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ABSTRACT

We report a clinical study that examines whether HIV infection affects *Streptococcus mutans* colonization in the oral cavity. Whole stimulated saliva samples were collected from 46 HIV-seropositive individuals and 69 HIV-seronegative control individuals. The level of *S. mutans* colonization was determined by conventional culture methods. The genotype of *S. mutans* was compared between 10 HIV-positive individuals before and after highly active antiretroviral therapy (HAART) and 10 non-HIV-infected control individuals. The results were analyzed against viral load, CD4+ and CD8+ T-cell counts, salivary flow rate, and caries status. We observed that *S. mutans* levels were higher in HIV-infected individuals than in the non-HIV-infected control individuals ($p = 0.013$). No significant differences in *S. mutans* genotypes were found between the two groups over the six-month study period, even after HAART. There was a bivariate linear relationship between *S. mutans* levels and CD8+ counts ($r = 0.412$; $p = 0.007$), but not between *S. mutans* levels and either CD4+ counts or viral load. Furthermore, compared with non-HIV-infected control individuals, HIV-infected individuals experienced lower salivary secretion ($p = 0.009$) and a positive trend toward more decayed tooth surfaces ($p = 0.027$). These findings suggest that HIV infection can have a significant effect on the level of *S. mutans*, but not genotypes.

KEY WORDS: HIV infections, *Streptococcus mutans*, genotype, saliva, CD8+ T-lymphocytes, HAART.

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HIV Infection Affects *Streptococcus mutans* Levels, but Not Genotypes

INTRODUCTION

According to UNAIDS, more than 34 million people worldwide were living with HIV in 2010 (UNAIDS, 2011). The availability of highly active antiretroviral therapy (HAART) has significantly changed the disease profile and prolonged the lifespan of HIV-infected individuals, suggesting, in turn, that those individuals will continue to battle HIV-associated diseases, particularly those affecting the oral cavity (Greenspan *et al.*, 2004; Hodgson *et al.*, 2006).

Previous studies demonstrated that HIV-infected individuals are at greater risk for caries compared with their non-HIV-infected counterparts (Baqui *et al.*, 1999; Mulligan *et al.*, 2004; Phelan *et al.*, 2004), as well as for enlargement of salivary glands and salivary gland hypofunction (Mulligan *et al.*, 2000; Navazesh *et al.*, 2009). It has been hypothesized that immunodeficiency and a progressive decrease in CD4+ T-lymphocytes resulting from HIV infection might alter salivary flow rate and impair the secretory immune system, thus contributing to increased bacterial colonization in the oral cavity (Lu and Jacobson, 2007; Nittayananta *et al.*, 2010), implying that cariogenic bacteria may also increase in the oral cavity. Investigators have continuously provided new evidence suggesting that high HIV viral loads and progressive decrease in CD4+ T-lymphocytes resulting from HIV infection are associated with the increased dental caries in HIV-infected individuals (Baqui *et al.*, 1999; Hicks *et al.*, 2000; Phelan *et al.*, 2004; Beena, 2011). However, it remains unclear if the increased risk for dental caries in HIV-infected individuals is associated with quantitative and qualitative changes in oral microbial colonization, salivary hypofunction resulting from immunosuppression, or other unexplored factors, such as dietary differences.

Streptococcus mutans is considered one of the principal pathogens associated with the development of dental caries (Loesche, 1986). Aas *et al.* reported that *Streptococcus* species were more commonly detected in supragingival plaque of HIV-infected individuals with low viral loads, compared with non-HIV-infected control individuals (Aas *et al.*, 2007). A recent study by Back-Brito and

co-workers reported that HIV infection could affect salivary microbial diversity (Back-Brito *et al.*, 2011). Madigan and colleagues found that levels of mutans streptococci and *Lactobacillus*, as cariogenic oral flora indicators, were correlated with HIV status in HIV-infected children (Madigan *et al.*, 1996). We currently know very little about the association between *S. mutans* colonization and HIV infection and the effect of HAART on the oral microbial community. This study, therefore, aimed to compare levels and genotypes of *S. mutans* in saliva samples of HIV-infected individuals and non-HIV-infected control individuals. We further explored changes in *S. mutans* colonization before and after HAART initiation over a six-month period.

MATERIALS & METHODS

Participant Recruitment

This study protocol was approved by the Institutional Review Board of the New York University School of Medicine for the College of Dentistry (NYUCD), Bellevue Hospital Center, and the New York City Health and Hospital Corporation for Activities Involving Human Subjects. A total of 115 individuals, 46 HIV-seropositive individuals and 69 HIV-seronegative control individuals, men and women, ages 18 yrs and older, were recruited from the NYU AIDS Clinical Trial Unit located at Bellevue Hospital Center and the Bluestone Center for Clinical Research at NYUCD. All HIV-infected participants were HAART-naïve or had been off therapy for at least 6 mos and enrolled shortly before the initiation of HAART. Individuals who were pregnant or taking any antimicrobials were excluded from the study. After enrollment, all participants were scheduled immediately for an oral examination and sample collection at the Bluestone Center of NYUCD. Data for this report were based on 3 visits, which included two baseline visits (visits 1 and 2) within 2 wks for assessment of the variability and reliability of all assays, and a follow-up visit performed 24 wks after the initiation of HAART.

Oral Examination and Sample Collection

Two standardized dentists conducted a comprehensive oral examination at visit 1. The caries status of all participants was examined at the tooth surface level according to NHANES III criteria and DMFT/S indices (Decayed, Missing, and Filled Teeth/Surfaces). After participants chewed a piece of paraffin for 30 sec, whole stimulated saliva samples were collected into a centrifuge tube on ice. Total volume was determined and recorded within 10 min to establish salivary flow rate. A 1-mL quantity of the whole saliva sample was immediately transferred on ice to the Microbiology Laboratory at NYUCD for analysis of *S. mutans* colonization. Demographic and medical data were obtained from medical records of all participants, including age, gender, ethnicity, HIV viral load, CD4+/CD8+ T-cell counts, and medication use.

S. mutans Cultivation, Isolation, and Genotyping

Detailed procedures for oral bacterial cultivation, *S. mutans* identification, chromosomal DNA isolation, and genotyping have been described elsewhere (Li and Caufield, 1995; Liu

et al., 2010; Appendix). Briefly, after a 72-hour anaerobic incubation (85% N₂, 10% CO₂, and 5% H₂) at 37°C, the colony-forming units (CFU) were counted for evaluation of *S. mutans* levels. In total, 812 *S. mutans* isolates, an average of 10.8 isolates *per* sample (7 to 14 colonies *per* participant), were randomly isolated for the first 10 HIV+ individuals and the first 10 HIV- control individuals who completed their six-month visits. The subset samples were used for *S. mutans* genotype comparisons.

Statistical Analysis

Data management and analyses were performed with the SAS/STAT program (SAS Institute Inc., Cary, NC, USA). *S. mutans* CFU values for each participant for visits 1 and 2 were averaged and logarithmically transformed to normalize the variance distribution. The non-parametric Mann-Whitney-Wilcoxon test was used to compare HIV+ and HIV- groups for continuous outcome measures, since many variables were non-normally distributed, even after transformation. The Pearson χ^2 test was used to compare the two groups for differences in categorical outcomes. The Spearman correlation coefficient was used to determine the correlation between *S. mutans* level and viral load, CD4+ and CD8+ T-cell counts, and salivary flow rate in HIV+ individuals. Significance (p value) is reported unadjusted. Multiple comparison adjustments by the Bonferroni method were used to maintain type 1 error rate at 0.05 over all tests addressing a hypothesis.

RESULTS

Among the 115 participants, 85% of HIV+ individuals and 72% of HIV- control individuals were non-Hispanic (Table 1). The average age was 39.5 yrs (SD = 12.2; range, 20-69 yrs). Although the HIV- control group appears to have more female members, it included individuals age-gender-race-matched to the HIV+ participants. Exploratory analysis with multivariate models was performed to assess the outcome measures for both gender and interaction effects with HIV status, but no gender effects were found on any of the outcome measures. Besides gender, no statistical differences in the demographic measurements were found between the two groups.

Among the HIV+ individuals, the HIV viral load ranged from 2.55×10^2 to 3.77×10^6 copies/mL. The mean CD4+ T-cell counts ranged from 6 to 925 cells/mm³, and the mean CD8+ T-cell counts ranged from 168 to 1,885 cells/mm³. *S. mutans* mean levels were higher in the HIV+ participants (5.01 ± 1.23) than in the HIV- participants (4.52 ± 1.44 , $p = 0.013$) (Table 2). We also found a bivariate linear relationship between *S. mutans* levels and CD8+ counts ($r = 0.412$; $p = 0.007$) (Appendix Fig. 1), but not with either CD4+ counts ($r = 0.088$; $p = 0.575$) or with viral load ($r = -0.154$; $p = 0.308$). Furthermore, compared with HIV- control individuals, HIV+ individuals experienced lower salivary secretion (1.35 mL/min vs. 1.72 mL/min; $p = 0.009$) and a significant trend toward more decayed tooth surfaces (DS score 7.2 vs. 4.5; $p = 0.027$); however, no difference in DFS scores between the groups could be demonstrated (Table 2).

At baseline visits, numerous morphological variations of *S. mutans* isolates were observed within and among the individuals

Table 1. Characteristics of the Study Population at Baseline Visits

Measurement ^a	HIV- Control	HIV+ Pre-HAART	Significance ^b HIV+ vs. HIV-
N	69	46	
Age (yrs)	39.7 ± 13.5	39.2 ± 10.1	p = 0.619
Ethnicity	10 (15%) Hispanic	13 (28%) Hispanic	p = 0.071
Race			
Asian	8 (11.6%)	1 (2.2%)	p = 0.318
Black	37 (53.6%)	28 (60.9%)	
White	18 (26.1%)	12 (26.1%)	
Other	6 (8.7%)	5 (10.9%)	
Gender ^c			
Male	41 (59.4%)	37 (80.4%)	p = 0.018
Female	28 (40.6%)	8 (17.4%)	
Transgender	0	1 (2.2%)	

^aUnless otherwise specified, the Table shows mean ± standard deviation values.

^bMultiple comparison adjusted alpha level was used for significance in this Table.

^cThere were more women in the HIV- group, in addition to age-gender-race-matched controls to the HIV+ individuals.

Table 2. Comparison of *S. mutans* Colonization at Baseline Visits

Measurement ^a	HIV- Control	HIV+ Pre-HAART	Significance ^a HIV+ vs. HIV-
N	69	46	
Viral load ^b , log ₁₀ value	-	4.48 ± 1.02	-
CD4 T-cell count ^c	-	287 ± 214	-
CD8 T-cell count ^c	-	903 ± 421	-
<i>S. mutans</i> , log ₁₀ CFU ^d	4.52 ± 1.44	5.03 ± 1.23	p = 0.013
Salivary flow rate ^e	1.72 ± 0.85	1.35 ± 0.61	p = 0.009
DS ^f	4.5 ± 9.2	7.2 ± 10.7	p = 0.027
DFS ^f	14.3 ± 13.5	18.7 ± 17.1	p = 0.209

^aUnless otherwise specified, the Table shows mean ± standard deviation values. A dash indicates that either the data were not measured or the test was not appropriate.

^bViral load, log₁₀ value of number of copies/mL of blood.

^cCD4/CD8 T-lymphocyte cells/mm³ of blood.

^dColony-forming units (CFU) per mL of saliva. The values were the mean of the two baseline visits, visit 1 and visit 2, which were 2 days to 2 weeks apart.

^eSalivary flow rate recorded as mL/min.

^fDS/DFS, number of Decayed, Filled Surfaces.

^g*S. mutans* is the primary outcome variable, and the alpha level for significance is 0.05. For the other measures of oral health, the multiple comparison adjusted alpha level is 0.017.

examined. Representative isolates were randomly obtained from the mitis-salivarius-bacitracin (MSB) culture plates of 10 HIV+ participants and 10 HIV- participants (Fig., A). The characteristics of the subset group are listed in Table 3. We observed that the viral load was significantly decreased ($p < 0.005$) and the CD4+ T-cell counts were significantly increased ($p < 0.005$) for the 10 HIV+ participants after 24 wks of HAART. Although the 10 HIV+ individuals experienced two-fold more tooth surfaces with caries and a decreased salivary flow rate at baseline visit, the statistical analysis was not significant for comparison of caries status between the two groups, mainly because of the small sample size.

As shown in the Fig. and the Appendix Table, of all 640 *S. mutans* isolates from the HIV+ participants, chromosomal DNA fingerprints identified 15 distinct genotypes; six individuals

(60%) had 1 genotype, and four (40%) had from 2 to 4 genotypes. Of all 172 *S. mutans* isolates from the HIV- participants, 13 unique genotypes of *S. mutans* were identified; four individuals (40%) had 1 genotype, and the others (60%) had 2 or 3 genotypes. We observed that each participant was colonized by 1 or more genotypically unique *S. mutans* strain(s), but that there was no significant difference in the mean numbers of genotypes between the two groups. The different morphologies did not necessarily correspond to the variations in genotypes, *e.g.*, 4 different colony morphologies were isolated for participant 1 (Fig. 1A), but all presented with an identical fingerprint (Fig. 1B, Lane 1, Genotype A). Additionally, *S. mutans* genotypes remained similar over the 6-month study period for the HIV+ group, even after HAART (Appendix Fig. 2).

Table 3. Characteristics of the Subset Study for *S. mutans* Colonization and Genotype

Measurement ^a	HIV-	HIV+ Pre-HAART	HIV+ Post-HAART	Significance ^{g,h} HIV+ vs. HIV-	Significance ^{g,i} Pre- vs. Post-HAART
N	10	10	10		
Age (yrs)	35.3 ± 12.3	41.2 ± 10.9	-	-	-
Ethnicity	20% Hispanic	10% Hispanic	-	-	-
Gender ^b	3M/7F/0T	7M/2F/1T	-	-	-
Viral load, log ₁₀ value ^c	-	4.47 ± 0.58	0.64 ± 1.46	-	p < 0.005
CD4 count ^d	-	294 ± 173	407 ± 213	-	p < 0.005
CD8 count ^d	-	868 ± 343	996 ± 615	-	p = 0.406
<i>S. mutans</i> log ₁₀ CFU ^e	5.10 ± 0.90	5.16 ± 0.89	5.02 ± 0.99	p = 0.884	p = 0.801
<i>S. mutans</i> genotype	13	15	15	-	-
Salivary flow rate ^f	1.39 ± 0.40	1.07 ± 0.36	1.56 ± 0.90	-	p = 0.149
DFS, mean	14.1 ± 12.6	26.0 ± 26.9	25.4 ± 23.9	p = 0.285	p = 0.756

^aUnless otherwise specified, the Table shows mean ± standard deviation values. A dash indicates that either the data were not measured or the test was not appropriate, given the sample size of the study.

^bGender: M = male; F = female; T = transgender.

^cViral load, log₁₀ value of number of copies/mL of blood.

^dCD4/CD8 T-lymphocyte cells/mm³ of blood.

^eColony-forming units (CFU) per mL of saliva. The values were the mean of the two baseline visits, visit 1 and visit 2, which were 2 days to 2 weeks apart.

^fSalivary flow rate recorded as mL/min.

^gTests performed were exact permutation tests. For group comparisons, the Fisher Pittman Test for Independent Samples was used. For pre- vs. post-HAART comparisons in the HIV+ group, the Fisher Pittman Test for Paired Samples was used.

^hMultiple comparison adjusted alpha level is 0.025.

ⁱMultiple comparison adjustment alpha level is 0.008.

DISCUSSION

It has been suggested that the loss of CD4+ T-lymphocytes and subsequent immunosuppression in HIV-infected individuals could compromise normal mucosal responses to oral micro-organisms, resulting in an elevated colonization of cariogenic and periodontal pathogens or an alteration of the oral microbial community (Patel *et al.*, 2003; Gonçalves *et al.*, 2004, 2007; Aas *et al.*, 2007; Saxena *et al.*, 2012). Those changes could be contributing factors for the development of HIV-associated oral diseases, including increased prevalence of dental caries. Previous studies also suggested that HIV+ individuals with elevated viral load experienced high caries scores (Baqui *et al.*, 1999). Immunocompromised women with CD4+ counts less than 200 had more DMF teeth, and the number of DMF surfaces increased more with decreasing CD4 counts (Mulligan *et al.*, 2004). Caries development etiologically involves a consolidated interaction among host, cariogenic microbe, and refined carbohydrate intakes. However, the effect of HIV infection on these convergence factors and the development of caries remain to be elucidated. In the meantime, an extensive literature search found either inconclusive or divergent data, suggesting more studies are required.

The present study aimed to ascertain the effect of HIV infection on *S. mutans* colonization in saliva. We found that HIV+ individuals experienced significantly higher levels of *S. mutans*. Interestingly, the level of *S. mutans* significantly correlated with CD8+ count, but not with viral load or CD4+ counts. It has been reported by Hicks' group that moderate to severe immunosuppression favored more cariogenic bacterial colonization and increased prevalence of caries in HIV+ children (Hicks *et al.*,

2000). In our study, 73% of the 46 HIV+ and HAART-naïve individuals presented CD4+ T-cell counts over 350/mL, and the mean CD4+ count was above 200 cells/mm³, suggesting that this HIV+ cohort could have had only slight or mild dysfunction of the immune system at the time of enrollment, according to the US Centers for Disease Control and Prevention (CDC) classification system (http://www.aidsinfo.org/aidsinfo?page=cg-205_hiv_classification). Therefore, the elliptically distributed scatterplot displaying the relationship between the CD4+ counts and *S. mutans* level was not surprising.

Our study also revealed decreased salivary flow rate in the HIV+ group. Saliva is strongly correlated with the colonization and clearance of pathogenic micro-organisms by means of antibacterial activities or other mechanisms. In the 1990s, Beighton and Loesche documented that the amount of saliva in the oral cavity could significantly influence the colonization of cariogenic bacteria, including *S. mutans* (Beighton *et al.*, 1991; Loesche *et al.*, 1995). Recently, Lin and colleagues demonstrated that HIV infection could decrease salivary flow rate and alter the salivary composition that may affect salivary antimicrobial activities (Lin *et al.*, 2006). Navazesh and co-workers suggested that HIV+ individuals are at a significantly higher risk for salivary gland enlargement and salivary gland hypofunction compared with HIV- control individuals (Navazesh *et al.*, 2009). Together, these results support the notion that HIV infection can affect salivary glands and alter salivary functions. However, additional research is needed to determine the biological implications of these HIV-associated alterations in oral microbial colonizers.

Cytotoxic CD8+ T-cells play a key role in cell-mediated immune response (Levy, 2009). In HIV infection, decreased CD8+

A

HIV+

HIV-

B

HIV+

HIV-

Figure. Morphological variation and chromosomal DNA fingerprint of *S. mutans* clinical isolates. **(A)** Illustration of a variety of *S. mutans* morphologies from 10 HIV+ participants (No. 1 to No. 10) and 10 HIV- participants (No. 11 to No. 20). **(B)** Fifteen unique genotypes (A to O) for the HIV+ group; 13 unique genotypes (a to m) for the HIV- control individuals. No difference was found in the mean numbers of genotypes between the two groups. The genotypes of D', C', a', b', and i' show that the genotypes were almost identical to D, C, a, b, and i, except for 1 or 2 different bands.

counts are associated with immune deficiency (Gulzar and Copeland, 2004), correlated with the absence of submandibular/sublingual saliva (Mulligan *et al.*, 2000), and the resulting opportunistic infections in the oral cavity (Fidel, 2006). A recent study demonstrated a significant correlation between CD8+ T-cell activation levels and total bacterial 16S rDNA present in stool samples of HIV+ individuals (Ellis *et al.*, 2011). Our study revealed a positive correlation between CD8+ counts and *S. mutans* levels and a significant difference in *S. mutans* levels between the HIV+ and the HIV- groups, clearly suggesting that other HIV-associated factors mechanistically mediate *S. mutans* colonization in saliva.

Our descriptive subset study revealed that the phenotypic variance of *S. mutans* was greater than its genotypic variance, e.g., 4 different colony morphologies were isolated for participant 1, but all presented with an identical fingerprint. Although morphological characteristics have proved to be a useful tool in *S. mutans* identification on mitis-salivarius agar with optimal concentrations of sucrose and bacitracin, Okahashi *et al.* previously illustrated that the same serotype of *S. mutans* could have different phenotypes based on the variations in glucans and fructans synthesis (Okahashi *et al.*, 1984). The findings of our study provide new evidence to support the notion that *S. mutans* morphologic characteristics on MSB agar do not necessarily correspond to genotypic variance and are therefore not a reliable means of delineating *S. mutans* genotypes. Furthermore, we demonstrated that each participant had unique genotype(s) of *S. mutans*, affected neither by HIV infection nor by HAART, suggesting that the genotypic characteristics of *S. mutans* are individually unique with limited variation in the oral cavity, even for individuals with compromised immune systems. The findings provide valuable insight for studying the potential cariogenicity of *S. mutans*, since a considerable degree of variation in virulence among different *S. mutans* strains has been observed.

In summary, this is the first study to examine the effect of HIV infection on the level and genotypic characteristics of *S. mutans* colonization. There are sufficient data to suggest that host immune status of HIV-infected individuals may play an important role in the colonization of *S. mutans* and consequently affect caries outcomes. We did not find differences in genotypic characteristics of *S. mutans* between HIV+ individuals and HIV- control individuals. In addition, no significant changes were observed in either levels or genotypes of *S. mutans* over the six-month study period, not even after HAART. Although HIV infection could accelerate *S. mutans* colonization, HAART apparently had no significant inhibitory effects on *S. mutans* colonization in the oral cavity of HIV+ individuals. Since more dental caries was evidenced in HIV+ individuals from this study and others, additional studies are required to elucidate and understand the correlation between the colonization of other cariogenic microbes, including *S. mutans*, and the status of immunosuppression at the advanced stages of HIV infection.

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