Salivary Cathelicidin (LL-37) in Children and Adolescents Living with HIV

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Abstract

Introduction: Human cathelicidin LL-37 is a salivary antimicrobial peptide (AMP) with broad-spectrum activity against oral diseases, but few studies have assessed its role in children and adolescents living with HIV (CALHIV). We assessed salivary LL-37 levels and correlates in a long-term cohort of Kenyan CALHIV followed since antiretroviral therapy (ART) initiation. ***Methods:*** Saliva was collected from 76 CALHIV who were recruited from two ongoing pediatric HIV studies in Nairobi, Kenya. Oral examinations documenting oral manifestations of HIV, dental caries, and gingivitis were completed. Additional variables included age, sex, HIV treatment (initial ART regimen) and disease parameters, caregivers' demographics, and oral pathologies were conducted. Data were statistically analyzed using the independent T test on the log-transformed LL-37. ***Results:*** At the oral exam visit, the mean age of participants was 13.3 years (±SD = 3.4), and the median CD4 count was 954 cells/mm^3. Mean salivary cathelicidin values of the cohort were 23.7 ± 21.1 ng/mL. Children with permanent dentition at time of oral examination, and children who initiated ART at ≥2 years old had higher mean LL-37 concentrations compared to those with mixed dentition and those who initiated ART <2 years old (p = 0.0042, 0.0373, respectively). LL-37 levels were not found to differ by initial type of ART regimen, CD4 count, or oral disease. ***Conclusion:*** Further research and longitudinal studies are necessary to evaluate and improve the innate immunity of CALHIV in Kenya.

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Published by S. Karger AG, Basel

Introduction

While the advent of antiretroviral treatment (ART) has significantly improved the quality of life for children and adolescents living with HIV (CALHIV), they remain at elevated risk for noncommunicable diseases, including

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oral diseases [1–3]. HIV infection causes global immune dysregulation, and this extends to the oral cavity. For example, HIV has been shown to disrupt the secretion and function of salivary antimicrobial peptides (AMPs) [2, 3]. Salivary AMPs are small, low-molecular weight, cationic proteins that act as a part of the innate immune response to oral pathogens [4, 5]. AMPs play a significant role in protecting the oral tissues from diseases arising from a broad-spectrum of pathogens within and without the oral cavity [4–12].

AMP LL-37 is the only human cathelicidin and an important AMP which aids in the prevention of both hard and soft tissue oral diseases [9–12]. LL-37 has both pro-inflammatory and anti-inflammatory activities [6–8, 13]. It is synthesized and secreted by neutrophils and its expression increases in response to the presence of periodontal pathogens and correlates with an increase in gingival crevice depth [4, 14, 15]. Previous studies have found correlations between lower salivary levels of LL-37 and higher incidence of caries besides the increase of its concentration with age [9, 16]. Periodontal disease is also one of the most prevalent of oral diseases among PWH, and concentrations of LL-37 in saliva have been found to be significantly higher in individuals with periodontal disease [4, 17].

While LL-37 has been well-studied in adults with HIV infection, much less research has been conducted in CALHIV, and the factors which may impact LL-37 secretion in childhood are poorly defined. Despite great strides in the medical management of CALHIV, less attention has been focused on oral pathologies in low-middle income countries such as Kenya [18, 19]. The purpose of the present study was to assess levels of salivary LL-37 and explore its association with oral diseases among CALHIV in Nairobi, Kenya. We hypothesized that children with oral diseases would have significantly higher levels of salivary LL-37 compared to those without, and that its distribution varies by age.

Methods

Study Cohort

The Institutional Review Board at the University of Washington and the Kenyatta National Hospital/University of Nairobi Ethics Research Committee approved this research. Caregivers provided written informed consent for participation, and children >8 years old were additionally asked for assent.

The current study was nested within an ongoing cohort study of Kenyan CALHIV who have been followed since ART initiation, and data pertaining to HIV treatment (age at time of ART initiation and first-line ART regimen) were derived from these. The Kenya Pediatric Studies (KPS) Cohort has been described in detail elsewhere and comprises two sub-cohorts of children who were enrolled and started ART according to contemporaneous treatment guidelines [20, 21]. The initial ART regimens were either non-nucleoside reverse transcriptor (NNRTI)- or protease inhibitor (PI)-based regimens. NNRTI regimens were nevirapine- or efavirenz-based regimens, and PI regimens were lopinavir/ritonavir-based regimens.

Oral Examinations

Participants were enrolled and received oral examinations between February and August 2019 [22]. Details of the examinations have been reported elsewhere [22]. Briefly, HIV-associated oral mucosal lesions were examined and classified according to the WHO Oral Health Surveys and Record Form for Oral Manifestations of HIV/AIDS [23]. Dental caries status was determined by presence (Y/N) and the total number of lesions. Dentition status was determined by age at time of oral examination (permanent dentition being >12 years old; mixed dentition ≤12 years old). We additionally screened for several oral diseases previously associated with salivary LL-37, including ulcers, xerostomia, unilateral or bilateral swelling of major salivary glands, gingivitis, periodontitis, candidiasis, and caries [24–26]. Gingival bleeding index was used to determine gingivitis status. The Gingival bleeding index was determined as the number of sites demonstrating bleeding on probing on the six surfaces of each tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual) and is calculated as a ≥10% percentage of affected sites [23].

Measurement of LL-37

The passive drool technique was used to collect non-stimulated saliva (5 mL), participants were asked to refrain from eating and drinking 30 min prior to collection. [27] Saliva samples were immediately stored at −80°C. ELISA (Hycult Biotech, Wayne, PA, USA) was used to measure LL-37 levels from 5 mL of saliva. The LL-37 standard included in the kit was prepared according to the manufacturer's instructions, and all samples were run in duplicate.

Analyses

SAS 9.4 was utilized for all analyses. Mean LL-37 levels were compared between groups using the independent T test on the log-transformed LL-37. All analyses used 2-tailed tests with alpha = 0.05.

Results

Seventy-six CALHIV were enrolled, received oral examinations, and had saliva collected for LL-37 measurement. Participant characteristics are provided in Table 1. The median age of children at the time of assessment was 11.4 years [IQR: 10.7, 16.3]. At the time of oral examination, the median CD4 count for all participants was 954 cells/mm^3 [IQR: 696, 1,290]. Of all enrolled CALHIV, 49 (64.5%) had mixed dentition while 27 (35.5%) had permanent dentition. The mean age at ART initiation was 0.6 years old [IQR: 0.4, 2.7] (Table 1).

The mean salivary concentration of LL-37 across all participants was 23.7 ± 21.1 ng/mL. Table 1 shows mean LL-37 levels comparing children by HIV disease

Table 1. Characteristics of the study population (N = 76)

Characteristic (N = 76)	N (%) or median (IQR)
Caregiver sex	
Female	67 (88.2)
Male	5 (6.6)
Not reported	4 (5.3)
Caregiver education	
Missing/none	5 (6.6)
Primary	30 (39.5)
Secondary	27 (35.5)
College or University	13 (17.1)
Caregiver age, years	40 (34, 44)
Caregiver education, years	10 (8, 12)
Child age, years	
At ART initiation	0.6 (0.4, 2.7)
At oral exam	11.4 (10.7, 16.3)
Child sex	
Female	36 (47.4)
Male	40 (52.6)
Child CD4 count at oral exam	
≥500 cells/mm^3	69 (90.8)
<500 cells/mm^3	7 (9.2)
Child CD4 count (cells/mm^3) at oral exam	954 (696, 1290)
Dentition status	
Mixed dentition	49 (64.5)
Permanent dentition	27 (35.5)
First-line ART regimen	
NNRTI-based regimen[a]	56 (73.7)
PI-based regimen[b]	20 (26.3)

[a]Nevirapine- or efavirenz-based regimens. [b]Lopinavir/ritonavir-based regimens.

parameters and prevalent oral conditions. Youth with permanent dentition had significantly higher LL-37 concentrations than children with mixed dentition (32.8 ± 25.4 ng/mL vs. 18.6 ± 16.5 ng/mL; p = 0.0042) (Table 2). Participants who initiated ART at <2 years of age had significantly lower salivary LL-37 values than those who initiated ART at ≥2 years of age (21.2 ± 20.4 ng/mL vs. 29.8 ± 22.1 ng/mL; p = 0.037) (Table 2). We did not find any association between LL-37 levels and sex, first-line ART regimen, or CD4 count. We did not find any oral health condition to be significantly associated with LL-37 levels. Children with gingivitis had higher LL-37 levels (25.1 ± 21.7 ng/mL) compared to those without gingivitis (12.8 ± 8.7 ng/mL) but was not a statistically significant difference (Table 2).

Discussion

We sought to assess levels of salivary LL-37 and evaluate associations with oral diseases among CALHIV in Nairobi, Kenya, and hypothesized that salivary LL-37 levels would be correlated with age and presence of oral diseases. The results of this study partially support the original hypothesis, as mean salivary LL-37 concentration was higher in CALHIV who were in permanent dentition and ≥2 years old at time of ART initiation. However, we did not find a relationship between oral diseases and LL-37 levels.

Literature describing the salivary concentration of LL-37 in children, healthy or otherwise, is sparse. The average salivary concentration of our cohort was similar to that reported by Davidopoulou et al. [9] who examined forty-nine healthy, gingivitis-free children ages 2–18 years. They found that the average salivary LL-37 concentration was 22 ng/mL, which was similar to our findings (concentration 23.7 ± 21.1 ng/mL) [9]. These data suggest that in the context of effective modern ART, LL-37 levels are not substantially elevated by HIV infection in children.

We found age to be a factor in the secretion of salivary LL-37 with children in mixed dentition having significantly lower levels than CALHIV in permanent dentition. Specifically, our results showed an increase

Table 2. Salivary concentration (ng/mL) of LL-37 by characteristics of sample and comparison of log LL-37 using two sample *t* test

	Mean (SD)	Range	*p* value
Sex			
Female	21.7 (15)	0.5–57.6	0.9455
Male	25.4 (25.4)	1.3–96.2	
Age at ART initiation			
<2 years of age	21.2 (20.4)	0.5–96.2	0.0373
≥2 years of age	29.8 (22.1)	3.6–82.5	
First-line ART regimen			
NNRTI-based regimen	24.5 (21.1)	1.6–96.2	0.2174*
PI-based regimen	21.4 (21.5)	0.5–80.6	
CD4 counts (cells/mm³) at oral examination			
≥500	23.3 (21.8)	0.5–96.2	0.1973
<500	27.6 (12.5)	11.2–44.8	
Dentition status			
Mixed dentition	18.6 (16.5)	0.5–80.6	0.0042
Permanent dentition	32.8 (25.4)	3.6–96.2	
Any oral diseases**			
No	20.5 (13.1)	1.6–44.8	0.5073
Yes	24.3 (22.2)	0.5–96.2	
Caries			
No	22.9 (18.6)	0.5–82.5	0.7813
Yes	24.1 (22)	1.3–96.2	
Ulcers			
No	23 (20.4)	0.5–96.2	0.3483
Yes	33.2 (25.9)	5.7–80.6	
Xerostomia			
No	24.1 (21.8)	0.5–96.2	0.8823
Yes	22.1 (19.1)	3.6–80.6	
Gingivitis			
No	12.8 (8.7)	3.1–31.9	0.2715
Yes	25.1 (21.7)	0.5–96.2	

*Using two sample *t* test on log scale of LL-37 with variance not equal. **Any oral diseases known to be related to LL37: herpetic stomatitis/gingivitis and/or labial (*n* = 1), aphthous ulcer(s) (*n* = 1), other ulcerations (*n* = 5), xerostomia (*n* = 17), unilateral or bi-lateral swelling of salivary glands (*n* = 0), gingivitis (*n* = 67), and dental caries (*n* = 51).

in age that peaks around puberty. While these changes are similar to those found in healthy children, our salivary cathelicidins findings are slightly higher in both dentition types when compared to other cohorts of HIV-unexposed children and adolescents [9]. Stukes et al. [28] reported the mean plasma concentration of LL-37 in 133 healthy children to be 28.1 ng/mL, with increasing age and male sex correlating with higher concentrations. Longitudinal studies of large cohorts of CALHIV will better answer the question whether these changes result from biological differences that persist or are transitory due to growth and development.

Cathelicidin LL-37 has been universally accepted to be a biomarker for periodontal diseases [4, 29, 30]. Several studies show significantly increased levels of LL-37 in adults with chronic periodontitis when compared to adults with and without gingivitis, and edentulous adults [29, 31]. Our finding that CALHIV with gingivitis had elevated LL-37 levels (while not statistically significant in this study) is thus consistent with previous research. In addition to having a documented relationship with periodontal diseases, LL-37 has been evaluated in correlation with mucosal diseases, such as oral lichen planus and aphthous ulcers [32]. LL-37 levels are also increased in patients with more severe stages of immune-mediated mucosal disease [17, 32]. In our cohort, CALHIV with oral ulcers (of unspecified origin) had elevated, but not significantly higher LL-37 levels than those without ulcers. Similarly, we also found elevations of cathelicidin by dental caries level when compared to CALHIV without cavities (24.1 ± 22 ng/mL and 22.9 ± 18.6 ng/mL respectively). However, the correlation between LL-37 and decayed teeth was not statistically significant (*p* = 0.763). Our findings are the first in reporting salivary cathelicidins levels in CALHIV. Yet, it is still not clear how this innate immunity-mediated response to infection and inflammation varied between CALHIV, exposed uninfected, and unexposed children.

This study had several limitations. First, the study design was cross-sectional. Second, the sample size was small, so while we found absolute differences in LL-37 levels by some diseases, and by sex, these were not statistically significant but may have been with a larger cohort. Third, the study site was the public hospital with the generally good infrastructure and the study population is a cohort of CALHIV receiving care since they were born or infants. Finally, it is not possible to disentangle the effects of permanent dentition and age since these are collinear (older children have permanent dentition), and our sample is too small to have sufficient sample size to detect effects in a stratified analysis. Yet, this is the first time that salivary LL-37 has been evaluated among CALHIV allowing us to establish relevant baseline data for this understudied HIV group of children and adolescents regarding oral health. Further longitudinal investigation that includes children exposed uninfected is needed to better understand the role of this AMP in the context of HIV infection and treatment.

Salivary cathelicidin levels in CALHIV were higher in older participants with permanent dentition. Further longitudinal studies evaluating immune factors and their relationship to oral health in CALHIV are necessary.

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Acknowledgments

Statement of Ethics

All methods were performed in accordance with the relevant guidelines and regulations for medical research involving human subjects laid forth by the Declaration of Helsinki. This study was approved by the Institutional Review Board at the University of Washington (STUDY00003298) and the Kenyatta National Hospital/University of Nairobi Ethics Research Committee (KNH/ERC.R/133). Written informed consent from all participants under age 18 and their parent/legal guardian/next of kin was obtained to participate in the study. Caregivers provided written informed consent for participation, and children >8 years old were also asked for assent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

Author Contributions

A.L.S. and A.K. contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. A.E.K. contributed with drafting and critically revised the manuscript. W.C. contributed to conception, design, laboratory analysis and interpretation and critically revised the manuscript. Y.W. contributed to data cleaning, analysis, and interpretation. G.J.-S., D.W., S.B.-N., and J.A.J. contributed to design and data interpretation, critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Data Availability Statement

Data cannot be shared publicly because of Institutional IRB restrictions. The study population is categorized as a vulnerable one (children with HIV). Data are available after permission granted by the University of Washington Institutional Review Board and by the Kenyatta National Hospital Ethics Research Committee (contact via email) for researchers who meet the criteria for access to confidential data. Contact info: University of Washington: Leah Miller (ude.wu@rellimel) Kenyatta National Hospital: Beatrice Amugune (ek.ca.ibnou@cre_hnknou). Further inquiries can be directed to the corresponding author.

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