Microbiological and Molecular Characteristics of Microorganisms of Importance in Dental Caries and Periodontal Disease: Research Contributions in Colombia*

Características microbiológicas y moleculares de microorganismos de importancia en caries dental y enfermedad periodontal: aportes de investigación en Colombia

Características microbiológicas e moleculares de microorganismos importantes na cárie dentária e doença periodontal: contribuições de pesquisa na Colômbia

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*Integrative review

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ABSTRACT

Background*:** Dental caries is an infectious and multifactorial pathological process that destroys hard dental tissues, and *Streptococcus mutans* is found as the main microorganism associated with this disease. Periodontal disease is a multifactorial infectious disease, and, in its etiology, diverse microorganisms are involved, among which *Porphyromonas gingivalis* is of vital importance due to its virulence factors and role in the development of periodontal pathology. ***Purpose and Method: This integrative review article built on the basis of previously published research experiences of

the authors of this document and contrasted with other studies worldwide, aimed to describe the microbiological and molecular characteristics of *S. mutans* and *P. gingivalis*, microorganisms, respectively, of great importance in dental caries and periodontal disease. Important studies were found with the use of the following keywords or medical subject headings (MeSH) in Pubmed, Scopus and Google Scholar: *"S. mutans"* and *"P. gingivalis"* and "phenotypic identification" and "genotypic characterization". ***Results*:** From *S. mutans*, different biotypes were identified according to the methodology used, wide antimicrobial susceptibility and genotypic variability by AP-PCR that depends on the nesting of the primers on the DNA. Of *P. gingivalis*, high susceptibility to antimicrobials and broad genotypic discrimination were found by the AFLP technique. ***Conclusion***: the deep phenotypic and genotypic knowledge of *S. mutans* and *P. gingivalis*, bacteria of great importance in dental caries and periodontal disease, together with the classical epidemiological study could be very important in the application of better prevention and control strategies that impact the oral public health in these two diseases.

Keywords

dentistry; molecular characterization; oral microbiology; *P. gingivalis*; *Porphyromonas gingivalis*; *Streptococcus mutans*; *S. mutans*

RESUMEN

***Antecedentes*:** La caries dental es un proceso patológico infeccioso y multifactorial que destruye los tejidos duros dentales, y *Streptococcus mutans* es el principal microorganismo asociado a esta enfermedad. La enfermedad periodontal es una enfermedad infecciosa multifactorial y en su

etiología *Porphyromonas gingivalis* es muy importante por sus factores de virulencia y papel en la patología periodontal. ***Objetivos y metodología***: este artículo de revisión integradora construido con base en experiencias de investigación ya publicadas de los autores de este documento y contrastadas con otros estudios a nivel mundial, tuvo como objetivo describir las características microbiológicas y moleculares de *S. mutans* y *P. gingivalis*, microorganismos, respectivamente, de gran importancia en caries dental y enfermedad periodontal. Se encontraron importantes estudios con el uso de las siguientes palabras clave o Medical Subject Headings (MeSH) en PubMed, Scopus y Google Scholar: "S. mutans" y "P. gingivalis" y "identificación fenotípica" y "caracterización genotípica". ***Resultados*:** De *S. mutans*, se identificaron diferentes biotipos de acuerdo a la metodología empleada, amplia susceptibilidad antimicrobiana y variabilidad genotípica por AP-PCR que depende del anidamiento de los primers sobre el ADN. De *P. gingivalis* se encontró alta susceptibilidad a los antimicrobianos y amplia discriminación genotípica por la técnica AFLP. ***Conclusión*:** el profundo conocimiento fenotípico y genotípico de *S. mutans* y *P. gingivalis*, bacterias de gran importancia en caries dental y enfermedad periodontal, unido al estudio epidemiológico clásico podría ser muy importante en la aplicación de mejores estrategias de prevención y control que impacten la salud pública oral en estas dos enfermedades.

Palabras Clave

caracterización molecular; estudio fenotípico; microbiología oral, odontología; *P. gingivalis*; *Porphyromonas gingivalis*; *Streptococcus mutans*; *S. mutans*

RESUMO

***Antecedentes*:** A cárie dentária é um processo patológico infeccioso e multifatorial que destrói os tecidos duros dentais, e onde o *Streptococcus mutans* é o principal microrganismo associado a esta doença. A doença periodontal é uma doença infecciosa multifatorial e sua etiologia envolve vários microrganismos, entre os quais *Porphyromonas gingivalis* é muito importante por seus fatores de virulência e papel na patologia periodontal. ***Objetivos e metodologia*:** este artigo de revisão integrativa construído com base em experiências de pesquisa previamente publicadas dos autores deste documento e contrastado com outros estudos em todo o mundo, teve como objetivo descrever as características microbiológicas e moleculares de *S. mutans* e *P. gingivalis*, microrganismos, respectivamente. de grande importância na cárie dentária e doença periodontal. Foram encontrados estudos importantes com o uso das seguintes palavras-chave ou cabeçalhos de assuntos médicos (MeSH) no Pubmed, Scopus e Google Scholar: "*S. mutans* "e" *P. gingivalis* "e" identificação fenotípica "e" caracterização genotípica ". ***Resultados*:** A partir de *S. mutans*, foram identificados diferentes biótipos de acordo com a metodologia utilizada, ampla susceptibilidade antimicrobiana e variabilidade genotípica por AP-PCR que depende do aninhamento dos primers no DNA. De *P. gingivalis*, alta suscetibilidade a antimicrobianos e ampla discriminação genotípica foram encontrados pela técnica AFLP. ***Conclusão*:** o conhecimento fenotípico e genotípico profundo de *S. mutans* e *P. gingivalis*, bactérias de grande importância na cárie dentária e doença periodontal, juntamente com o estudo epidemiológico clássico podem ser muito importantes na aplicação de melhores estratégias de prevenção e controle que impactam a saúde pública bucal nessas duas doenças.

Palavras-Chave

caracterização molecular; microbiologia oral; estudo fenotípico; odontologia; *P. gingivalis*; *Porphyromonas gingivalis*; *Streptococcus mutans*; *S. mutans*

INTRODUCTION

The balance of oral microbial diversity is of vital importance in the development of the functions of the different oral ecosystems, and in an adequate immune response to the invasion of pathogens that lead to diseases (1-3). Ecologically speaking, dental caries is the consequence of an imbalance in the oral environment that leads to the predominance of a flora previously considered normal in the oral cavity (1,2). Dental caries is an infectious, multifactorial, and communicable disease process that destroys hard dental tissues (4). The main microorganisms associated with caries production are, in order of frequency, *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus gordonii* (*S. gordonii*), and *Lactobacillus* and *Actinomyces* species (1,2,5). On the other hand, periodontal disease is a multifactorial infectious disease, and its etiology involves various microorganisms, among which *Porphyromonas gingivalis* (*P. gingivalis*) is of vital importance due to its virulence factors and role in the development of periodontal pathology. Factors inherent to the host, smoking and environmental factors are important and determining in its evolution and severity (6-9).

Molecular biology techniques have greatly expanded knowledge on oral microbiology and the human oral cavity is currently considered to be the ecological niche with the greatest diversity in genera and species, known to date (10) and in the one that there are great possibilities of entering a huge bank of biodiversity, which is still in the process of knowledge and characterization (10).

In general terms, the recognition and characterization of oral microorganisms immersed in infectious processes requires the performance of four activities: the first, identification of microorganisms, begins with the culture of samples from the oral cavity, in culture media and conditions specific, followed by microscopic examination with Gram stain and standard biochemical tests performed on the isolated microorganism; the second activity involves the detection of phenotypic characteristics (enzymes, metabolism products, proteins, bacteriocins, etc.). The third activity involves the molecular epidemiological association or comparison of the isolates by genotypic characteristics, in order to control the infection or analyze the population (1,2). This activity has been enriched with molecular typing techniques for microbial DNA, which lead to the identification of clones (strains that have a high degree of genetic relationship) isolated from different sources and circumstances (1,2). Finally, in the fourth activity and thanks to the latest massive sequencing techniques, especially second-generation ones, and without depending on the limitations of microbiological cultures and standard identification procedures, a large number of microorganisms has been identified. with which the oral microbiota present in the different ecosystems has been built and structured in a better way.

MATERIALS AND METHODS

This integrative review article built on the basis of previously published research experiences of the authors of this document and contrasted with other studies worldwide, aimed to describe the microbiological and molecular characteristics of *S. mutans* and *P. gingivalis*, microorganisms, respectively, of great importance in dental caries and periodontal disease. To this end, important studies were searched and found with the use of the following keywords or medical subject

headings (MeSH) in PubMed, Scopus and Google Scholar: "*S. mutans*" and "*P. gingivalis*" and "phenotypic identification" and "genotypic characterization." Regarding *S. mutans*, the microbiological characteristics, isolation and identification, biotyping, antimicrobial susceptibility and genotyping by AP-PCR are presented in sequence, and in relation to *P. gingivalis*, the microbiological characteristics, isolation and identification, antimicrobial susceptibility and genotyping are described. by AFLP.

RESULTS AND DISCUSSION

S. mutans Microbiological Characteristics

In taxonomic terms, the members of the genus Streptococcus belong to the Streptococcaceae family, in which the genera Leuconostococcus, Aerococcus, Pediococcus, Peptococcus, Peptostreptococcus, Gemella, Ruminococcus, Coprococcus, and Sarcina are also found (11). Streptococci are Gram-positive cocci on Gram stain and negative behavior on the catalase test, which are grouped in pairs or chains. In the Streptococcus genus, the presence of organisms of the *Streptococcus viridans* (*S. viridans*) group stands out, as they are capable of producing 30-40% of cases of subacute bacterial endocarditis with an impact on highly significant bacteremia (11). Streptococcus viridans are made up of the following groups: *S. mutans* group (*S. mutans*, *S. sobrinus*, *S. cricetus*, and *S. rattus* species), *S. sanguinis* group (*S. sanguinis* species, *S. parasanguinis*, *S. gordonii*, and *S. crista*), *S. mitis* group (*S. mitis* and *S. oralis* species), and *S. salivarius* group (*S. salivarius* and *S. vestibularis* species). These groups are differentiated by the production of acetoin, hydrolysis of esculin, production of arginine dehydrolase and urease, and the production of acid from mannitol and sorbitol (11).

S. mutans and *S. sobrinus* species of the S. mutans group are the main microorganisms associated with the initiation and pathogenesis of dental caries. In general, in the scientific community, there is consensus in pointing out *S. mutans* as the most important microorganism in dental caries, and therefore the isolation, phenotypic and molecular characterization, prevention and control strategies are mainly directed towards it. *S. mutans*, a Gram-positive coccus and negative catalase test, is named for the mutant forms in which it occurs, coccobacillus (oval shape) in an acid medium and coco (round shape) in an alkaline medium (11). In blood agar cultures, the colonies of this microorganism are alpha or gamma hemolytic (11). It is facultative anaerobic; however, its optimal growth occurs in anaerobiosis for 48-72 hours at 37 ^{o}C. Concomitantly with the synthesis of dextran from sucrose (a dietary disaccharide made up of glucose and fructose), colonies of this microorganism emit an aqueous exudate on the surface of the culture medium with sucrose, often abundant enough to that forms a puddle around the colony (11).

In culture media with sucrose, this microorganism can produce extracellular polysaccharides, acquiring an opaque, rough and white appearance, not adherent to the culture medium and occasionally surrounded by glucan polymers with a moist appearance. *S. mutans* produces extracellular polysaccharides from sucrose by the action of two enzymes, glucosyltransferase (GTF) and fructosyltransferase (FTF). GTF is able to synthesize glucan from glucose, and FTF, fructan from fructose. The insoluble glucans formed by this microorganism allow it to adhere to the smooth surfaces of the teeth and form the matrix of the biofilm. Specific and nonspecific adherence of *S. mutans* and other organisms to insoluble glucan bound to the tooth and subsequent acid formation leads to demineralization of tooth enamel and the initiation of carious lesions. The

most common medium for the isolation of *S. mutans* is Mitis Salivarius Agar supplemented with bacitracin 0.2 U / ml and 20 % sucrose, which allows the selection of other streptococci (11). Currently, studies are not only directed towards the search for *S. mutans* in saliva and dental plaque, but also towards the quantification of this microorganism (11-15). Different studies have shown a correlation between the counts of this microorganism in the oral cavity with the prevalence and incidence of caries (11-15). However, other studies have not found a correlation between the amount of *S. mutans* and the incidence of caries (11-13). Currently, the finding of a high count of S. mutans is a risk factor to consider in the prevention and control of dental caries.

S. mutans Isolation and Identification

For this purpose, samples of dental plaque and spontaneous or stimulated saliva are serially diluted 10 in 10 in 0.05 M phosphate buffer saline. After mixing the samples with the 0.05 M phosphate buffer saline in vortex for 30 seconds, they are taken 100 ul of each dilution and planted on mitis salivarius bacitracin agar (MSB) in order to selectively isolate and count *S. mutans*. MSB agar (Difco; Detroit, MI) contains digested pancreatic casein, no proteose peptone. 3, proteose peptone, dextrose, 20 % sucrose, dipotassium phosphate, trypan blue, crystal blue, agar, Chapman's tellurite, and bacitracin 0.2 U / ml. Petri dishes with MSB agar are incubated in anaerobiosis (H_2: CO_2: N_2; 10:10:80) for 2 days at 37 ^{o}C. After growth, the colonies with morphology characteristic of *S. mutans* are counted, and the respective calculation is made.

The final count is expressed in colony forming units (CFU) per ml of saliva or gram of dental plaque (16). After bacterial count, colonies with characteristics of *S. mutans* are examined by Gram stain and subjected to biochemical tests. *S. mutans* has the following biochemical profile: positive

fermentation of raffinose, mannitol, melobiose, trehalose, inulin, cellobiose, arbutin and amygdalin; positive hydrolysis of starch, negative hydrolysis of esculin in the presence of bile, and positive hydrolysis of esculin in the absence of bile; negative urease; negative hydrolysis of arginine and hippurate; and resistance to 2 U of bacitracin (16, 17). In the study by Gamboa et al. performed in 206 children between the ages of 3 and 5 years (18), *S. mutans* was identified in 30 of the 79 children without dental caries (38 %) and in 56 of the 127 children with caries (44.1 %). In total, 121 *S. mutans* strains were identified in the 86 children: 43 strains in the 30 children without dental caries and 78 in the 56 children with dental caries.

S. mutans Biotyping

At present, *S. mutans* biotyping is being carried out according to their enzymatic profile, with the api-ZYM system (bioMérieux, France) (17,19). This typing system has made it possible to establish differences in intra and interindividual *S. mutans* strains (20). The first evaluation of the api-ZYM system as a biotyping system was carried out in 1999 by De la Higuera et al. (17). In this study, 8 different phenotypes were identified in 160 clinical isolates of *S. mutans* only on the basis of the action of three enzymes of the microorganisms. Subsequently, the system was used to evaluate the enzymatic action on the 19 substrates included in the test (19-22). The study by Lamby et al. (19) in children 3 to 6 years of age with incipient dental caries identified 17 phenotypes and the most frequent phenotype was 15 with 10 *S. mutans* strains. It is important to note that the greatest number of positive enzymatic reactions with substrates occur at an acidic pH (pH = 5.4), a situation that may be related to some of the specific characteristics of *S. mutans*, the ability to constantly produce acids at a pH low or acidic (acidity) and its property to tolerate and survive in acidic environments (acidophility) (11).

In the study by Gamboa et al. (21) *S. mutans* strains were grouped into 10 phenotypes, of which the most frequent phenotypes were 10 and 15, with 9 and 8 strains respectively, and it was possible to clearly establish inter- and intra-individual differences. In another study by Gamboa et al. (22) 119 *S. mutans* strains were grouped into 85 phenotypes: 33 phenotypes in strains isolated from children without cavities and 52 phenotypes in strains isolated from children with cavities. Two patients included in the study had 4 phenotypes in common (phenotypes 5, 6, 9 and 12). The most frequent phenotypes in children without caries were 6, 9, 5 and 3, and in patients with caries the most frequent phenotypes were 37,39, 6 and 9. In the study by Gamboa et al. (22) a large number of phenotypes represented by a single strain are highlighted. In another study (18), the grouping of 121 strains of *S. mutans* is reported in 38 biotypes: 24 in the caries group and 14 in the caries-free group. The study draws attention to the difference found in the most frequent biotypes in children with and without dental caries. In the dental caries group the most frequent biotypes were XV, XI and XII, respectively, with 26, 12 and 11 strains and in the group without caries the most frequent biotypes were XXVI, XX and XXXVI, respectively, with 14. 8 and 7 strains.

Very likely the presence of unique profiles found in children with dental caries compared to children without dental caries may suggest a greater virulence capacity of *S. mutans* strains. In this sense, in the study by Napigoma et al. (23) the relationship between clonal diversity and some virulence factors of *S. mutans* isolated from individuals with and without dental caries was evaluated; the results showed 44 different profiles, with a maximum of 8 profiles in an individual. They also found a large number of genotypes of *S. mutans* with greater ability to synthesize insoluble glucans, one of the important points of contribution in the genesis and development of

dental caries (23). On the other hand, in the study by Krzysciak et al. (24) the usefulness of biotyping in the recognition of pathogenic determinants in *S. mutans* is discussed. They demonstrated that the biotype I prephenate dehydrogenase enzyme (typified with the STREPTOtest system) present in most of the *S. mutans* strains of children with dental caries had greater activity than that presented by S. mutans strains isolated from children without dental caries. Due to all the above and due to the usefulness of biotyping in the discrimination of *S. mutans* in children with and without dental caries, it is very necessary to continue with research to demonstrate the implications and relationships with the pathogenicity of *S. mutans* and its role in the genesis and development of dental caries and the indirect implications in maintaining balance in oral microbial ecology.

Regarding bacteriocins in *S. mutans*, the metabolic capacity of this microorganism to synthesize glucans from sucrose and produce bacteriocins is of great importance in the process of initiation and development of dental caries (25). Bacteriocins are antibiotic peptides or proteins, with strong bactericidal properties, produced by a wide variety of bacterial species. Hamada and Ooshima (26) demonstrated that many *S. mutans* strains are producers of bacteriocins that possess a wide range of activity against Gram-positive microorganisms and closely related species (26-28). The survival and proliferation of a microorganism can occur if it manages to eliminate or displace a competent organism in its ecological niche, where competition is very strong due to the diversity of species (29). It has been suggested that the function of bacteriocins is to allow the establishment and permanence of the bacterium that produces it in the niche that it colonizes. Most *S. mutans* strains produce bacteriocins, which are specifically called mutacins and which are those that exert antagonistic or inhibitory action on other microorganisms in the oral environment (29-32). For

many years, the search for *S. mutans* strains with antagonistic capacity and their application in replacement therapy or bacteriological control to displace virulent native strains of S. mutans has continued (29).

Different investigations indicate that the antagonistic capacity of *S. mutans* is due to its production of bacteriocins, which could confer a great ability to displace native strains of the same species in the oral cavity (16,19). In the study by Gamboa et al. (21) the presence of 8 *S. mutans* strains producing mutacins with complete activity (100%) is reported on all *S. mutans* strains used as indicators. In the work of Balakrishnan and et al (30), the finding of 39 strains is reported, of the genera *Streptococcus*, *Enterococcus* and *Staphylococcus*, producing bacteriocins, which represent 14.3% of the 272 strains evaluated. In another study (27) on 319 *S. mutans* strains isolated from 8 patients with caries and from 8 patients without caries, the finding of 254 (79.62%) bacteriocin-producing *S. mutans* strains that have a diversity of action is reported. on the 12 *S. mutans* strains used as indicators.

The diversity in the production of bacteriocins by *S. mutans* strains evaluated in the different studies is probably due to the different conditions in which the tests are carried out and the inherent susceptibility of the indicator strains used. Regarding the strains used as indicators, in the work of Gamboa et al. (20) for the search of bacteriocins in *S. mutans* strains, 12 indicator strains were used, 6 of which were the most representative biotypes of Colombian clinical isolates and 6 reference strains, in order to have a close idea of the spectrum of the bacteriocins.

S. mutans Antimicrobial Susceptibility

In addition to dental caries and related pyogenic infections, *S. mutans* is also a very important infectious agent in endocarditis (33). The participation of this microorganism in oral and non-oral infections has generated interest in the knowledge of its susceptibility to antimicrobial agents. One of the most appropriate procedures to determine the antimicrobial susceptibility of *S. mutans* strains is the determination of the minimum inhibitory concentration (MIC) (34). The most widely used antimicrobial agents are: penicillin, amoxicillin, cefazolin, erythromycin, clindamycin, imipenem, and vancomycin. MIC is done using the agar dilution method. The protocol is briefly described below: 1. MIC is performed with the aforementioned antimicrobials, at concentrations between 0.003 and 32 ug / ml (13); 2. With a replicator, on Wilkins-Chalgren agar (Oxoid) or Mueller-Hinton Agar (Oxoid), standardized suspensions of 10,000 CFU / ml of the bacteria to be evaluated are applied; 3. After 48 hours of incubation at 35 ºC in an anaerobic atmosphere (H_2: CO_2: N_2 10:10:80), the MIC is determined according to the lowest concentration of the antimicrobial agent that inhibits the visible growth of the evaluated bacteria. (16.34).

In the study by Gamboa et al. (20) the high sensitivity of *S. mutans* strains to penicillin, amoxicillin, cefazolin, erythromycin, clindamycin, imipenem and vancomycin is reported; 50 and 90 % of *S. mutans* strains were inhibited by all antibiotics at concentrations lower than 0.12 and 0.5 ug / ml, respectively. The lowest average value was that of penicillin. The MIC of all the strains were very similar to those observed by other authors (17,34). In another study (18) the antimicrobial susceptibility of *S. mutans* strains to penicillin, amoxicillin, cefazolin, erythromycin, clindamycin, imipenem, vancomycin and teicoplanin was evaluated. All strains were highly sensitive to the antimicrobials tested; 50 % and 90 % of the strains were inhibited, respectively, by concentrations

lower than 0.12 and 1 ug / ml for all the antimicrobials evaluated. For penicillin, erythromycin, and imipenem, the lowest average MIC values were obtained, and all susceptibility patterns are in close agreement with those observed by other authors (14, 34-36). Although the treatment of dental caries is not done with antimicrobials, since they can lead to the nonspecific destruction of many microorganisms of the oral cavity, which leads to the alteration of the balance of the oral microbiota and a reinfection with *S. mutans* with the possibility of returning at pretreatment levels, it is important to make it clear that isolated Colombian *S. mutans* strains (18) remain sensitive and that in the event of systemic infections or endocarditis caused by *S. mutans*, a good number of antimicrobials are available that will have high inhibitory activity on this microorganism.

S. mutans Genotyping with Arbitrarily Primed PCR (AP-PCR)

One of the objectives of molecular typing is the identification of specific virulent clones within bacterial species and the study of the clones involved in an epidemiological event. Several molecular methods have allowed the study of oral streptococci (37,38), of which the analysis by restriction enzymes, ribotyping and typing with arbitrarily primed PCR (AP-PCR) have revealed considerable genetic heterogeneity between S. mutans strains (39,40). In recent years, AP-PCR has been widely applied in the genotypic characterization of different bacterial species, including oral pathogens (41,42). In recent years, a consensus has emerged on the use of the AP-PCR technique to detect different DNA profiles in clinical isolates of *S. mutans* (23,43-46). With AP-PCR, random DNA segments from the organism under study are amplified with simple primers of arbitrary sequence. The greatest advantage that AP-PCR offers for its development is that it is not necessary to know in advance the DNA sequence of the bacterial species to be amplified. It is very

well established that there are different genotypes of *S. mutans* in children with dental caries and that genotyping with AP-PCR is useful in demonstrating the diversity of genotypes (23,43-46).

In the study by Gamboa et al. (47) shows a great diversity in the genotypes of *S. mutans* with AP-PCR, 22 genotypes were found in the group of patients with dental caries and only 9 genotypes in the caries-free group. The 27 genotypes present made it possible to clearly establish inter and intra-individual differences. In the study by Napigoma et al. (23) there is also a greater number of genotypes in patients with caries in relation to those presented in patients without caries; established the relationship between clonal diversity and some virulence factors of *S. mutans* isolated from 8 patients with dental caries and 8 caries-free patients, and found 44 different genotypes, with a maximum number of 8 genotypes in an individual. They also found a large number of *S. mutans* genotypes with a high capacity to synthesize insoluble glucans (23). Emanuelsson et al. (48) analyzed the distribution and persistence of *S. mutans* in different sites of the teeth in the oral cavity and found a maximum of 7 different genotypes in the same individual. Pieralis et al. (49) evaluated *S. mutans* genotypic diversity in preschoolers with and without dental caries in Brazil and found 62 different genotypes in the 28 children included in the study. All these results clearly illustrate the complexity and heterogeneity of *S. mutans* colonization and its persistence in the oral cavity of individuals with dental caries. In order to increase the discriminatory power of the AP-PCR technique, different primers have been used (23,27,46,49). There are two strategies for using primers: one in which the primers OPA 02 and OPA 03 (23,27,46,49,50) are used and another in which the first OPA 05 is used (46,51-53) . In order to determine the discriminatory potential of the primers, Machado et al. (46) evaluated the genotypic diversity in *S. mutans* using primers OPA 02, OPA 03, OPA 05 and OPA 13; the genotypic

variability and heterogeneity found makes it clear that any of the 4 primers are useful for typing *S. mutans.*

In the study by Gamboa et al. (47) with the use of the first OPA 05, 27 profiles were obtained from the 69 *S. mutans* isolates. These latter results are in close agreement with those of Saarela et al. (51) who used the first OPA 05 on 81 *S. mutans* strains, obtaining 33 genotypes. Thus, genotypic variability in the population depends on the random nesting of the single primers on the DNA of the bacteria studied. Regarding reproducibility, when evaluating in duplicate, Truong et al. (54) and Gamboa et al. (47) reported that the results were consistent. Truong et al. (54) conclude that because AP-PCR is rapid and reproducible, it is of great value in distinguishing species of *S. mutans* and *S. sobrinus* and other strains of oral streptococci.

P. gingivalis **Microbiological Characteristics**

Periodontal disease is a multifactorial oral infectious disease that affects a large number of people in the world population (6.7) and in Colombia this disease, evaluated by loss of clinical attachment affects a large number of the population (55). Periodontitis corresponds to a form of periodontal disease. Due to its insidious and asymptomatic behavior, its diagnosis is almost always made in advanced age and even in terminal stages of the disease (6-9). This disease leads to progressive attachment and bone loss and is also characterized by the formation of pockets that can affect a variable number of teeth with different stages of progression (6-8). Factors inherent to the host, smoking and environmental factors are important and determining factors in its evolution and severity (6-9). Various microorganisms are involved in its etiology, among which *P. gingivalis* is of vital importance due to its virulence factors and role in the development of periodontal

pathology (6-9,56). *P. gingivalis* is a strictly anaerobic, Gram-negative bacillus, capable of developing brown-black colonies on anaerobic blood agar, and in the oral cavity it is found mainly immersed in the subgingival microflora (6-8). This microorganism meets the requirements to be considered a pathogen: it stimulates the host's immune response, evades defense mechanisms and destroys host tissues by secreting its own substances (6,57-59). Different studies have shown that the frequency and distribution of periodontal microorganisms and in particular of *P. gingivalis*, in the subgingival microflora, is variable according to geographical region, race, diet, level of development and living conditions, among others (6,7,56-60).

P. gingivalis Isolation and Identification

For the correct isolation and identification of strict anaerobic microorganisms, it is very important to pay great attention to the selection, collection and transport of clinical samples (61-65). Processing of clinical specimens, selection of culture media, inoculation and incubation methods, and inspection of positive cultures are laboratory procedures that should carry excellent quality control. The incorrect development of any of the previous stages mentioned can lead to erroneous results and consequently to misinformation in the anaerobic species involved in the infectious process (63-66).

Regarding the isolation and identification of *P. gingivalis* from crevicular fluid samples, for the collection of samples in general, 2 to 5 sites with pocket depth ≥ 4mm and clinical insertion level ≥ 2 mm are selected. the supragingival biofilm with a sterile gauze, the area is isolated with sterile cotton and the paper cones are introduced into the periodontal bag for 1 minute. After this time, the cones are removed and placed in eppendorf tubes with 900 uL of thioglycollate broth (BBL™

Fluid, Becton Dickinson and Company) supplemented with hemin (5 ug / ml) and menadione (1 ug / ml) (62-65), and are placed in jars with anaerobic sachets (Anaerogen, Oxoid) (no more than 4 hours) until reaching the laboratory. In the laboratory, the samples in the anaerobic jars are incubated for 4 hours at 37 ° C in order to achieve enrichment and consequently multiplication of the anaerobic microorganisms (64,65). After incubation, centrifugation (Eppendorf ® Centrifuge) is carried out at 4000 r.p.m for 10 minutes. 300 µl are removed from the centrifuged product and the remaining 600µl are stirred in Vortex (Maxi mix II Thermolyne ®) to achieve a homogeneous mixture of the sample. Subsequently, from the remaining thioglycollate broth, five serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) are made in thioglycollate broth in order to isolate *P. gingivalis*. Continuing with the process, 50 uL of the last three dilutions (10^{-3}, 10^{-4}, 10^{-5}) are massively reseeded in Wilkins-Chalgren anaerobic agar (Oxoid) supplemented with hemin and 1% menadione (v / v) and lamb blood at 5 % (v / v) and left in incubation at 37 ° C for 8 days in an anaerobic atmosphere.

Once the incubation time has elapsed, the types of colonies present (brown-black pigmented and non-pigmented) are observed in the culture medium and subjected to Gram staining (66-68). The presumptive colonies of *P. gingivalis* are seeded again to practice the aerotolerance test on Wilkins-Chalgren agar (Oxoid) supplemented with hemin and menadione at 1 % (v / v) and lamb blood at 5% (v / v) and they are left in incubation at 37 °C for at least 8 days. Finally, strict anaerobic colonies are selected, and their purity is confirmed by Gram staining. The identification of pure isolates of strict anaerobes is carried out among other commercial tests with the RapID^{TM} ANA II system (Remel).

In the work carried out in Colombia by Gamboa et al. (69) in 30 of the 87 patients with chronic periodontitis included in the study, *P. gingivalis* was identified, which represents a frequency of 34.5%. Of the 87 patients with a diagnosis of chronic periodontitis included in the study, 48 (55.2%) were female and 39 (44.8%) male. Regarding age, the 87 patients were distributed as follows: 10 (11.5%) were in an age range between 18-30 years, 42 (48.3%) were in an age range between 31-50 years and the remaining 35 (40.2%) were found in an age range between 51-70 years. The age values (mean ± standard deviation) found in the patients with *P. gingivalis* and without *P. gingivalis* were, respectively, 45.63 ± 12 and 46.73 ± 12 years, and no statistically significant differences were found (P> 0.834). The 30 isolates of *P. gingivalis* were distributed as follows: in the range of 18-30 years there were 15 isolates (50%), between 31-50 years there were 11 isolates (36.7%) and between 51-70 years 4 isolates were presented (13.3%). Regarding gender, there were no statistically significant differences between patients with or without the presence of *P. gingivalis* (P> 0.05).

Regarding the clinical parameters, the values (mean ± standard deviation) of pocket depth found in patients with and without *P. gingivalis* were, respectively, 5.62 ± 1.4 mm and 5.77 ± 1.6 mm, and not statistically significant differences were found (P> 0.6514). According to probing depth in the 30 patients with chronic periodontitis and the presence of *P. gingivalis*, 13 patients (43.3%) were found in a pocket depth range of 4-5mm, 12 (40%) in a range of pocket depth between 5-7 mm, and 5 patients (16.7%) in a pocket depth range greater than 7 mm. Regarding the level of clinical attachment, it could be determined that in patients in whom *P. gingivalis* was detected, a value (mean ± standard deviation) of attachment level loss of 5.77 ± 3.1 was found and in patients in those for which *P. gingivalis* was not identified was 5.56 ± 2.6 mm. These differences were not

statistically significant (P> 0.8687). Nor were statistically significant differences (P> 0.7511) found in terms of the severity of periodontitis between the group of patients with *P. gingivalis* (5.56 ± 1.33) compared to the group of patients without *P. gingivalis* (5.43 ± 1.18).

Regarding the percentage of sites with bleeding on probing, in the group of patients with chronic periodontitis and presence of *P. gingivalis* and in the group of patients with chronic periodontitis and absence of *P. gingivalis*, values of (mean ± standard deviation) of 97.3 ± 14 and 95.9 ± 12, with a P value> 0.672 by the chi-square test indicating non-statistically significant differences. In relation to the extent of periodontal destruction, localized chronic periodontitis occurred in 71.3% of patients. Of the 87 patients (48 women and 39 men) with chronic periodontitis, 40 had moderate chronic periodontitis and 47 had severe chronic periodontitis, representing 46% and 54% respectively. Of the 48 women included in the study, 26 had moderate chronic periodontitis and 22 had severe chronic periodontitis. On the contrary, in the 39 men, 14 had moderate chronic periodontitis and 25 had severe chronic periodontitis. In the 30 patients with chronic periodontitis in whom *P. gingivalis* was found, 14 had moderate chronic periodontitis and 16 had severe chronic periodontitis; and in the 57 patients with chronic periodontitis in whom *P. gingivalis* was not found, 26 presented moderate chronic periodontitis and 31 presented severe chronic periodontitis. According to the U-Mann Whitney statistical analysis, no statistically significant differences were found between these two groups (P> 0.5063). According to other studies, the frequency found by Gamboa et al. (69) was below those reported since 2007 in patients with chronic periodontitis for Iran (41.7%) (70), Spain (77.8%) (53), Japan (78.5%) (71) and Chile (83.8%) (70). In previous studies conducted in Colombia, frequencies of *P. gingivalis* in patients with chronic periodontitis of 60.7, 65.9, 67.1, 68.2 and 76.47% (6,7,57,72,73) have been reported.

Everything seems to indicate that the variations found in the frequency of these microorganisms are due to differences in the taking, transport and processing of samples and isolation by bacteriological culture, as well as the use of molecular techniques, sociocultural and demographic aspects and particular living conditions (6.57.70-75). In the study by Sanai et al. (75) makes it clear that the transport of the samples may have led to the loss of viability of *P. gingivalis, P. intermedia*, and *P. nigrescens*. Differences in the sensitivities (recovery and identification of the bacteria in culture) obtained by culture could also be due to situations that generate changes in the subgingival microflora, among which are: poor hygiene habits and attitudes, chronic underlying diseases, smoking, alcoholism and previous antimicrobial therapies (7,57,58,72,76). In the study by Urban et al. (77), the detection of periodontopathogenic bacteria is made by the traditional anaerobic culture method and by commercial PCR. The PCR test detected almost the same number of positive samples for *P. gingivalis* as those detected by culture (77), with only two discrepant results and reached a concordance of 94%. According to these last results, it can be deduced that commercial PCR can be recommended for its speed (2-3 hours), sensitivity and specificity in the routine of an oral microbiological diagnostic laboratory. However, the culture method despite being tedious, slow and requires expertise, allows to evaluate antimicrobial susceptibility and carry out other studies that require the bacteria in live form to be used in typing or in virulence and pathogenicity studies. When choosing any of these methodologies, laboratories must assess their needs, the impact they may have on the diagnosis, and the limitations of the two methodologies. From the results seen with the culture and the PCR, everything seems to indicate that it is possible that the joint use of the two methodologies is required due to the individual contributions of each technique (74,77,78).

P. gingivalis Antimicrobial Susceptibility

If antimicrobial therapy is necessary in patients with periodontal disease, to control *P. gingivalis* or another strict anaerobic periodontopathogen, its antibiotic resistance or susceptibility profile must be known (58,79,80). In this sense, various susceptibility patterns have been found in this microorganism (70,75,81-84). Through in vitro antimicrobial susceptibility tests, the profiles and changes in the behavior of microorganisms can be determined against the different therapeutic alternatives in periodontal treatment, in order to contribute to the development of appropriate antibiotic management policies and consequently delay the emergence of antimicrobial resistance (58,70,84,85). In the microbiological process, after the isolation and identification of *P. gingivalis*, the antibiotic susceptibility (MIC) for different antimicrobials is evaluated using the M.I.C. Evaluator (M.I.C.E., Oxoid) or the EtestR test (bioMérieux).

In the case of the M.I.C. Evaluator proceeds to make a fresh isolation of the *P. gingivalis* strains in Wilkins Chalgren anaerobic agar (Oxoid) supplemented with hemin and menadione at 1% (v / v) and lamb blood at 5% (v / v). All isolates are left in incubation at 37 ° C for 5 days in an anaerobic atmosphere, in order to obtain fresh colonies for the subsequent mounting of susceptibility tests according to the CLSI M100-S22 standards and the recommendations for the evaluation of susceptibility with the MIC system Evaluator. After the 5 days of incubation, the purity of the isolates is examined again and suspensions of each of the isolates are prepared in sterile isotonic saline and adjusted to the McFarland scale 1. Subsequently, by means of a swab, massive sowing of all suspensions is carried out in anaerobic Wilkins-Chalgren agar (Oxoid) supplemented with hemin and menadione at 1% (v / v) and lamb blood at 5% (v / v), the different

MIC system antibiotic strips Evaluator and test media are incubated at 37 ° C for 5 days in an anaerobic atmosphere. Finally, the MIC is read, according to the recommendations of the manufacturer of the system used and according to the cut-off points for the evaluated antibiotics, it is defined whether the strains are sensitive or resistant.

In the study carried out by Gamboa et al. (69) in the evaluation of antimicrobial susceptibility to metronidazole and tetracycline in 30 strains of *P. gingivalis*, a very diverse susceptibility was found. All 30 isolates (100%) were sensitive to metronidazole with MIC values ranging from 0.015 to 4 ug / ml; the highest frequency of sensitivity (n = 3 strains) occurred at MIC values of 0.015, 0.03 and 0.06 ug / ml. Regarding tetracycline, 27 isolates (90%) were sensitive with MIC values ranging from <0.015 to 4 ug / ml; the highest frequency of sensitivity (n = 3 strains) was presented at MIC values of 0.015, 0.03 and 0.06 ug / ml. The remaining 3 isolates (10%) were resistant to tetracycline with MIC values of 8 ug / ml. The M.I.C. Evaluator used in the study by Gamboa et al. (69) offers fast and reliable results in the determination of MICs since it does not require the development of dilutions of the antimicrobial and avoids the excessive consumption of culture media (86). Similarly, in the study by Andrés et al. (81) in 1998, 31 isolates of P. gingivalis were found to have 100% susceptibilities to metronidazole and tetracycline, with MIC for metronidazole of <0.125-2 ug / ml and for tetracycline of <0.125.0.5 ug / ml. Kulik et al. (84) among other antimicrobials, evaluated the antimicrobial susceptibility of 152 strains of *P. gingivalis* to metronidazole and tetracycline. All isolates were 100% susceptible to these antimicrobials, with MICs for metronidazole between <0.016-0.016 ug / ml and for tetracycline <0.016-2 ug / ml. Japoni et al. (70) reported 100% and 94% susceptibilities, respectively, to doxycycline and metronidazole in 50 P. gingivalis strains isolated in Iran from patients with chronic periodontitis.

Van Winkelhoff et al. (87) reported 100% susceptibilities for metronidazole and tetracycline in clinical isolates of *P. gingivalis* from the Netherlands and Spain. In contrast to the previous high susceptibilities, in the work of Ardila et al. (83) the resistance was reported in 21.56% (11/51) of *P. gingivalis* to metronidazole with MIC values between 0.08-16 ug / ml. With the exception of this high resistance to metronidazole, all other studies showed a high sensitivity to this antimicrobial. The high resistance to metronidazole and other antimicrobials could be due to the excessive use and misuse of antimicrobials that promote the development of bacteria with high resistance (83). In this sense, these situations that lead to the generation of bacterial resistance should be avoided every day (78,79). In the study by Gamboa et al. (69) the resistance presented by 10% to tetracycline is striking. The most common mechanism of resistance to this antimicrobial is through the synthesis of efflux pump proteins, which in Gram-negative microorganisms are encoded by *tet* genes (82). Sanai et al. (75) in 2002 determined the presence of the tetracycline resistance gene (tet-Q) in 3 of 5 isolates (60%) of *P. gingivalis* isolated from children and that seem to belong to the same clone of origin. It is important to consider that antimicrobial resistant bacteria residing in the oral cavity can be an important source of transmission of antimicrobial resistance genes to other pathogenic bacteria (75).

P. gingivalis Genotyping with Amplified Fragment Length Polymorphism (AFLP)

The genetic diversity of *P. gingivalis* in the population suffering from periodontal disease is high and it is possible to find an association between the clonal type of *P. gingivalis* and the type or severity of the disease (88-90). Multiple clonal types have recently been reported in an Indonesian population with varying degrees of periodontal disease (88). The typing of oral cavity bacteria

allows the identification of specific virulent clones within a species and the study of bacterial clones involved in an epidemiological event (88-90). Different methods or techniques such as biotyping, phagetyping, serotyping and methods based on DNA technology have been used for this purpose. Among the DNA methods are: 1. Restriction enzymatic studies (REA), 2. Random polymorphic analysis of DNA with amplification by polymerase chain reaction (RAPD / AP-PCR), 3. Enzyme multilocus electrophoresis, 4. PCR identification of repetitive extragenic palindromic sequences, 5. Ribotyping, and 6. Detection of the ribosomal intergenic region. In recent years, the Amplified Fragment Length Polymorphism (AFLP) technique has been used and a consensus is beginning to be found in its use in the study of the genetic variability of isolated strains (88). The fundamental advantages of this technique are: 1. A small amount of DNA is required to be digested by the enzymes in a short time, and 2. The possibility of optimizing the number of fragments using different nucleotides selected with the amplification primer. On the other hand, knowledge of the genotypes and genetic variability due to AFLP of P. gingivalis isolates allows to adjust prevention and control strategies.

The group of Gamboa et al. (article in preparation) conducted an investigation to typify 65 isolates of *P. gingivalis* by AFLP. The entire procedure (5 steps) followed for the study by AFLP is described below:

- Step 1. Digestion of bacterial DNA. All 65 P. gingivalis isolates and the reference strain P. gingivalis ATCC were processed to lyse the bacteria, obtain and quantify DNA. In order to digest the DNA, 300 ng of genomic DNA were taken, 5 ul of NB4 buffer and 0.5 ul of BSA were added, and then 1 unit of MSEI (New England) and 5 units of ECORI (New England)

were added. Subsequently, DEPC water (Invitrogen) was completed at 50 ul of final volume, incubated at 37 ºC for 1 hour, to finally leave at 65 ºC for 20 minutes. Digestion was verified by taking 4 ul of the digestion and run on 1% agarose gel%.

- Step 2. Digested DNA ligation. To this end, 20 ul of the DNA digestion product of all 66 bacteria were treated with restriction enzymes EcoRI and MseI, 1 ul of the T4 ligase enzyme (New England) was added together with 2.5 of 10X buffer. Then 0.5 ul of EcoRI and MseI adapters (Applied Biosystem) (previously heated at 95ºC for 56 minutes, cooled to room temperature and centrifuged) and 2.5 ul of ATP were added to make up a final volume of 25 ul. Finally, the mixed products were incubated at 16 ° C overnight and a 1/10 dilution was made as a working solution.

- Step 3. Pre-selective PCR amplification. To analyze the variability, an Applied Biosystem amplification kit (AFLP microbial fingerprinting) was used that contains specific primers for the EcoRI and MseI adapters. Initially 4.0 ul of the digestion and ligation product were added, with 0.5 ul of each preselection primer (EcoRI-0 and MseI-0) and 15.0 ul of amplification buffer for AFLPs (amplification core mix). The entire reaction was run in a BioRad thermal cycler under the following conditions: 72 ° C-2min, followed by 20 cycles of 94 ° C - 20 sec, 56 ° C-30 sec and 72 ° C-2min; finally cooled to 4 ºC and verified on 1.5% agarose gel.

- Step 4. Selective PCR amplification. With the use of the Applied Biosystem amplification kit (AFLP microbial fingerprinting), primers modified with additional nucleotides were used and, after standardization, the different combinations that indicated greater variability were used

(EcoRI-A / MseI-C, EcoRI-T / MseI- C). 1.5 ul of the pre-selective amplification product was taken and 0.5 ul of each first MseI (5M) were added together with 0.5 ul of first EcoRI (1M) fluorlabelled with FAM and JOE. Finally, 7.5 ul of amplification buffer for AFLPs (amplification core mix) were added and the reaction was run in a BioRad thermal cycler under the following conditions: 10 cycles of (94 ºC- 2min, 94 ºC- 20 sec, 66 ºC / 57 oC -30 sec, 72 ºC- 2 min), 20 cycles of 94 ºC- 2 min, 94 ºC-20 sec, 56 ° C-30 sec, 72 ºC-2 min, 1 cycle of 60 ºC-3 min, 1 cycle of 4 ºC-inf.

- Step 5. Sequencing of the selective amplification products. The products of the selective amplification were dissolved in DPEC water (1/10) and sent for sequencing. The information obtained was analyzed using the bioinformatics tool GeneMapper and SPSS.

Regarding results, the dendrogram analysis of the products generated by digestion with MSE1C-ECOR1A allowed clustering into two larger clusters A and B. In cluster A, 28 isolates were grouped and subclusters A1 and A2 were presented. In subcluster A1, there were two subgroups called A1a and A1b; Subgroups 1 (13 strains) and 2 (1 strain) were derived from subgroup A1a. In subcluster A2, two subgroups called A2a and A2b were found. Subgroups 1 and 2 were derived from subgroup A2a, subgroup 1 was affiliated with 6 strains and subgroup 2 was affiliated with only 1 strain. Two subgroups called 1 and 2 were derived from subgroup A2b, and in each of these 1 strain was affiliated. In cluster B 38 isolates were grouped and subclusters B1 and B2 were presented. In subcluster B1, two subgroups called B1a and B1b were found; from subgroup B1a, subgroups 1 (15 strains) and 2 (a single strain) were derived. In subcluster B2 there were two subgroups called B2a and B2b; Subgroups 1 (6 strains) and 2 (14 strains) were derived from subgroup B2a. On the other hand, the dendrogram analysis of the products generated by digestion

with MSE1C-ECOR1T also allowed the grouping of bacteria into two larger clusters A and B. In cluster A, 56 isolates were grouped and subclusters A1 and A2 were presented; Subgroups 1 and 2 were derived from subgroup A1a. 15 strains were affiliated with subgroup 1 and three strains affiliated with subgroup 2. Subgroups 1 (13 strains) and 2 (24 strains) were derived from subgroup A1b. Only one strain affiliated to subcluster A2. In cluster B 10 isolates were grouped and subclusters B1 and B2 were presented. In subcluster B1, two subgroups called B1a and B1b were found; from subgroup B1a, subgroups 1 and 2 were derived. Subgroup 1 was affiliated with 1 strain and subgroup 2 was also affiliated with a single strain. Subgroups 1 (3 strains) and 2 (1 strain) were derived from subgroup B1b. Only one strain affiliated to subcluster B2. Regarding the discriminatory capacity of the two digestion systems, MSE1C-ECOR1A and MSE1C-ECOR1T, both allowed the grouping of P. gingivalis strains into two large clusters, A and B. However, the MSE1C- ECOR1A allowed greater discrimination in both groups, since it distributed 28 strains in cluster A and 38 strains in cluster B. In contrast, the MSE1C-ECOR1T digestion system allowed greater discrimination of the strains within subcluster A1.

In conclusion, the work carried out by Gamboa et al. (article in preparation) allowed to typify the 65 isolates by AFLP, in two larger clusters (A and B) and in several smaller clusters, and obtain a high discrimination between the isolates, which consequently indicates the importance of the AFLP technique as a useful tool. in molecular epidemiology.

CONCLUSIONS

In this article, fundamental aspects of the microbiological and molecular characterization of *S. mutans* and *P. gingivalis*, microorganisms of great importance in dental caries and periodontal disease, were discussed, based on the research experiences of the authors of this review compared to other important findings from the world literature. Complete isolation strategies from clinical samples, dilutions, culture media, and biochemical identification protocols were described for both microorganisms. Regarding *S. mutans*, different biotypes of importance and associated with pathogenicity were found according to the methodology used, wide antimicrobial susceptibility of the isolates and genotypic variability by AP-PCR that depends on the random nesting of the simple primers on the DNA of the bacteria studied. The AP-PCR genotyping technique, because it is fast and reproducible, is also of great value in distinguishing species of *S. mutans* and *S. sobrinus* and other oral streptococci. From *P. gingivalis*, clinical isolates were highly susceptible to antimicrobials, including metronidazole and tetracycline, and high genotypic discrimination between isolates by the AFLP technique, which indicates the importance and usefulness of this in molecular epidemiology. The deep phenotypic and genotypic knowledge of *S. mutans* and *P. gingivalis*, together with the classical epidemiological study, could be very important in the application of better prevention and control strategies that impact oral public health in dental caries and periodontal disease.

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REFERENCES

1. Liébana J. Microbiología Oral. 2a ed. Madrid (España): McGraw-Hill Interamericana; 2002.
2. Acosta A, Agudelo CM, Barrientos S, Chávez M, Cuellar A, Durán C, Gamboa F, Gamboa LF, Gómez OL, Gómez M, Gómez SI. Fundamentos de ciencias básicas aplicadas a la odontología. 1a ed. Bogotá (Colombia): Pontificia Universidad Javeriana; 2006.
3. Dong L, Yin J, Zhao J, Ma S, Wang H, Wang M, Chen W, Wei W. Microbial similarity and preference for specific sites in healthy oral cavity and esophagus. Frontiers in Microbiology. 2018 jul; 9: 1603. https://doi.org/10.3389/fmicb.2018.01603
4. Rodríguez A, González OA. Fisiopatología de la caries dental. Bogotá (Colombia). Univ Odontol; 2000; 20(1): 21-27.
5. Crossner CG, Claesson R, Johansson T. Presence of mutans streptococci and various types of lactobacilli in interdental spaces related to development of proximal carious lesions. Scand J Dent Res. 1989 ; 97(4): 307-315. https://doi.org/10.1111/j.1600-0722.1989.tb01618.x
6. Mayorga-Fayad I, Lafaurie GI, Contreras A, Castillo DM, Barón A, Aya MdelR. Microflora subgingival en periodontitis crónica y agresiva en Bogotá, Colombia: un acercamiento epidemiológico [Subgingival microbiota in chronic and aggressive periodontitis in Bogotá, Colombia: an epidemiological approach]. *Biomédica*. 2007; 27(1): 21-33.
7. Herrera D, Contreras A, Gamonal J, Oteo A, Jaramillo A, Silva N, Sanz M, Botero JE, León R. Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. J Clin Periodontol. 2008 oct; 35(2): 106-113. https://doi.org/10.1111/j.1600-051X.2007.01170.x
8. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontology. 2000. 1999; 20(1): 168-238.

9. Joss A, Adler R, Lang NP. Bleeding on probing: A parameter for monitoring periodontal conditions in clinical practice. J Clin Periodontol. 1994 jul; 21(6): 402-408. https://doi.org/10.1111/j.1600-051X.1994.tb00737.x
10. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. The human oral microbiome. J Bacteriol. 2010 oct; 192(19): 5002-5017. https://doi.org/5017.10.1128/JB.00542-10
11. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986 dic; 50(4): 353-380.
12. Lang NP, Hotz PR, Gusberti FA, Joss A. Longitudinal clinical and microbiological study on the relationship between infection with *Streptococcus mutans* and the development of caries in human. Oral Microbiol Immunol. 1987 mar; 2(1): 39-47. https://doi.org/10.1111/j.1399-302X.1987.tb00268.x
13. Beighton D, Manji F, Baelum V, Fejerskov O, Johnson NW, Wilton JM. Associations between salivary levels of *Streptococcus mutans*, *Streptococcus sobrinus*, lactobacilli, and caries experience in Kenyan adolescents. J Dent Res. 1989; 68(8): 1242-1246. https://doi.org/10.1177/00220345890680080601
14. Macpherson LMD, MacFarland TW, Geddes DAM, Stephen KW. Assesment of the cariogenic potential of *Streptococcus mutans* strains and its relationship in vivo caries experience. Oral Microbiol Immunol. 1992 jun; 7(3): 142-147. https://doi.org/10.1111/j.1399-302X.1992.tb00527.x
15. Marsh PD, Featherstone A, Mckee AS, Hallsworth AS, Robinson C, Weatherell JA, Newman HN, Pitter AFV. A microbiological study of early caries of approximal surfaces in

schoolchildren. J Dent Res. 1989 jul; 68(7): 1151-1154. https://doi.org/10.1177/00220345890680070301

16. Gamboa F, Estupiñán M, Galindo A. Presence of *Streptococcus mutans* in saliva and its relationship with dental caries: antimicrobial susceptibility of the isolates. Univers Scient. 2004; 9: 23-27.
17. de La Higuera A, Gutiérrez J, Liébana J, García-Mendoza A, Castillo A. A new biotyping method for *Streptococcus mutans with the* api-ZYM system. Clin Microbiol Infect. 1999 feb; 5(2): 88-91. https://doi.org/10.1111/j.1469-0691.1999.tb00108.x
18. Gamboa F, García DA, Lamby CP, Sarralde AL. Biotipos y susceptibilidad antimicrobiana de *S. mutans* en niños con y sin caries dental. Rev Colomb Cienc Quím Farm. 2016 jul; 45(2): 139-147. http://dx.doi.org/10.15446/rcciquifa.v45n2.59944
19. Lamby C, Gamboa F, Chaves M, Valdivieso C. Fenotipificación bioquímica del *Streptococcus mutans* en cavidad bucal en población escolar de Cota, Cundinamarca. Trib Odontol. 2005; 2(3): 73-77.
20. Gamboa F, Chaves M, Estupiñan M, Galindo A. Bacteriocins in *S. mutans* strains isolated from children with and without dental caries: biotypes and sensitivity to antibiotics. Acta Odontol Latinoam. 2008; 21(1): 97-104.
21. Gamboa F, Chaves M, Estupiñan M, Galindo A. Detección de mutacinas en biotipos de cepas *S. mutans* aisladas de niños prescolares con y sin caries dental. Universitas Odontológica. 2006 jun-dic; 25(57): 7-13.
22. Gamboa F, Chaves M, Lamby C, Fajardo A, Arévalo A. Antagonistic action of indiginous *Streptococcus mutans* strains. Acta Odontol latinoam. 2009 sep; 22(2): 129-138.

23. Napigoma MH, Kamiya RU, Rosa RT, Rosa EV, Hofling JF, Mattos-Graner RO, Gonçalves RB. Genotypyc diversity and virulence traits of *Streptococccus mutnas* in caries-free and caries-active individuals. J Med Microbiol. 2004 jul; 53(7): 697-703. https://doi.org/10.1099/jmm.0.05512-0
24. Krzysciak W, Pluskwa KK, Piatkoski J, Krzyściak P, Jurczak A, Kościelniak D, Skalniak A. The usefulness of biotyping in the determination of selected phathogenicity determinants in *Streptococcus mutans.* BMC Microbiology. 2014 aug; 14: 194. https://doi.org/10.1186/1471-2180-14-194
25. Hamada S, Slade HD. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol Rev. 1980 jun; 44(2): 331-384.
26. Hamada S, Ooshima T. Production and properties of bacteriocins (Mutacins) from *Streptococcus mutans*. Arch Oral Biol. 1975; 20(1): 641-648. https://doi.org/10.1016/0003-9969(75)90131-4
27. Kamiya RU, Napimoga MH, Rosa RT, Höfling JF, Goncalves RB. Mutacin production in *Streptococcus mutans* genotypes isolated from caries-affected and caries-free individuals. Oral Microbiol Immunol. 2005 feb; 20(1): 20-24.
28. Rogers AH. Bacteriocinogeny and the properties of some bacteriocins of *Streptococcus mutans*. Arch Oral Biol. 1976; 21(2): 99-104. https://doi.org/10.1016/0003-9969(76)90079-0
29. Hillman JD, Brooks TA, Michalek SM, Harmon CC, Snoep JL, van Der Weijden CC. Construction and characterization of an effector strain of *Streptococcus* mutans for replacement therapy of dental caries. Infect Immun. 2000. 2004 mar; 68(2): 543-549. https://doi.org/10.1128/IAI.68.2.543-549.2000

30. Balakrishnan M, Simmonds RS, Tagg JR. Diverse activity spectra of bacteriocin-like inhibitory substances having activity against Mutans Streptococci. Caries Res. 2001; 35(1): 75-80.
31. Hillman JD; Dzuback AL, Andrews SW. Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. J Dent Res. 1987 jun; 66(6): 1092-1094.
32. Gamboa F, Acuña BH, Martínez MC. Control microbiológico sobre *Streptococcus mutans* y su acción acidogénica. Univers Scient. 2004 Jan-Jun; 9: 45-55.
33. Ullman RF, Miller SJ, Strampfer MJ, Cunha BA. *Streptococcus mutans* endocarditis: report of three cases and reviw of the literature. Hearth Lung. 1988 mar; 17(2): 209-212.
34. Liébana J, Castillo A, Peis J, Baca P, Piedrola G. Antimicrobial susceptibility of 1042 strains of *Streptococcus mutans* and *Streptococcus sobrinus:* comparison from 1985 to 1989. Oral Microbiol Immunol. 1991 jun; 6(3): 146-150. https://doi.org/10.1111/j.1399-302X.1991.tb00469.x
35. De La Higuera A, Castillo A, Gutiérrez J, García-Mendoza A, Liébana J. In-vitro susceptibility, tolerance and glycocalix production in *Streptococcus mutans.* J Antimicrob Chemother. 1997 Sep; 40(3): 359-63. https://doi.org/10.1093/jac/40.3.359
36. Pasquantonio G, Condò S, Cerroni L, Bikiqu L, Nicoletti M, Prenna M, Ripa S. Antibacterial activity and various antibiotics against oral streptococci isolated in the oral cavity. Int J Immunopathol Pharmacol. 2012 jul; 25(3): 805-809. https://doi.org/10.1177/039463201202500331

37. Coykendall AL, Gustafson KB. Taxonomy of *Streptococcus mutans*. In Hamada S, Michalek SM, Kiyono H, Menaker L, McGhee JR editors. Molecular microbiology and immunobiology of Streptococcus mutans. Amsterdam, The Netherlands: Elsevier Science. 1986: 61-70.
38. Schleifer KH, Kilpper-Bälz R. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci, and lactococci: a review. System Appl Microbiol. 1987 Nov; 10(1): 1-19. https://doi.org/10.1016/S0723-2020(87)80002-4
39. Kulkarni GV, Chan KH, Sandham HJ. An investigation into the use of restriction endonuclease analysis for the study of transmission of mutans streptococci. J Den Res. 1991 Jul; 68(7): 1155-1161. https://doi.org/10.1177/00220345890680070401
40. Saarela M, Alaluusua S, Takei T, Asikainen S. Genetic diversity within isolates of mutans streptococci recognized by and rRNA gene probe. J Clin Microbiol. 1993 Mar; 31(3): 584-7.
41. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990 Nov; 18(22): 6531-6535. https://doi.org/10.1093/nar/18.22.6531
42. Chen C, Slots J. Arbitrarily primed polymerase reaction analysis of periodontal pathogens: discriminative primers and genetic diversity. Clin Infect Dis. 1995 Jun; 20(2): S301-S303.
43. Grönroos L, Alaluusua S. Site-specific oral colonization of mutans streptococci detected by arbitrarily primed PCR-Fingerprinting. Caries Res. 2000 Nov-Dec; 34(6): 474-480. https://doi.org/10.1159/000016626
44. Li Y, Caufield PW, Emanuelsson IR, Thornqyist E. Differentiation of *Streptococcus mutans* and *Streptococcus sobrinus* via genotypic and phenotypic profiles from three different populations. Oral Microbiol Immunol. 2001 Feb; 16(1): 16-23. https://doi.org/10.1034/j.1399-302x.2001.160103.x

45. Cogulu D, Sabah E, Uzel A, Ozkinay F. Genotyping of *Streptococcus mutans* by using arbitrarily primed polymerase chain reaction in children with Down Syndrome. Arch Oral Biol. 2006 Mar; 51(3): 177-182. https://doi.org/10.1016/j.archoralbio.2005.07.008
46. Machado CP, Sousa MK, Arthur RA, Mattos-Graner RO, Del Bel Cury AA, Cury JA. Evaluation of genotypic diversity of *Streptococcus mutans* using distinct arbitrary primers. J Appl Oral Sci. 2008 Dec; 16(6): 403-407. https://doi.org/10.1590/S1678-77572008000600009
47. Gamboa F, Chaves M, Valdivieso C. Genotypic profiles by AP-PCR of *Streptococcus mutans* in caries-active and caries-free preschoolers. Acta Odontol Latinoam. 2010; 23(2): 143-149.
48. Emanuelsson IM, Carslsson P, Hamberg K, Brathall D. Tracing genotypes of mutans streptococci on tooth sites by random amplified polymorphic DNA (RAPD) analysis. Oral Microbiol Immunol. 2003 Feb; 18(1): 24-29. https://doi.org/10.1034/j.1399-302X.2002.180104.x
49. Pieralisi FJ, Rodrigues MR, Segura VG, Maciel SM, Ferreira FB, García JE, Poli-Frederico RC. Genotypic diversity of *Streptococcus mutans* in caries-free and caries-active preschool children. Intern J Dent. 2010 Nov; 2010. https://doi.org/10.1155/2010/824976
50. Nascimento MM, Höflin JF, Gonçalves RB. *Streptococcus mutans* genotypes isolated from root and coronal caries. Caries Res. 2004 Sep-Oct; 38(5): 454-463. https://doi.org/10.1159/000079627
51. Saarela M, Hannula J, Mattö J, Asikainen S, Alaluusua S. Typing of mutans streptococci by arbitrarily primer polymerase chain reaction. Arch Oral Biol. 1996 Aug-Sep; 41(8-9): 821-826. https://doi.org/10.1016/S0003-9969(96)00049-0

52. Hames HH, Ucar FB, Ersin NK, Uzel A, Alpöz AR. Colonization and vertical transmission of *Streptococcus mutans* in turkish children. Microbiol Res. 2008 Mar; 163(2): 168-172. https://doi.org/10.1016/j.micres.2006.03.016

53. Ersin NK, Kocabas EH, Alpöz AR, Uzel A. Transmission of *Streptococcus mutans* in a group of Turkish families. Oral Microbiol Immunol. 2004 Dec; 19(6): 408-410. doi; https://doi.org/10.1111/j.1399-302x.2004.00163.x

54. Truong TL, Menard C, Mouton C, Trahan L. Identification of mutans and other oral streptococci by random amplified polymorphic DNA analysis. J Med Microbiol. 2000 Jan; 49(1): 63-71. https://doi.org/10.1099/0022-1317-49-1-63

55. Ministerio de Salud y Protección Social. IV ESTUDIO NACIONAL DE SALUD BUCAL 2013-2014. ENSAB IV, Situación en Salud Bucal. Bogotá (Colombia): MinSalud; 2014.

56. Kamma JJ, Nakou M, Manti FA. Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters. J Periodontol. 1994 nov; 65(11): 1073-1078. https://doi.org/10.1902/jop.1994.65.11.1073

57. Lafaurie GI, Contreras A, Barón A, Botero J, Mayorga-Fayad I, Jaramillo A, Giraldo A, González F, Mantilla S, Botero A, Archila LH, Díaz A, Chacón T, Castillo DM, Betancourt M, Aya MR, Arce Roger. Demographic, Clinical, and Microbial Aspects of Chronic and Aggressive Periodontitis in Colombia: A Multicenter Study. J Periodontol. 2007 Apr; 78(4): 629-639. https://doi.org/10.1902/jop.2007.060187

58. Tanner AC, Kent J. R, Kanasi E, Lu SC, Paster BJ, Sonis ST, Murray LA, Van Dyke TE. Clinical characteristics and microbiota of progressing slight chronic periodontitis in adults. J Clin Periodontol. 2007 Sep; 34(11): 917-930. https://doi.org/10.1111/j.1600-051X.2007.01126.x

59. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent JR. Microbial complexes in subgingival plaque. J Clin Periodontol. 2005 Dec; 25(2): 134-144. https://doi.org/10.1111/j.1600-051X.1998.tb02419.x
60. Cao CF, Aeppli DM, Liljemark WF, Bloomquist CG, Bandt CL, Wolff LF. Comparison of plaque microflora between Chinese and Caucasian population groups. J Clin Periodontol. 1990 Feb; 17(2): 115-118. https://doi.org/10.1111/j.1600-051X.1990.tb01072.x
61. Axelsson P, Albandar JM, Rams TE. Prevention and control of periodontal diseases in developing and industrialized nations. Periodontol 2000. 2002 Apr; 29(1): 235-246. https://doi.org/10.1034/j.1600-0757.2002.290112.x
62. Brewer JH. A clear liquid medium for the "aerobic" cultivation of anaerobes. J Bacteriol. 1940 Aug; 115(8): 10-15.
63. Vera HD. Comparative study of materials suitables for the cultivation of clostridia. J Bacteriol. 1944; 47(1): 59-65.
64. Gibbons RJ, MacDonald JB. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J Bacteriol. 1960 Aug; 80(2): 164-170.
65. Wilkins TD, Chalgren SL, Jimenez-Ulate F, Drake CR, Johnson JL. Inhibition of *Bacteroides fragilis* on blood agar plates and reversal of inhibition by added hemin. J Clin Microbiol. 1976 Mar; 3(3): 359-363.
66. Slots J, Reynolds HS. Long-wave UV light fluorescence for identification of black-pigmented *Bacteroides* spp. J Clin Microbiol. 1982 Aug; 16(6): 1148-1151.

67. Shah HN, Collins DM. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Evol Micr. 1990 apr; 40(2): 205-208.

68. Condorelli F, Scalia G, Calì G, Rosetti B, Nicoletti G, Lo Bue AM. Isolation of *Porphyromonas gingivalis* and detection of immunoglobulin A specific to fimbrial antigen in gingival crevicular fluid. J Clin Microbiol. 1998 Aug; 36(8): 2322-2325.

69. Gamboa F, Acosta A, García DA, Velosa J, Araya N, Ledergerber R. Ocurrence of *Porphyromonas gingivalis* and tetracycline in patients with chronic periodontitis. Acta Odontol Latinoam. 2014; 27 (3): 137-144.

70. Japoni A, Vasin A, Noushadi S, Kiany F, Japoni S, Alborzi A. Antibacterial susceptibility patterns of *Porphyromonas gingivalis* isolated from chronic Periodontitis patients. Med Oral Patol Oral Cir Bucal. 2011 oct; 16(7): e1031-1015. https://doi.org/doi:10.4317/medoral.17174

71. Amano A, Nakagawa I, Kataoka K, Morisaki I, Hamada S. Distribution of *Porphyromons gingivalis* Strains with *fim A* Genotypes in Periodontitis Patients. J Clin Microbiol. 1999 feb; 37(5): 1425-30.

72. Botero JE, Contreras A, Lafaurie G, Jaramillo A, Betancourt M, Arce RM. Ocurrence of Periodontopathic and Superinfecting Bacteria in Chronic and Aggressive Periodontitis Subjects in a Colombian Population. J Periodontol. 2007 apr; 78(4): 696-704. https://doi.org/doi:10.4317/medoral.1717410.1902/jop.2007.060129

73. Ardila CM, López MA, Guzmán IC. Positive correlations between presence of Gram-negative enteric rods and *Porphyromonas gingivalis* in subgingival plaque. Acta Odontol Latinoam. 2011; 24(1): 15-19.

74. Colombo AP, Teles RP, Torres MC, Souto R, Rosalém JW, Mendes MC, Uzeda M. Subgingival microbiota of Brazilian Subjects with Untreated Chronic Periodontitis. J Periodontol. 2002; 73(4): 360-69.

75. Sanai Y, Persson GR, Starr JR, Luis HS, Bernardo M, Leitao J, Roberts MC. Presence and antibiotic resistance of *Porphyromonas gingivalis, Prevotella intermedia,* and *Prevotella nigrescens* in children. J Clin Periodontol. 2002 nov; 29(10): 929-934.

76. American Academy of Periodontology. International Workshop for a Classification of Periodontal Diseases and Conditions. Ann Periodontol. 1999 oct-nov; 4(1): 1-112.

77. Urbán E, Terhes G, Radnai M, Gorzó I, Nagy E. Detection of periodontopathogenic bacteria in pregnant women by traditional anaerobic culture method and by a commercial molecular genetic method. Anaerobe. 2010 Jun; 16(3): 283-288. https://doi.org/doi:10.1016/j.anaerobe.2010.02.005

78. Eick S, Pfister W. Comparison of microbial cultivation and a commercial PCR based method for detection of periodontopathogenic species in subgingival plaque samples. J Clin Periodontol. 2002 jul; 29(7): 638-644.

79. Sweeney LC, Dave J, Chambers PA, Heritage J. Antibiotic resistance in general dental practice-a cause for concern? J Antimicrob Chemother. 2004 apr; 53(4): 567-576. https://doi.org/10.1093/jac/dkh137

80. Herrera D, Alonso B, León R, Roldan S, Sanz M. Antimicrobial therapy in periodontitis: the use of sytemic antimicrobials against the subgingival biofilm. J Clin Periodontol. 2008; 35: 45-66.

81. Andrés MT, Chung WO, Roberts MC, Fierro JF. Antimicrobial susceptibilities of *Porphyromonas gingivalis, Prevotella intermedia,* and *Prevotella nigrescens* spp. Isolated in Spain. Antimicrob Agents and Chemother. 1998 Aug; 42(11): 3022-3023.
82. Larsen T. Susceptibility of *Porphyromonas gingivalis* in biofilms to amoxicillin, doxycycline and metronidazole. Oral Microbiol Immunol. 2002 Sep; 17(5): 267-271. doi: https://doi.org/10.1034/j.1399-302X.2002.170501.x
83. Ardila CM, López MA, Guzmán IC. High resistance against clindamycin, metronidazole and amoxicillin in *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* isolates of periodontal disease. Med Oral Patol Oral Cir Bucal. 2010 nov; 15(6): e947-951. https://doi.org/10.4317/medoral.15.e947
84. Kulik EM, Lenkeit K, Chenaux S, Meyer J. Antimicrobial susceptibility of periodontopathogenic bacteria. J Antimicrob Chemother. 2008 May; 61(5): 1087-1091. https://doi.org/10.1093/jac/dkn079
85. Bascones A, Caballero A. *Actinobacillus actinomycetemcomitans* y *Porphyromonas gingivalis* como principales patógenos periodontales. Avances en Periodoncia e Implantología. 2000 Sep; 12(2): 69-75.
86. Rennie RP, Turnbull L, Brosnikoff C, Cloke J. First comprehensive evaluation of the MIC Evaluator device compared to Etest and CLSI reference dilution methods for antimicrobial susceptibility testing of clinical strains of anaerobes and other fastidious bacterial species. J Clin Microbiol. 2012 Jan; 50(4): 1153-57. https://doi.org/10.1128/JCM.05397-11
87. Van Winkelhoff AJ, Herrera D, Oteo A, Sanz M. Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in the Netherlands and Spain. J Clin Periodontol. 2005 Mar; 32(8): 893-898. https://doi.org/10.1111/j.1600-051X.2005.00782.x

88. Rijnsburger MC, Delwel S, Boutaga K, van Steenbergen TJM, Savelkoul PHM, van Winkelhoff AJ. Comparison of restriction enzyme analysis and amplified fragment length polymorphism typing of Porphyromonas gingivalis isolated from spouses. Oral Microbiol Immunol. 2007 Sep; 22(6): 369-373. https://doi.org/10.1111/j.1399-302X.2007.00372.x
89. Enersen M, Olson I, van Winkelhoff AJ, Caugant DA. Multilocus sequence typing of *Porphyromonas gingivalis* strains from different geographic origins. J Clin Microbiol. 2006 Jan; 44(1): 35-41. https://doi.org/10.1128/JCM.44.1.35–41.2006
90. Tran SD, Rudney JD. Multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. J Clin Microbiol. 1996 nov; 34(11): 2674-2678.