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Original Article

Time-related Increase of Staphylococci, Enterobacteriaceae and Yeasts in the Oral Cavities of Comatose Patients

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BACKGROUND/PURPOSE: The composition of oral microbiota in comatose patients remains uncertain. Some pulmonary pathogens may be found in dental biofilms or as part of the saliva microbiota. It is supposed that some pneumopathogenic microorganisms may overgrow in the mouths of comatose patients and spread to their lungs.

METHODS: The oral colonization dynamics of staphylococci, Enterobacteriaceae and yeasts in nine comatose patients (group 1), and in 12 conscious patients that brushed their teeth at least twice a day (group 2) was evaluated. Both groups were followed up for 7 days after hospitalization. Daily samples of saliva were obtained, dispersed and plated on selective culture media and colony forming units of each microbial group were obtained.

RESULTS: For patients in group 1, the counts of total viable bacteria, staphylococci, Enterobacteriaceae and yeasts progressively increased in a time-dependant manner. For the conscious patients of group 2, there was no increase.

CONCLUSION: It would appear that concomitant consciousness and brushing teeth are determinants in controlling the selected pneumopathogen counts in resting saliva. The increase in microbial counts in comatose patients is understandable because these microorganisms could spread to the lungs.

KEYWORDS: coma, Enterobacteriaceae, saliva, staphylococci, yeast

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Introduction

The oral microbiota are known to be the most diverse of those that colonize the epithelia and mucosa.[1] Some oral bacteria may disseminate to internal organs and provoke systemic infections, especially in the case of pneumonia.[2,3] In many pneumonia cases, the involved oral microorganisms may reach the lungs as the result of aspiration of saliva.[4] Apart from the organisms commonly found in the oral microbiota (streptococci, lactobacilli and Gram-negative

anaerobic rods), transitory entities such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* may also grow in oral biofilms and be carried by saliva to the lungs when cell numbers are high, provoking acute pneumonia.[5–7] Oral yeasts may also be associated with acute pneumonia, mainly in mechanically ventilated patients.[8]

Comatose patients, and those with severe traumatic brain injury, are not in control of their oral hygiene. Therefore, it is reasonable to suppose that these pulmonary pathogens may easily overgrow in their mouths and reach the lungs, making the patients more prone to pulmonary diseases.[9] Moreover, many comatose patients require mechanical ventilation, meaning that maintaining adequate oral hygiene is very difficult for nursing staff, and this may increase the risk of pulmonary infection.[10,11] It is also believed that some oral cleansers do not benefit patients and do not decrease the incidence of nosocomial pneumonia in patients.[12,13]

Regardless of the potential importance of this matter, very little information regarding normal or hospital-acquired oral microbiota composition in comatose patients is available; very few publications report the prospective oral-pulmonary infection pathway in these patients. The aim of this study was to investigate the impact of a lack of oral hygiene and time-dependent oral growth of some selected pneumonia-related microorganisms in institutionalized comatose patients. We opted to investigate the growth of staphylococci, Enterobacteriaceae and *Candida* sp. because they are important species involved in nosocomial infections.

Methods

A formal agreement according to the guidelines established by the Local Ethical Committee for Research Involving Human Beings was required for each patient in this study. Nine institutionalized adult patients from two hospitals in Curitiba (Brazil) that were diagnosed as being in a comatose state (Glasgow scale ≥ 6 and ≤ 8) and undergoing antibiotic therapy were enrolled in this study (group 1; Table).

Table. Descriptive data for patients and hospital history

Group	Patient no.	Coma etiology	Age (yr)/sex	Glasgow score	Antibiotic therapy	Coma evolution
1	1	CVS	42/M	8	Ceftriaxone + clindamycin	Death from pneumonia
	2	Trauma	77/F	8	Cefepime + clarithromycin	Woke from coma
	3	Trauma	28/F	7	Cefazolin	Woke from coma
	4	MIC	42/M	6	Ceftazidime + clarithromycin	Woke from coma
	5	CVS	23/M	6	Ceftriaxone + clarithromycin	Death from pneumonia
	6	Trauma	40/M	7	Cefazolin	Woke from coma
	7	Trauma	30/M	7	Cefazolin	Death from pneumonia
	8	Trauma	37/M	8	Ceftriaxone + metronidazole	Death from pneumonia
	9	MIC	48/F	7	Ceftriaxone	Woke from coma
2	10	NC	53/F	–	–	–
	11	NC	64/F	–	–	–
	12	NC	23/M	–	–	–
	13	NC	34/M	–	Cefazolin	–
	14	NC	22/F	–	–	–
	15	NC	45/M	–	–	–
	16	NC	44/M	–	–	–
	17	NC	51/F	–	Cefazolin	–
	18	NC	67/M	–	Ceftriaxone	–
	19	NC	19/M	–	–	–
	20	NC	33/M	–	–	–
	21	NC	23/F	–	–	–

CVS = cerebral vascular stroke; MIC = medically induced coma; NC = non-comatose; M = male; F = female.

In parallel, 12 conscious patients hospitalized for surgeries or trauma treatment were followed up for 7 days and formed the control group (group 2). These patients were able to brush their teeth at least twice a day and were not using any antibacterial mouth washes.

Saliva samples from patients were collected from the sublingual region using prewet sterile cotton balls and avoiding the dental plaque, tongue or lips. Baseline collections were taken immediately after the coma diagnoses or hospital admission and subsequently at 24 hour intervals. After sampling, the wet cotton balls were immediately sent to the laboratory and their mass were determined. After that, microbial cells were disrupted in 2 mL sterile saline by sonication (50 KHz, 100 W, 3 minutes).

Each bacterial suspension was serially diluted in sterile saline. Aliquots of 100 µL from each dilution were applied onto blood agar, mannitol salt agar (Difco Laboratories, Detroit, MI, USA), CHROMOcult Coliform Agar (Merck Diagnostics, Darmstadt, Germany) and CHROMagar Candida (CHROMagar, Paris, France) to determine the total number of viable staphylococci, Enterobacteriaceae and yeasts. Petri dishes with blood agar and mannitol salt agar were incubated at 35°C for 24 hours. The CHROMOcult Coliform Agar and CHROMagar Candida plates were incubated at 28°C for 24 hours. Following incubation, plates with 30–300 colonies were chosen and the microbial counts determined. Colonies showing phenotypic differences in each culture medium were taken and submitted to complementary biochemical and physiological identification tests.[14]

When *Klebsiella pneumoniae, K. oxytoca,* or *Escherichia coli* was identified, a double-disk synergy test for extended spectrum β-lactamase (ESBL) detection was carried out. Disks containing ceftazidime (30 µg) and cefotaxime (30 µg) were placed 15 mm apart (edge to edge) from an amoxicillin (20 µg)-clavulanate (10 µg) disk. Imipenem (10 µg), meropenem (10 µg) and cefepime (30 µg) disks were also placed on this plate. Following incubation at 35°C for 18–20 hours, a clear extension of the zone of inhibition between ceftazidime and/or cefotaxime and the amoxicillin (20 µg)-clavulanate (10 µg) disk was interpreted as positive for ESBL production.[15] The *K. pneumoniae* ATCC 700603 (ESBL positive) and *E. coli* ATCC 25922 (ESBL negative) strains were used for control.

The normality of all data were checked using the Kolmorogov–Smirnov and Shapiro–Wilk tests; subsequently, the nonparametric test of Kruskal–Wallis was applied to calculate any statistical differences between the microbial counts in the saliva collected from the patients in both groups. Differences between the baseline and the last days of sample collection were also evaluated by the nonparametric test of Kruskal–Wallis. The growth of different microbial groups was assessed by the correlation product, based on the Pearson moment in relation to the total viable microorganism counts.

Results

Of nine patients in group 1 (comatose patients), four could be followed up for 7 days, one for 6 days, one for 5 days and three for 4 days. After these periods, five patients had woken from their coma and the remaining four had died from pneumonia, as confirmed by a physician after necropsy. The microbial counts in the patients who died were lower than that in the comatose patients ($p = 0.634$). Patients from group 2 (control group) were followed up for 6 days after their hospitalization, with the exception of patient 19, who left hospital after 5 days.

The total viable bacteria counts in patients from group 1 varied from $1.1 \pm 0.9 \times 10^4$ CFU/mL at the baseline up to $5.2 \pm 2.8 \times 10^6$ CFU/mL, 6 days later. Differences between the first and last days were statistically significant ($p < 0.001$). Patients in group 2 exhibited counts that varied from $3.3 \pm 2.4 \times 10^3$ CFU/mL (on the day of hospitalization) to $4.1 \pm 2.1 \times 10^3$ CFU/mL (6 days later). No statistical differences were observed between these days ($p = 0.401$) for patients from group 2 (Figure 1).

Figure 2 shows that all patients in group 1 harbored staphylococci in their mouths initially ($8.0 \pm 3.7 \times 10^1$ CFU/mL), with the counts increasing ($3.2 \pm 1.9 \times 10^4$ CFU/mL) over the time of hospitalization ($p < 0.001$). The same progression was not observed in the patients from group 2 ($p = 0.235$). For patients from both groups, 10 mannitol fermentative colonies were randomly chosen and further identified as *S. aureus*.

Progressive colonization ($p < 0.001$) of Enterobacteriaceae (Figure 3) was observed in all patients from group 1 ($4.1 \pm 2.8 \times 10^2$ CFU/mL increased to $4.7 \pm 2.3 \times 10^4$ CFU/mL), but not in patients from group 2 ($p = 0.110$). Among the bacteria of this group, species from the genus *Klebsiella* were predominant in patients 1, 5 and 6. Ten to 15 colonies

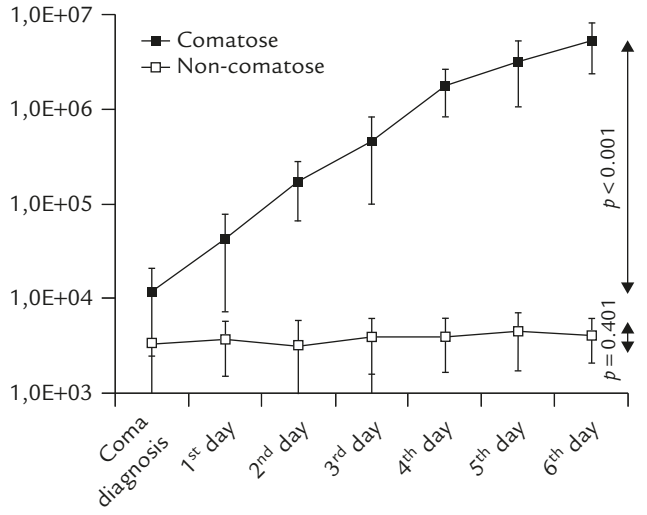

Figure 1. Six-day oral colonization dynamics of total countable bacteria.

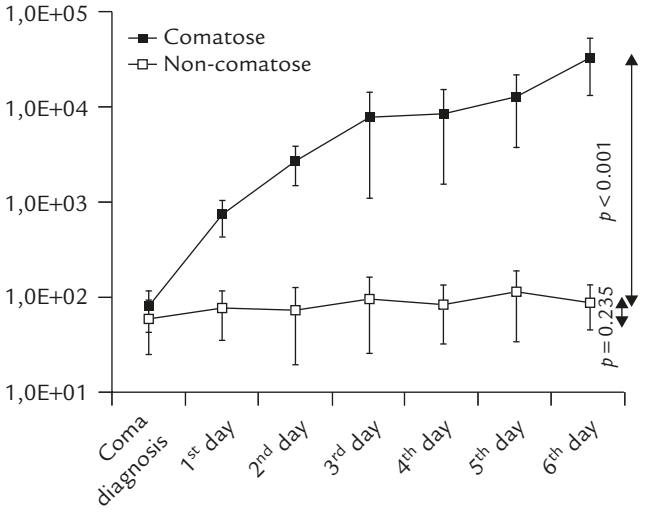

Figure 2. Six-day oral colonization dynamics of staphylococci.

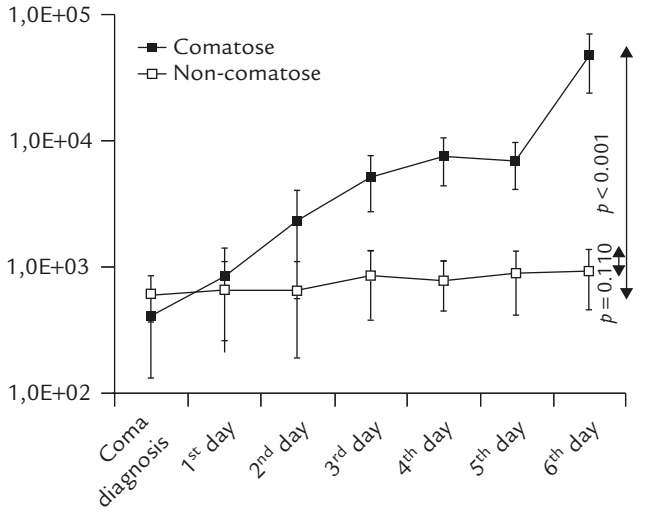

Figure 3. Six-day oral colonization dynamics of Enterobacteriaceae.

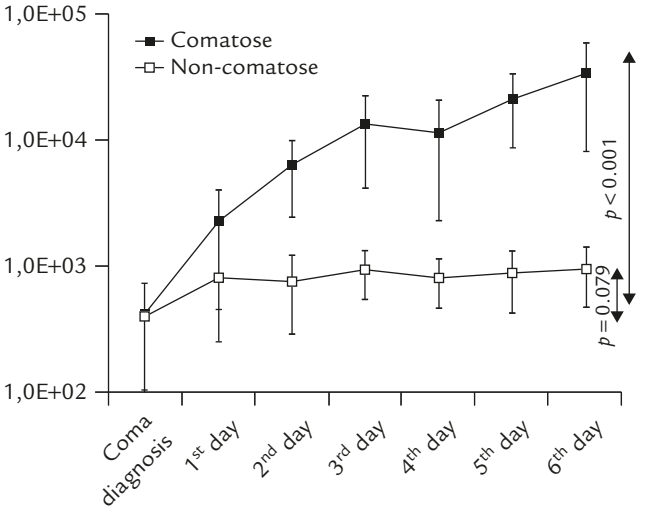

Figure 4. Six-day oral colonization dynamics of yeasts.

from each of these patients were tested for indole/Voges-Proskauer. All of these specimens were negative for both tests. These were interpreted as putative results for *K. pneumoniae*. The remaining patients presented with a predominance of *Klebsiella*-like colonies (approximately 90–95%). Colonies suggestive of *E. coli* were also detected (approximately 5–10%) in both patient groups. No ESBL strains were obtained amongst the Enterobacteriaceae.

The number of colonies grown on CHROMagar Candida increased from $4.1 \pm 3.3 \times 10^2$ CFU/mL to $3.4 \pm 2.5 \times 10^4$ CFU/mL during the hospitalization period ($p < 0.001$) for patients in group 1 (Figure 4). For patients in group 2, differences in the baseline counts and towards the last days of the study were not statistically significant ($p = 0.079$). In both groups, the phenotypic aspect of colonies was predominantly indicative of *Candida albicans*. The unique exception one patient from group 2 who exhibited mixed colonization, presumably by *C. albicans* and *C. tropicalis*; these increased simultaneously throughout the hospitalization period. Further identification tests were not carried out.

All microorganism groups showed a positive linear correlation when compared with the total viable microorganism counts for both patient groups. For group 1, correlation

coefficients were 0.5911 for staphylococci ($p < 0.001$), 0.5493 for Enterobacteriaceae ($p < 0.001$), and 0.393 for yeasts ($p = 0.004$). For group 2, the correlation coefficient values were 0.988 for staphylococci ($p < 0.001$), 0.924 for Enterobacteriaceae ($p < 0.001$), and 0.949 for yeasts ($p < 0.001$).

Discussion

The results obtained were not unexpected but warrant discussion. We hypothesized that some incremental increase in microbial loads should be expected; however, the results showed the worst scenario where microorganisms with known pneumopathogenic potential[9,12] tended to increase exponentially during the hospitalization time.

It was observed that the hospital nursing staff gave little and inadequate attention to the oral hygiene of those comatose patients with dental plaques obviously visible. Besides the increase in microbial population, intense halitosis was observed after 2 or 3 days of hospitalization (data not shown). As soon as these results were obtained, the hospitals' Committees for Infection Control and the Nursing Care Service were informed, and both hospitals altered their conduct in relation to the oral hygiene of unconscious patients.

As shown in Figure 1, there was a time-dependent increase in the total microbial cell numbers recovered from comatose patients. This result could be expected because such patients did not receive any oral care throughout their time in hospital. The group 2 patients that brushed their teeth had an unchanged microbial population during the course of hospitalization. Myrianthefs et al[16] reviewed the factors associated with nosocomial pneumonia and pointed out that patients must receive good oral attention, including frequent suctioning of oral secretions and application of oral rinses twice daily, especially in those patients under pulmonary ventilation.

The choice of microorganisms evaluated in this study was based on the findings of other groups.[8,17–22] Some of these microorganisms are supplementary to normal oral microbiota. Pseudomonades and other glucose non-fermentative Gram-negative bacilli were expected but not detected, probably because of technical problems associated with culture medium handling. It has been shown that *S. aureus*, *P. aeruginosa* and Enterobacteriaceae might participate in the dental biofilm composition of patients from intensive care units.[7,23] In this study, respiratory pathogens were detected initially in all patients, which reinforces the idea that the oral cavity of comatose patients may become a favorable source for pathogens associated with pneumonia. Patients 4 and 9 presented with high counts for staphylococci when coma was induced, possibly because these patients had been institutionalized for several days before medical coma induction.

Significant positive correlation among the total viable microorganisms and the three different groups (staphylococci, Enterobacteriaceae and yeasts) reinforced our opinion that such microorganisms, known to be pneumonia-provoking agents, encountered favorable conditions for growth in the mouth of patients in comas, and may become a steady fraction of the oral microbiota. The positive correlation between bacterial counts in dental biofilm and saliva has been established previously.[24,25] Thus it is reasonable to propose that lack of hygiene led to plaque accumulation, which served as a food source for salivary microbiota.[26,27] The role of oral bacteria in lung diseases has received more attention recently. It has been reported that a great variety of bacteria, including anaerobes such as *Prevotella* spp. and *Fusobacterium* spp., could be isolated from bronchoalveolar samples.[2] The authors of that study found a positive correlation between the high values of plaque indexes and positive pulmonary recovery of anaerobes in institutionalized elderly people undergoing severe aspirative pneumonia.

The antibiotic regimen of the comas patients was based on empirical experience in both hospitals. Apparently, the anti-pneumonia antibiotics prescribed did not affect the bacterial growth in the oral cavity of comatose patients. Although these drugs are effective against a broad bacterial range, salivary clearance is not significant for clindamycin and cefoxitin.[28,29] Recently, it was demonstrated that although detectable cefazolin levels in serum have significant effect against salivary bacteria, the concentrations are low in the saliva and do not reach the minimum inhibitory concentration.[30] Ceftriaxone, which has a good salivary secretion rate,[31] did not prevent pathogen development in the oral environment, probably due to bacterial resistance and/or salivary rate decrease. Although all nine comatose patients were colonized by *Candida* sp., none were put on an antifungal regimen. Some drugs, especially the azoles, have shown efficacy against yeast cells due to their good salivary concentration levels.[32–35]

The prescription of azoles could be recommended since it is well known that *Candida* spp. are associated with pneumonia in hospitalized patients.[36,37]

Notwithstanding the importance of our results, there are limitations of this study. We did not state whether the microorganisms evaluated in this study were community or hospital strains. To determine this, molecular fingerprinting needs to be employed. We supposed that those strains that increased in number during the hospitalization period were present in patients prior to their hospitalization, with the numbers already high when comas were diagnosed. Additionally, in the first 24 hours the numbers of these bacteria increased in an exponential fashion. The profiles of sensitivity to antibiotics for these microorganisms were not evaluated. If they were identified, we could also establish the time-related resistance increase, if any occurred. Also, in those patients whose cause of death was diagnosed as pneumonia, we had no access to the biological (pulmonary) material. Thus it was not possible to determine whether these microorganisms were actually involved or responsible for those deaths.

Despite these missing data, our results lead us to conclude that some pulmonary pathogens colonize the oral cavity of comatose patients and grow, as a result of the cessation of brushing and normal oral hygiene. This growth is progressive and may hypothetically disseminate to the lungs.[38] Complementary studies must be carried out to establish colonization patterns of other pathogens such as *P. aeruginosa*, *Acinetobacter baumannii*, *Streptococcus pneumoniae* and *Haemophilus influenza*, among others. Molecular typing of microorganisms simultaneously harvested from the oral cavity and lungs may contribute to the elucidation of this putative oro-pulmonary infection pathway.

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