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Identification of microorganisms in biofluids of individuals with periodontitis and chronic kidney disease using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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RATIONALE: Chronic kidney disease (CKD) and periodontitis (PD) are important health issues. There is a large variety of microorganisms related to the pathogenesis of periodontitis, and optimising the time and the cost of laboratory assays to detect these organisms is highly valuable in the medical field.
METHODS: Bacteria were isolated from saliva and oral biofilm of 30 adolescents and young adults with definite medical and dental diagnosis of CKD and PD, respectively, and proteins were extracted for microorganism identification by means of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) technique.
RESULTS: The results showed that the most incident microorganisms were *Actinomyces dentalis* (43%), *Acinetobacter ursingi* (60%), *Aggregatibacter actinomycetencomitans* (60%), *Corynebacterium argentoctens* (63%), Staphylococcus aureus (93%), *Streptococcus salivarius* (97%) and *Tannerella forsythensis* (43%). The analysis of oral biofilm showed higher incidences for *Actinomyces dentalis* (33%), *Acinetobacter ursingi* (50%), *Aggregatibacter actinomycetencomitans* (50%), *Corynebacterium argentoctens* (70%), *Pseudomonas aeruginosa* (40%), Staphylococcus aureus (73%) and *Streptococcus salivarius* (87%).
CONCLUSIONS: Based on these results, we concluded that the MALDI Biotyper protocol proves useful as a rapid and reliable assay for distinguishing different microorganisms possibly related to CKD and PD. Copyright © 2016 John Wiley & Sons, Ltd.

A large community of microorganisms resides in the oral cavity and constitutes a dynamic and symbiotic ecosystem that is constantly interacting with host metabolic processes.[1] Inefficient oral hygiene, as well as other local and/or systemic factors, can potentially increase the severity of the disease of an individual.

In order to evaluate the composition of the oral ecosystem, technological advances have simply enabled more specific analyses. These increases in breadths and throughput have optimized the time taken for sample examination, enhancing smaller costs for the process.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) is a technique that has been used for microbial species identification and is based on the comparison of specific mass spectra of the mixture of cellular components, mainly proteins and peptides, directly obtained from 'whole' cells without preliminary separation of cellular components.[2] The technique approaches are particularly promising in periodontology research due to the broad impact an oral ecosystem has on the severity of periodontitis.

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Gram-negative bacteria have been related to chronic periodontitis (CP), an infectious disease that destroys the supporting tissues of the teeth. The presence of these bacteria causes the release of proteolytic enzymes that are capable of degrading the gingival tissue. Several periodontal pathogens not only induce inflammation and local tissue damage, but can also be associated with systemic inflammation.[3]

Chronic kidney disease (CKD), a public health problem worldwide, can be defined based on the presence of kidney damage or glomerular filtration rate (GFR) <60 mL/min per 1.73 m^2 for 3 months, regardless of the cause.[2] Fisher *et al.*, in a cross-sectional study, identified periodontal disease (PD) as a potential risk for CKD, among other factors.[4]

Oral microorganisms have usually been identified by traditional methodologies[4] other than MALDI-TOFMS. This technique holds the potential to provide a fast method for both genus and species identification of several microorganisms.[5] Therefore, one important feature of this technique is that it may beneficially facilitate the monitoring of the oral ecosystem.

A prerequisite for a successful analysis is use of adequate software tools with bioinformatics algorithms, able to compare and to automatically differentiate the spectra of different bacterial strains.[4]

Although CP is not trivial among pediatric individuals, based on our clinical experience, those under medical treatment for chronic diseases are noticed not to give the same attention to oral health as they do for other medical care.

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Hence, gingival inflammation and periodontal disease become more severe, and we speculate that PD, more specifically CP,[3] represents a potential and preventable cause of deprived health outcomes in people with CKD.

To the best of our knowledge, there have not been studies on oral microbiota of individuals with CKD and PD previously performed by means of the MALDI-TOFMS technique. On account of that, the aim of this study was to identify microorganisms present in saliva and in oral biofilm (supragingival plaque) of CKD patients, which could possibly be related to PD, by means of the MALDI-TOFMS technique.

EXPERIMENTAL

The study was undertaken after obtaining ethical clearance from the Ethics Committee of the Dental School and from the Children Institute (Medical School) – University of São Paulo (Brazil) – (Registration No. 078109/2013).

Inclusion and exclusion criteria

Thirty adolescents and young adults, aged 12–18 years, with definite medical diagnosis of CKD (stages 1 to 5) and attending the CAPE (Center of Attendance for Special Needs Patients) of the Dental School, University of São Paulo, Brazil, and the Children Institute of the Medical School, University of São Paulo, Brazil, were non-randomly selected to be included in the study.

Patients who presented a number of caries lesions >3 per tooth, received periodontal treatment within the six months prior to the study entry; smokers and those who were undergoing orthodontic treatment were excluded from the study. Additional exclusion criteria were the use of antibiotics and/or anti-inflammatory medication in the previous six months.

Clinical evaluation and specimen collection

Complete medical and dental histories were obtained from the patients' records.

Periodontal evaluation was firstly carried out by means of the visible plaque index and recorded in a clinical form.[6] Patients were then required to expectorate 3 cm^3 of unstimulated whole saliva into a plastic universal tube (Falcon®, Rio de Janeiro, Brazil) for about 5 min in the morning and samples were frozen at –80°C until further analysis.[7] Participants were asked to refrain from oral activities for 2 h prior to saliva collection.

Next, relative isolation was set and oral biofilm collected by using sterile spoon-shaped excavators. All of the samples collected were stored in a plastic tube (Eppendorf, Germany) and frozen at –80°C.

Lastly, the second part of the periodontal evaluation (Kappa intraexaminer =0.84)[8] was performed and the examiners recorded scores of simplified periodontal screening examination for each subject.[6]

Bacteria isolation

Bacteria were isolated from saliva and oral biofilm using Brain Heart Infusion (BHI) agar 37 g L^{-1}. Samples were grown at 37°C for 4 days. In order to purify different bacteria morphologically, this process was repeated as many times as necessary to obtain single colonies. An average of four different bacteria was obtained in each primary agar plate.

MALDI-TOFMS analysis

Both in-tube extraction and a direct smear protocol were tested and the higher scores were observed for the in-tube extraction. The extraction protocol is described below.

Bacterial cells were transferred from the plate to an extraction tube (Eppendorf, Germany) containing 200 µL of distilled water. Then, 900 µL of ethanol was added and the sample centrifuged (17900 *g*, 2 min). The supernatant was discharged, and residual ethanol removed after repeated centrifugation. The pellet was suspended in 50 µL of 70% formic acid. Acetonitrile (50 µL) was added, and the sample was mixed using a vortex and centrifuged. The α-cyano-4-hydroxycinnamic acid matrix was prepared as a saturated solution in 50% acetonitrile and 2.5% trifluoroacetic acid. Subsequently, 1 µL of sample extract was spotted onto a steel target plate and allowed to dry at room temperature. Finally, 1 µL of matrix solution was added and left to air dry (Fig. 1).

Mass spectrometry analyses were performed using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) operating in the linear positive ion mode. Mass spectra were acquired in a mass range from 2 to 20 kDa with ions formed by irradiation of smartbeam using a frequency of 2000 Hz, PIE 100 ns, 7 kV lens. The voltages for the first and second ion sources were 25 kV and 23 kV, respectively. Bacteria were identified by means of the Biotyper 3.1 database. The identification cut-off values higher than 2 and 1.7 were used for species and genus identification, respectively.

RESULTS AND DISCUSSION

The MALDI Biotyper identifies microorganisms using MALDI-TOFMS to measure a molecular fingerprint of an organism. The identification is based on the protein composition of microbial cells. A wide use of this technique in routine laboratories became more frequent when successful species identification for different genus was shown. Pérez-Sancho *et al.*[9] demonstrated that MALDI-TOFMS represents a rapid, accurate and cost-saving method and a reliable alternative to polymer chain reaction (PCR)-based methods for routinely identifying some microorganism isolates from both human and animal origins.[10,11]

A few studies have been published focusing on oral microbiota of patients with CKD and PD. However, the majority of them use the PCR technique to identify and to evaluate the frequency of microorganisms in subgingival, supragingival plaque and in gingival crevicular fluid related to PD.[12,13]

In the current study, we used the MALDI-TOFMS technique. The development of this technique has shown a significant change in the identification of microorganisms in clinical microbiology laboratories. Also, MALDI-TOFMS measures highly abundant proteins that are found in all microorganisms, is highly accurate, is applicable to a wide range of microorganisms, is much faster than traditional

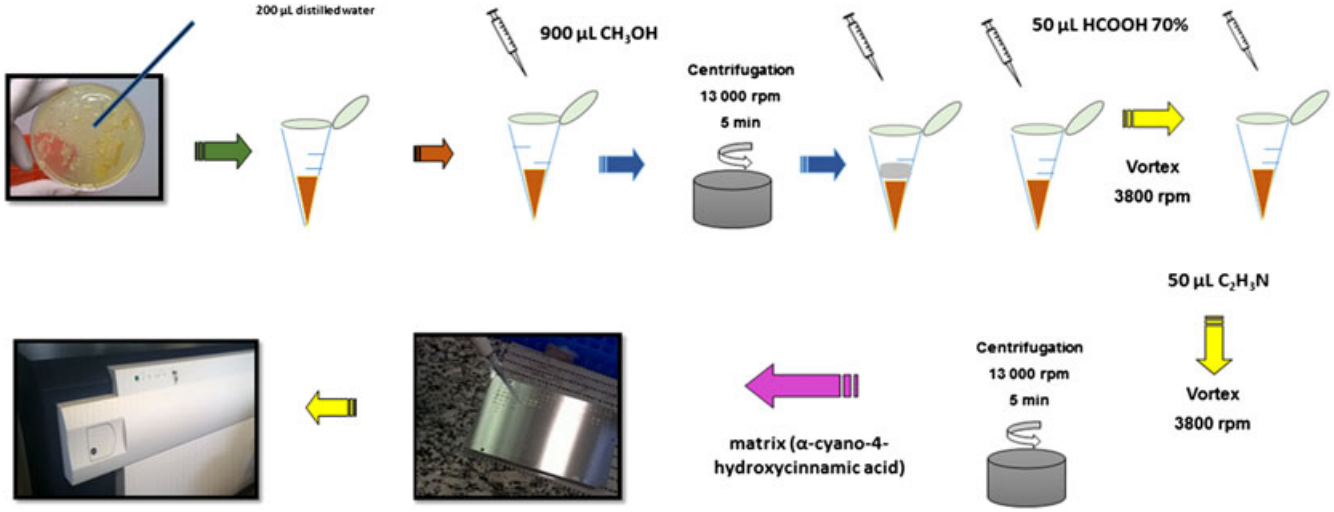

Figure 1. MALDI-TOFMS technique: sample preparation.

methods and is cost-effective.[10,14,15] The fast identification process of microorganisms can favor the optimization of clinical management and antimicrobial therapy.

Several microorganisms have been identified by means of this technique, although in some cases the correct identification of microorganisms was observed to possibly require some extra molecular analysis to provide more identification information.[10]

Bizzini et al.[16] evaluated strains of *Corynebacterium argentoctens* by means of the MALDI Biotyper technique and showed that all strains, except for one, were correctly identified with scores ≥2000, corroborating the potentiality of the technique.

Another important biofluid, saliva, can be used as a diagnostic tool for several diseases and a number of promising biomarkers have been already identified and correlated with the clinical parameters of periodontitis.[16] The flora of periodontal pockets is characterized by a high proportion of motile Gram-negative bacteria (30%) and spirochetes.[17]

The microbiology results found in this study of saliva showed that the most incident microorganims were *Actinomyces dentalis* (43%), *Acinetobacter ursingi* (60%), *Aggregatibacter actinomycetencomitans* (60%), *Corynebacterium argentoctens* (63%), Staphylococcus aureus (93%), *Streptococcus salivarius* (97%) and *Tannerella forsythensis* (43%). The analysis of oral biofilm showed higher incidences for *Actinomyces dentalis* (33%), *Acinetobacter ursingi* (50%), *Aggregatibacter actinomycetencomitans* (50%), *Corynebacterium argentoctens* (70%), *Pseudomonas pertucinogena* (40%), *Staphylococus aureus* (73%) and *Streptococcus salivarius* (87%). Staphylococcus aureus and *Streptococcus salivarius* have been found to be present in both saliva and oral biofilm as trivial components of the oral microbiota. (Table 1).

The high frequent percentage of *Corynebacterium argentoctens* corroborates another finding in the literature,[17] which demonstrated the relationship between the presence of this microorganism, aggressive and chronic periodontitis. This genus contains more than 100 species isolated mostly from animals or humans and it has been traditionally considered a contaminant when isolated from various clinical materials and recognized as an opportunistic pathogen,[18] etiological factor of pneumonia,[19] vertebral osteomyelitis,[20] septicaemia[21] and endocarditis.[22]

No single species has been verified as the primary pathogen for PD; instead, the onset of periodontitis has been reported as polymicrobial, and in the majority of the cases, microorganisms of the red-complex bacteria – *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerela forsythia* – are the most prevalent. Our results showed an incidence of 43% for *Tannerella forsythia* in saliva of CKD and PD patients.

Despite not presenting a high incidence in our results, *Filifactor alocis* was also identified. This microorganism has been reported in the literature as one of the microorganisms related to infections in kidney-transplanted individuals. Additionally, several recent studies[23–26] have found *Filifactor alocis* at increased frequency and in higher numbers in PD sites than in healthy sites, leading to the hypothesis that *Filifactor alocis* should be included as a diagnostic indicator of PD.[26]

Table 1. Percentage of microorganisms identified in the studied population

Microorganism (genus and species)	Saliva (n = 30)	Oral biofilm (n = 30)	Saliva (%)	Oral biofilm (%)
Actinomyces dentalis	13	10	43	33
Acinetobacter ursingi	18	15	60	50
Aggregatibacter actinomycetencomitans	18	15	60	50
Candida albicans	1	7	3	23
Clostridium inocuum	1	4	3	13
Corynebacterium argentoctens	19	21	63	70
Enterococus faecium	2	5	7	17
Filifactor alocis	5	3	17	10
Klebsiella pneumoniae	7	2	23	7
Kocuria varians	1	3	3	10
Neisseria flavescens	2	2	7	7
Pseudomonas pertucinogena	8	12	27	40
Rhotia dentocariosa	2	1	7	3
Staphylococus aureus	28	22	93	73
Streptococus salivaris	29	26	97	87
Tannerella forsythensis	13	8	43	27

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Subgingival plaque has also been related as polymicrobial and the main etiologic agent of PD. Different combinations of bacterial species have been reported as leading to periodontitis. Salivary and oral biofilm *Acinetobacter ursingi*, *Aggregatibacter actinomycetencomitans*, *Porfiromonas gingivalis* and *Tannerella denticola* have been shown to contribute to deepened pockets in some individuals.[13,24,27]

The periodontal decay process does not depend only on Gram-negative microorganisms, but also on Gram-positive anaerobic bacteria.[28] Cell wall components of Gram-positive bacteria have been reported in the literature to induce an anti-inflammatory response. Also, peptidoglycan, the main cell wall component in Gram-positive bacteria, can potentialise the release of anti-inflammatory cytokines.[29]

Many chronic inflammatory conditions are a consequence of imbalanced interactions between host and microorganisms. Although the renal impairment is the base pathology of CKD patients in this study, most individuals displayed some comorbities, such as cardiovascular diseases, also related to CP. PD has been associated to endothelial dysfunction and a lower bioavailability of nitric oxide, which, together with other factors, can affect systolic and diastolic blood pressure of patients with CKD. Overall, these conditions lead to a dysbiotic community, a variety of disease outcomes and dysregulated immune responses.[30]

PD is currently diagnosed using radiography and clinical measurements of probing pocket depth, bleeding at probing and clinical attachment level.[16] However, these traditional clinical measurements may display false positive results; laboratory tests are required to predict susceptible individuals who might be at risk of PD in the future, especially in the paediatric population. In the post-identification period, individuals will be submitted to clinical sessions of scaling and root planning of the teeth. In most cases, the young population does not display chronic periodontitis. Therefore, no medication is required together with the clinical procedure. The mechanical intervention is the only procedure performed to prevent initial inflammation conditions from evolving to a chronic periodontal process.

CONCLUSIONS

We have shown that MALDI Biotyper is an efficient methodology for microorganism identification, and it has largely been able to implicate specific microorganisms in disease pathogenesis such as periodontitis. Furthermore, a combined data analysis set may provide increased statistical power for integrating this identification methodology with other throughput techniques. The current methodology was efficient and rapid to identify microorganisms that have been well recognised and mentioned in the literature as risk potential for CKD patients, especially for those who will be referred to dialysis treatment or kidney transplantation. These oral microorganisms may enter the circulatory system and destroy the endothelial cells of the kidney. In dentistry, the rapid identification of microorganisms displays a relevant significance with regards to the clinical treatment proposed for patients that can be mechanical only or associated with pharmacological protocols.

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