osterhaus¹, Anna Wald^{1,4,5}, and Georges M.G.M. Verjans^{1,*}

¹Department of Viroscience, Erasmus MC, 's-Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands ²Department of Laboratory Medicine, University of Washington, Seattle, WA, USA ³Rotterdam Eye Hospital, Rotterdam, the Netherlands ⁴Department of Medicine, University of Washington, Seattle, WA, USA ⁵Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Abstract

Primary herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) infection leads to a life-long latent infection of ganglia innervating the oral mucosa. HSV-1 and VZV reactivation is more common in immunocompromised individuals and may result in viral shedding in saliva. We determined the kinetics and quantity of oral HSV-1 and VZV shedding in HSV-1 and VZV seropositive individuals infected with HIV and to assess whether HSV-1 shedding involves reactivation of the same strain intra-individually. HSV-1 and VZV shedding was determined by real-time PCR of sequential daily oral swabs (n=715) collected for a median period of 31 days from 22 individuals infected with HIV. HSV-1 was genotyped by sequencing the viral thymidine kinase gene. Herpesvirus shedding was detected in 18 of 22 participants. Shedding of HSV-1 occurred frequently, on 14.3% of days, whereas solely VZV shedding was very rare. Two participants shed VZV. The median HSV-1 load was higher compared to VZV. HSV-1 DNA positive swabs clustered into 34 shedding episodes with a median duration of 2 days. The prevalence, duration and viral load of herpesvirus shedding did not correlate with CD4 counts and HIV load. The genotypes of the HSV-1 viruses shed were identical between and within shedding episodes of the same person, but were different between individuals. One-third of the individuals shed an HSV-1 strain potentially refractory to acyclovir therapy. Compared to HSV-1, oral VZV shedding is rare in individuals infected with HIV. Recurrent oral HSV-1 shedding is likely due to reactivation of the same latent HSV-1 strain.

*Corresponding author: Georges M.G.M. Verjans MSc PhD, Viroscience lab (Ee1720a), Erasmus MC, 's-Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands. Tel: +31-10-7044004, Fax: +31-10-7044760, g.verjans@erasmusmc.nl.

Competing interests

None of the authors declare conflicts of interest apart from ADME Osterhaus who is a part time employee of Viroclinics Biosciences BV (for details go to www.viroscielab.org). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

Keywords

human herpesvirus-1; human herpesvirus-3; reactivation; saliva; immunocompromised; genotyping

Background

Herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) are closely related neurotropic human alpha-herpesviruses (α HHV) that are endemic worldwide. A hallmark of both viruses is the ability to establish a lifelong latent infection of sensory ganglia with intermittent reactivation and neuronal spread of the virus to innervating tissues [Roizman et al., 2007]. α HHV shedding in bodily secretions and fluids, particularly saliva, contributes to virus transmission throughout the population [Wald et al., 1995; Koelle and Wald, 2000; Kaufman et al., 2005; Cohrs et al., 2008]. Virus shedding is commonly asymptomatic, but may lead to recurrent herpetic lesions most commonly as cold sores for HSV-1 and as shingles for VZV. Recurrent HSV infections of the same anatomical location may be due to reactivation of the latent virus strain or superinfection with an exogenous strain. Whereas most sequential HSV-1 isolates from the same anatomical location of an individual are identical, HSV-1 isolates with different genome profiles have been described in patients with oral, genital and corneal herpesvirus infections [Roest et al., 2004; Umene et al., 2007; Duan et al., 2009; Liljeqvist et al., 2009]. Alternatively, multiple virus strains may have established latency in the same ganglion. Indeed, different HSV-1 strains have been detected in the same latently infected human ganglion, including the oral mucosa innervating trigeminal ganglion, indicating that recurrent oral HSV-1 shedding may be due to reactivation of genetically different latent HSV-1 strains [Lewis et al., 1984; van Velzen et al., 2012]. The host immune system is pivotal to limit reactivation from its ganglionic stronghold [Miller, 1980; Griffin et al., 2008; Birlea et al., 2011]. As such, individuals infected with HIV experience more severe and persistent herpetic lesions and may be at risk for central nervous system disease [Cinque et al., 1998; Schacker et al., 1998; Kim et al., 2006; Birlea et al., 2011]

Previous studies have described high oral HSV shedding frequencies in individuals infected with HIV compared to healthy persons seropositive for the respective herpesviruses [Kim et al., 2006; Mark et al., 2008; Mark et al., 2010]. Oral lesions present in individuals infected with HIV have been associated with shedding of herpesviruses in the oral cavity [Contreras et al., 2001; Miller et al., 2006]. Moreover, oral detection of herpesviruses is decreased in individuals treated with anti-retroviral or anti-herpesvirus drugs [Ceballos-Salobrena et al., 2000; Posavad et al., 2004; Miller et al., 2005]. Limited number of studies have reported on VZV shedding in saliva or oral swabs of herpes zoster patients [Mehta et al., 2008; Nagel et al., 2011], healthy individuals [Nagel et al., 2011] and in one study on individuals infected with HIV [Wang et al., 2010]. These studies were mainly restricted to the detection of VZV only and limited to the analysis of one or a few consecutive saliva samples per individual. The trigeminal ganglion, which innervates the oral mucosa and eye, commonly contains both latent HSV-1 and VZV in co-infected individuals. Hence, the aim of this study was to determine the prevalence and kinetics of oral HSV-1 and VZV shedding in HSV-1 and VZV

seropositive individuals infected with HIV and to assess whether HSV-1 shedding involves reactivation of the same strain intra-individually.

Materials and Methods

Study Population

Individuals infected with HIV-1 were recruited between 1995 and 2007 at the University of Washington Virology Research Clinic (Seattle, WA) from a pool of unrelated research study participants known to comply with an intensive study protocol and asked to collect oral swab specimens at home daily for at least 30 days (Table I). The median duration of HIV infection was 8 years (range 3 months to 18 years). Participants were instructed to rub a Dacron swab across the buccal mucosa and tongue in the morning prior to showering or brushing their teeth, to place the oral swab in 1 mL of PCR transport medium and to store the sample at -20°C until laboratory processing [Mark et al., 2010]. Participants were eligible if they were HSV-1 and VZV seropositive, and agreed not to use anti-herpesvirus drugs, such as acyclovir (ACV) during the study. The use of anti-herpesvirus drugs was only monitored if prescribed by the University of Washington Virology Research Clinic (Seattle, WA). Participants #11 and #16 used famciclovir bidaily for 60 days in the year prior to collection of the oral specimens, but treatment was stopped two weeks before start of the study. At baseline, plasma HIV RNA load and blood CD4 T-cell counts were determined as described [Mark et al., 2010]. A log book recording symptomatic (herpetic) oral lesions was filed. Participants had routine clinic visits at the start and end of the study and irregularly during the study. During these visits brief visual oral exams were performed and a history of suspected herpetic oral lesions since the last visit was reviewed by the clinician and noted in the participant's chart. Except for participant #6, no evident abnormalities of the mouth (e.g. bleeding gum) or the neck were recorded and reported by the participants themselves during the sampling period. Written informed consent was given by the participants and the protocol was approved by the Institutional Review Board at the University of Washington (Seattle, WA). The study was performed according to the tenets of the Declaration of Helsinki.

Quantitative α HHV PCR Analyses and HSV-1 Thymidine Kinase Sequencing from Oral Swabs

DNA was extracted from swab medium as described [Mark et al., 2010]. Quantitative PCR (qPCR) assay for HSV-1 and VZV DNA was performed using an ABI prism 7500 and Taqman Universal Master Mix (both from Applied Biosystems, Foster City, CA) as reported [Remeijer et al., 2009]. The HSV-1 and VZV qPCR used published virus-specific primers and probes [van Doornum et al., 2003]. For standardization of HSV-1 and VZV Taqman assays, electron microscopy counted high-titer virus preparations and commercially available quantified DNA control panels (Advanced Biotechnologies) were used [van Doornum et al., 2003]. The lower limit of detection of both qPCR assays was 50 genome equivalent copies (geq)/mL. Cycle threshold values outside the linear range of the qPCR assay were considered as positive results, but could not be reliably quantified.

From a selected number of HSV-1 positive swabs (n=39), the entire HSV-1 thymidine kinase (TK) gene was amplified and sequenced as described [Duan et al., 2008]. The TK sequences were aligned to the consensus TK sequence of reference HSV-1 strain H129 (GenBank: GU_734772). The obtained HSV-1 TK sequences were deposited in the GenBank database under accession numbers JQ895543-JQ895556. Phylogenetic analysis was performed by estimating a maximum-likelihood unrooted tree of HSV-1 TK nucleotide sequences under the Kimura 2-parameter model and 1,000 bootstrap replications (MEGA 5.0 software).

Statistical Analysis

Herpesvirus shedding episodes were defined as one virus DNA positive swab or a series of DNA positive swabs that were collected before and after at least two negative swabs. Any shedding episode could include one missing or one negative swab within the episode [Mark et al., 2010]. Statistical analyses were done using GraphPad Prism 4. Spearman's correlation tests were used to determine correlations between herpesvirus shedding frequency, HIV viral load, CD4 T-cell counts or highly active anti-retroviral therapy (HAART). Mann-Whitney tests were used to compare shedding rates and median HSV-1 viral loads in HAART versus non-HAART persons and among shedding episodes of variable length. Differences were considered significant if $P < 0.05$.

Results

Oral HSV-1 and VZV Shedding in Individuals Infected with HIV

Twenty-two HSV-1 and VZV seropositive individuals infected with HIV were enrolled in the study. The median age was 42 years (range 22-61 years) and 20 were male. Fifteen participants were HSV-2 seropositive, and 6 persons (27%) were taking HAART (i.e. participants #1 to #6) (Table I and Fig. 1). Participants had a median CD4 T-cell count of 268 cells/mL with an interquartile range (IQR) of 202-476 cells/mL and a median HIV RNA load of 37,900 copies/mL (IQR: 15,656-109,316 copies/mL) (Table I). Whereas the CD4 T-cell counts were not different (Mann-Whitney test; $P = 0.45$), the HIV RNA load was significantly lower in persons taking HAART compared to those not receiving HAART (Mann-Whitney test; $P = 0.01$), respectively. A total of 715 oral swabs were obtained and analyzed for the presence and amount of HSV-1 and VZV DNA by qPCR. Samples were collected for a median of 31 days (IQR: 28-33 days), with 19 participants collecting for 30 days (Fig. 1). Except for individual #6, none of the participants reported symptomatic herpetic oral lesions during the study period.

Four of the 22 (18%) persons shed neither HSV-1 nor VZV DNA during the study. From the 18 remaining persons, HSV-1 DNA was detected on 102 out of 715 sample days (14.3%) (Table II). The HSV-1 DNA load was quantified in 97 swabs, with a median DNA load of 5,603 geq/mL (IQR: 1,073-56,050 geq/mL). Very low VZV DNA levels were detected in 7 swabs from two persons, and could be quantified in 1 sample (participant #4; 58 geq/mL). The median number of episodes of HSV-1 shedding was 2 and 1.5 episodes per 30 days among participants receiving HAART and those who were not receiving HAART (Mann-Whitney test; $P = 0.48$), respectively. The frequency of HSV-1 and VZV shedding, and the

maximum detected HSV-1 load, did not correlate with the participants' CD4 T-cell counts or HIV RNA load (Fig. 1 and data not shown). All VZV DNA positive swabs were HSV-1 negative, with the exception of one swab from participant #17 in which both HSV-1 and VZV were detected (Fig. 1).

The 102 HSV-1 positive swabs clustered into 34 distinct shedding episodes, with a median duration of 2 days (range 1-20 days) (Table II). During the study period, 14 episodes (41%) of 1 day duration were detected with a median HSV-1 DNA load of 256 geq/mL (IQR: 124-3,555 geq/mL). Eleven episodes (32%) lasted 4 days, and 7 episodes were of unknown duration because swabs were positive at the beginning or end of the study (Fig. 1 and Table II). The median HSV-1 load of 3-day episodes (909,842 geq/mL) or 4-day episodes (106,000 geq/mL) was significantly higher compared to one-day episodes (Mann-Whitney test; $P=0.005$ and $P=0.0001$, respectively) (Fig. 2). One of the participants (#6) reported symptomatic herpetic oral lesions at days 15 to 20 of the study. HSV-1 DNA was detected in mucosal swabs at the start of symptoms and was undetectable during the resolution phase. Notably, the participant's second HSV-1 shedding episode, with a 2-log higher HSV-1 DNA load, was asymptomatic (Fig. 1).

Genotyping of Oral HSV-1 in Individuals Infected with HIV

To determine if oral HSV-1 shedding involves reactivation of the same latent strain within and between shedding episodes, the entire TK gene from a selected set of HSV-1 DNA positive oral swabs was sequenced (Fig. 1). Besides the causative role of TK mutations in ACV resistance (ACV^R), the hypervariability of the TK gene provides insight into the genetic composition of a virus isolate [Morfin and Thouvenot, 2003; Duan et al., 2008; van Velzen et al., 2012]. The HSV-1 TK genotype was determined from 14 participants with a median of 2.5 (range 1-8) oral swabs analyzed per person. The analyzed sequential oral swabs were obtained during one (n=10 participants) or of two subsequent HSV-1 shedding episodes (n=4 participants) (Table III). Alignment of the TK sequences obtained with the corresponding sequence of the HSV-1 reference strain H129 revealed numerous TK gene nucleotide substitutions, including those resulting in amino acid mutations in the encoding TK protein. Notably, HSV-1 TK sequences of sequential oral swabs from each individual, both within and between shedding episodes, were identical suggesting reactivation and subsequent oral shedding of the same endogenous HSV-1 strain (Table III). Most of the viruses shed by each individual had a unique TK nucleotide sequence clustering into distinct participant-specific phylogenetic clades (Fig. 3). However, HSV-1 shed by participants #2 and #18, and participants #6 and #13, could not be differentiated based on the TK gene genotypes (Table III and Fig. 3). The TK sequence homology was not due to contamination, since the participants' samples were processed at different time points, all sequential swabs of each participant were identical (Table III), and none of the aforementioned participants were family members or in any way related.

ACV^R HSV-1 is predominantly due to specific mutations in the drug-targeted TK protein leading to its defective or limited ability to convert ACV to ACV-monophosphate necessary to block HSV-1 replication [Morfin and Thouvenot, 2003]. Whereas the majority of the HSV-1 TK amino acid changes identified in the oral swabs were natural polymorphisms, 4

of 14 (29%) participants (#6, #10, #13 and #17) shed HSV-1 strains expressing ACV^R-associated TK protein variants suggesting that the respective viruses are unresponsive to ACV therapy (Table III) [Graham et al., 1986; Morfin and Thouvenot, 2003; Sauerbrei et al., 2010; van Velzen et al., 2012]. Notably, participants #6 and #17 shed the respective HSV-1 strain on two subsequent shedding episodes suggesting that this inferred ACV^R virus had reactivated from latency. For participant #6, one of these shedding episodes coincided with symptomatic oral lesions.

Discussion

The aim of this study was to examine the kinetics and quantity of oral HSV-1 and VZV shedding during a one-month daily sampling in individuals infected with HIV. It was found that shedding of HSV-1 occurs frequently, on 14.3% of days, whereas VZV shedding is very rare and at significantly lower genome copies. Based on the TK genotypes of sequential HSV-1 DNA positive oral swabs it was demonstrated that the participants shed genetically identical HSV-1 viruses, within and between HSV-1 shedding episodes, which were generally patient-unique. One-third of the participants shed a virus with an ACV^R TK genotype that potentially results in an HSV-1 strain refractory to ACV therapy.

Oral shedding of α HHV likely contributes to the epidemic spread within the human population [Wald et al., 1995; Koelle and Wald, 2000; Kaufman et al., 2005; Cohrs et al., 2008]. Estimates on the frequency of HSV-1 shedding in immunocompetent individuals range from 0.5-76%. The sensitivity of virus detection techniques used, the number of persons and consecutive swabs sampled, and the time course of the studies may have attributed to the high variation reported [Scott et al., 1997; Knaup et al., 2000; Kaufman et al., 2005; Gilbert, 2006; Miller and Danaher, 2008]. Studies that sampled saliva multiple times a day have revealed that 39% of oral HSV-2 reactivations are cleared within 12 hours [Mark et al., 2008]. A study in individuals infected with HIV demonstrated that HSV reactivations are also of short duration and usually resolve before the onset of symptoms [Mark et al., 2010]. Consistent with previous reports, the current study detected HSV-1 shedding in 82% of the individuals infected with HIV at a frequency comparable to immunocompetent individuals [Scott et al., 1997; Knaup et al., 2000; Kaufman et al., 2005; Gilbert, 2006; Miller and Danaher, 2008; Mark et al., 2010]. The results corroborate with earlier data describing that a large proportion of the oral shedding episodes were cleared within 2 days and maximal viral loads per episode were significantly higher in episodes of prolonged duration [Mark et al., 2008; Mark et al., 2010]. In this study, one participant (#6) reported oral lesions during one shedding episode that coincided with the detection of HSV-1 DNA in the mucosal swabs. Notably, the concurrent shedding episode of this participant, which was asymptomatic, had 2-log higher HSV-1 DNA copy numbers suggesting that the viral DNA load was not related to symptomatic herpetic oral lesions [Miller et al., 2006].

Subclinical reactivation of VZV has been less well studied and is largely evident in the elderly, in immunocompromised individuals and in herpes zoster patients [Ljungman et al., 1986; Schunemann et al., 1998; Mehta et al., 2008; Nagel et al., 2011]. One study evaluated the prevalence of VZV in saliva of individuals infected with HIV and demonstrated low

copy numbers in 3 of 59 (5.1%) participants [Wang et al., 2010]. A similarly low incidence and low copy numbers of oral VZV shedding was reported in the current study. The low prevalence of VZV shedding did not allow investigation of the potential interrelatedness of oral HSV-1 and VZV reactivation and shedding in individuals infected with HIV. Deprived VZV-specific T-cell immunity, as seen in immunocompromised individuals and the elderly, is a risk factor for VZV reactivation [Miller, 1980]. Future studies on more severe immunocompromised individuals, e.g. stem cell transplant patients, are warranted to study a potential interrelation between oral HSV-1 and VZV shedding [Ljungman et al., 1986; Wang et al., 2010; Birlea et al., 2011].

HSV-1 and VZV are closely related human herpesviruses that establish a lifelong latent infection of sensory ganglia, yet HSV-1 shedding is much more frequent compared to VZV shedding. The different patterns in virus shedding resemble the differences observed in recurrent symptomatic HSV-1 and VZV infections. Recurrent HSV-1 lesions occur frequently, whereas individuals typically develop herpes zoster only once in a life time [Steiner et al., 2007]. In contrast to HSV-1, VZV establishes latency in ganglia along the entire neuraxis, hence VZV reactivation from ganglia other than the trigeminal ganglia may be undetectable in saliva. However, previous studies have shown that VZV DNA and infectious virus can be detected during asymptomatic reactivation in astronauts and in zoster patients, irrespective of the affected dermatome [Cohrs et al., 2008; Mehta et al., 2008; Nagel et al., 2011]. Likewise, elderly vaccinated with the live-attenuated VZV vaccine shed viral DNA in saliva, suggesting that VZV reaches the saliva by viremic spread upon vaccination or viral reactivation [Pierson et al., 2011].

It was previously shown that HSV-1/VZV co-infection correlates with the detection of both viruses in human trigeminal ganglia [Theil et al., 2003a; Verjans et al., 2007]. Both viruses can be detected in the same trigeminal ganglion and even HSV-1 and VZV double-infected neurons have been described [Theil et al., 2003b]. The higher ganglionic HSV-1 DNA load compared to VZV DNA load [Theil et al., 2003b; Cohrs et al., 2005; Verjans et al., 2007] may account for a higher HSV-1 reactivation frequency. Alternatively, viral determinants of HSV-1 latency, including intra-neuronal expression of latency-associated transcripts and microRNAs [Stevens et al., 1987; Tang et al., 2008; Umbach et al., 2008; Umbach et al., 2009], which are not shared by VZV, may contribute to the differential α HHV reactivation patterns. Finally, CD8 T-cells are considered pivotal to control HSV-1, but not VZV latency in human trigeminal ganglia suggesting that different immune mechanisms act on the control of latency of both α HHVs [Theil et al., 2003a; Verjans et al., 2007].

In addition to the genetic hypervariability of the HSV-1 TK gene, ACV resistance is mostly associated with specific mutations in the drug-targeted viral TK protein [Morfin and Thouvenot, 2003]. The current study demonstrated that for each participant the sequential HSV-1 strains shed were identical within and between oral HSV-1 shedding episodes and expressed an overall individual-unique genotype. The data are in line with a recent study demonstrating that paired trigeminal ganglia are latently infected with a person-specific HSV-1 strain [van Velzen et al., 2012]. Recurrent symptomatic ACV^R HSV-1 infections have been described in ocular infections demonstrating that ACV^R HSV-1 can reactivate from latency [Duan et al., 2009]. Four of 14 (28.6%) participants shed HSV-1 strains with

ACV^R-associated TK protein variants, including 2 patients on two different episodes, suggesting that the inferred ACV^R HSV-1 strains have established latency and reactivated from latency [Duan et al., 2009; van Velzen et al., 2012]. Recurrent therapy-induced ACV^R HSV-1 have been described in immunocompromised individuals, illustrating the importance of local immunity in viral clearance from infected mucosal tissues [Saijo et al., 1999; Morfin et al., 2000; Levin et al., 2004; Zhu et al., 2007]. Due to the low prevalence of ACV^R HSV-1 in the population [Bacon et al., 2003], the participants likely developed ACV^R HSV-1 during the course of ACV treatment prior to study entry. Alternatively, ACV^R HSV-1 could arise locally as the result of natural variation. HSV-1 TK genotyping can be an additional diagnostic tool to determine the anti-viral sensitivity of clinical samples [Duan et al., 2008; Duan et al., 2009]. Rationalized selection of the appropriate antiviral agents may prevent the development of severe herpetic disease in immunocompromised patients, including individuals infected with HIV.

The limitations of the current study are the relatively small sample size with male predominance and the relatively long swabbing interval. Assessment of herpesvirus shedding in the oral cavity in persons infected with HIV can be improved by a dense time interval of mucosal sampling in addition to a detailed description of clinical symptoms (e.g., cold or flu-like symptoms) and dental procedures that may be associated with herpesvirus reactivation. Moreover, a next generation sequencing approach would allow monitoring of drug-resistant minority virus variants present in the isolate.

In summary, the current study demonstrated that short episodes of oral HSV-1 reactivations occur frequently in individuals infected with HIV. Within a daily swabbing interval, HSV-1 shedding episodes were detected with a median of 2 days. HSV-1 TK genotyping demonstrated that each individual sheds a unique HSV-1 strain among episodes, which can express ACV^R-associated mutations. VZV shedding was detected in two of 22 participants at very low copy numbers, demonstrating the low incidence of VZV shedding even in immunocompromised individuals. Future studies should address the mechanism underlying the different shedding kinetics between these two closely related human alpha-herpesviruses.

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Fig. 1.

Oral herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) shedding patterns in individuals infected with HIV. Viral loads [genome equivalent copies (geq) per mL] are plotted on the y-axis and the days on study on the x-axis. For each patient, CD4 T-cell counts (cells/μL) and HIV RNA loads (geq/mL) are specified. Individuals #1 to #6 were taking anti-retroviral therapy (HAART). The dotted line represents the lower limit of detection of the qPCR. Blue bars indicate HSV-1 shedding, red bars indicate VZV, and black bars below the x-axis indicate missing swabs. The solid box (graph of patient #6) indicates the presence of symptomatic herpetic oral lesions. Arrowheads denote the end of the swabbing period per individual and arrows indicate swabs that were used for HSV-1 thymidine kinase-based genotyping (see Table III).

Fig. 2.

Oral herpes simplex virus 1 (HSV-1) shedding characteristics of individuals infected with HIV. Episode duration (in days) is plotted against the peak HSV-1 viral load per individual (log-transformed geq/mL). Bars indicate the median viral load. The dotted line represents the lower limit of detection of the qPCR. The Mann-Whitney test was used to compare median viral loads and significant differences are indicated.

Fig. 3.

Distinct oral herpes simplex virus 1 (HSV-1) thymidine kinase (TK) genotypes in individuals infected with HIV. Maximum likelihood unrooted phylogenetic tree of HSV-1 TK sequences was estimated under the Kimura 2-parameter model. The HSV-1 TK variants shown are coded by the participant's number, episode number (1 or 2), and swab within an episode (A to H). Selected bootstrap values are given. Scale bar represents number of nucleotide substitutions per site. *The TK variants from participant #17 are identical to those of individuals #1 and #6, except for a 3-nucleotide deletion (see Table III).

TABLE I

Demographic and Clinical Characteristics of Study Subjects.

Baseline Characteristics ^a	n = 22 subjects
Median age (range) in yrs	42 (22-61)
Male, n (%)	20 (91)
HSV serostatus, n (%)	
HSV-1 only	7 (32)
HSV-1 and HSV-2	15 (68)
Race/ethnicity, n (%)	
White	18 (82)
Black	2 (9)
Other	2 (9)
Anti-retroviral use during study, n (%)	6 (27)
Median (IQR) CD4 count, cells/ μ L	268 (202-476)
HAART treatment: yes	367 (153-645)
HAART treatment: no	240 (202-412)
Median (IQR) HIV RNA, geq/mL	37,900 (15,656-109,316)
HAART treatment: yes	7,141 (25-46,608)
HAART treatment: no	59,500 (25,400-113,500)

^aHSV-1, herpes simplex virus 1; VZV, varicella-zoster virus; HAART, highly active anti-retroviral therapy; IQR, interquartile range; geq, genome equivalent copies

TABLE II

Proportion of Days, Time Points, and Study Participants with HSV-1 or VZV Detected in at Least One Oral Swab^a.

Baseline Characteristics ^b	n = 22 subjects
Days sample collected, n	715
VZV DNA positive participants, n (%)	2 (9)
Days VZV detected, n (%)	7 (1)
HSV-1 DNA positive participants, n (%)	18 (82)
Days HSV-1 detected, n (%)	102 (14)
Duration of HSV-1 DNA positive episodes	34
1 day (%)	14 (41)
2 days (%)	5 (15)
3 days (%)	4 (12)
4 days (%)	11 (32)

^a HSV-1, herpes simplex virus 1; VZV, varicella-zoster virus

^b n (%), indicates the number, between parentheses the percentage, of the indicated parameters within the whole study group or the total number oral swabs obtained.

Table III

Herpes Simplex Virus 1 Thymidine Kinase Variants Detected in Sequential Oral Swabs of HIV Patients.

Subject ID	Sampling day ^a	Thymidine kinase (TK) protein amino acid changes ^b	GenBank Accession No. ^c
2	16, 17 & 19	I138V	JQ895543
3	6	S23N, E36K, Q89R, I138V, G240E & R281Q	JQ895544
4	21, 25 & 29	S23N, E36K, Q89R, I138V, G240E & R281Q	JQ895545
5	2, 3 & 5	L42P, Q89R, I138V, G251C, V267L, P268T, D286E & N376H	JQ895546
6	17, 25 & 26	C6G, R41H , Q89R, I138V, A192V, G251C, V267L, P268T, D286E & N376H	JQ895547
9	8	I138V & <u>A316V</u>	JQ895548
10	24	S23N, E36K, Q89R, I138V, G240E, R281Q & C336R	JQ895549
11	25 & 28	C6G, L42P, Q89R, I138V, <u>L267T</u> , P268T & D286E	JQ895550
12	15, 16, 17 & 19	I138V & G240E	JQ895551
13	14, 16, 19, 22, 23, 27, 29 & 31	C6G, R41H , Q89R, I138V, A192V, G251C, V267L, P268T, D286E, N376H	JQ895552
15	21 & 22	C6G, I138V & G240E	JQ895553
16	54 & 56	I138V	JQ895554
17	2, 4, 7 & 26	C6G, del36E , R41H , Q89R, I138V, A192V, G251C, V267L, P268T, D286E & N376H	JQ895555
18	30, 50	I138V	JQ895556

^a Sampling day refers to the oral HSV-1 shedding day of which the corresponding TK sequence of the indicated subject was determined. The analyzed sampling days are also depicted with arrows in Fig. 1.

^b Amino acid changes are listed that are different from the HSV-1 TK reference sequence (GenBank No.: GU_734772). All sequential HSV-1 DNA positive swabs of the indicated days were identical within each subject. Underlined and bold/italic TK residue changes are unknown to affect acyclovir (ACV) sensitivity and published TK mutations leading to an ACV-resistant phenotype of the respective HSV-1 strain, respectively [Graham et al., 1986; Morfin and Thouvenot, 2003; Sauerbri et al., 2010; van Velzen et al., 2012]. All other residue changes are TK polymorphisms that are described not to affect ACV sensitivity. The mutation "del36E" refers to a deletion of "glutamic acid" at residue position 36 of the TK protein. HSV-1 DNA positive oral swabs of subjects #2 and #18, and #6 and #13, had identical HSV-1 TK nucleotide and protein sequences, respectively. In contrast, the TK sequences of the HSV-1 shed by subject #16 was different at the nucleotide level compared to the TK sequences of subjects #2 and #18.

^c The GenBank Accession numbers of the HSV-1 TK sequences of the indicated oral swabs are provided.