Virulence Profiles of Bacterial Strains Isolated From Periodontal Lesions

Received for publication, February 20, 2015
Accepted, July 10, 2015

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Abstract

Periodontitis is a chronic inflammatory disease of polymicrobial etiology that could lead to the destruction of bones and tissues that support the teeth. In spite of the growing data on the pathogenic consortia associated with the disease, currently this information is poorly used to improve diagnostics or developing methods for monitoring the treatment outcome. The investigation of the microbial composition of periodontal pocket or the gingival sulcus in 23 patients with periodontal diseases showed that only 39% of the total analysed samples were harbouring microbial strains etiological linked to periodontal disease or frequently encountered in pathological periodontal conditions, i.e. Actinomyces naeslundii, A. viscosus, Prevotella oralis, Fusobacterium mortierferum, Streptococcus mitis and Bacteroides sp. The microbial strains isolated from the periodontal lesions expressed virulence factors that enable them to colonize and damage the host tissues (i.e. ability to adhere to inert and cellular substratum, pore forming toxins, proteases, DN-ase and siderophore-like molecules).

Keywords: periodontal disease, microbial strains, dental biofilm

Introduction

Periodontitis is a chronic inflammatory disease of polymicrobial etiology that can lead to the destruction of bones and tissues that support the teeth including the alveolar bone, periodontal ligament, cementum, and gingiva (1, 2). The treatment of periodontitis involves elimination or at least control of pathogenic bacteria associated with the disease. However, in spite of the growing data on the pathogenic consortia associated with the disease, currently this information is poorly used to improve diagnostics or developing methods for monitoring the outcomes of treatment success (3).

The increased prevalence of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus and Treponema sp. in different forms of periodontal disease has earned them the recognition as diagnostic markers in the disease...
process. The purpose of this study was the investigation of the microbial composition of the periodontal pockets and gingival sulcus in Romanian patients with periodontitis, including aggressive and chronic periodontitis, and to determine the association the between microbiological profile, virulence microbial determinants and clinical status.

**Material and methods**

**2.1. Isolation and identification of the microbial strains**

A total of 23 patients, aged between 27 years to 60 years old (9 men and 14 female) with pathology of periodontal tissues confirmed by clinical and radiographic examinations consented to participate in this investigation. They underwent periodontal treatment or routine check-ups at three dental offices in Bucharest. The criteria used for inclusion in the study were the following: age over 18 years old; clinical signs of suggestive periodontal diseases: gingivitis, bleeding gums, gingival retractions, tooth mobility or tooth loss; no antibiotic or periodontal treatment in the last 6 months; absence of severe systemic diseases or major changes in diet or lifestyle. The intraoral clinical examination achieved by inspection and palpation, followed close the oral mucosa appearance and possible pathogenesis of gingival recession, the plaque and tartar index, the papillary bleeding index, the gingival retraction, the tooth mobility and the existing depth of periodontal pockets.

Samples from periodontal pockets and gingival sulcus were collected from patients suffering from periodontal diseases during stomatological treatment. The tooth was wiped with sterile gauze and the subgingival environment was accessed through the periodontal pocket. Samples from each patient were placed in thioglycollate broth. The predominant cultivable microbiota was investigated by classical microbiological methods which included cultivation on enriched non selective media (Brucella blood agar), differential and selective media (MacConkey agar, Slanetz Bartley, Manitol Salt agar, Cetrimide agar). The microbial colonies were examined microscopically to establish the morphology and Gram stain affinity. Conventional biochemical tests (oxidase and catalase) were used to select the appropriate API microtube system [API Staph, API Strep, API 20E, API NE, API 20A] for the identification of the microbial isolates.

**2.2. Investigation of cell-associated and soluble virulence microbial factors**

**2.2.1. Assessment of the adherence capacity to the inert substratum**

The adherence capacity to the inert substratum of the microbial strains was determined by quantifying the production of slime factor using the microtiter plate method (4). The microbial suspensions corresponding to 0.5 Mac Farland density were seeded in Schaedler Anaerobic Broth, distributed in 96 multi-well plates, then incubated in anaerobic condition at 37°C for 48 h. Visible microbial biofilms, formed at the bottom of wells, were washed three times with tap water, fixed with cold methanol for 5 minutes, coloured with 1% violet crystal for 15 minutes, washed again with tap water and resuspended in 33% acetic acid. Schaedler Anaerobic Broth was used as a negative control. Biofilm formation was judged qualitatively by the observation of the colour intensity of the obtained suspension, proportional with the number of microbial cells adhered to the inert substratum and estimated as absent (0), weak (+), moderate (+++) or strong (++++). All assays were done in triplicate.

**2.2.2. The evaluation of the adherence capacity to the cellular substratum**

This microbial virulence parameter was assessed by the Cravioto’s adapted method. The Hep-2 line cells were cultivated for 24 hours at 37°C in MEM (Eagle Minimal Essential Medium) supplemented with 10% fetal bovine serum (Gibco BRL). 1 ml of microbial suspension with a density corresponding to the 0.5 Mac Farland nephelometric standard,
prepared in PBS using a 18-24 hours culture of was added over the cellular monolayer. The inoculated plates were incubated at 37°C for 2 hours, allowing the bacterial cells to adhere to the cellular substratum. After incubation, the cellular monolayer was rinsed with PBS, fixed with methanol for 5 minutes and coloured with 1% Giemsa solution for 20 minutes. After staining, the wells were washed with tap water, dried at room temperature and submitted to microscopic examination with the 100x immersion objective in order to establish the adherence patterns (localized, diffuse or aggregative) of the microbial isolates and to determine the adherence index (expressed as the ratio between the number of eukaryotic cells exhibiting adhered microbial cells per 100 cells counted on the microscopic field).

2.2.3. Study of the soluble virulence factors production

Culture media containing specific enzymatic substrates were used to highlight the expression of different metabolic enzymes: hemolysins, amylases, caseinases, gelatinases, esculin hydrolysis, DN-ase, as previously reported (5). Briefly, 18 h microbial culture was spotted onto agar plates with specific enzymatic substrata, i.e. 5% sheep blood (hemolytic activity), 1% starch (amylase activity), 1% casein and 0.4% gelatine (proteolytic activity), 1% esculine (esculinase activity), 0.2% DNA (DNase production). All media were prepared in house. Enzyme production was detected after 48-72h of incubation at 37°C, by macroscopic observations of specific modifications of the media around the culture spot (hemolysis, precipitation, clearing, blackening). All assays were done in triplicate.

Results

3.1. Microbial composition of the periodontal pockets and gingival sulcus

The microbiological analysis of the 23 periodontal pockets and gingival sulcus samples led to the recovery of forty microbial isolates. The preliminary results indicated a predominant gram positive microbiota that consists of various cocci and lactobacilli (70% of the microbial strains) and gram negative rods (30% of the microbial strains). *Actinomyces* sp. was the most most frequently isolated microorganism constituting 20% of the total isolates followed by *Pasteurella haemolytica* (15%) and *Streptococcus* sp. (12.5%). Gram negative microbiota detected in the examined samples was represented by *Pasteurella haemolytica* (15%), *Chryseomonas luteola* (7.5%), *Aeromonas hydrophila* (2.5%) and *Acinetobacter iwoffii* (2.5%). *Actinomyces naeslundii* was the most prevalent microorganism, which was found in 21.73% of the samples. Other microorganisms, frequently encountered in pathological periodontal conditions (6), were *Prevotella oralis*, *Fusobacterium mortiferum*, *Streptococcus mitis* and *Bacteroides sp.*., which were identified in 4.34% of the samples.

3.3. Microtiter plate assay for assessment of biofilm production

The experiments performed in our study enabled us to screen the adherence and biofilm formation capacity of the isolated bacteria. The results ranged from complete absence of biofilm to moderate biofilm formation according to the microorganism and sample site. 60.86% of the periodontal pockets and gingival sulcus samples contained microbial strains with strong capacity of biofilm formation on inert substratum. The majority (92.85%) of the isolates, with strong capacity to adhere and to colonize the inert substrata, belonged to *Actinomyces* sp. and *Streptococcus* sp., regarded as pioneer colonizers of tooth surfaces (7). Only two gram negative strains, i.e. *Chryseomonas luteola* and *Pasteurella haemolytica* exhibited a strong capacity to develop biofilm on inert substrata. The reputed secondary colonizer *Fusobacterium sp.*., identified in one sample, did not exhibit the capacity to adhere and form biofilm on inert substrata.
3.2. Evaluation of adherence to cellular substrata

Many of the suspected periodontopathogens have surface structures necessary for attachment, including fimbriae, capsules and lipopolysaccharides, which facilitate the adhesion of the bacteria to a surface but also the coaggregation with other plaque organisms, and are highly regulated to respond to environmental changes (8). In order to highlight the presence of such surface attachment structures, the bacterial strains isolated from periodontal pockets and gingival sulcus samples were tested for their ability to adhere to cellular substratum represented by eukaryotic cells belonging to Hep-2 cell line. The isolates exhibited different levels of a attachment to eukaryotic cells, the experimental results showing that 66.66% of the analyzed strains were able to adhere, with an adherence index ranging from 15 to 100%. The adherence patterns observed were: diffuse, localized, localized-aggregative and localized-diffuse.

3.3. Soluble enzymatic factors expression

Many pathogenic bacteria produce extracellular products that have tissue-damaging effects. Some of the diverse armamentarium of products from pathogenic bacteria serve as virulence factors in the pathogenesis of disease by facilitating the spread of bacteria or toxins through tissues (9). Our results revealed that the studied strains showed the capacity to produce pore forming toxins, including haemolysins (61.1%), lecithinases (14.81%) and lipases (14.81%); proteases, i.e. caseinase (18.51%) and gelatinase (14.81%); amylase (46.29%); DN-ase (18.51%) and the production of siderophore-like molecules resulted from the in vitro hydrolysis of aesculin (51.85%) (Figure 1).

![Fig. 1. Expression levels of different soluble virulence factors in the analyzed strains](image)

**Discussion**

Periodontal disease result from mixed bacterial infections, in which both host resistance barriers and bacterial interactions are important (10). The bacteria associated with periodontal diseases are predominantly gram-negative anaerobic bacteria and may include *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Campylobacter rectus, Eubacterium nodatum, Peptostreptococcus micros, Streptococcus intermedius* and *Treponema sp.* (11).

Today, periodontal therapy remains targeted toward removal of the plaque mass as opposed to elimination of specific pathogens. But, with the continuing evolution of the specific plaque hypothesis, there is increased interest in chemical antimicrobials, which have the ability to suppress and/or eradicate the pathogenic players (12). Microbiological analysis in periodontitis is therefore an important tool in monitoring the outcomes of antimicrobial treatments.
Microbial characterization of the twenty-three periodontal pockets and gingival sulcus samples from our study revealed the presence of bacterial species that are strongly implicated with various forms of periodontal disease like: *Actinomyces naeslundii*, the most prevalent isolated microorganism, *Prevotella oralis*, *Fusobacterium mortiferum*, *Bacteroides sp.* although species that cannot yet be cultivated are likely also to be relevant. Nevertheless the microbiologic data from the present study suggest that microbiological diagnosis of periodontal pathogens could be helpful in the prevention and treatment of periodontal disease.

Interactions between oral bacteria and gingival epithelial cells are essential aspects of periodontal infections. The induction and progression of the periodontal tissue destruction is a complex process, which implies accumulation of dental plaque, release of microbial components and inflammatory reactions of the host (13, 14, 15, 16, 17).

The microbial species must first adhere to a surface (dental enamel, periodontal pocket epithelium, extracellular matrix substances) in order to colonize a periodontal site, or to other bacteria that had previously adhered to that surface by coaggregation and coadhesion (18, 19, 20, 21, 22, 23). The term of slime was used initially by Christensen et al. (1982) referring to the glycocalix produced by the strongly adherent strains of *Staphylococcus epidermidis* isolated from the infected surface of medical implants, and subsequently, to other microbial species, producing a hydrophilic exopolysaccharides for facilitating the adherence to inert abiotic, surfaces. Slime represents an indicator of the resistance and survival capacity of the bacterial strains in the external environment, but also a virulence factor, in case of an infection of a host organism, by blocking the phagocytosis. Our experimental results showed that the slime factor production, a necessary element in the colonization of subgingival bacteria, was present in 61% of the analyzed strains; these results demonstrate their potential to adhere, an essential required step for pathogenesis identified for many other periodontal pathogens (12).

Using an *in vitro* tissue culture model, a selected group of bacteria isolated from periodontal pockets and gingival sulcus samples, including *Actinomyces naeslundii*, *Streptococcus acidominimus*, *Pasteurella haemolytica*, *Streptococcus intermedius*, and *Micrococcus sp.*, were examined for their attachment characteristics using Hep-2 eukaryotic cells. Our results showed that these microorganisms exhibited the ability to colonize cellular substrata, with adherence indexes ranging from 15% to 100% and different adherence patterns.

New technologies have shown that pathogenic bacterial species are present in defined complexes within subgingival plaque, thus identifying specific targets for therapeutic intervention. Multidisciplinary investigations using new approaches will help understand the ways in which bacteria move and interact in a variety of surface microenvironments during biofilm development. Such knowledge will enhance our knowledge of dental biofilm formation and of the sophisticated processes of interaction between prokaryotes and eukaryotes.

Although the periodontopathogenic bacteria rarely invade the tissues and cause acute infections, they could release substances that penetrate the gum and directly causes the tissue destruction by the enzymes and endotoxins action, or indirectly through the induction and maintenance of the chronic inflammatory process, leading to the progressive destruction of collagen in the connective tissue that fixes the teeth in the gum (24, 25, 26).

The analyzed bacterial strains exhibited different expression patterns of soluble virulence factors involved in the destruction of the periodontal tissue. The collagen and other components of the perivascular extracellular matrix are destroyed, either by the release of collagenases and cytokines activating the bone resorption, or by the bacterial proteases (27), with gelatinase activity (28, 29). Thus, the protease production by the analyzed strains, especially those with gelatinase activity, can determine the cleavage of type I collagen, inducing the bone resorption (30, 31).
The iron acquisition is an essential virulence factor due to the restriction of free Fe, by producing compounds that tie and transport Fe like ferritin, hemin, lactoferrin and transferrin. However, the periodontal pathogens do not produce siderophores (32), nor directly interact with Fe binding proteins (transferrin) (33). In exchange, esculethol that results after the hydrolysis of esculin could chelate iron with great affinity and can play the role of classical siderophores, providing in this manner the Fe ions necessary for the bacterial cells to activate some genes and express their virulence potential (34).

The pore forming toxins, like hemolysins, lipases and lecithinases, due to the lesional effects induced in the plasmatic membrane, can play a role in the tissue destruction and inactivation of immune cells.

The amylases can provide a nutritional advantage to the producing bacteria, and at the same time, can play an important role in the constitution of the polysaccharide extracellular matrix of the dental biofilm.

The production of DN-ases, mediating the hydrolysis of the DNA released after the cellular destruction, could provide nucleotides for the bacterial cells own synthesis, offering them a competitive advantage for the colonization of a certain ecological niche.

Conclusions

The investigation of the microbial composition of periodontal pocket or the gingival sulcus in patients with periodontal diseases showed that approximately 39% of the total analysed samples were harbouring microbial strains etiological linked to periodontal disease or frequently encountered in pathological periodontal conditions i.e. A. naeslundii, A. viscosus, P. oralis, Fusobacterium mortiferum, Streptococcus mitis and Bacteroides sp. This result suggests that the isolation and monitoring of specific periodontal pathogens could aid in management and treatment by determining the etiology, and monitoring of progression of periodontal infection and deciding on recall intervals. Also the virulence profile of the microbial isolates evaluated by the ability to adhere to inert and cellular substratum and by production of soluble enzymatic factors (i.e. pore forming toxins, including haemolysins, lecithinases and lipases; proteases: caseinase and gelatinase; amylase; DN-ase and the production of siderophore-like molecules resulted from the in vitro hydrolysis of aesculin) showed that the periodontal bacteria possessed factors that enables them to colonize and damage the host tissues. In light of increasing antibiotic resistance amongst oral bacteria, new strategies for control of periodontal bacteria must be developed that inhibit the bacterial factors necessary for colonization (microbial attachment structures) and destruction of host tissues by the production of a large spectrum of soluble virulence factors involved in the progression and chronicization of periodontal lesions.
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