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Oral shedding of Herpesviruses in HIV+ patients with varying degrees of immune status

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Abstract

Objective—Herpesvirus shedding in the oral cavity was analyzed to determine if presence in the oral compartment correlates with systemic changes in HIV-associated immune deficiency as measured by CD4+ counts, plasma HIV viral load (VL) and presence of AIDS-defining events.

Design—A5254 is a multicenter, cross-sectional, single-visit study to evaluate oral complications of HIV/AIDS and determine the association between clinical appearance, herpesvirus shedding and immune status as ascertained by CD4 count and HIV viral load. 307 HIV infected individuals were evaluated and throat wash collected.

Methods—Fisher's exact test and Kruskal-Wallis test were used to assess the association between presence of herpes viruses and the state of immunodeficiency as stratified by a combination of CD4+ count and HIV VL. Relationship between pathogens and HIV VL in plasma was modeled by logistic regression.

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Results—The presence of cytomegalovirus CMV and Herpes Simplex Virus-1 (HSV-1) in throat wash was associated with decreased CD4 counts. By contrast Kaposi Sarcoma-associated herpes virus (KSHV) and EBV were similarly detectable across all levels of CD4 counts. One unit increase in $\log_{10}$(HIV VL) was associated with 1.31 times higher odds of detecting CMV in throat wash when controlling for oral candidiasis, CD4 count, and sites (95% CI 1.04 –1.65, P=0.02).

Conclusions—Oral CMV shedding was significantly higher in highly immunocompromised HIV+ participants. Our finding supports the recommendations to start antiretroviral therapy (ART) independent of CD4 count as this may have the added benefit to lower the risk of herpes virus transmission among persons infected with HIV and their partners.

Keywords

Introduction

Combined clinical observations and pathologic evaluations represent the standard to diagnose oral complications of immunodeficiency and are used to make treatment decisions. By comparison, the treatment of human immunodeficiency virus (HIV) relies heavily on HIV plasma viral load (VL). Current recommendations are to initiate combination antiretroviral therapy (ART) as early as possible. Starting ART independently of CD4 count is justified, in part, in order to prevent HIV transmission [1]. It depends on an extensive health care infrastructure to supply ART and to provide for early detection of HIV. In populations with limited access to ART, Kaposi Sarcoma (KS), oral hairy leukoplakia (OHL), and oral candidiasis (OC) signify advanced HIV disease. This study asked whether the presence of herpes simplex virus (HSV-1), Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), or Kaposi's sarcoma-associated herpesvirus (KSHV) in the throat wash of persons with HIV infection correlated with immune status as measured by CD4 count and plasma HIV viral load. If this was true, monitoring the levels of oral pathogens could help in the diagnosis of deteriorating immune status. Since sampling the oral cavity is minimally invasive compared to blood sampling and can be done by self-collection this may present an option to reach underserved populations and to improve HIV care in resource limited settings.

Observing cutaneous KS is part of the definition of AIDS (reviewed in [2]). KS disease is associated with declining immune status in the setting of untreated HIV infection. In HIV-negative transplant recipients KS is a complication of iatrogenic immune suppression and the relative risk of KS in transplant recipients is estimated at 200 fold compared to the general population [3–5]. Oral KS in addition to cutaneous or systemic KS is considered an indicator of advanced KS disease (T1); it is associated with poor outcome in response to cytotoxic therapy. Oral KS may benefit from intralesional application of cytotoxic agents [6]. Aspects of the AIDS KS epidemic in US have changed since the introduction of ART. One third of KS now develops in patients on effective ART with plasma HIV VL below the limits of detection [7, 8]. The decline of KS incidence as observed concurrent to the introduction of ART has leveled off since 2010 and KS remains the single most prevalent cancer occurring

in persons with HIV today [9, 10]. KSHV is the etiological agent of KS. All cases of KS are associated with prior or concurrent KSHV infection regardless of HIV status. KSHV is detected in saliva and an inhibitor of KSHV viral replication (ganciclovir) reduces oral shedding and lifetime KS risk [11–17]; whether or not established KS lesions respond to inhibitors of viral replication is unclear [18, 19].

OHL is an AIDS defining condition [20]. EBV is the etiological agent of OHL and all cases of OHL are associated with concurrent permissive EBV lytic replication in the lesion [21, 22]. OHL has also been found in HIV-negative, EBV-positive organ transplant patients [23]. It tends to resolve upon immune reconstitution in the setting of HIV infection and responds to acyclovir, an inhibitor of the EBV DNA polymerase [24]. EBV is detected at high levels in saliva and an inhibitor of EBV viral replication (acyclovir) reduces oral shedding [25–27].

OC was the most common oral complication of HIV/AIDS prior to the introduction of ART (reviewed in [28, 29]); it still is in many low and middle-income countries. OC can be caused by multiple *Candida* species in the setting of HIV [30]. OC is associated with severe immune suppression and extremely low CD4+ cell counts. Systemic therapy is indicated though studies suggest that local agents are efficacious as well [31, 32].

The primary objective of the ACTG A5254 trial was to describe the prevalence of oral lesions in people with HIV infection and to test the hypothesis that after training non-professional oral health specialists can accurately diagnose oral lesion frequently found in this population. This work has been published, as have the details of the clinical data and socioeconomic characteristics of the study population [33]. As oral complications of HIV/AIDS were previously detected at lower CD4+ counts, this cohort was geared towards enrollment of persons with low CD4 counts. This design allowed evaluation of associations between different pathogens in the oral cavity in participants with defined immunologic and HIV plasma VL status.

Methods

Study Design

Detailed study design, patient population and sample collection were previously published [33]. In brief, ACTG A5254 is a cross-sectional study that enrolled HIV-1-infected adults 18 years or older with or without prior ART from five locations in the US and one location in Haiti between 2009 and 2012. Institutional Review Boards or Ethics Committees of each participating institution approved the study, and each patient gave written informed consent.

As previously reported [33] there were 128 (42%) cases of OC, 39 (13%) cases of OHL, and 31 (10%) cases of oral KS in the A5254 cohort. OC was most common in stratum A (A: 66%, B: 18%, and C/D: 14%, *P* value for the Fisher's exact test across strata < 0.001). OHL seemed less common in stratum B (A: 24 (15%); B: 5 (5%); C/D 10 (17%), p value for the Fisher's exact test across strata < 0.05). Oral KS was most common in stratum A (A: 29 (18%); B: 2 (2%); C/D (0%), p value for the Fisher's exact test across strata < 0.001). HSV-1 and CMV oral lesions could not be discerned from oral lesions of non-viral origin. In sum,

OC and oral KS were more common in participants with lower CD4+ cell count overall and within the context of immunodeficiency associated higher HIV VL (comparing stratum A and B).

Throat wash Collection

A 5-minute unstimulated whole saliva (UWS) flow rate was recorded, and collected [34]. A 1-minute oral rinse/throat wash using 10 mL of sterile saline was collected afterwards. Both saliva and throat wash specimens were frozen in aliquots at −80°C at the site laboratory, and banked. Only soluble throat wash was used for the current study.

Candida analysis

Before the throat wash was processed further 2.5 mL sterile saline was aliquoted and processed for *Candida* detection by culture. A culture was defined as positive, and confirming the clinical diagnosis of OC, among individuals with clinical features of OC and a number of colony forming units (CFUs).

CD4+ count and plasma HIV VL

Phlebotomy was performed at the time of the visit and CD4+ cell count and plasma HIV-1 VL were measured. Plasma HIV-1 VL was measured using the Abbott real-time HIV-1 quantitative PCR Assay on 0.6–1.0 ml input volume (sensitivity of 40 copies/mL, linear range 1.6 log copies/mL to 7.0 log copies/mL, specificity >99% as reported by the manufacturer).

Throat wash herpesvirus load (CMV, EBV, KSHV, HSV-1)

Real-time qPCR was used to detect multiple herpesviruses as described previously [29]. The assay measures KSHV, CMV, EBV, HSV-1 independently. This was important, since we expected multiple herpesviruses be present at widely differing levels in the same sample, which may have impact the sensitivity of multiplexed PCR. Herpesvirus load were categorized either as present or absent (based on endpoint result regardless of viral load) and as copies/ml if >500 copies/ml, which was the lower limit of the linear range for this assay.

Data Availability

Analyses were performed by the Statistical Data Analysis Center for the ACTG, which is located within the Center for Biostatistics in AIDS Research, Harvard School of Public Health, Boston, MA. This is where the datasets are stored. Consistent with NIH regulations and ACTG policy, the data is publically available. Requests for data are to be submitted to sdac.data@sdac.harvard.edu. Data are de-identified prior to distribution. Additional analyses were performed at the University of North Carolina Lineberger Comprehensive Cancer Center, Chapel Hill, NC.

Statistical procedures

Sample characteristics were summarized using proportions for categorical variables, and mean, median with 1st and 3rd quantiles (Q1 and Q3) for continuous variables. The results were presented for all the strata, and for each CD4+ cell count / plasma HIV-1 viral load

stratum separately (strata C and D were combined). The frequency of OC was confirmed by a positive culture (1 CFU/mL) was computed and the difference across strata using the Fisher's exact test was explored. CD4+ cell count and $\log_{10}$ (plasma HIV-1 VL) was compared among participants across strata using the Kruskal-Wallis test. $\log_{10}$ (VL) of individual herpesviruses in saliva was also calculated and summarized across strata for participants with detectable herpesviruses. The frequency of virus-detectable cases was computed by CD4+ cell count/plasma HIV-1 VL stratum, and the difference across strata was compared using the Fisher's exact test. Logistic regression was used to model the relationship between the presence of herpesvirus oral shedding and HIV-1 VL in plasma controlling for CD4+ cell count, oral candidiasis and sites. A detailed description of the methods of diagnosis of oral manifestations of disease in this study has been reported previously [33]. The model yielded adjusted odds ratios with 95% confidence intervals.

Results

Oral disease was associated with severe immunodeficiency

The salient features of the A5254 study design were as follows: A5254 was a single time-point, cross sectional study, which enrolled its first participant in October 2009 and its last participant in September 2012 [33]. All participants were evaluated by oral health specialists, and throat wash samples were taken. Participants were initially enrolled across four strata. We combined participants for two strata (C and D) with CD4 counts > 200 cells/mm^3 regardless of HIV VL in order to obtain a more balanced design (Table 1). Stratum A encompassed N=157 participants with a CD4+ cell count   200 cells/mm^3 and plasma HIV-1 VL > 1,000 copies/ml. Of these, 99 (63%) were enrolled in Haiti and 58 (37%) in the continental US. 88% of participants in stratum A were black, non-Latino. The proportion of black, non-Latino was significantly larger than 60% and 47% in stratum B and C/D, respectively (P value for the Fisher's exact test across strata < 0.001). The majority (93%) of participants from Haiti were enrolled only in stratum A. Stratum B encompassed N=91 participants with CD4+ cell count   200 cells/mm^3 and a plasma HIV-1 VL   1,000 copies/mL. Stratum C/D encompassed N=59 participants with CD4+ cell count   200 cells/mm^3 regardless of HIV plasma VL. The majority of participants were male in stratum B (85%) and C/D (68%), significantly larger than 55% in stratum A (P value for the Fisher's exact test across strata < 0.001). The median age was 44 (Q1 – Q3: 38–51) with participants in stratum B being somewhat older compared to stratum A and C/D.

Ninety-nine (32%) participants were not on combination antiretroviral therapy (ART) at study entry. Almost all patients in stratum B were on ART. 52% of patients in stratum A also reported being on ART, yet >90% of those did not recount a prior AIDS defining illness (data not shown). Presumably this reflects participants, who only recently started on ART.

Immune status associated shedding in the oral cavity differs among different herpesviruses

HSV-1 was only detectable in stratum A participants (8%) (Table 2). The median (Q1, Q3) of HSV-1 VL ($\log_{10}$ copies/mL) for all virus positive samples was 4.25 (3.90, 4.40). CMV was detectable in throat wash of 50 % of stratum A participants, 15% in stratum B, and 10%

in stratum C/D. There was a significant association between the presence of CMV and stratum (p < 0.001). The median (Q1, Q3) of detectable CMV VL (Log_{10} copies/mL) was 3.60 (3.40, 3.90). KSHV, the etiologic agent of KS, was detectable in throat wash of 3% of stratum A participants, 4% of stratum B participants, and 7% of stratum C/D participants. There was no association between the presence of KSHV and stratum. Overall the median (Q1, Q3) of detectable KSHV VL (Log_{10} copies/mL) was 3.35 (3.20, 3.70). EBV was detectable in throat wash of 87% of stratum A participants, 77% of stratum B participants, and 83% of stratum C/D participants. There was no significant association between the presence of EBV and stratum. Overall the median (Q1, Q3) of detectable EBV VL (Log_{10} copies/mL) was 4.60 (4, 5). We found no statistically significant evidence for an association of EBV VL or CMV VL with candida burden.

The overall frequency of HSV-1 or KSHV positive samples was very low (4%), thus the study was inadequately powered to detect possible associations of HSV-1 or KSHV with candida burden, i.e., colony count units. EBV was detectable in 83% of samples and CMV in 32% and their VLs spanned a large range (Figure 1). Evidence for an association between EBV VL or CMV VL with candida burden was not statistically significant *(P*=0.98, *P*=0.60; data not shown). No significant association was found between EBV and CMV VL in the oral cavity (*P*=0.63; data not shown), suggesting that independent factors foster oral replication and shedding of these two herpesviruses.

We used logistic regression to evaluate the relationship between CMV shedding in oral cavity and HIV plasma VL. (Table 3). The odds of detecting CMV was 1.31 times higher with one unit increase in log_{10}(HIV VL) when controlling for OC, CD4 count, and sites (95% CI 1.04 –1.65, p=0.02), suggesting a significant association between detection of CMV in throat wash and HIV VL in plasma. The association between the presence of EBV in throat wash and log_{10} (HIV VL) in plasma was marginally significant with odds ratio 1.26 (95% CI 0.99 –1.61, *P*=0.06; Data not shown). CMV and HSV-1 were more often detected in stratum A than in stratum B, which had a similar level of immunodeficiency as ascertained by CD4+ count but suppressed HIV replication.

Discussion

Oral complications of HIV/AIDS manifest primarily in stages of advanced HIV disease. OHL was one of the first described clinical manifestations of AIDS and oral KS became widely recognized during the early AIDS epidemic in the US and Europe. CMV-associated diseases, in particular retinitis, pneumonia and encephalitis also are part of the clinical manifestations that signify end-stage AIDS, as is HSV-1 disease. The etiological agent for KS is KSHV and the etiological agent for OHL is EBV. CMV, EBV, KSHV, HSV-1 are present in oral secretions and transmitted via the oral route [12, 35–37]. CMV plasma VL is closely linked to immune suppression, whether by HIV or iatrogenic drug regimen [35, 38]. A close association between plasma VL and disease burden also holds true for EBV-associated post-transplant lymphoproliferative disease and some EBV-positive lymphoma [39, 40], but not always for KSHV and KS [41]. A5254 found that oral shedding of HSV-1 and CMV, but not of EBV and KSHV, were correlated with immune deficiency.

The VL of each of the four herpesviruses did not correlate with each other and not always with immune status. EBV was shed consistently independent of immune status, and independent of CMV VL (Figure 1). EBV oral VLs were much higher than those of the other herpesviruses consistent with prior work [25, 35, 37, 42]. Our single point detection rate for KSHV was low and not associated with immune status, whereas oral KS disease was. This lack of association may be the result of sampling limitations as outlined below or it may be due to the fact that for KSHV, like for other herpesviruses, the majority of shedding occurs in the absence of overt lesions. KSHV VLs in the oral cavity tended to fluctuate, necessitating the need to sample frequently over extended periods to detect virus [36, 43]. Longitudinal sampling of the same person and detailed biopsies may be needed to establish associations among the sporadically shed herpesviruses (HSV-1, KSHV), lesion phenotype, and clinical parameters. HSV-1 shedding and disease has been associated with overall immunodeficiency and more directly with HIV replication [35, 44]. In the A5254 trial, HSV-1 was only detected in the oral cavity of participants in stratum A, i.e. in the setting of high HIV VLs and CD4 < 200 cell/mm^3, though we did not have enough events to establish a robust association. This may be due to the previously described episodic nature of HSV-1 shedding [43, 44].

CMV in the oral cavity was closely linked to immune suppression in the setting of HIV. This is consistent with prior studies [35, 38]. In addition, we found that CMV VL in the oral cavity was independently associated with HIV VL in the setting of immunodeficiency. This suggests a relation between CMV and HIV replication above and beyond immune status. It may be feasible to develop CMV saliva VL into a biomarker for immunodeficiency analogous to CMV plasma VL in transplant patients. Such an assay would be minimally invasive and lend itself repeat sampling as well as self-sampling.

This study had a number of limitations, such as the cross sectional nature of sample collection, which did not allow us to distinguish primary infection from reactivation. This design also does not allow us to make inferences about persistence. As the primary objective of A5254 was to evaluate oral examination by non-oral health specialists [33], we did not include a blood draw. Hence, we can only infer the underlying seroprevalence for each of the herpesviruses based on published reports in these populations. For CMV, seroprevalence in MSN, and in the general population has been consistently estimated at 80%, levels for EBV and HSV-1 are estimated at 90% and 67% in the general population globally and consistently higher among HIV+ [45, 46]. For KSHV the data are more variable [47–49]. Most recently, the ALLRT study estimates a seroprevalence of 38% in ACTG clinic attendants [50]. Lastly, a large proportion of patients in stratum A enrolled in Haiti, whereas few of the other strata enrolled at this location because of the state of HIV care on the island. This represents a potential confounder, which was included in the multivariable logistic regression model that demonstrated a direct association between CMV oral VL and HIV systemic VL.

In sum, we find that HIV-induced deficiency in the adaptive immune response, as ascertained here by CD4+ counts, does not elevate oral shedding among herpesviruses equally. These findings are consistent with a model in which local trigger/pathogen pairings synergize for herpesvirus replication, infection, reactivation, transmission, and disease. In

case of CMV one of the triggers may be HIV replication itself. If we were to understand the forces that govern virus reactivation in the oral cavity, it would open up new avenues of intervention, prophylaxis, and diagnosis.

Acknowledgments

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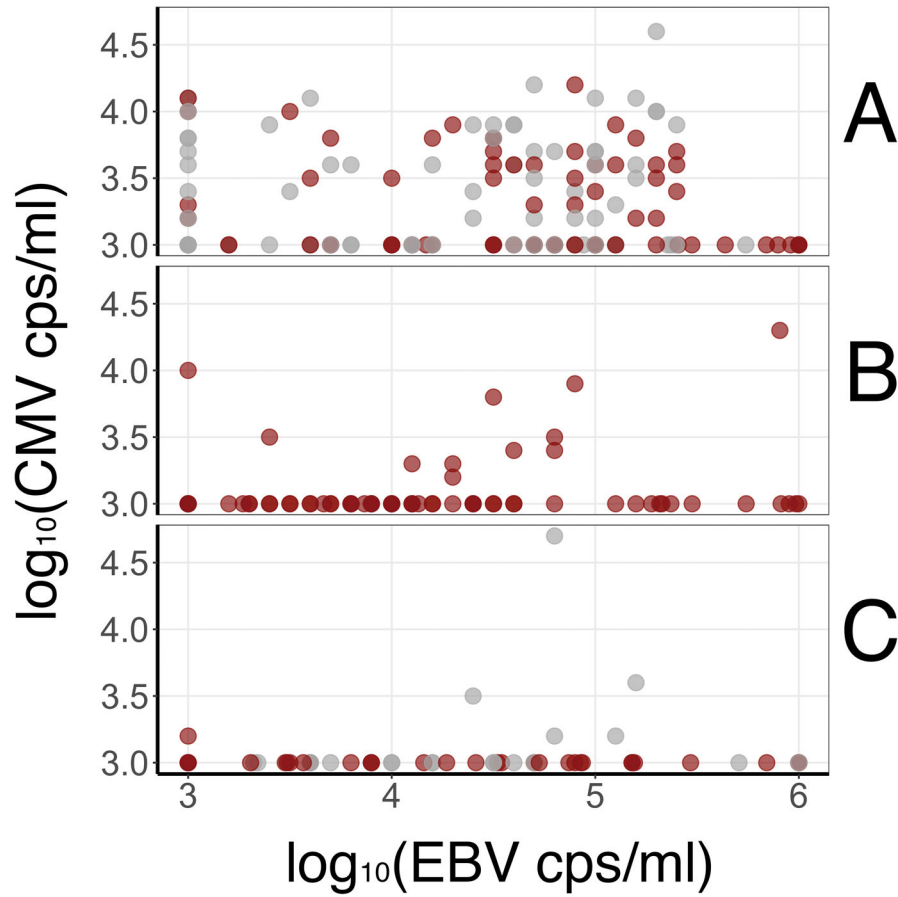
Figure 1.
Association between very high EBV and CMV levels in throat wash. The data are separated by stratum. The three panels represent the three study strata A, B, and C/D. The top panel shows the measurements for stratum A (high HIV VL, CD4   200 cells/μl), the middle panel stratum B (low HIV VL, CD4   200 cells/μl), and the bottom panel stratum C/D (CD4 > 200 cells/μl). Shown in each panel is $\log_{10}$ EBV copies/ml on the horizontal axis compared to $\log_{10}$ CMV copies/ml on the vertical axis. EBV is detectable at all strata across a wide range of VL. CMV is highly enriched in stratum A (top panel). This reflects the significant association with HIV viral load as described in Table 3. To aid visualization the threshold was set at $3 \times \log_{10}$ so that participants with detectable VL lower than $3 \times \log_{10}$ were coded as $3 \times \log_{10}$, and participants with detectable VL higher than $6 \times \log_{10}$ were coded as $6 \times \log_{10}$. Lastly, ART status is reflected in the color. Colored in gray are participants not on ART and shown in red are participants who self-reported being on ART, though we do not know the time of therapy initiation, nor did we detect a correlation between ART usage and CMV or EBV VL.

Table 1

Demographics and baseline characteristics

Cohort Characteristics (non-viral). IV drug history and history of an AIDS-defining illness were recorded, but did not differ significantly among the three strata.

HIV VL		>1000	1000	any		
CD4		200	200	CD4 >200		
Characteristic		A (N=157)	B (N=91)	C/D (N=59)	Total (N=307)	P-Value
Sex	M	87 (55%)	77 (85%)	40 (68%)	204 (66%)	<.001 *
	F	70 (45%)	14 (15%)	19 (32%)	103 (34%)	
Location	US	58 (37%)	83 (91%)	59 (100%)	200	<.001 *
	Haiti	99 (63%)	8 (9%)	0 (0%)	107	
Race/Ethnicity	White non-Latino	8 (5%)	28 (31%)	13 (22%)	49 (16%)	<.001 *
	Black non-Latino	138 (88%)	55 (60%)	28 (47%)	221 (72%)	
	Latino (regardless of race)	7 (4%)	8 (9%)	15 (25%)	30 (10%)	
	Other	4 (3%)	0 (0%)	3 (5%)	7 (2%)	
Oral Candidiasis	No	51 (32%)	72 (79%)	51 (86%)	174 (57%)	<.001 *
	Yes	104 (66%)	16 (18%)	8 (14%)	128 (42%)	
	Missing	2 (1%)	3 (3%)	0 (0%)	5 (2%)	
Oral Hairy Leukoplakia	No	133 (85%)	83 (91%)	49 (83%)	265 (86%)	.047 *
	Yes	24 (15%)	5 (5%)	10 (17%)	39 (13%)	
	Missing	0 (0%)	3 (3%)	0 (0%)	3 (1%)	
Kaposi Sarcoma	No	128 (82%)	86 (95%)	59 (100%)	273 (89%)	<.001 *
	Yes	29 (18%)	2 (2%)	0 (0%)	31 (10%)	
	Missing	0 (0%)	3 (3%)	0 (0%)	3 (1%)	
Currently on ART?	No	75 (48%)	2 (2%)	22 (37%)	99 (32%)	<.001 *
	Yes	82 (52%)	89 (98%)	37 (63%)	208 (68%)	
Age at study entry	N	157	91	59	307	<.001 **
	Median	42	48	43	44	
	Q1, Q3	37, 50	43, 54	34, 49	38, 51	
CD4 (cells/µL)	N	157	88	59	304	<.001 **
	Missing	0	3	0	3	

| | | HIV VL >1000 | 1000 | any | | |
| | | CD4 200 | 200 | CD4 >200 | | |
Characteristic		A (N=157)	B (N=91)	C/D (N=59)	Total (N=307)	P-Value
	Median	93	142	525	138	
	Q1, Q3	38, 143	100, 172	339, 657	71, 189	
Log10[HIV RNA(cp/ml)]	N	157	88	59	304	<.001 **
	Missing No.	0	3	0	3	
	Median	5.12	1.70	1.88	4.40	
	Q1, Q3	4.70, 5.53	1.60, 1.89	1.68, 4.43	1.70, 5.18	
Colony Unit Count (CFU/ml)	N	155	88	59	302	<.001 **
	Missing	2	3	0	5	
	Median	1,660	100	200	720	
	Q1, Q3	400, 2,800	1, 1,223	1, 980	8, 2,360	

*
Fisher's Exact Test

**
Kruskal-Wallis Test

Table 2

Herpesvirus loads by stratum

Characteristic		A (N=157)	B (N=91)	C/D (N=59)	Total (N=307)	P-Value[a]
CMV (Log$_{10}$ cps/ml) of detectable	N (%[b])	78 (50%)	14 (15%)	6 (10%)	98 (32%)	0.001
	Median	3.65	3.50	3.35	3.60	
	Q1, Q3	3.50, 3.90	3.30, 3.80	3.20, 3.60	3.40, 3.90	
EBV (Log$_{10}$ cps/ml) of detectable	N (%[b])	136 (87%)	70 (77%)	49 (83%)	255 (83%)	0.147
	Median	4.70	4.20	4.50	4.60	
	Q1, Q3	4.20, 5.10	3.80, 4.80	3.90, 4.94	4, 5	
HSV (Log$_{10}$ cps/ml) of detectable	N (%[b])	12 (8%)	0 (0%)	0 (0%)	12 (4%)	0.001
	Median	4.25	n/a	n/a	4.25	
	Q1, Q3	3.90, 4.40	n/a	n/a	3.90, 4.40	
KSHV (Log$_{10}$ cps/ml) of detectable	N (%[b])	4 (3%)	4 (4%)	4 (7%)	12 (4%)	0.327
	Median	3.50	3.55	3.19	3.35	
	Q1, Q3	3.35, 3.60	3.25, 3.95	3.15, 3.70	3.20, 3.70	

[a] Fisher's Exact Test

[b] percent of samples with detectable virus

Table 3

Logistic regression model exploring the association between detection of CMV in throat wash and HIV VL in plasma controlling for CD4 cell count, oral candidiasis, and sites.

Independent variables	aOR (95% CI)	P value
Log_{10}(HIV VL)	1.31 (1.04, 1.65)	0.02
Oral candidiasis (yes vs. no)	0.75 (0.37, 1.53)	0.43
Sqrt(CD4)	0.95 (0.90, 1.01)	0.13
Sites (international vs. domestic)	5.30 (2.56, 10.97)	<0.001

aOR = adjusted odds ratio; CI = confidence interval; Sqrt(CD4) = square root of CD4.