ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF ADHESION AND DEVELOPMENT CAPACITY OF BIOFILMS IN MICROBIAL STRAINS INSULATED IN PATIENTS FUNCTIONALLY REHABILITATED WITH FIXED PROSTHETIC RESTORATIONS

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Abstract: *Microbial aggressions resulting in acute or chronic infections have generated in time major problems for human species. Bacterial strains are present in the environment but also on the surface and within the human body. Microbial development at the passive surfaces that come in contact with the human body on medium and long term increases the risk of developing serious infections over time. The oral cavity, one of the body's non-sterile cavities, is a good developmental environment for many bacteria, about 600-800 species. Therefore, careful consideration should be given to microbial biofilms relevant to human health present in the bacterial plaque but also on the surface of various materials used in dentistry.*

INTRODUCTION

Since ancient times, the human species has been the victim of very varied microbial aggressions, represented in most cases by acute epidemic infectious diseases, most often caused by bacterial species living together in the human body as well as in the environment, causing different chronic infections over time, some of which are of great gravity. Thus, the occurrence of these acute or chronic infections is caused by microbial biofilms, which develop mainly on inert surfaces, on dead tissues, on medical devices, including the surfaces of dental restorations, orthodontic devices (fixed, mobile and / or mobile) and / or coronary restorations, etc., which we find in the medium and long term in the oral cavity.(1,2,3,4)

PURPOSE

The objective of our research is to obtain scientific information on microbial biofilms relevant to human health in order to identify and improve the prophylactic and therapeutic methods for their aggressions (infections). This research focuses on the study of microbial plaque attached to dental surfaces, but also on various materials used in dental prosthodontics.

The present study comprises of several stages, namely:

1. Determining a base of bacterial and fungal strains in dental plaque (supra- and subgingival) from patients functionally rehabilitated by fixed prosthetic restorations.
2. Isolation and identification of species of microorganisms present in the microbial dental plaque. Some of these will then be used to conduct the study in the next subchapter.
3. Determination of adhesion and formation capacity of microbial biofilms.

MATERIALS AND METHODS

1. Determining a base of bacterial and fungal strains in dental plaque (supra- and subgingival) from patients functionally rehabilitated by fixed prosthetic restorations.

This first stage of the study consisted in the collection of supra- and subgingival dental microbial plaque samples from a number of 63 patients rehabilitated with fixed prosthetic restorations. The study was conducted between January and June 2017. A total of 312 patients were examined in a preliminary phase, but in order to collect the dental microbial plaque, we selected only 63 patients aged between 25-68 years, 41 female (65.07 %) and 22 males (34.92%), rehabilitated with fixed prosthetic restorations. These fixed prosthetic restorations were metal-ceramic, metal-polymeric (metal-acrylic and metal-composite) and metallic, with ages between 2 and 5 years. In the visual inspection of these patients as well as at the interview, it was found that the metal alloys used were Ni-Cr and / or Co-Cr inoxidable alloys. We have avoided as far as possible high noble and noble alloys, as the antimicrobial potential of Au-Ag-Pd compounds is known from the literature. After taking, samples were maintained and transported sterilely into a RTM (Reduced Transport Medium, thioglycollate broth) to the microbiology lab with which we collaborated, and where these samples were processed.

2. Isolation and identification of species of microorganisms present in the microbial dental plaque. Some of these will then be used to conduct the study in the next subchapter.

Samples of microbial dental plaque were placed in non-selective rich medium (blood agar) and incubated at 37 ° C.

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The culture characteristics and cell morphology were determined by analysis of dimensions, synthesized pigments, form, colony development pattern, as well as determination of Gram character, aspect which allowed classification of isolated strains according to tinctorial affinity and morphological type. Depending on morphothinctorial characteristics and the results of oxidase and catalysed rapid enzymatic assays, the types of API identification strips were selected for a certan indentification of the strains.

Microscopic examination of Gram stained smears

The principle of Gram staining: by treating with a basic purple dye of the pararosaniline series (e.g., methyl violet, purple crystal) and iodine, the bacteria behave differently under the action of a bleach agent (eg alcohol, acetone):(5)

- In some bacteria, iodine exhibits mordant effect, fixation of the dye by protoplast structures and prevents discoloration, so the bacteria remain colored in purple. These bacteria have been called Gram positive.
- Other bacteria discolorise under the action of the alcohol / acetone bleach mixture by permeability of the cell wall and, to be observed, must be re-colored with a different dye (eg fucin, safranin, and neutral red); these bacteria have been called Gram negative.

Different behavior of bacteria in Gram staining is related to the existence of differences in the cell wall structure: the three-dimensional peptidoglycan tight mesh network further reduced by the desiccant action of the decolorizing solution may explain the retention of the violet-iodine colored complex by Gram positive bacteria; the two-dimensional double-sided peptidoglycan looser mesh network produced by dissolving the wall-associated lipid components under the action of the decolorizing solution may explain the discoloration of Gram negative bacteria.(5)

Enhancement of enzymes involved in respiratory metabolism

Highlighting cytocromic respiratory enzymes - the oxidase test: is based on a biochemical reaction produced by oxidase-positive species to produce enzymes capable of transferring electrons from an electron donor (usually NADH) to an electron acceptor, usually atmospheric oxygen, during aerobic respiration, aerobic microbial species, as well as aerobic optional anaerobes.(5,6) To perform this test, chromatographic type filter paper strips are used, impregnated in an aqueous solution of N, N, N'N'-tetramethyl 1-4, phenylenediammonium dichloride, applied with a tweezers on a glass slide and lightly moistened with a few drops of distilled water. The test samples, consisting of fragments from an isolated colony taken with platinum loop or a Pasteur pipette tip, were applied over a 2-3 mm band area, resulting in the rapid appearance of the color reaction due to the presence of oxidase and indophenol formation; the positive reaction is indicated by the appearance of blue color in 10-20 sec.(5,6)

Emphasis of non-cytotoxic respiratory enzymes (detoxifying enzymes that protect the bacterial cell from O_2 partial reduction products): catalase production is evidenced by the addition of a 3% H_2O_2 solution over a bacterial culture fragment for 24h deposited on a glass slide, the positive reaction being visualized by efervescence as a result of O_2 release. (7) This enzyme converts H2O2 into O2 in bacteria that are not strictly anaerobic and aerotolerant.(5,6,7)

Identifying the certainty of microbial strains by establishing the biochemical profile with API assays (Analytical Profile Index)

The BioMérieux API tests are micro-test systems used for the biochemical identification of microbial strains. The microtest galleries were inoculated, incubated and interpreted according to the manufacturer's instructions. In this study, galleries *Staph API, API 20 Strep, API 20 A, API 20E, API 20NE* were used.

3. Determination of adhesion and formation capacity of microbial biofilms.

Adhesion to the inert substrate. Adhesion capacity to inert substrate was evaluated by the slime test by quantitative method. The term slime designates the biofilm matrix material, a lax material, easy to separate by centrifugation, consisting of exopolysaccharides, hydrophilic glycoproteins secreted by some strains, and which support the adherence of bacterial cells to inert, abiotic surfaces. It is an indicator of the degree of resistance / tolerance and survival capacity of bacterial strains in the external environment, but also a virulence factor of opportunistic / pathogenic bacteria during the infection of a host organism, mainly by its antifungal effect.

Bacterial suspensions were inoculated into 2 ml broth in hemolysis tubes and incubated for 24 h at 37° C. After incubation, the tubes were emptied of the bacterial culture, washed 3 times with sterile saline solution, fixed with 2 ml methanol for 5 minutes and safranin stained for 15-20 minutes. The presence of a red ring on the inner walls is considered a positive result. The adhesion strength to the inert substrate, directly proportional to the color intensity, was assessed semi-quantitatively in: absence [0], poor [1], moderate [2] and strong (3).(6,7)

Adhesion to the cellular substrate. This test was performed by the adapted Cravioto method, using cell line Hep-2 (Human Epithelioma) as the cell substrate. Hep-2 cells were cultured for 24 hours at 37° C in MEM (Eagle Minimal Essential Medium) supplemented with 10% fetal bovine serum (Gibco BRL), 0.1 mM amino acids (Gibco BRL) and 0.5 ml gentamicin solution 50 μg / ml) (Gibco BRL). Hep-2 cell monolayers obtained in six-well plastic plates were washed three times with PBS (Phosphate Buffered Saline), and then 1 ml of bacterial suspension was added to each well. The plates were washed, dried at room temperature, examined under a microscope immersion objective (100X), to determine the semi-quantitative adhesion level and adhesion pattern (localized, diffuse or aggregate). The adhesion index was calculated as being equal to the percentage of adherent bacteria cells in 100 cells counted.(6,7)

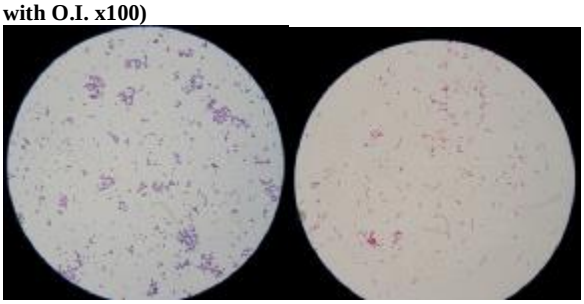
RESULTS

1, 2. Results obtained for the first two parts of the study [Determining a base of bacterial and fungal dental plaque (supra- and subgingival); Isolation and identification of some species of microorganisms present in the dental microbial plaque] will be presented below. It is known that the oral cavity provides a unique habitat for many bacterial species, being colonized with one of the most diverse bacterial flora (microbiota) existing in the non-sterile cavities of the human body, consisting of approximately 600-800 bacterial species.(8,9) Up to now, 200-300 species have been grown from the human oral cavity, and another approximately 400 have been detected by a variety of analysis methods independent of their culturing. Of the approximately 700 possible colonizers, it is estimated that 100-200 species are constantly present in a healthy human being.(8-10) The identification of microorganisms was performed based on the examination of culture and colony characters, Gram character (figure no. 1), conventional biochemical tests (catalase, oxidase) and standardized biochemical identification systems API (BioMérieux, France), (API Strep, API 20A) (figure no. 2).

The identified strains belonged to following species *Staphylococcus sp., Streptococcus sp., Actinomyces sp.,*

Enterococcus sp., Gemella sp., Pasteurella sp.

Figure no. 1. Morphological type and Gram affinity of isolated microbial strains (Gram stained smear, observed with O.I. x100)



3. Determination of adhesion and formation capacity of microbial biofilms

The initial phase of dental plaque formation is the formation of dental conditioning film, which is the result of adsorption of biopolymers to the tooth-saliva interface and is processed very quickly, starting in vivo a few seconds after tooth cleaning. The film is made up of salivary components, gingival crevicular fluid, fibrin, bacteria, mucosa and possibly some dietary components, more than 180 peptides, proteins and glycoproteins, including keratin, mucins, proline-rich proteins, phosphoproteins, histidine rich proteins and other molecules that function as bacterial adherence sites (receptors).(11)

Bacterial colonization of salivary film begins with the selective absorption of bacteria from saliva on the surface of the salivary film as isolated cells or small groups.(11)

The first organisms that adhere to this conditional film will be those that have adhesins and the ability to bind to the molecules receptors present in the film. Within a few hours, between 12% and 32% of the enamel surface is covered with bacteria, and those species that can grow and reproduce under the conditions of the environment are the "pioneer" species. Adhesion is provided by adhesion molecules (polysaccharides, lipoteic acids, glucoziltransferases and lectins) present on the cell surface or associated with cellular structures (fimbriae, capsules) and receptors located on buccal surfaces (salivary components: glycoproteins, mucins, amylases, lysozyme, immunoglobulin A and G, proline and staterine rich proteins, bacterial components: glucans, glycosyltransferases).(12)

The studied strains showed the ability to produce slim factor (adhesion to the surface of the inert substrate), being divided according to the intensity of the ring formed in high and moderately adherent or non-adherent strains (figure no. 3).

Figure no. 2. Biochemical API profiles of isolated microbial strains.

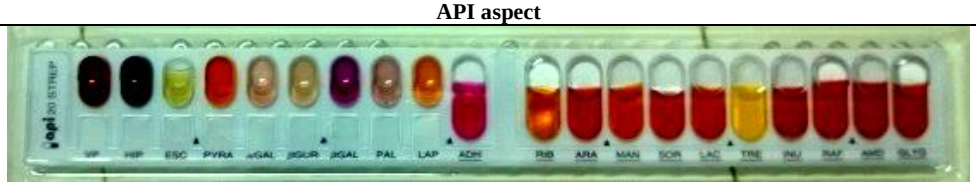
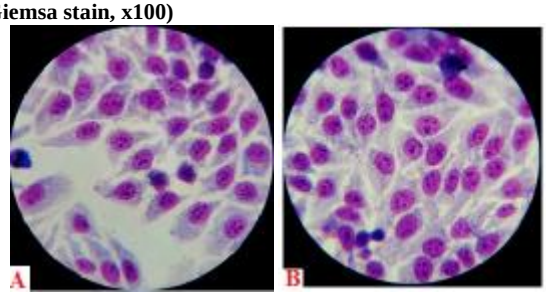
Isolated strain	API aspect
Streptococcus acidominimus	
Staphylococcus epidemidis	
Gemella morbillorum	
Actinomyces naeslundii	
Enterococcus faecium	

Figure no. 3. The appearance of the positive reaction of different intensities for the slime test - the red ring is represented by the microbial adherent cells to the glass

Bacterial adherence is mediated by specialized structures of the bacterial surface known as adhesins and provides a major environmental advantage for pathogenic bacteria, regarding insurance of nutrient, protection against host defence mechanisms. Their multiplication, after adhesion, takes place at a higher rate than that of non-adherent cells. The strains included in this study showed diffuse, localized diffuse, aggregative diffuse patterns of adhesion (figure no. 4).

Figure no. 4. Types of cell substrate adherence: A diffuse adhesion, B: localized-diffuse adhesion (Hep-2 cell monolayer smear infected with different microbial strains, Giemsa stain, x100)



DISCUSSIONS

As is well known, oral streptococcs are among the species that originally colonize the dental surfaces due to the ability to adhere directly to the salivary film and represent 60-90% of the bacteria that colonize the teeth in the first 4 hours after their professional cleansing.(13,14) Other primary colonizers are the genus species of Actinomyces, Veillonella, Gemella, Granulicatella, Prevotella, Rothia and Neisseria, which are part of the normal microbiota (bacterial flora) and very few are responsible for the development of periodontal disease, but may be responsible for other infections of the body, more or less serious.(15-17)

CONCLUSIONS

Following the analysis of the results obtained in this study, several conclusions could be drawn, among which we would like to mention the ones that we have appreciated as the most interesting:

- The determining of a base of bacterial and fungal strains based on microbiological culturing from the dental plaque (supra- and subgingival) was carried out according to a very rigorous program, we did not encounter difficulties during culturing, transport and / or identification.
- The large number of samples collected from the supra and subgingival dental plaque allowed us a very large microbiological analysis of good quality, the increased variety of isolated microbial strains, allowing us a very serious double selection, the final result after both the first screening and the second, being in full consistency with the information in the literature.
- This study allows us to select a range of microbial strains that can be used in further studies of sensitivity of microorganisms to contact with impression materials used in dental prosthodontics.
- Microbiology studies were carried out with very high accuracy and competence, the results obtained being in accordance with specialized literature.

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