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Identification by MALDI-TOF MS of bacterial microorganisms associated with dental caries

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Abstract—It is essential to understand bacterial succession during the development of dental caries and determine the microorganisms related to this disease. This work aimed to identify bacteria associated with cariogenic pieces and carry out an antibiogram assay. First, we isolated bacterial isolates using MALDI TOF-MS and species-specific PCR techniques. Next, we implemented an antibiogram test to evaluate antimicrobial susceptibility events in *Streptococcus oralis*, *Staphylococcus epidermidis*, and *Bacillus cereus*. Our results showed that the bacterial species frequently associated with cariogenic processes was *S. oralis*. The antibiogram analysis determined that the most effective antibiotic was streptomycin, with a minimal significant difference of 0.52. Furthermore, the phylogenetic reconstruction allowed confirming the identity of the Colombian isolates that were sequenced, corresponding to the species *S. oralis*. Finally, we describe the complex etiology of dental caries where not only one bacterial agent is related to the development of this pathology. It is a complex of species.

Keywords—Dental caries, mass spectrophotometry, antibiogram test, oral microbiology

I. INTRODUCTION

Dental caries is one of the common bacterial etiology diseases among humans, and it is still a public health problem because it affects the quality of life of individuals who suffer from it [1] It is the disease with the highest oral morbidity worldwide and regionally [2]. In the oral cavity, we find between 200 to 300 species of microorganisms involved in the formation of bacterial plaque, which follows a colonization sequence called autogenic succession [3]. Specifically, several described bacteria cause dental caries, such as genera: Lactobacillus, Neumococci, Neisseria, and Haemophilus. Meanwhile, the first to settle and the most numerous are Streptococcus [4]. The first colonizers of the tooth are the species, *Streptococcus sanguis*, *Streptococcus mitis*, *S. mutans* and *S. oralis*, followed immediately by *Actinomyces naeslundii.* These microorganisms are the first to form dental plaque [5]. Streptococcus genus subclassifies into several serotypes based on immunological, biological, and genetic properties. The serotypes present for the *S. mutans* bacterium are c, e, f, and k [2]. Streptococcus represents a broad group of microorganisms. Some are part of the normal microbiota without their pathogenicity having been demonstrated. Others, on the contrary, behave as saprophytes, pathogenic commensals producing various infections in humans [6]. Streptococcus has been identified in all deep caries lesions, including *S. oralis* and *S. mutans*, indicating a strong association of these species with advanced caries lesions [7]. In the clinical cases, it can result in these microorganisms being resistant or even multi-resistant to different antibiotics. The genus Streptococcus, mainly the species *S. oralis* and *S. mutans*, present high rates of resistance, and its importance lies in the fact that it is related to Infective endocarditis requires knowing the types of serotypes present, the degree of resistance to beta-lactams such as amoxicillin and the mechanism by which microorganisms create resistance [8]. We hypothesize that, i. The *S. mutans* bacterium is the principal etiological agent of dental caries in the study population, and other bacterial agents are associated with this pathology. ii. Bacterial isolates may present resistance or susceptibility to the antibiotics evaluated.

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II. METHODOLOGY

A. Collection of biological material

We collected 30 samples during the first semester of 2017 from cariogenic pieces of patients between the ages of 12 and 50, supplied by the "Odontoamigo" dental clinic in Bogotá-Colombia. Each patient declared through informed consent that she was aware of and authorized the use of the biological material for academic and research purposes. For the pre-isolation of the pathogen, we prepared 30 test tubes containing 5 ml of Oxoid® brand nutrient broth. Later, the entire dental piece was deposited inside each nutrient broth and incubated at 37°C for 24 hours [9]. Followed, by growth in the liquid medium, we inoculated *Mitis Salivarius* Bacitracin-MSB Oxoid® in the solid culture medium. The bacterial colonies grown in MSB medium were picked and purified in 5% Oxoid® blood agar solid medium and incubated at 37ºC for 24 hours [10].

B. *Bacterial identification using MALDI-TOF MS*

We carried out the identification procedure through MALDI-TOF MS following the standardized protocols within the Human Proteomics and Mycoses Research Unit of the Pontificia Universidad Javeriana in Bogotá. Bacterial isolates obtained from marine agar were reactivated in a culture medium at 37ºC for one day [11], [12].

We identified using either the transfer methodology with direct extended plate extraction or ethanol and formic acid extraction. For direct extraction, we picked a single colony with a wooden stick, and a fragile layer of the microorganism was applied to the MALDI steel plate, allowing it to dry at room temperature. Next, we added 1µl of 100% formic acid and covered it with 1µl of HCAA matrix (α-cyano-4-hydroxycinnamic acid-HCCA), drying it at room temperature [13]. Ethanol and formic acid extraction were performed only for isolates with a low score with the direct methodology, following the manufacturer's instructions (Bruker Daltonik GmbH, Bremen, Germany) [14].

To visualize the profiles, we use FlexControl software (version 3.0) and MALDI Biotyper RTC. For calibration and as a positive control, we used the standard bacterial test (BTS), which contains the protein profile of an *Escherichia coli* strain (DH5a) (Bruker Daltonik GmbH, Bremen, Germany). To read the results, we use the following identification criteria:

- A score ≥ 2.0, identification to species level
- A score between 1.7 and 1.9, identification at the gender level
- A score < 1.7, no identification.

Scores between 1.7 and 2.0 were considered valid for identification at the genus and species level.

C. *Antibiogram assay*

Mueller Hinton Oxoid® Agar was used to develop susceptibility tests for antimicrobials [11]. We established the antibiogram test for the bacteria *S. oralis*, *S. epidermidis*, and *B. cereus*. These were the most frequently identified bacteria by the MALDI-TOF MS method. The experiment design was randomized complete blocks, where we took 20 bacteria corresponding to each bacterial group. The experiment was replicated for 40 evaluations and conducted in two different time spaces. Four antibiotics, amoxicillin (10 µg), penicillin (10 µg), clindamycin (20 µg), and streptomycin (2 µg), OXOID® brand, were chosen for the determination of resistance/susceptibility events. We took the reading of the inhibition halos at four different appointments: 12, 24, 28, and 48 hours. We determined the effect of the variables, time, antibiotic, and diameter, making multiple comparisons to establish which antibiotic is more effective against the activity of the group of bacteria and the combination of experimental factors using the R software for data analysis. Considering all of the above, the equation or mathematical model that adjusts to the data is the following:

$$Diamijkl = Mici + Antibj + Timemk + Antib(Time)jk + \epsilon l,$$

Antib (Time)jk is the interaction between the antibiotic and the time that denotes a relationship in effect on the diameter variable. At the same time, the two factors are present in different combinations, and ϵl is the random error component that the model cannot effectively explain.

D. *Molecular detection and amplification of species-specific primers*

For molecular confirmation of the bacteria, *S. oralis*, *S. epidermidis*, and *B. cereus*, they were initially inoculated in nutrient broth, Oxoid®, and incubated at 25°C for 24 to 48 hours. Then, following the manufacturer's instructions, we performed DNA extraction from bacterial biomass with the Hig Pure PCR Template Preparation-ROCHE® Kit. Finally, we implemented a molecular detection method through the use of species-specific primers for the taxonomic identification of *S. oralis*, *S. epidermidis*, and *B. cereus* [13], [14]

The final concentration conditions for the PCR-Polymerase Chain Reaction were: 1X PCR Buffer, 3mM MgCl2, 0.2 mM dNTPs, 0.24 µM of each Primer, 1 U/µl of Taq polymerase, Bioline® and 2µl of template DNA (50-100 ng/µl) in a final volume of 25 µl. Following the PCR program: 95ºC 2 min. 35 cycles (95ºC 40s; 50ºC 1 min; 72ºC 1:00 min), final extension at 72ºC 10 min. We performed the reactions in a PTC100 MJ-Research Thermocycler, and the PCR products were visualized in 1.5% electrophoresis gel and revealed with Ethidium Bromide. Then, to confirm the results obtained with MALDI-TOF MS, the resulting amplification products were sequenced at Macrogen-Korea, using the Applied Biosystems 3700 (ABI-3700) system.

E. *Phylogenetic reconstruction*

For the phylogenetic analyses, we compared 39 16S rDNA gene sequences; 5 sequences of *S. oralis* generated in this work, 31 sequences from other species belonging to the genus Streptococcus and 3 external controls, housed in the NCBI Genebank (https://www.ncbi.nlm.nih.gov/), using the BLASTN subroutine (nucleotide-nucleotide) v. 2.2.27 [15]. We reconstructed a phylogenetic tree from sequences corresponding to the detected bacterial group. The sequences were analyzed with the Geneious program (Biomatters) and aligned with the ClustalW program [16].

We calculated the nucleotide substitution model that best fits the data with the Mega 7.0 program. Using Bayesian analysis, we also performed the phylogenetic reconstruction and implemented the General Time Reversible Substitution Model (GTR) with 30,000,000 million generations. We conducted the analyses using the MrBayes program [17].

III. RESULTS

A. *Bacterial identification using MALDI-TOF MS*

The MALDI-TOF MS analysis establishes that the most prevalent samples were *S. oralis* at 30%, *S. epidermidis* with a percentage of 23%, and finally, *B. cereus* at 17%. We identified

the rest of the bacteria that, in less frequency, were *Streptococcus salivarius* and *Enterococcus faecium*, with a 3% percentage for both pathogens.

Consistent categories were essential for determining taxonomic species, with complete isolation reliability. For the 30 samples, we established that 63% were up to species and 37% to genus. The dendrogram given by the MALDI TOF MS analysis found four main groups represented by the different genera identified. Those other isolates are far, and the similar ones are closer, which makes it possible to locate groupings characterized by a single species, an example case, the group of *S. oralis* (Figure 1).

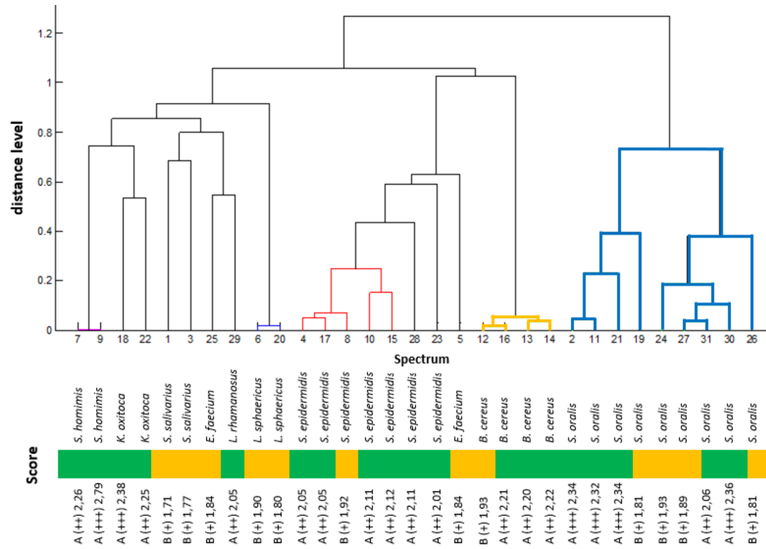

Figure 1. MALDI-TOF MS dendrogram of bacteria isolated from dental caries samples.

B. *Antimicrobial susceptibility test*

We performed this assay with the following bacterial groups; *S. oralis*, *S. epidermidis*, and *B. cereus*. The antibiotic streptomycin presented the most significant inhibition halo with a diameter of 22 mm, compared to amoxicillin and clindamycin, which was 20 mm. Penicillin was the antibiotic that showed a lower inhibition halo with a diameter of 19 mm. The concentrations of each of the antibiotics were as follows: amoxicillin (10 µg), penicillin (10 µg), clindamycin (20 µg), and streptomycin (2 µg) (Figure 2).

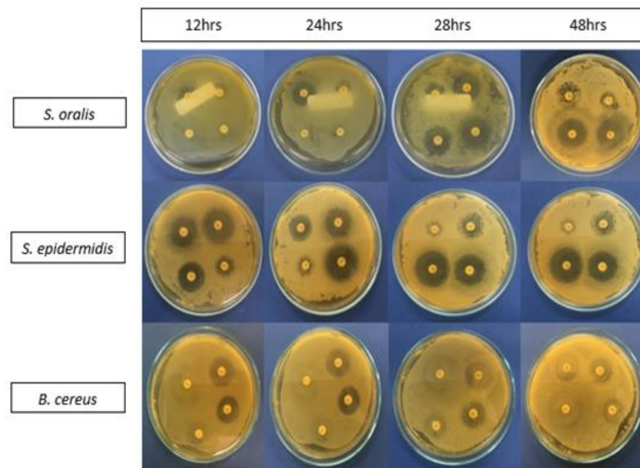

Figure 2. Growth on Mueller Hinton Agar, and response to antimicrobial susceptibility assay.

By applying Fisher's minimum significant difference test, we established which treatments are more effective or which differ statistically from each other. Although we found different groups that allowed us to classify the Fisher test if the letters we saw in the groups were the same, as with penicillin and clindamycin, they do not differ significantly or statistically. Still, when applying this test, we found that streptomycin is the most effective antibiotic. with a minimal significant difference of 0.527. However, amoxicillin presented a lower response to antimicrobial action in the present study.

C. *Molecular detection and amplification of species-specific genes*

The electrophoresis gel is shown below for molecular detection, we observed the following amplification-PCR products: 575Pb, 124Pb, and 1584Pb for *B. cereus*, *S. epidermidis*, and *S. oralis*, respectively (Figure 3). It allowed us to confirm the findings obtained by the MALDI-TOF MS method.

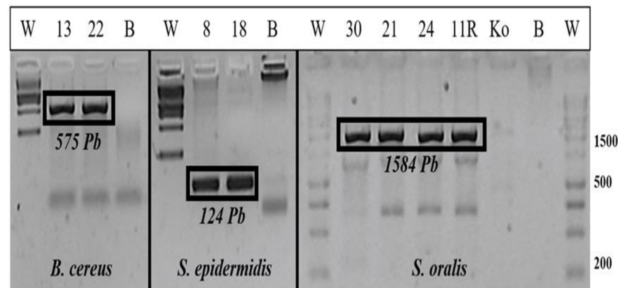

Figure 3. Agarose gel electrophoresis using species-specific primers for *B. cereus* (BCFomp1/BCRomp1), *S. epidermidis* (SE1/SE2) and *S. oralis* (sL1/ sL13). Ko: external control (*Klebsiella oxitoca*), B: control reaction, W: Promega® DNA marker-1 Kb.

D. *Phylogenetic reconstruction of the 16s rDNA gene for Streptococcus sequence*

We evidenced two clades, which presented supports with bootstrap indices of 100% and 99% for clades 1 and 2, respectively. We grouped the sequences of *S. oralis* and *Streptococcus mitis* in the first clade. We observed a high similarity, corroborated by the bootstrap index and sequence homology. Likewise, in this clade, we obtained the four sequences of *S. oralis* in the present study. On the other hand, in the second clade, we used only the sequences belonging to the species that as external controls (*Enterobacter* Sp., *Salmonella enterica*, *Haemophilus influenzae*) were found. The mentioned phylogenetic analysis confirmed the identity of the sequenced Colombian isolates, confirming that they belonged to the species *S. oralis*. We observed that the species *S. mitis* is the closest to *S. oralis*. The two species presented a high sequence homology supported by a bootstrap index of 100%. The presence of an unresolved polytomy or clade is due to the low variability for the sequences of *S. mitis* and *S. oralis.*

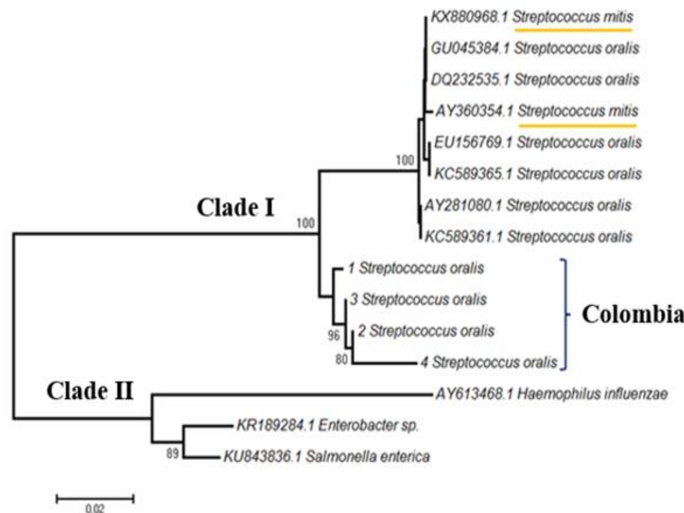

Figure 4. Phylogenetic reconstruction of the *16S rDNA* gene using Bayesian analysis to determine the taxonomic identity of the Colombian *S. oralis* strain.

IV. DISCUSSION

The present study identified the *S. oralis* bacterium associated with dental caries. Other investigations [15]. describe the presence of this bacterium also associated with caries and its high prevalence in the dental pathological process. We characterized the disease as a complex etiology. Several bacterial agents are associated with a species complex in the oral cavity. MALDI-TOFMS is a successful technique that we can routinely implement in clinical diagnosis, but without diminishing the importance of the biochemical tests used in identifying cocci for bacterial characterization as previously reported [16], [18]. They stated that these biochemical tests are conventional methods for determining Gram-positive cocci and that these tests require more than 72 hours, compared to MALDI-TOF MS, which is a tool that spends less time, on average, 2 hours.

On the other hand, some antimicrobials fail to control the development of the pathogen, which can lead to long-lasting resistance. Several recent studies have focused on the relationship between various pathologies and antibiotic resistance, including dental caries, periodontal diseases, and oral bacteria [17]. In this work, we examined which antibiotics are the most effective against the three groups of bacteria and whether some of these bacteria present antimicrobial sensitivity. In the results of our work, it was possible to establish significant differences between the different treatments reflected in the p-value response. It allows us to make a series of considerations regarding the effectiveness and resistance that suggests extensive research in the clinic and dental processes, the World Health Organization (WHO) states that the development of research on antimicrobial resistance is urgently required [18], [19].

The 16S rDNA phylogenetic reconstruction demonstrates a phylogenetic closeness for the species *S. oralis* and *S. Mitis* of almost 100% for their sequence homology [20], performs a phylogeny for the glucose-6-gene Phosphate dehydrogenase (gdh), in which the genetic closeness between *S. mitis* and *S. oralis* confirmed. It agrees with what we found in the present study.

We described the complex etiology of dental caries where not only a bacterial agent is related to the development of this pathology, but it is probably a complex of species.

V. CONCLUSIONS

Identification by MALDI-TOF MS allowed establishing of the presence of *S. oralis* and determining that other bacterial agents are present in the development of oral pathology.

We evidenced resistance in the three groups of bacteria exposed to the antibiotics under study, the efficiency of the antibiotic is multifactorial, and its success will depend on parameters or dosing compliance, time, and dose supplied.

Finally, we observed resistance on the part of *S. oralis*, which is one of the main bacteria that cause dental caries and other pathologies associated with the oral cavity, against the antibiotic amoxicillin, the most widely prescribed beta-lactam in the treatment of various pathologies, followed by antibiotics penicillin and clindamycin.

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