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Staphylococcus spp., Enterobacteriaceae and Pseudomonadaceae oral isolates from Brazilian HIV-positive patients. Correlation with CD4 cell counts and viral load

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ABSTRACT

The aim was to evaluate the presence of *Staphylococcus* spp., *Enterobacteriaceae* and *Pseudomonadaceae* in the oral cavities of HIV-positive patients. Forty-five individuals diagnosed as HIV-positive by ELISA and Western-blot, and under anti-retroviral therapy for at least 1 year, were included in the study. The control group constituted 45 systemically healthy individuals matched to the HIV patients to gender, age and oral conditions. Oral rinses were collected and isolates were identified by API system. Counts of microorganisms from HIV and control groups were compared statistically by a Mann–Whitney test ($\alpha = 5\%$). The percentages of individuals positive for staphylococci were similar between the groups ($p = 0.764$), whereas for Gram-negative rods, a higher percentage was observed amongst HIV-positive ($p = 0.001$).

There was no difference in *Staphylococcus* counts between HIV and control groups ($p = 0.1008$). Counts were lower in the oral cavities of patients with low viral load ($p = 0.021$), and no difference was observed in relation to CD4 counts ($p = 0.929$). *Staphylococcus aureus* was the most frequently isolated species in HIV group, and *Staphylococcus epidermidis* was the prevalent species in the control group. Significantly higher numbers of enteric bacteria and pseudomonas were detected in the oral cavities of the HIV group than in the control ($p = 0.0001$). *Enterobacter cloacae* was the most frequently isolated species in both groups. Counts of enteric bacteria and pseudomonas were significantly lower in patients with low CD4 counts ($p = 0.011$); however, there was no difference relating to viral load. It may be concluded that HIV group showed greater species diversity and a higher prevalence of *Enterobacteriaceae/Pseudomonadaceae*.

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1. Introduction

The oral cavity is inhabited by more than seven hundred microbial species. Many intrinsic and extrinsic factors have effects on the composition, metabolic activity, and pathogenicity of the oral microflora.^{1,2} The oral microflora are remarkably stable in healthy subjects, but significant changes may occur in subjects facing serious systemic disease and its treatment. An imbalance in the commensal flora may occur in immunosuppressed individuals or those under antibiotic therapy, favouring the growth of some microorganisms and causing opportunistic infections.^{3–5}

Considerable controversy remains as to whether *Staphylococcus* spp. play a role in the ecology of the normal oral flora. The role of *S. aureus* in several diseases of the oral mucosa merits further investigation. Smith et al.⁶ stated that staphylococci are not infrequent colonizers of the oral cavity, and that this site may serve as a potential reservoir for transmission to other body sites. Zuanazzi et al.⁷ reported that the most prevalent bacteria in the saliva of hospitalized individuals were *Staphylococcus* spp. (85.7%), *Pseudomonas* spp. (83.8%), and *Acinetobacter* spp. (53.3%). The results of another study suggested a possible peroral route for staphylococci, as the provision of microorganisms from the nasal cavity was shown.⁸

Staphylococcus species are amongst the most frequent causes of bacteremia in mechanically ventilated patients.⁹ Further work in this area may lead to benefits such as improved decolonization regimens for eradication of MRSA and acknowledgement of the mouth as a source of bacteremia-causing staphylococci.¹⁰

Microorganisms of the *Enterobacteriaceae* and *Pseudomonadaceae* families have been thoroughly investigated by the medical field and are known for their pathogenicity in humans; however, these bacteria have not been considered pathogenic in the oral cavity. Despite this, the presence of these bacteria in the oral cavity can serve as a reservoir, and can severely compromise the lives of immunocompromised individuals.^{11–14} Some studies have reported an association between *Enterobacteriaceae* and oral ulcerations in HIV-positive patients, but this association may not necessarily be causal, as enterobacteria may be secondary invaders.^{15,16}

Local and systemic factors appear to be correlated with increased oral prevalence of *Enterobacteriaceae* and/or *Pseudomonas*. However, the percentage of oral isolates containing these species differs amongst reports from different groups and remains controversial.^{3,14,17,18}

Despite the importance of this subject, few studies of *Enterobacteriaceae* and/or *Pseudomonas* in the oral cavities of HIV-positive patients have been performed in Brazil. Most of the published studies have focused on *Candida* spp. Because oral reservoirs of potential pathogens such as enterobacteria and staphylococci may also cause local or systemic infections and compromise the lives of immunosuppressed individuals, the aim of this study was to evaluate the presence of *Staphylococcus* spp., *Enterobacteriaceae* and *Pseudomonadaceae* in the oral cavities of HIV-positive patients.

2. Materials and methods

This study was approved by the Local Ethics Committee (protocol number 012-PH/CEP) and was undertaken with the informed written consent of each subject.

Forty-five individuals (23 female and 22 male), aged 22–66 years, HIV-positive as diagnosed by ELISA and confirmed by Western blot, undergoing treatment at the Day Hospital of Taubaté Medical School or Medical Specialties Center (São Paulo State; ARE), and having undergone anti-retroviral therapy for at least 1 year were included in the study. Of the total, 43% of the patients were undergoing highly active antiretroviral therapy (HAART), and the remaining patients were treated only with a protease inhibitor. For the control group, 45 healthy individuals were selected amongst the patients under treatment at São José dos Campos Dental School. They were aged 23–66 years, with similar age (± 2 years), gender and oral conditions (use of dentures or orthodontic devices and smoking; salivary flow was not evaluated) to the HIV-positive individuals.

The most recent data for the values of the CD4 cell count, viral load, antiretroviral treatment and antibiotic use were obtained from the medical records of the HIV group. Antimicrobial/antifungal therapy during the 3 months preceding the sampling, diabetes mellitus, use of antidepressant drugs, pregnancy and use of orthodontic appliances were considered exclusion criteria.

Samples from each individual were collected by oral rinses with phosphate-buffered saline (PBS; 0.1 M, pH 7.2) for 10 min.¹⁹ The samples were centrifuged for 10 min at $8000 \times g$ and the supernatant was discarded. The pellets were resuspended in 2.5 ml of PBS. Dilutions of 10^{-1} and 10^{-2} in PBS were made, and an aliquot (0.1 ml) of each dilution was plated on mannitol agar (Difco, USA) and MacConkey agar (Difco, USA) in duplicate. Plates were incubated at 37 °C for 48 h. After this period, colonies were counted and the number of colony-forming units per millilitre (cfu/ml) was obtained. Colonies with different morphologies were subjected to microscopic confirmation and were isolated and stored in gelose agar at room temperature.

Coagulase-positive *Staphylococcus* isolates were identified according to the phenotypic tests proposed by Koneman et al.²⁰ Coagulase-negative isolates were identified using the API Staph system (Biomerieux, France). Isolates of Gram-negative rods were identified using the API 20E system (Biomerieux, France), according to the manufacturer's instructions.

2.1. Analysis of data

The proportions of individuals positive for the studied microorganisms in the control and experimental groups were compared by a Z-test. Counts of the microorganisms obtained for HIV-positive and control groups were compared by a Mann–Whitney test. The Kruskal–Wallis ANOVA was used to compare the counts of microorganisms according to CD4 cell count and viral load in HIV-positive patients. Values of $p \leq 0.05$ were considered statistically significant.

For comparison purposes, patients were classified into 3 subgroups according to counts (cells/mm³) of CD4 lympho-

cytes (<200, 200–500 and >500), based on the anti-retroviral therapy guidelines for adults and adolescents infected with HIV.^{21,22} Patients were also divided into subgroups based on viral load (<400, 400–20,000 and >20,000 copies/ml of serum).

3. Results

Similar numbers of HIV-positive patients were positive for staphylococci (84.4%) compared to the control group (86.6%) ($p = 0.764$). There was no statistically significant difference in the staphylococcus counts obtained from the oral cavities of control subjects and HIV-positive patients ($p = 0.9839$) (Table 1).

S. aureus was the most frequently isolated species in the HIV-positive group (30.2%). In contrast, *Staphylococcus epidermidis* (31.4%) was the most frequently isolated species amongst the controls (Table 2).

No significant differences in staphylococcus counts were observed amongst the subgroups for CD4T cells; however, counts were significantly lower in the subgroup with a viral load of less than 400 copies/mm³ (Table 3).

The HIV-positive group showed a higher percentage of individuals positive for *Enterobacteriaceae* and *Pseudomonadaceae* (77.7%) than the control (44.4%) ($p = 0.001$). Also, the counts of these microorganisms were significantly higher amongst HIV-positive patients than in the control group ($p = 0.0001$) (Table 1).

Table 1 – Oral levels of the microorganisms (colony-forming units per millilitre; cfu/ml) obtained for the control and HIV groups.

	Median		Interquartile range		<i>p</i>
	HIV	Control	HIV	Control	
S	1140	1300	3110	2180	0.9839
E/P	620	0	1850	90	0.0001

S: *Staphylococcus* spp. E/P: *Enterobacteriaceae*/*Pseudomonadaceae*, and *p* = *p*-value.

Table 2 – Staphylococci isolates identified in HIV and control groups.

Species	HIV		Control	
	<i>n</i>	%	<i>n</i>	%
<i>Staphylococcus aureus</i>	73	92.4	48	54
<i>Staphylococcus epidermidis</i>	69	47	68	61.8
<i>Staphylococcus haemolyticus</i>	14	9.5	4	3.6
<i>Staphylococcus saprophyticus</i>	13	8.8	2	1.8
<i>Staphylococcus warneri</i>	12	8.1	3	2.7
<i>Staphylococcus capitis</i>	10	6.8	7	6.4
<i>Staphylococcus hominis</i>	9	6.1	5	4.5
<i>Staphylococcus xylosum</i>	7	4.7	4	3.6
<i>Staphylococcus schleiferi</i>	6	7.6	40	45.9
<i>Staphylococcus chromogenes</i>	6	4	0	0
<i>Staphylococcus simulans</i>	5	3.4	0	0
<i>Staphylococcus sciuri</i>	2	1.3	7	6.4
<i>Staphylococcus lugdunensis</i>	0	0	10	9

Enterobacter cloacae was the most frequently isolated species in both groups (18.8% in the HIV-positive group and 16.32% in the control group). Amongst *Pseudomonadaceae* species, *Chryseomonas luteola* was the most common in both studied groups (7.3% in the HIV-positive group and 6.1% in the control group). Other species identified are shown in Table 4.

Counts of *Enterobacteriaceae* and *Pseudomonadaceae* were significantly lower in the subgroup with <200 CD4 cells/mm³. With respect to viral load, significantly lower counts of staphylococci in the subgroup with <400 copies/mm³ were observed (Table 3).

4. Discussion

One of the most challenging problems involving staphylococci has been their increasing resistance to methicillin, vancomycin and other antibiotics.^{23–25} Oral reservoirs of these microorganisms may be potential sources for infection in immunosuppressed patients.²⁶

In this study, staphylococci were isolated from 86.6% of the control group and 84.4% of HIV-positive patients. Previous studies reported a variable presence of staphylococcus in systemically diseased patients. These values varied from 28% amongst patients with malignant neoplasias³ to 96% in patients with rheumatoid arthritis.²⁷ High percentages of patients positive for staphylococci in the oral cavity have been reported in the literature, with values from 94%²⁷ to 95.6%²⁸ amongst adults. Jackson et al.²⁹ also observed a higher frequency of isolation in the oral cavities of healthy children (92%).

The results obtained in this study confirm the conclusion of Smith et al.¹⁰ that staphylococcus species can often be isolated from the oral cavities of healthy or diseased children and adults. Although staphylococci have been considered part of the normal oral microbiota,^{27,29} their presence in the oral cavity may be associated with local and systemic infections, especially in immunosuppressed patients.¹⁰

With respect to the species identified in this study, *S. epidermidis* and *S. aureus* were the most prevalent coagulase-negative and coagulase-positive species, respectively, in both groups. The isolation of these species in the oral cavity and periodontal sites has been reported in the literature.^{27–31} The HIV-positive group showed a greater diversity of coagulase-negative species; the presence of *S. warneri*, *S. capitis*, *S. haemolyticus*, *S. xylosum*, *S. saprophyticus* and *S. hominis* have been reported in previous studies.^{26,27,29–32} In our results, *S. sciuri*, *S. simulans* and *S. chromogenes* were identified. These species were not found in previous studies of oral samples. Some of these species, although isolated infrequently, may cause infections in humans, such as urinary tract infections, bacteremia, endocarditis, osteomyelitis, cellulitis and cerebral empyema.^{33,34}

Counts of staphylococci were lower in the oral cavities of patients with low viral load (<400 copies/ml), but no difference was observed in relation to CD4 cells. No previous studies were found with which to compare these data.

Enterobacteria and *pseudomonas* were identified in the oral cavities of 77.7% of the HIV-positive group. The control group showed a lower isolation frequency (44.4%) in the oral

Table 3 – Descriptive analysis of data for the counts of microorganisms (cfu/ml) of the oral cavity according to the subgroups related to CD4 cells (cells/mm³) and viral load (copies/ml).

CD4 T cells	Median			Interquartile range			KW	p
	<200	200–500	>500	<200	200–500	>500		
S	1030.0	620.00	640.00	2750	1720	4780	0.5523	0.7587
E/P	10,000	1680.0	60,000	4780	3090	1440	4.1778	0.1238

Viral load	Median			Interquartile range			KW	p
	<400	400–20,000	>20,000	<400	400–20,000	>20,000		
S	420.00	1420.0	690.00	1575	5775	3130	1.7137	0.4245
E/P	90,000	930.00	90,000	1405.0	2072.5	2310.0	0.7594	0.6841

S: *Staphylococcus* spp., E/P: *Enterobacteriaceae/Pseudomonadaceae*, KW: Kruskal–Wallis statistics test, and p: p-value.

cavity. The increased oral prevalence of these microorganisms seems to be associated with systemic and local factors. However, data in the literature are still controversial. Jobbins et al.³ reported isolation of coliforms from 49% of patients with malignancy. A low prevalence of these microorganisms in the elderly and mentally disabled patients was reported.^{17,18} Senpuku et al.¹⁴ isolated *Enterobacteriaceae* from 16% of elderly patients and from 6% of controls. A higher prevalence of enterobacteria in the oral cavity was observed by Santos and Jorge¹¹ in healthy Brazilian individuals (51%). Hägg et al.³⁵ reported a significant increase in the prevalence of enterobacteria after insertion of fixed orthodontic appliances. Zhu et al.,³⁶ studying stroke patients at three different stages (acute phase, upon discharge from the hospital and 6 months later), observed that the oral carriage rate of coliforms was

significantly lower at 6 months after hospital discharge, but found no significant relationship between the presence of coliforms and other variables studied (age, gender, plaque index, bleeding index, DMFT, denture wearing, dysphagia, smoking, diabetes and tooth brushing difficulty).

Other studies with HIV-positive patients in different countries found a low prevalence of enterobacteria and/or pseudomonas in the oral cavity. Schmidt-Westhausen et al.³⁷ obtained an enterobacteria prevalence of 22%. Tsang and Samaranyake¹⁵ reported the isolation of *Enterobacteriaceae* (26.3%) and *P. aeruginosa* (15.1%). The only Brazilian study regarding these microorganisms in the oral cavities of HIV-positive patients was conducted by Figueirêdo et al.,¹⁶ who found *Enterobacteriaceae* in 96.4% of isolates and *P. aeruginosa*, the only *Pseudomonas* identified, in 3.6% of isolates. According

Table 4 – Enterobacteria and pseudomonas isolates in HIV and control groups.

Species	HIV		Control	
	n	%	n	%
<i>Enterobacter cloacae</i>	27	22.3	10	18.1
<i>Klebsiella oxytoca</i>	13	10.7	5	9
<i>Klebsiella pneumoniae</i>	7	5.7	9	16.4
<i>Enterobacter sakazakii</i>	8	6.6	4	7.2
<i>Escherichia coli</i>	9	7.4	1	1.8
<i>Serratia marcescens</i>	8	6.6	0	0
<i>Pantoea</i> spp.	3	2.4	4	7.2
<i>Citrobacter freundii</i>	6	5	0	0
<i>Serratia liquefaciens</i>	2	1.6	4	7.2
<i>Klebsiella ornithinolytica</i>	2	1.6	4	7.2
<i>Enterobacter asburiae</i>	4	3.3	1	1.8
<i>Enterobacter aerogenes</i>	2	1.6	2	3.6
<i>Serratia odorifera</i>	3	2.4	0	0
<i>Kluyvera</i> spp.	3	2.4	0	0
<i>Serratia ficaria</i>	2	1.6	1	1.8
<i>Salmonella</i> spp.	2	1.6	0	0
<i>Klebsiella terrigena</i>	1	0.8	1	1.8
<i>Citrobacter koseri</i>	1	0.8	1	1.8
<i>Enterobacter amnigenus</i>	1	0.8	1	1.8
<i>Shigella</i> spp.	0	0	1	1.8
<i>Serratia fonticola</i>	1	0.8	0	0
<i>Enterobacter intermedius</i>	1	0.8	0	0
<i>Chryseomonas luteola</i>	10	8.2	5	9
<i>Pseudomonas aeruginosa</i>	2	1.6	1	1.8
<i>Pseudomonas fluorescens</i>	2	1.6	0	0
<i>Burkholderia cepacia</i>	1	0.8	0	0

to Santos and Jorge,¹¹ some authors have noted discrepancies in the prevalence of these microorganisms in the oral cavities of individuals from developed and developing countries, hypothesizing that the incidence of these microorganisms in the oral cavity may be related to high numbers of coliforms in drinking water and foods.

In the present study, *E. cloacae* and *C. luteola* were the most prevalent species amongst isolates of *Enterobacteriaceae* and *Pseudomonaceae* from both groups. *E. cloacae* has been frequently identified amongst clinical oral isolates.^{11,12,26} However, *C. luteola* is rarely isolated from the oral cavity, and infections caused by this microorganism include sepsis, meningitis, endocarditis, osteomyelitis and peritonitis.^{20,38}

The HIV group showed the greatest diversity of *Enterobacteriaceae* and *Pseudomonas* species, many of which can cause opportunistic infections, such as *Escherichia coli* infections, gastro-intestinal infections,³⁹ endocarditis,⁴⁰ urinary infections⁴¹ and *Klebsiella pneumoniae* infections that are often involved with aspiration pneumonia.⁴²

With respect to CD4 cell count, lower counts of enterobacteria and *Pseudomonas* were observed amongst patients in the subgroup with <200 CD4 cells/mm³. This unexpected result warrants further study. The viral load values were distributed equally amongst the subgroups evaluated. Considering that the studies reporting the prevalence of enterobacteria and *Pseudomonas* in the oral cavities of HIV-positive patients did not correlate their findings with clinical variables, it was not possible to compare our results to the literature. However, increased oral carriage of Gram-negative bacilli in the HIV group compared to the control group confirms that carriage rates tend to increase in medically compromised individuals, and the presence of such bacteria in the oral cavity as a putative reservoir of infection should be considered.⁴³

Regarding treatment, it was not possible to have a fixed therapy protocol for all patients because the treatment strategy would change according to the CD4 lymphocyte level and viral load as well as other relevant clinical variables, such as the occurrence of opportunistic diseases and adverse reactions to medication. HAART is correlated with lower occurrence of oral diseases. It promotes inhibition of viral replication as well as redistribution and restoration of immunity, resulting in an increase in CD4 cell counts. Within the limits of this study, we could not assess the effect of HAART on the oral microflora. The few studies on this subject report that protease inhibitors may have anti-*Candida* activity by inhibiting the protease of this microorganism.^{1,44}

5. Conclusion

Based on the results obtained, it may be concluded that the HIV-positive group showed a higher prevalence of *Enterobacteriaceae* and *Pseudomonadaceae*. No difference in staphylococci counts was found between the studied groups.

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