Opportunistic Microorganisms in Patients with Head and Neck Trauma

Microrganismos oportunistas em pacientes com trauma de cabeça e pescoço

Microorganismos oportunistas en pacientes con trauma de cabeza y cuello

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The use of antimicrobial agents has facilitated the dissemination of multi-resistant microorganisms, compromising dental and medical treatment. The aim of this study was to evaluate the distribution of different opportunistic microbial species in patients who suffered head and neck trauma, under temporary maintenance in nosocomial environment, particularly intensive care units, on the occurrence of such microorganisms in the oral cavity of the patients. It was selected 38 patients subjected to head and neck traumas. After emergency surgical procedures, clinical samples of saliva, sub and supragingival biofilms and mucosal surfaces were collected at two different moments: just after stabilization of the patient and soon after patients' release from medical units. The presence of opportunistic and superinfecting microorganisms was evaluated by culture on selective and non-selective media, and the presence of the family *Enterobacteriaceae*, as well as genera *Enterococcus*, *Pseudomonas*, and *Staphylococcus* was assessed by PCR. It was found that the use of antimicrobials, even for short periods of time was sufficient to facilitate colonization by microorganisms of the families *Enterobacteriaceae* and *Pseudomonadaceae*, as well as yeasts and enterococci. These results support the concept that medical and dental teams should make a periodically change of antimicrobials used in treatment protocols in hospital for head and neck trauma patients, in order to minimize dissemination of opportunistic or superinfecting microorganisms.

Keywords: Antimicrobial Agents; Head and Neck Trauma; Opportunistic Infections; Hospital Units**;** Polymerase Chain Reaction**;** Culture Media.

INTRODUCTION

Oral biofilm might become a major reservoir for opportunistic microorganisms, particularly enteric microorganisms and yeasts, which are responsible for many serious infections in hospitalized patients[1,2], and often carry resistance markers to various antimicrobials used in medical and dental clinics[3,4].

Recent studies have presented data about microorganisms detected in oral cavity from patients

subjected to medical treatment in nosocomial environment[5]. Besides oral bacteria, many opportunistic and superinfecting microorganisms have been identified, particularly enteric bacteria, yeasts, staphylococci and enterococci[5,6]. The occurrence of opportunistic infections is particularly relevant for procedures and patients maintained in intensive care units (ICU), since ICU is a sector where high risk patients are attended, such as multiple trauma. In order to minimize risks of infections, the routine treatment has its own characteristics and peculiarities[7].

Nosocomial infections resistant to antimicrobial can be caused by the patient's resident microflora or by exogenous microorganisms from nosocomial environment[8], which might become lodged in the dental biofilm[9]. Some evidences support that the longer the period of hospitalization, the greater the risk of acquisition of microorganisms resistant to most frequently antimicrobials, since nosocomial usage of antimicrobial drugs is able to create extremely favorable conditions for these microorganisms[10,11].

The literature has shown the oral cavity and oral biofilm are large reservoirs of microorganisms associated with nosocomial sepsis[2,7,9]. Oral microorganisms have been associated with various systemic diseases, such as cardiovascular disease[12,13], chronic obstructive pulmonary infections[14] and the occurrence of lung infections of nosocomial origin in intensive care units[15,16]. Moreover, control of dental biofilm can reduce colonization by pathogens associated with nosocomial pneumonia and is a procedure for which the medical professionals do not usually give due importance[5,7,9].

In Brazil, nosocomial infections are a current concern but scarce data are available about participation of antimicrobial resistant microorganisms in patients with head and neck trauma, which difficult the establishment of measures in order to minimize the risks of sequelae. Then, this study aimed evaluate the occurrence of opportunistic and superinfecting microorganisms in oral microbiota of patients with head and neck trauma and the effects of antimicrobial use and period of hospitalization on this phenomenon.

MATERIAL AND METHODS

1. STUDY POPULATION AND COLLECTION OF CLINICAL SAMPLES

A total of 38 patients (28 males and 10 females) seen at the Santa Casa de Misericórdia de Araçatuba and Santa Casa de Birigui, São Paulo, Brazil, aged 18-52 years (mean age 26.4 years), with oral or head and neck trauma were included in the study. The patients who receive dental or medical treatment prior 3 months of this study were excluded, as well as patients presenting fewer than 10 teeth. This study was approved by the Institutional Review Board of School of Dentistry of Araçatuba-UNESP (No. 00312/2011).

Clinical samples were collected from deepest periodontal sites presenting bleeding on probing, supragingival biofilm, saliva, tongue dorsum, soft mucosa and floor of the mouth. Sample collection were carried out immediately after patient hospitalization and immediately before the end of period of hospitalization. Supragingival biofilm samples were collected by using sterile periodontal curettes; subgingival samples were obtained with sterile paper points that were introduced to the bottom of periodontal pockets or gingival crevices and maintained for 30s. Soft mucosa was collected with swabs and saliva samples were obtained by mean of Salivettes (Aktiengesellsschaft, Nümbrecht, Germany). Clinical samples from traumatized tissues were also collected. All clinical samples were transferred to ultrapura Milli Q water to DNA extraction and to 300µL of PBS (phosphate buffered solution) for culture procedures. Clinical specimens were collected from 1993 to 2012.

2. MICROBIAL ISOLATION AND SPECIATION

Clinical samples were inoculated in peptone water and EVA broth (Difco, Rochester, NY) and incubated 3-7 days at room temperature. From specimens with microbial growth in peptone water, aliquots of 0.1 ml

were transferred to Eosin Methylene Blue agar, Sabouraud Dextrose agar with 100 μg/ml of chloramphenicol, MacConkey agar, Brilliant Green agar and Brain Heart Infusion agar supplemented with defibrinated horse blood, and incubated at 37°C, for 24-72h. From tubes containing EVA broth, aliquots of 0.1 mL were transferred to Bile Esculin agar and incubated in aerobiosis for 48h. at 37°C[17].

Speciation of bacterial isolates was carried out by morphocolonial and morfocellular evaluation (Gram-staining), fermentation of carbohydrates, reduction of nitrate, production of gas from glucose, production of indole, hydrolysis of esculin. Some bacterial isolates were identified using the BBL Crystal Enteric/Nonfermenter system (Becton Dickinson Microbiology Systems, Cockeysville, MD). The yeast identification was performed by carbon and nitrogen assimilation tests, fermentation of carbohydrates, germ tube formation (at 37°C and at 39°C), colonial morphology and growth at 37°C and 42°C. In the carbohydrate assimilation tests[17].

3. DETECTION OF TARGETED MICROORGANISMS BY PCR

The DNA of all clinical samples transported in sterile ultrapure water was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Concentrations of bacterial DNA were determined with a spectrophotometer at A_{260} nm.

The presence of *Enterococcus* spp., *E. faecalis*[18], *E. faecium*[19], pseudomonads[20], *Staphylococcus* sp.[21] and family *Enterobacteriaceae*[22] was evaluated by PCR using specific primer pairs and conditions[17]. DNA from *P. aeruginosa* ATCC 10145, *P. putida* ATCC 49128, *E. coli* ATCC 35218 and ATCC 25922, *Acinetobacter haemolyticus* ATCC 19002, *E. faecium* ATCC 35667, *E. faecalis* ATCC 29212 and *S. aureus* ATCC 25923 was used as positive controls.

PCR amplification was performed in volumes of 25 μL containing 1X PCR/Mg^{++} buffer (Boehring Mannheim, Indianapolis, IN, USA), 0.2 mM each of dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.5 U Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil), 0.4 μM of each primer pair (Invitrogen) and 10 ng of template. Amplification was performed in a DNA Thermal Cycler (Axygen, New York, NY) programmed for 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds; annealing temperature specific for each primer pair, ranging between 30 seconds and 1 minute; 72°C for 1 minute, then 72°C for 5 minutes to allow completion of DNA extension. Amplification products were compared by electrophoresis in a solution of 1% agarose gel in 1X TBE buffer stained with ethidium bromide (0.5 mg/mL).

STATISTICAL ANALYSIS

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS Incs v.13, Chicago, IL, USA). Variables that had three or more categories were subjected to Chi-square test, and those with two categories were evaluated through Mann-Whitney test and Fisher's exact test. The differences in the distribution of different microorganisms were evaluated using analysis of variance with repeated measures for categorical data (ANOVAmr), according to Brunner, Langer[23]. Test of Spearman's correlations was used to determine the existence of inter-relationships between the clinical and microbiological parameters. Differences of $p<0.05$ was considered statistically significant.

RESULTS

Among the subjects who underwent clinical examination, there was heterogeneity with respect to gender (Chi-square, $p= 0.099$) and age (Chi-square, $p= 0.157$), with vast predominance of young males (Chi-square, $p <0.001$), tobacco (Chi-square, $p <0.001$) and/or alcohol consumers (Chi-square, $p = 0.012$). In general, patients treated presented modest formal education and almost invariably hospitalizations occurred due to car accidents (34.2%), motorcycles (55.3%), or injury by firearms (10.5%).

Table 1 presents some data on the patients studied, showing that the bones most affected by facial

trauma were, in descending order, nasal and zygomatic bones, followed by the jaw. The occurrence of complex fractures could be observed in 28.9% of patients. Like most trauma patients showed less complicated and closed fractures, bone fragments without exposure to the external environment, the length of hospital stay was reduced. The use of antimicrobial drugs in the hospital reflected the availability of these agents within public health system as well as the etiology of the vast majority of infectious processes, usually linked to resident microbiota microbiota, and good distribution in the bone tissue, like most of the β-lactam and clindamycin. Cephalosporins were the most commonly prescribed drug (36.8%), followed by penicillins (29.9%), clindamycin (13.2), aminoglycosides (2.6%), vancomycin (2.6%), β-lactamases resistant penicillins (2.6%), associations of penicillin-metronidazole (7.9%), aminoglycoside-metronidazole (2.6%), and cephalosporin-metronidazole (2.6%).

Table 1- General characteristics of the studied population

Characteristic	Frequency N(%)
[1]Tobacco consumption	17 (44.7%)
[2]Alcohol consumption	21 (55.3%)
Ethnicity (self-declared)	
Euro-Brazilian	19 (50.0%)
Afr+o-Brazilian	1 (2.6%)
Asiatic	1 (2.6%)
Miscellaneous	17 (44.7%)
Traumatized areas in the skull	
Mandible	6 (15.8%)
Maxilla	2 (5.3%)
Nasal	9 (23.7%)
Zygomatic	9 (23.7%)
Frontal	1 (2.6%)
Le Fort/complex	11 (28.9%)
Trauma involving teeth	21 (55.3%)
Open fractures	12 (31.6%)
Closed Fractures	26 (68.4%)

[1]At least 10 cigarettes per day over the past five years;
[2]At least two daily doses of *cachaça*, a distilled alcoholic beverage made from the fermentation of sugarcane juice in Brazil, with an alcohol content of 38-48% v / v.

The results of the microbial culture and detection of targeted microorganisms by PCR were similar and were discussed together (tables 2-4). The test of Spearman correlations demonstrated that to the existence of numerous schemes of antimicrobial therapy, it was not possible to establish possible links between antibiotics used and their effects on the opportunistic microbiota. However, if the different regimens are evaluated together, the members of the families *Enterobacteriaceae* and *Pseudomonadaceae*, *Candida* species, as well as cocci of the genus *Enterococcus* had a higher occurrence after the use of antimicrobials.

The number of species and the prevalence of the family *Enterobacteriaceae* (ANOVAmr, p= 0.01), occurrence of enterococci (ANOVAmr, p = 0.026) and yeasts (ANOVAmr, p= 0,04) showed a significant increase. In addition, no species of the families *Enterobacteriaceae* and *Pseudomonadaceae* showed a significant increase, but collectively, these microorganisms showed a pronounced elevation, regardless of antimicrobial drugs used.

Table 2- Occurrence of selected microorganisms in oral specimens. Data obtained by culture.

Microorganism	Sample N(%)	
	Beginning	Conclusion of Hospitalization
family *Enterobacteriaceae*[1]	12	31
Enterococcus faecalis	8	19
E. faecium	1 (2.6)	2 (5.3)
Enterococcus sp.	9	22
Pseudomonas aeruginosa	4 (10.5)	13
Pseudomonas sp.	6 (15.8)	10
Staphylococcus aureus	4 (10.5)	6 (15.8)
S. epidermidis	1 (2.6)	4 (10.5)
S. haemolyticus	0 (0.0)	4 (10.5)
S. hominis	1 (2.6)	5
S. pasteuri	1 (2.6)	0 (0.0)
S. saprophyticus	6 (15.8)	11
Staphylococcus sp.	6 (15.8)	17

[1]The occurrence of species of the family *Enterobacteriaceae* are presented separated in Table 4.

Table 3- Occurrence of family *Enterobacteriaceae* in the oral cavity of head and neck trauma. Data obtained by culture at the beginning of the treatment and at the end of period of hospitalization.

Microorganism	Sample N(%)	
	Beginning	Conclusion of Hospitalization
Citrobacter freundii	1 (2.6)	4 (10.5)
Enterobacter cloacae	4 (10.5)	6 (15.8)
E. intermedius	2 (5.3)	4 (10.5)
Klebsiella oxytoca	1 (2.6)	5 (13.2)
E. sakazakii	0 (0.0)	1 (2.6)
Klebsiella oxytoca	0 (0.0)	3 (7.9)
Morganella morganii	1 (2.6)	3 (7.9)
Pantoea agglomerans	1 (2.6)	2 (5.3)
Pasteurella pneumotropica/haemolytica	1 (2.6)	1 (2.6)
Proteus mirabilis	2 (5.3)	4 (10.5)
P. vulgaris	0 (0.0)	4 (10.5)
Proteus sp.	0 (0.0)	2 (5.3)
Serratia marcescens	1 (2.6)	0 (0.0)
Providencia alcalifaciens	0 (0.0)	4 (10.5)
Total	12 (31.6)*	31 (81.6)

*Some patients were colonized by two or more species of *Enterobacteriaceae*.

Table 4- Occurrence of selected microorganisms in oral specimens. Data obtained by PCR.

Microorganism	Sample N(%)	
	Beginning	Conclusion of Hospitalization
family *Enterobacteriaceae*	17 (44.3)	35 (92.1)
Enterococcus faecalis	19 (50.0)	31 (81.6)
E. faecium	1 (2.6)	3 (7.9)
Enterococcus spp.	22 (57.9)	31 (81.6)

Pseudomonas aeruginosa	8 (21.1)	21 (55.3)
Pseudomonas spp.	15 (39.5)	24 (63.2)
Staphylococcus spp.	17 (44.3)	22 (57.9)

Table 5 shows the occurrence of *Candida* species, and the most prevalent was *C. albicans*. During and after antibiotic therapy, most patients was colonized by yeast. The increase in the prevalence of yeasts was linked to *C. albicans* and non-*C. albicans* species as well.

Table 5- Species of the genus *Candida* in the mouth of patients undergoing hospital treatment to orofacial trauma.

Microorganism	Sample N (%)	
	Beginning	Conclusion of Hospitalization
C. albicans	8 (21.1)	17 (44.7)
C. tropicalis	2 (5.3)	5 (7.1)
C. krusei	1 (2.6)	2 (5.3)
C. glabrata	0 (0.0)	1 (2.6)
C. *parapsilosis*	1 (2.6)	4 (10.5)
Candida sp.	0 (0.0)	5 (13.2)
Total[1]	9 (23.7)	21 (55.3)
Mean a± SD[2]	31 ± 22.5	212 ± 143

[1]Some patients were colonized by two or more species of yeasts.
[5]Mean counts (colony forming units, CFU) ± standards deviation

DISCUSSION

The pattern of the patients examined, young adults, males, low education, is consistent with the profile described by other authors in the literature[24]. In this sense, the low average age is linked to the type of trauma usually associated with accident with frequent involvement of motorcycles and injuries with firearms.

The factors associated with colonization of the oral cavity by opportunistic microorganisms are varied, but the selection of microorganisms resistant to antimicrobials, biofilm accumulation and immunosuppression have been frequently implicated.[2,25]. Several species of these opportunistic pathogens are associated with multidrug-resistant infections, such as surgical wounds, respiratory infections, bacteremia and septicemia, urinary tract infections, and others[1,26], with high mortality.

The impact of antimicrobial use on the occurrence of enteric microorganisms resistant to these drugs suggests that these compounds might be used for the shortest time to minimize its effects on microbiota[27]. Although various external sources for opportunistic organisms might be evaluated, such as consumption of contaminated water or food, as suggested by Slots et al.[28], possibly the patient's own intestinal microbiota associated to poor oral hygiene standards are the major source of enteric microorganisms and pseudomonads. The suppression of anaerobes and other members of resident oral microbiota might create conditions for the colonization of the oral cavity by superinfecting organisms. Moreover, it is possible that the deterioration of oral hygiene after orofacial trauma could allow the dissemination of oral surfaces by these microorganisms. It is possible that drug resistance ultimately facilitate the spread of such pathogens[26].

The oral colonization by microorganisms of the families *Pseudomonadaceae* and *Enterobacteriaceae* and staphylococci and enterococci may be transient[29]. In order to confirm this hypothesis, the data about oral persistence of such opportunistic pathogens must be assessed in extended period, perhaps months or years. Thus, the association between oral colonization by opportunistic pathogens and occurrence of infections resistant to antimicrobials must be evaluated[1,30-32].

Candida species are frequently recovered from oral cavity of periodontally healthy adults, but they usually occurs in low numbers. However, in severely debilitated patients or individuals with poor standards of oral hygiene, as detected in the present investigation, these yeasts have been associated with opportunistic and severe infections[33], and Table 5 shows that the populations of fungi increased significantly after initiation of treatment with antibacterials, as also described by Soysa et al.[34]. These authors evidenced the association between consumption of broad-spectrum antimicrobials for longer periods and suppression of bacterial microbiota, but in the present investigation the regimens of antimicrobials were short-lived. The results presented here show that the use of antibiotics, even for a few days, can affect the occurrence of opportunistic fungi.

Factors associated with dissemination of oral yeasts remains unclear, but it is possible that the

removal of part of the oral bacterial microflora may provide nutrients and create new habitats for these microorganisms[35]. Some evidences support this hypothesis. For example, the presence of some microorganisms, such as *P. gingivalis* and α-hemolytic streptococci appear to inhibit the adhesion of *Candida* spp. to its host[35], and the presence of these bacterial groups was severely depressed in the studied population (data not presented here). Moreover, some antimicrobial drugs (tetracyclines) might directly increase the adhesion of *Candida* spp. the surface of epithelial cells[36], but no data are available regarding the β-lactams, nitroimidazoles and macrolides-lincosamides, which constitute the majority of prescriptions of antibiotics in dentistry.

The presence of these opportunistic microorganisms in patients hospitalized for longer periods increases the risk of severe infections, which suggests the need for a proper control of oral biofilms, using for example chlorhexidine. This compound is able to reduce the oral carriage of opportunistic pathogens[5].

CONCLUSIONS

The use of antimicrobials even for short periods of time may affect temporarily the composition of the oral microbiota, and he most commonly detected opportunistic pathogens belonged to families *Enterobacteriaceae* and *Pseudomonadaceae*, as well as yeasts and enterococci. However, the follow-up period proved short to assess the effects of antimicrobials on the oral microbiota of the patients, which may be aggravated by the difficulty of re-establishing contact with patients after completion of treatment of trauma.

RESUMO

O uso de agentes antimicrobianos tem facilitado a disseminação de microrganismos multirresistentes, comprometendo o tratamento odontológico e médico. O objetivo deste estudo foi avaliar a distribuição de diferentes espécies microbianas oportunistas em pacientes que sofreram trauma de cabeça e pescoço, mantidos temporariamente em ambiente hospitalar, particularmente em unidade de terapia intensiva, sobre a ocorrência de tais microrganismos na cavidade bucal dos pacientes. Foram selecionados 38 pacientes com trauma de cabeça e pescoço. Depois dos procedimentos cirúrgicos de emergência, espécimes clínicos de saliva, biofilmes sub e supragengivais e superfícies de mucosas foram coletados em dois diferentes momentos: logo após a estabilização do paciente e logo após a liberação da unidade médica. A presença de microrganismos oportunistas e superinfectantes foi avaliada por cultura em meios seletivos e não-seletivos, e a presença da família Enterobacteriaceae, bem como os gêneros Enterococcus, Pseudomonas, and Staphylococcus foram avaliados por PCR. Verificou-se que o uso de antimicrobianos, mesmo em pequenos períodos de tempo foi suficiente para facilitar a colonização por microrganismos das famílias Enterobacteriaceae e Pseudomonadaceae, bem como leveduras e enterococos. Estes resultados apoiam o conceito que equipes médicas e odontológicas devem fazer uma alteração dos antimicrobianos utilizados nos protolocos de atendimento em hospital para pacientes com trauma de cabeça e pescoço periodicamente, de modo a minimizar a disseminação de microrganismos oportunistas e superinfectantes.

Palavras chave: Agentes Antimicrobianos; Traumatismos Craniocerebrais e de Pescoço; Infecções Oportunistas; Unidades Hospitalares; Reação em Cadeia da Polimerase; Meios de Cultura.

RESUMEN

El uso de agentes antimicrobianos ha facilitado la difusión de microorganismos multirresistentes, poniendo en peligro el tratamiento dental y médico. El objetivo de este estudio fue evaluar la distribución de las diferentes especies microbianas oportunistas en pacientes que han sufrido un trauma de cabeza y cuello, bajo mantenimiento temporal en ambiente hospitalario, unidades de cuidados intensivos en particular, en la ocurrencia de tales microorganismos en la cavidad bucal de los pacientes. Fue seleccionado 38 pacientes sometidos a traumas en la cabeza y el cuello. Muestras clínicas de la saliva, biofilms sub y supragingival y las mucosas fueran obtuvo en dos momentos diferentes: sólo después de la estabilización del paciente y poco después del lanzamiento de los pacientes de las unidades médicas. La presencia de microorganismos oportunistas era evaluada mediante cultivo en medios selectivos y no selectivos, mientras que la presencia de la familia Enterobacteriaceae, así como géneros Enterococcus, Pseudomonas, y Staphylococcus se evaluó por PCR. Se encontró que el uso de antimicrobianos, incluso durante cortos periodos de tiempo era suficiente para

facilitar la colonización por microorganismos de las familias Enterobacteriaceae y Pseudomonadaceae, así como levaduras y enterococos. Estos resultados apoyan el concepto de que los equipos médicos y dentales debe hacer un cambio periódicamente de los antimicrobianos utilizados en los protocolos de tratamiento en el hospital para trauma de cabeza y cuello, a fin de minimizar la diseminación de microorganismos oportunistas.

Palabras clave: Antiinfecciosos; Traumatismos Craneocerebrales y del Cuello; Infecciones Oportunistas; Unidades Hospitalarias; Reacción en Cadena de la Polimerasa; Medios de Cultivo.

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