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PREVOTELLA SPECIES – THE MOST PREVALENT BLACK-PIGMENTED ANAEROBIC BACTERIA AMONG OROPHARYNGEAL ISOLATES OBTAINED FROM A SAMPLE OF HEALTHY YOUNG ADULTS

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ABSTRACT

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Introduction The black-pigmented *Prevotella - Porphyromonas* group comprises members of the normal flora of the oral cavity, oropharyngeal, intestinal and genitourinary tract, but may be associated with various infections too. The purpose of this study was to identify the species of this anaerobic group which frequently colonize the oropharynx in clinically healthy young adults.

Methodology The microbiological investigation was carried out on a strain collection of 93 dark-pigmented anaerobic isolates originated from the oropharynx of healthy dental students, at the Department of Microbiology, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy - Bucharest. All isolates of Gram-negative bacilli were identified at the genus and species level by conventional methods, MASTRING ID (MAST Group Ltd., U.K.) and Rapid ID 32 A system (BioMérieux, France), while the isolates of anaerobic cocci were tested only by MASTRING ID.

Results The microscopy of the Gram-stained smears and the results of the MASTRING ID test performed with the 93 black-pigmented anaerobic isolates indicated that 77 strains were Gram-negative bacilli and/or coccobacilli, while 16 strains were Gram-positive cocci. The identification of the Gram-negative bacilli at the species level concluded that 57 strains belonged to *Prevotella denticola*, 18 strains to *Prevotella melaninogenica* and 2 strains to *Prevotella intermedia*.

Conclusion *P. denticola* and *P. melaninogenica* might be considered the main species of the black-pigmented *Prevotella-Porphyromonas* group which colonize the oropharynx in healthy young adults. These species are usually beta-lactamase producers and their high rate of oropharyngeal colonization should be considered when antibiotics are needed in oral infections therapy.

KEYWORDS

Oropharynx, Microbiota, Anaerobes, Black-Pigmented Bacteria, *Prevotella*.

1. INTRODUCTION

The oropharynx comprises the palatine tonsils, tongue base, soft palate and posterior pharyngeal wall and is lined by a nonkeratinized stratified squamous epithelium [1]. The oropharynx mucosa comes in contact with saliva and nasopharyngeal secretion. The oropharynx microbiota is complex and comprises hundreds of microbial species [2,3], most of them organized in biofilms associated with the respective microenvironment [4]. About 80% of the local normal flora is represented by the viridans streptococci and commensal species of *Haemophilus* and *Neisseria* [5,6]. Besides different

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species of *Mycoplasma*, *Corynebacterium* and staphylococci, the oropharynx may harbor also pathogenic or potentially pathogenic bacteria like: *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae subsp. equisimilis*, *S. pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae* or *Moraxella catarrhalis* [6-14]. Some studies reported that the dominant bacteria of the normal oropharyngeal flora are the anaerobes [15]. The persistence of anaerobic bacteria is favored by the anaerobic conditions found into the depth of the biofilm covering the oropharyngeal mucosa, mainly due to the oxygen consumption by aerobic and facultative anaerobic bacteria. The mucosal convolutions and especially the tonsils crypts may be considered important anaerobic microhabitats. At these sites, the strictly anaerobes are found in high proportion with *Prevotella* being the most frequently isolated species [16,17]. Most Gram-negative anaerobic bacteria colonizing the oropharynx in healthy adults belong to the following genera: *Prevotella*, *Fusobacterium*, *Leptotrichia* and *Veillonella* [2,18-22]. Some researchers also found *Porphyromonas* at this site, but except for *Prevotella* spp., the other anaerobes were isolated in much smaller percentages than the facultative anaerobes, such as: streptococci, staphylococci and diphteromorphs [16]. The conclusion of a Norwegian-American research team that applied cultural-independent molecular techniques to determine the microbial diversity of the oral flora was that there is a distinctive dominant oral flora in healthy subjects, with site-specificity and high diversity [23]. The same team found an unexpected high diversity at tonsils level (more than 50 different species) with high variation between subjects [23].

Thus, *Prevotella* and *Porphyromonas* spp. were isolated from some subjects, but were missing in others who were harboring mostly bacteria belonging to the phylum *Firmicutes* [23].

Some bacterial community analysis indicated that the main group associated with the throat microbiota comprises: species belonging to *Streptococcus* and other genera of *Firmicutes*, species belonging to the family of *Pasteurellaceae*, *Fusobacterium* spp. and *Actinomyces* spp., while the main group associated with the tonsils includes species of *Streptococcus, Mogibacterium* and other *Firmicutes*, *Fusobacterium* spp., *Prevotella* spp. and members of the family *Pasteurellaceae* [24]. In healthy persons, the core microbiome plays a major role in homeostasis [25]. In children, an oropharyngeal microbiome similar to that of adults was described, but richer in *Prevotella*, *Neisseria*, *Granulicatella*, *Porphyromonas* and *Fusobacterium* [26]. At present high efforts are required for advanced research of normal flora of the oral cavity and oropharynx, estimating that future findings may substantially contribute to understanding the role played by the microorganisms with oral or oropharyngeal habitat in human pathology [23]. The present study intended to contribue to the investigation of black-pigmented Gram-negative anaerobic bacilli, since the anaerobic bacteria are not commonly cultivated by many microbiology laboratories. Thus, the aim of this study was to identify the species belonging to this bacterial category which colonize the oropharynx in healthy young adults most frequently.

2. METHODOLOGY

The microbiological investigation was performed on a collection of 93 black-pigmented anaerobic bacterial strains stored at -70^{0}C, at the laboratory of the Department of Microbiology, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy (CDUMP) Bucharest. The respective strains originated from oropharyngeal swab samples collected from 29 male dental students and 64 female dental students, aged 20-34 years, at the same Microbiology department, in April 2018. They were second-year students, were clinically healthy and had not taken antibiotics in the last 6 months. Twenty-four of them were smokers: 7 male subjects and 17 female subjects. Prior to the present study, cultures from the 93 oropharyngeal swab samples were performed on Schaedler agar with 5% sheep blood (BioMérieux, France), which were incubated in GENbox, with GENbox anaer sachet and *Anaer* indicator (BioMérieux, France), at 35^{0}C, for 10 days, with examination every 48h. All isolates that developed black pigment and showed negative results in the aerotolerance test were stored in cryobiles, at -70^{0}C and constituted the collection of strains used in the present study. At the beginning of this study conducted in the second semester of 2018, the black-pigmented anaerobic strains belonging to the above mentioned collection were checked for their morphotinctorial characteristics by microscopic examination of Gram-stained smear. All Gram-negative bacilli strains were further identified by the conventional methods, MAST ID MID8 ANAEROBE ID RING/MASTRING ID (MAST Group Ltd., U.K.) and Rapid ID 32 A system (BioMérieux, France). The presumptive identification of the Gram-negative bacilli strains was performed by testing their ability to grow in the presence of 20% bile (by streaking them on *Bacteroides* bile-esculin agar, BBE) and by testing their susceptibility to: vancomycin (5 µg), kanamycin (1000 µg), colistin sulphate (10 µg), erythromycin (60 µg), penicillin G (2 units) and rifampicin (15 µg) by applying the MASTRING ID on blood agar plates (BioMérieux, France) seeded with bacterial inoculum adjusted to the turbidity of 2 McFarland standard. The plates were incubated in anaerobic atmosphere, at 35^{0}C, for 48h. *Bacteroides fragilis* ATCC 25285 was used as quality control. These tests were interpreted based on the indication mentioned in the textbooks of diagnostic microbiology [27], completed with the recommendation given by the MASTRING ID

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Figure 1. MASTRING ID test applied on a strain of black-pigmented Gram-negative anaerobic bacilli.

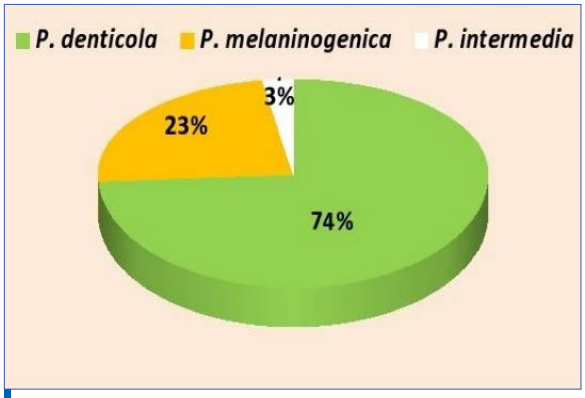

Figure 2. The distribution of the 77 strains of black-pigmented Gram-negative anaerobic bacilli by species.

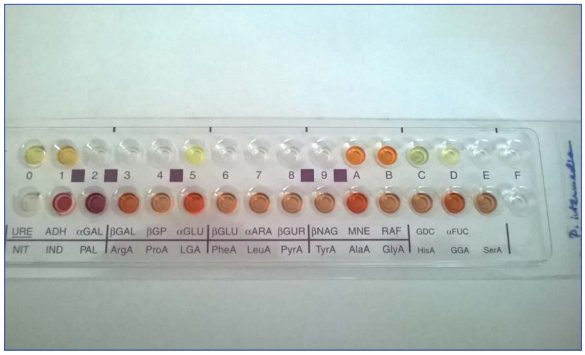

Figure 3. *P. intermedia* strain identified by the Rapid ID 32 A system.

producer. In addition, the MASTRING ID test was also applied to the coccus-shaped anaerobic isolates.
The Fisher exact test was used to find any statistically significant association between smoking (data on this habit being received from students prior to this study, when oropharyngeal swab samples were collected) and oropharynx colonization with black-pigmented Gram-negative anaerobic bacilli species. The chosen significance level was $p \leq 0.05$.

3. RESULTS

The microscopic examination of the Gram-stained smears from the culture of dark-pigmented anaerobic strains indicated that 77 of the 93 isolates were Gram-negative bacilli and/or coccobacilli. The microscopy showed that the other 16 strains were Gram-positive cocci (arranged mainly in irregular clusters) and this was in complete agreement with the results of the MASTRING ID. Figure 1 illustrates the result of the MASTRING ID test applied to a strain of black-pigmented Gram-negative anaerobic bacilli (Fig. 1). The microbiological investigation carried out on the dark-pigmented Gram-negative anaerobic bacilli and/or coccobacilli isolates showed that all of them were susceptible to 20% bile, rifampicin and erythromycin, but resistant to kanamycin and vancomycin. The results concerning the susceptibility to colistin and penicillin G varied, 45 strains being found susceptible and 32 strains resistant to colistin, while only 19 strains were found susceptible to penicillin. The interpretation of the tests according to the textbook of diagnostic microbiology [27] and MASTRING ID producer indicated that all these bacilli strains belonged to the genus *Prevotella*. The Rapid ID 32 A system indicated that 57 strains were *Prevotella denticola*, 18 strains were *Prevotella melaninogenica* and 2 strains were *Prevotella intermedia* (Fig. 2). In 5 subjects, pairs of *P. denticola* - *P. melaninogenica* were isolated. Twenty of the 72 students with black-pigmented *Prevotella* oropharyngeal colonization were smokers. Figure 3 presents the biochemical profile of a *P. intermedia* strain obtained with the Rapid ID 32 A gallery (Fig. 3). A *p*-value of 0.5737 was found when applying the Fisher exact test, indicating no statistically significant correlation between smoking and colonization of the oropharynx with black-pigmented Gram-negative anaerobic bacilli.

4. DISCUSSION

The normal oropharyngeal flora may show variability among healthy individuals due to external factors and ecological relationship. The investigation of the microbial relationship may contribute to clarifying many underlying aspects [28]. Anaerobic bacteria are not currently investigated by many laboratories due to the laborious and expensive work required. *Porphyromonas* and *Prevotella* (previously belonging to the genus *Bacteroides*) also include, besides non-pigmented species, dark-pigmented species like: *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Porphyromonas asaccharolytica*, and *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella melaninogenica*, *Prevotella loescheii*, *Prevotella denticola* and *Prevotella corporis*, respectively [29]. This study focused on the investigation of the oropharynx colonization by dark-pigmented *Prevotella* and *Porphyromonas* species. The culture method allowed the isolation of the black-pigmented anaerobic strains. Because the development of dark-pigment is usually a delayed process, primary cultures (which were obtained prior to this study) were checked throughout the incubation period. The present study began with the selection of

the bacilli strains among the 93 black-pigmented anaerobic isolates and continued with the species identification, based on both conventional and rapid methods. *P. denticola*, *P. melaninogenica* and *P. intermedia* were the only black-pigmented anaerobic bacilli species isolated from the oropharynx in this group of 93 healthy young dental students. The results obtained with the Rapid ID 32 A system showed very good to excellent identification for all but 6 strains. The identification at species level of the 6 strains mentioned above indicated *P. denticola* as the first choice and *Prevotella oralis* as the second choice. These isolates were considered to belong to *P. denticola* because *P. oralis* is known to not produce colonies with dark pigment. Atypical phenotypic characteristics of isolates can sometimes lead to misidentification of species. Therefore, the culture-independent methods are highly recommended for the detection of anaerobic species. However, both culture-based method and detection by molecular techniques also have advantages and limitations. Previous studies performed in adults and children reported *P. melaninogenica* in a higher percentage in patients with tonsilar crypts who suffer from recurrent tonsillitis, compared to healthy subjects with adenotonsillar hyperplasia [17]. Recent studies reported that *P. melaninogenica* colonizes the tonsils in large quantities [30]. Isolates of this species are usually beta-lactamase producers [31] and this may explain the failure of penicillin treatment in recurrent tonsillitis [32]. The bacteria that produce enzymes that destroy the beta-lactam ring of penicillin indirectly protect penicillin-sensitive bacteria such as *S. pyogenes* [33]. Changes in the oropharyngeal ecology can variably affect the oropharyngeal microbiota. Smoking is one of the main external factors that can directly influence the composition of the upper airway microbiota [2]. Both active and passive smoking can contribute to colonization of the upper respiratory tract with pathogenic microorganisms [34,35] by impairing the immune response and favoring bacterial colonization, either by stimulating microbial adhesion to the epithelium or by other means [36,37]. *Prevotella* comprises commensal species from the human microbiota, which usually protect the upper respiratory tract against colonization of pathogenic bacteria [24,35]. Some studies have reported that nasopharynx in smokers frequently hosts *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, while *Prevotella* is absent [24,35]. It has been observed that the oral cavity and the nasopharynx became colonized again with the normal resident flora when smoking was abandoned [38,39]. Recent research using univariate analysis and machine learning approaches has concluded that smokers compared to non-smokers are colonized with a greater number of species belonging to the genera: *Megasphaera*, *Streptococcus, Veillonella, Atopobium, Eggerthella, Dorea, Anaerovorax, Eubacterium* and to the family *Erysipelotrichaceae* [24]. Many of these bacteria can also be involved in oral infections. Some researchers found that significant changes occurring in the resident microbial population from oral and nasopharyngeal microbiota in smokers are correlated with higher frequencies of infections than in non-smokers [40]. Oropharyngeal flora varies due to many other factors, such as age and health status. In most studies focusing on the upper respiratory tract flora, mainly oral and nasopharyngeal flora have been investigated in children, the elderly and patients suffering from various diseases [41-47].

However, updated data on changes in oropharynx flora in healthy young adults are also needed.

Influenced by data from recent specialty literature, special attention was paid in the present research to analyze the association between smoking and colonization of oropharynx with black-pigmented Gram-negative anaerobic bacilli in the group of healthy young adults from whom the strains were isolated. Although the findings showed no statistically significant correlation, for a more accurate interpretation of the results, this study should be continued on a larger sample size.

It is important to understand that maintaining a normal oropharyngeal and nasopharyngeal microbiota is an important step in maintaining good health.

5. CONCLUSIONS

The findings of this study indicated *P. denticola* and *P. melaninogenica* as the main species of black-pigmented Gram-negative anaerobic bacilli that colonize oropharynx in healthy young adults. The well-known beta-lactamase activity of these bacteria should be strongly considered when antibiotics are needed in oral infection therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors had equal contribution to the paper.

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Questions

1. Prevotella originated from the genus?

❑a. *Bacteroides;*
❑b. *Fusobacterium;*
❑c. *Tannerella;*
❑d. *Porphyromonas.*

2. Prevotella comprises species of?

❑a. Gram-positive anaerobic bacilli;
❑b. Gram-negative anaerobic bacilli;
❑c. Gram-negative anaerobic cocci;
❑d. Gram-negative aerobic bacilli.

3. Exemples of black-pigmented anaerobic species?

❑a. *Prevotella melaninogenica, Prevotella oralis and Prevotella nigrescens;*
❑b. *Porphyromonas gingivalis, Porphyromonas endodontalis and Prevotella oralis;*
❑c. *Prevotella melaninogenica, Prevotella intermedia and Prevotella nigrescens;*
❑d. *Prevotella intermedia, Porphyromonas gingivalis and Prevotella oralis.*

4. The beta-lactamase?

❑a. Is never produced by black-pigmented anaerobic strains;
❑b. Enhances the penicillin effect against anaerobic bacteria;
❑c. Enhances the penicillin effect against aerobic bacteria;
❑d. Cleaves the beta-lactam ring of beta-lactam antibiotics.