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Low Prevalence of Varicella Zoster Virus and Herpes Simplex Virus Type 2 in Saliva from Human Immunodeficiency Virus-Infected Persons in the Era of Highly Active Antiretroviral Therapy

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Abstract

Objectives—Human herpesviruses (HHVs), *e.g.* herpes simplex virus (HSV) type 1, Epstein-Barr virus and cytomegalovirus, appear in saliva at greater frequency in persons infected with human immunodeficiency virus (HIV) than healthy individuals. However, it is not known if varicella zoster virus (VZV) and HSV-2 appear simultaneously during HIV infection at greater frequency in saliva during this era of highly active antiretroviral therapy (HAART). The aim of this study was to investigate the prevalence and amounts of VZV and HSV-2 in the saliva of HIV-infected, orally asymptomatic patients.

Study Design—Quantitative polymerase chain reaction was used to investigate the prevalence, quantity, risk, and correlates of salivary VZV and HSV-2 from 59 HIV-seropositive individuals and 53 healthy controls in a case-control, cross-sectional study. Seventy-eight percent of the HIV-seropositive patients (46/59) were taking HAART.

Results—VZV DNA was detected in the saliva of 5.1% (3/59) of the HIV-positive group and in only one healthy control 1.9% (1/53; P = 0.62). The amount of VZV DNA in the expressors was low, generally less than 1,100 copies/mL with no observed difference between the HIV-positive group and the controls (P= 1.0). HSV-2 DNA was not detected in either group. In the HIV-infected group, VZV shedding occurred in those on HAART, but was not associated with oral lesions, specific $CD4^+$ or $CD8^+$ T-cell levels, or demographic factors.

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Conclusions—VZV was detected at low prevalence in the saliva of HIV-infected persons whereas HSV-2 was not detected in the saliva of this cohort. HAART does not appear to diminish the risk for asymptomatic VZV shedding.

Keywords

human immunodeficiency virus infection; saliva; herpesvirus; varicella; shedding; transmission

Human herpesviruses (HHVs) are ubiquitous viral pathogens that cause significant morbidity, especially in immunosuppressed persons.[1–5] These large DNA viruses are spread by contact with infected secretions usually early in life and also later with the onset of sexual activity. Of the bodily secretions and fluids documented to harbor HHVs, saliva appears important for transmission of several HHVs.[6–10] Factors regulating cellular tropism, reactivation from latency, and the immune response dictate the frequency certain HHVs appear in saliva.[11,12] The amount and frequency of HHVs in saliva is currently thought to influence risk for transmission.[13, 14]

We previously reported that cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV)-1 and Kaposi sarcoma associated herpes virus (KSHV) are more prevalent in the saliva of persons infected with human immunodeficiency virus (HIV) than healthy controls.15 Less frequently studied in the saliva of HIV-seropositive persons are HSV-2 and varicella zoster virus(VZV). These two members of the Herpesviridae family both replicate in the epithelium, enter latency in sensory ganglia and are spread by contact with infected secretions. Data from a limited number of studies indicate that these two viruses are shed in saliva infrequently and asymptomatically in healthy individuals.[11,16–18] Men that have sex with men (MSM) and HIV-positive persons are known to have higher rates of oral HSV-2 shedding than healthy individuals.[18–20] Less well understood are the rates and amounts of both VZV and HSV-2 shed orally in asymptomatic, immunocompromised persons.

Current evidence supports that lower leukocyte counts and altered cell mediated immunity influenced by stress, disease and certain drug therapies contribute to higher rates of shedding of HSV-2 and VZV.[11,20–24] However, no study at present has defined the combined rates of salivary VZV and HSV-2 shedding in HIV-seropositive persons compared with healthy individuals during this era of highly active antiretroviral therapy (HAART). Studies on this topic are important in that both HIV infection and HAART are known to influence rates of opportunistic infections,[25–27] however not all HIV-infected persons receive HAART due to availability as well as demographic and economic reasons. Thus, the purpose of this study was to test the hypothesis that HSV-2 and VZV are more prevalent and in greater amounts in the saliva of persons infected with HIV than healthy controls. A secondary aim was to evaluate if HAART and the degree of cellular immunosuppression influenced shedding of these viruses and if multi-HHV infections occurred more frequently in HIV-positive persons.

PATIENTS AND METHODS

Study population and procedures

This cross-sectional study was performed after approval by the University Institutional Review Board and all patients provided written informed consent. The sample included 63 HIV-seropositive subjects who were recruited from the outpatient clinic of the University of Kentucky, in Lexington, Kentucky without respect to HAART administration. Fifty five age- and sex-matched control subjects were recruited from campus volunteers and from patients in the Neurology Clinic at the University of Kentucky. Enzyme linked immunosorbent assays (ELISAs) for HIV were performed on all controls to insure that they were HIV-seronegative. All subjects were 18 years or older. All controls were in good general health and did not have

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histories of liver or kidney dysfunction in order to limit the possibility of enrolling patients with other potentially immunocompromising conditions unrelated to HIV infection. All subjects were free of symptoms of acute illness (i.e., fever, sore throat, body aches, and diarrhea), malignancies and were not pregnant at the time of enrollment. Use of immunosuppressant medications and anti-herpetic therapy one week before the study enrollment date were additional exclusion criteria. Patients were considered to be using HAART when a protease inhibitor was taken with another antiretroviral drug.

Oropharyngeal examinations were performed by a study physician, who was calibrated for the detection of common oral mucosal diseases, and a dentist trained in oral medicine. Subjects were asked specifically about and inspected for gum bleeding, mouth ulcers, mouth pain, dry mouth, presence of tonsillar tissue, oral mucosal lesions, and cervical lymphadenopathy. Unstimulated whole saliva (5 mL) was collected at a single visit by the method described by Navazesh.[28] All samples were maintained on ice, divided into 1 mL aliquots, placed into bar code labeled cryotubes, and frozen at −85°C until use. Saliva samples were thawed, centrifuged and the cell pellet used for DNA isolation as we previously reported.14 Venous blood was collected, processed, and analyzed from all subjects for HIV serostatus using ELISA. Lymphocyte subsets were assayed using blood only from the HIV-posivite patients by flow cytometry.

Polymerase chain reaction (PCR) primers and probes

The primers and probes used for the detection of HHVs were derived from published sources as we have previously reported.[14] Briefly, HSV-2 primers and probes were designed for glycoprotein G as described by Ryncarz *et al.*[29] Primers and probes for VZV were directed to the open reading frame 62 (ORF62) as described by Pevenstein *et al.*30 All probes were labeled at the 3' end with the quencher fluorochrome, 6-carboxy-tetramethyl-rhodamine (TAMRA) (PE Applied Biosystems, Foster City, CA) and at the 5' end with the reporter fluorochrome, 6-carboxy-fluorescein (6-FAM). These primers and probes have been found to reliably detect as few as 10 copies of target HHV DNA per reaction14 and are specific when tested with known HHVs (i.e., cross-reactivity is not observed between the viral assays).29,30 To control for the presence of amplifiable DNA, each DNA sample was also evaluated with host DNA primers and probes designed for the cellular gene GAPDH as described by Cohrs *et al.*[31]

Real time quantitative PCR

Real time quantitative PCR was used for the detection and quantification of HHVs in saliva. The DNA from 1 mL of each saliva specimen was centrifuged and the DNA was isolated from the cell pellet using the Masterpure DNA Purification Kit (Epicentre Biotechnolognies, Madisom, WI) in that HHVs are more readily detected in the cell pellet than in the supernatant.[14] Each 50 μl-PCR mixture contained 10 μL purified DNA template in a final volume consisting of 1X TaqMan Universal PCR master mix (PE Applied Biosystems, Foster City, CA), 900 nM primers and 250 nM TaqMan probe. Real time-PCR was performed on an PCR on ABI 7500 Real-Time PCR system (PE Applied Biosystems, Foster City, CA). Cycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Each PCR run contained negative controls, including reaction mixtures without DNA template as well as several specimens that were known to contain no HHV DNA, a positive amplicon control, and a 10-fold dilution series (1×10^0 to 1×10^6 genome equivalents per reaction) of either genomic HHV DNA or cloned HHV sequences. The positive control standards were HSV-2 genomic DNA and plasmid containing the EcoRI-A fragment containing ORF62 of VZV strain Ellen cloned into pGEM kindly provided by S. Straus from NIH, Bethesda, Maryland. Each specimen was analyzed in duplicate. Results were scored positive if exponential amplification was detected in either reaction. Viral genome copy number results are reported as the means of the duplicate reactions.

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Statistical analysis

Titer levels were compared between controls and HIV patients using the Wilcoxon rank sum statistic. Mean ages were compared using a two sample t statistic. Proportions were compared using Fisher's exact test. All data were analyzed with use of the SAS statistical analysis software (SAS Institute, Cary, NC). Statistical significance was determined at the 0.05 level.

RESULTS

Fifty-five HIV-seronegative healthy and 63 HIV-seropositive individuals were enrolled and contributed whole expectorated saliva in this cross-sectional study. Of these, amplifiable cellular DNA (GAPDH) was present in 53 of the 55 control saliva samples and 59 of 63 samples from HIV-positive individuals. Samples with non-amplifiable cellular DNA were excluded from analysis. The demographics of the two groups (Table I) were similar (P = 0.31 for gender, 0.18 for age, and 0.91 for race). The majority of subjects in both groups were Caucasian men. None of the participants in either group had a clinical malignancy and none were receiving drugs approved for treatment against HHVs (e.g., acyclovir, valacyclovir, famciclovir, ganciclovir). Seventy-eight percent of the HIV-seropositive patients (46/59) were taking HAART. The majority of HIV-positive patients taking HAART were adequately controlled and immune reconstituted as determined by HIV loads (median: 61, range: 0–750,000/mL) and $CD4^+$ counts (median: 388, range: 38–2,427 cells/mm^3). Oral lesions were noted in seven individuals, all were HIV-seropositive. Two had hairy leukoplakia of the tongue, one had an aphthous-like ulcer on the labial mucosa and four had eythematous candidiasis of the tongue and palate.

The prevalence of VZV and HSV-2 was determined by real-time PCR under conditions in which all positive controls were detected and all negative controls proved negative. These assays showed the prevalence of VZV in saliva was low regardless of HIV serostatus. VZV DNA was detected in 5.1% (3/59) of the HIV-positive group and in only one healthy control 1.9% (1/53); the difference not being statistically significant (P = 0.62). HSV-2 DNA was not detected in either group. Interestingly, individuals shedding VZV in saliva in the HIV-positive group were all taking HAART. The immune status, VZV and HIV loads are shown in Table II. VZV shedding was associated with a range of $CD4^+$ and $CD8^+$ T-cell levels. One patient exhibited a low $CD4^+$ count with an inverted $CD4^+/CD8^+$, one exhibited a low $CD8^+$ count, and a third exhibited $CD4^+$ and $CD8^+$ counts within the normal reference range but had an inverted $CD4^+/CD8^+$. Of note, although 20 HIV-positive patients had $CD4^+$ counts of $\leq$ 210/μL, of which four of these persons were not on HAART, none were found to have detectable salivary levels of VZV or HSV-2. HIV loads were less than 10,000/mL for all VZV shedders and no specific demographic factor correlated with shedding.

In as much as we previously performed PCR studies on the majority of these samples for select HHVs,[14] our previous data was compiled with the current data to assess the multi-HHV profile of these patients. In this analysis, VZV was detected concurrently with EBV in the saliva of one healthy and one HIV-positive person. VZV shedding was concurrent with EBV and CMV in another HIV-positive person. The third HIV-positive individual who shed VZV also shed Kaposi sarcoma-associated herpes virus. None of the VZV shedders had oral lesions. Concentrations of VZV DNA in the expressors were low (Table II), generally less than 1,100 copies/mL. The amount of VZV shed by the control patient was similar to that observed in the VZV shedders in the HIV-positive group (P= 1.0 by Wilcoxon statistic).

DISCUSSION

This study is one of the first to report the prevalence and viral loads of HSV-2 and VZV in the saliva of HIV-positive patients during the HAART era. Our analyses demonstrate that salivary

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shedding of VZV is infrequent in HIV-positive patients but at a slightly higher rate than healthy controls. Salivary shedding of HSV-2 was not detected in either group. In all cases, shedding of VZV occurred in asymptomatic individuals who lacked visible oral lesions. Risk factors for salivary VZV shedding were not readily evident due to the small number of persons who shed in this population in this cross-sectional study.

Salivary VZV shedding has been reported to occur in ≤ 1% of the healthy population on any given day.11,14,[16] Consistent with this we detected VZV in the saliva of one of 53 healthy patients (1.9%). However, VZV is known to be present in saliva of up to 100% of patients with herpes zoster (HZ),[10] a disease that is often triggered by immune alterations.32 HIV is a disease characterized by immune T-cell deficits and a propensity for increased risk of HZ.33 However, HAART has reduced the incidence of HZ,34 and paradoxically has been associated with the induction of HZ during immune restoration.[35] Further, a low $CD4^+$ count in HIV-seropositive individuals is a significant risk factor for HZ.[34, 36] Thus, one could predict that HIV-infected individuals with low $CD4^+$ counts or recent immune reconstitution might demonstrate a higher prevalence of VZV in their saliva. However, none of the 20 HIV-positive patients in this study who had $CD4^+$ counts of ≤ 210/μL had detectable salivary levels of VZV. Even the four HIV-positive persons with $CD4^+$ counts < 210/μL who were not on HAART did not have detectable levels of VZV in saliva. In contrast, we observed that the HIV-infected persons who were shedding VZV were on HAART. These data suggest that VZV shedding is infrequent in asymptomatic HIV-positive persons when samples are procured randomly on any given day, and HAART does not appear to diminish the risk for asymptomatic VZV shedding.

The amount of VZV detected in the saliva of asymptomatic individuals, when present, ranged from 534 to 1094 copies/mL and were similar whether patients were HIV infected or not. These levels are considered low with respect to the mean levels observed during HZ. In a study of HZ patients, salivary VZV levels exceeded 10 million copies/mL with the mean approximately 3 logs higher than the mean levels detected during asymptomatic shedding.[10] However, the low levels of asymptomatic VZV shed in these study volunteers may be sufficient for transmission and interaction with the epithelium and other microbes within the oral cavity that should be considered in future studies.

The absence of HSV-2 shedding determined in this study of HIV-positive and healthy persons is similar to the low rate of detection reported in other studies.[14,16,37–40] In these studies, the rate of detection from asymptomatic healthy individuals is less than 1% on any given day. In MSM and HIV-positive individuals, the rate of asymptomatic shedding in saliva is reported to be slightly higher, *i.e.*, 2% of days when consecutive day sampling is employed.[18,20,23,41] Our lower rate is likely reflected by the fact that we did not perform consecutive multi-day sampling as performed by others, a clear limitation of our study. Our study was also limited by the fact that we did not obtain the serostatus of HSV-2 and VZV. In North America, about 17% to 20% of HIV-negative persons[42, 43] and ≥ 50% of HIV-positive persons are typically infected with HSV-2,[44–46] whereas > 90% of the general population carry antibodies against VZV, albeit many from receiving a live attenuated vaccine.[47] Thus, a significant portion of our population may not harbor these viruses and/or may not be able to shed these viruses. Nevertheless, our study cohort reflects the general population who could present to the dental office and the likelihood of these viruses appearing in saliva during a routine dental procedure.

Based on these data and our previous analyses,[15] multi-virus shedding was an additional finding. We observed that patients who shed VZV also shed CMV, EBV and KSHV in saliva on the same day, even in the absence of clinically visible lesions. This is consistent with our report that multi-herpesvirus shedding in saliva is common in asymptomatic HIV-positive persons,[15] and brings to light the need for studies to better understand the factors that differentially regulate HHV shedding.

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In conclusion, our data suggest that HIV infection with or without HAART did not significantly alter the frequency of salivary HSV-2 and VZV shedding compared with healthy controls when measured in a cross-sectional study design. In the future, longitudinal studies should be performed involving HIV-positive patients, before and after initiating HAART, to achieve a better understanding of the salivary shedding patterns that might be important for predicting orofacial disease caused by these viruses and if oral HHV infections facilitate the HIV epidemic much like genital HSV-2 infection does.[48,49]

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Table I

Demographic characteristics of the study population

Serostatus	Total	Gender	Age range (average)	Race
HIV negative	53	42 M / 11 F	18–75 (36.8)	34 C; 14 AA; 5 O
HIV positive	59	51 M / 8 F	22–69 (40.2)	40 C; 15 AA; 4 O

M=male; F=female; C=Caucasian; AA=African-American; O=Other

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Table II

Laboratory characteristics of those shedding VZV in saliva

Age	Race	Gender	Status	HSV-2	VZV	CD4	CD8	Ratio	HIV Load	Oral Lesions
27	C	F	Healthy	0	534	N/A	N/A	N/A	N/A	No
22	Asian	M	HIV+	0	1094	179	641	0.3	1060	No
43	C	M	HIV+	0	119	666	299	2.2	<400	No
40	C	F	HIV+	0	593	795	1132	0.7	9800	No

Viral loads are reported per mL. CD T-lymphocyte counts are reported per μL.

Not applicable = N/A.

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