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Intraoral microbiome components identified in Polish patients assessed in terms of threats to human health with infectious factors

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Abstract

Introduction and Objective. The human oral cavity, the main part of masticatory system, is a dynamic environment still requiring quality research. The aim of the study is assessment of the status of the oral cavity and composition of intraoral microbiome of Polish patients in terms of threats to human health with infectious factors.

Materials and Method. The study utilised the data of generally healthy persons: 30 young aged 16–26 years and 30 middle-aged patients, aged 42–52 years. Intraoral swabs were assessed microscopically and by *in vitro* culture methods to detect/identify microbiota.

Results. Different microorganisms occurr in the oral cavity, including non-resident species. Parasitic protozoans *Trichomonas tenax* and *Entamoeba gingivalis*, facultative parasitic *Acanthamoeba* strains, yeast-like fungi of *Candida* albicans group, opportunistic and pathogenic bacteria, including endosymbionts, were identified with various frequency in particular regions of the oral cavity. Higher prevalences of bacteria and fungi strains occurred in middle-aged patients.

Conclusions. The relationship between microbiota of the human oral cavity remains a rare subject of research. This study has shown the ability of different microorganisms to coexist intraorally. These components may pose clinically important threat that should be taken into account as infectious factors. Recognition of microbiome components as potentially contagious, early identification/monitoring/assessment of concomitant species, preventive elimination of the infectious strains during the treatment should be taken into consideration. Further quality research on the intraoral microbiome species that may pose severe local/general clinical diseases are needed to reduce the risk to human health.

Key words

intraoral microbiome, infectious factors, parasitic oral protists, amphizoic amoebae, opportunistic and pathogenic bacteria and fungi, increasing threats to human health

INTRODUCTION

The human oral cavity is the main component of the human masticatory system, a morphologically and functionally complex, and an open, dynamic environment with diverse conditions of specialized surfaces of soft and hard structures. Numerous endo- and exogenous factors may be included in the deterioration of oral cavity status including poor sanitary circumstances and difficulties with the maintenance of good oral health which may influence the hygiene of individual structures of the mouth and dental condition. Among possible factors that can influence the mouth environment in generally healthy persons is the age dependent immunogenetic ability changing during the human ontogenesis.

During the last decades, in many countries including Poland, the oral cavity microbiome of different population groups have often been the subject of research [1–7]. It is

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considered that the mouth microbiome is heterogenous in origin that can include both endogenous and the exogenous species. Components of the oral microbiome can form a multilayer biofilm with complex interactions between intraoral strains and human organisms [8–11].

The study describes the relationship between various species of bacterial plaque and the prevalence and dynamics of tooth decay in various periods of human ontogenesis. Special emphasis has been placed on the Gram-positive bacteria of the S*treptococcus* '*viridians*' group, believed to be resident species participating in the development of biofilm. They are typical oral bacteria inhabitants of healthy oral cavities, not related to any specific clinical pathological symptoms [11, 12].

The majority of studies concern pathogens associated with social diseases – caries and periodontitis. The role attributed to microorganisms in the etiopathogenesis of the periodontal diseases are still under investigation. In the oral microbiome, pathogens from the genera *Streptococcus* are the etiological agents of caries. Simultaneously, as the list of cariogenic bacteria has been expanded (e.g. with *Scardovia wiggsiae*) it was confirmed that caries is a polymicrobial disease [3, 5, 13–18].

The etiological agents of inflammatory periodontal diseases are species from the genera *Actinobacillus, Porphyromonas*, and *Campylobacter.* Some types of periodontal disease have a specific etiology associated with certain bacterial species, for example, in aggressive periodontitis the main role is attributed to *Actinobacillus* and *Porphyromonas gingivalis.* The bacterial components of the oral microbiome that are the etiological agents of the most common social diseases, periodontopathies and tooth decay, have been the subject of research on health and disease in various human populations [4, 14, 17, 19–21]. Since dental caries and periodontitis are the major oral health problems in Poland, most of the research in our country pertains to the bacteria as etiological factors of these important social diseases.

The oral microbiota not connected with these two groups of social illness, has been studied less frequently. Simultaneously, it is noteworthy that disturbances of the oral microbiome homeostasis that the impact the microbial composition may promote a colonization of the mouth with other exogenous species, including the potentially pathogenic.

It is considered that, among others, yeast-like fungi of *Candida albicans* group, that are widespread in the human environment, are often found in the oral cavity [19, 22]. Two species of the parasitic protozoans: *Trichomonas tenax* and *Entamoeba gingivalis*, that are spread between people by direct contact, and also indirectly by commonly used utensils, may occur in the oral cavity microbiome [23]. Some strains of amphizoic *Acanthamoeba* genus that complete their life cycle in the natural environment as free-living amoebae, are also able to exist as facultative parasites in the human oral cavity [24].

Currently, there is a little data on the effects of disturbances of oral ecology on the human organism, and on components of the mouth microbiome in both the young and the middle-aged. A deterioration of labile oral homeostasis may lead to excessive multiplication of the intraoral microorganisms, including these connected with the plaque and mucous membrane diseases.

The role attributed to the microorganisms in the etiopathogenesis of periodontal diseases still requires enhanced investigations. It is taken into account that the plaque microbiota accumulation may induce the intensified development of the bacterial species that are mainly related to tooth decay. It should be emphasized that the impact of the ecological changes, some ontogenetic factors in mouth status, as well as colonization the microbiome by exogenous opportunistic and pathogenic species, still require enhanced research.

Therefore, the aim of the presented qualitative study was to identify the microbiome components of the oral cavities of Polish patients, and assessment of the microrganisms in terms of threats to human health as infectious factors**.**

MATERIALS AND METHOD

The interdisciplinary study pertained to the data of generally healthy, conservatively treated patients who reported to Clinics and Departments of Medical University in Warsaw, Poland. Thirty young patients aged 16–26 years, and 30 middle-aged patients aged 42–52 years. Both groups consisted of 50% males and 50% females. All patients underwent clinical assessment for general health, including their oral cavity condition. The clinical evaluation of oral health status and data from the direct microscopical examinations, as well as *in vitro* culture techniques obtained in the bacteriological parasitological and pharmaceutical laboratories, were assessed. Data of the clinical evaluation of the oral cavity: status of periodontal and gingival tissues, the presence of inflammatory processes and tooth decay were also assessed. Values of Plaque Index and Bleeding Index were routine calculated to assess oral cavity hygiene and gingival inflammation.

The study was performed in accordance with the tenets of the Declaration of Helsinki.

The detection and identification of microbiome components was performed with a light microscopic and *in vitro* culture methods, using parasitological, bacteriological and mycological techniques.

From each persons, swabs were collected directly from 10 sites on the surface of the periodontium, dental plaques and dental pockets, according to a previously used procedure [11]. The material was placed in sterile tubes containing 0.9% NaCl isotonic solution with osmotic pressure equivalent to that of body fluid, at pH 6.8, and incubated at 36 °C. Samples of the biofilm material were used for the preparation of wet and permanent slides for light microscopic examinations. The Giemsa and trichrome stained slides were examined for preliminary determination of bacteria strains and detection of protozoans Gram staining was used for differentiation of bacteria into Gram-positive and Gram-negative strains. The microscopic and *in vitro* culture techniques were applied to isolate and identify/verify qualitatively the oral cavity microbiota for bacteriological, mycological and parasitological assessment.

Samples of the swab material were grown aerobically on bacteriological agar and on agar with 5% defibrinated sheep blood, and then tested for further specific determination. For the detection and isolation of staphylococci, Chapman's plate growth medium was used, and McConkey's medium applied to identify Enterobacteriaceae.

For the isolation of fungi, Sabouraud medium (bioMerieux) was used, and the laboratory identification of species from

the *Candida* genus was performed with Chromagar Candida BBL tests (Becton Dickinson).

To detect parasitic oral protozoans, swab material taken from the surface of the gingiva, dental pockets, amorphous plaque, periodontium and saliva, was examined. Live protozoans were observed in wet-mounted slides under a light microscope. Confirmation of the identification of species based on the morphology was made by permanent smears stained with Giemsa and Trichrome.

The qualitative composition of the oral microbiota and prevalences of particular detected strains were compared and statistically assessed (Statistica, FFisher, HSD-Tukey test). P-value 0.05 was considered significant.

RESULTS

In the presented study, patients from the population groups included in the analysis were without systemic disorders and without indications for surgical procedures.

The clinical assessment of the condition of the oral cavity showed changes in periodontal and gingival tissues, the presence of inflammatory processes and tooth decay. Certain differences were revealed in the status of the oral cavity of patients from particular age groups.

Although conservative/restorative dental treatment was conducted in all patients, comparative examination showed some deterioration of oral health expressed by a various extension e.g. poor status of the mucous membrane, dental caries, pathological pockets, loose teeth, gingival bleedings, and periodontitis noted more frequently in the older patients.

Results of the microscopic examination and *in vitro* assessment of oral swab samples showed that different bacteria, fungi and protozoan species were present in the oral cavities of both groups of patients. Microorganisms identified as the microbiome components in the oral cavities of the young and mid-age patients are presented in Table 1. In all assessed patients, among the bacteria, the strains of the *Streptococcus viridans* group and *Moraxella* genus were identified and considered as typical inhabitants of the human oral cavity – resident species, and thus were not the subject of the current study.

Examinations of the superficial layer of periodontium and dental pockets showed the presence of various bacteria strains belonging to six Gram-positive and five Gram-negative opportunistic or pathogenic strains. Among those Gram-positive strains, in both patients groups the faecal bacteria *Enterococcus faecalis* and *E. faecium* were detected. In the oral cavity of some patients, potentially pathogenic staphylococci: *Staphylococcus epidermidis* and *Staphylococcus aureus* were found.

Among Gram-negative bacteria, *Escherichia coli* occurred in the oral cavities of both groups of patients, whereas *Pseudomonas aeruginosa* and *Klebsiella* spp. were identified only in older patients. Gram-positive and Gram-negative bacteria strains isolated from the microbiome of the oral cavities of young and mid–age patients are presented in Table 1.

In both patient groups analyzed, in the material collected from oral cavities and used for Chromagar-Candida BBL cultivation, yeast-like fungi were isolated and identified as species from the *Candida* genus, predominantly various strains of *Candida albicans* group. These fungi were detected in patients from both groups assessed. Comparative, qualitative analysis revealed some differences in the prevalences of fungal strains detected in the oral cavities of young and mid–age patients. Prevalences of fungi as intraoral microbiome components are presented in Table 1.

Table 1. Oral cavity microbiome components identified in young patients 16–26 years and mid-age patients 42–52 years

MICROORGANISM	YOUNG PATIENTS n=30 N / %	MID AGE PATIENTS n=30 N / %
Gram-positive bacteria		
Enterococcus faecalis	**3 / 10**	**5 / 16.6**
Enterococcus faecium	**2 / 6.6**	**0**
Staphylococcus epidermidis	**0**	**3 / 10**
Staphylococcus aureus	**2 / 6.6**	**7 / 23.3**
Micrococcus luteus	**0**	**1 / 3.3**
Bacillus sp	**0**	**1 / 3.3**
Gram-negative bacteria		
Enterobacter cloacae	**0**	**1 / 3.3**
Escherichia coli	**2 / 6.6**	**4 / 13.3**
Klebsiella oxytoca	**0**	**1 / 3.3**
Klebsiella pneumoniae	**0**	**1 / 3.3**
Pseudomonas aeruginosa	**0**	**3 / 10**
Yeast-like fungi		
Candida albicans group	**3 / 10**	**7 / 23.3**
Parasitic protozoans		
Entamoeba gingivalis	**3 / 10**	**3 / 10**
Trichomonas tenax	**2 / 6.6**	**7 / 23.3**
Amphizoic amoebae		
Acanthamoeba spp.	**2 / 6.6**	**4 / 13.3**

N / % = *number/percent of patients in whom the microorganisms have been identified*

In some patients, live protozoans were found between the oral epithelial cells and blood cells. Flagellates moving relatively fast, were identified based on their morphophysiology (nucleus, axostyle, four free flagella, the fifth flagellum associated with undulating membrane, 10–15 μm long and 5–11 μm wide, with vacuoles containing multiplying bacteria) as *Trichomonas tenax* (Fig. 1 and Fig. 2).

The moderately active amoebae (with characteristic ectoplasm, blunt pseudopodia, measured 5–15 μm × 20–35 μm, with vacuoles containing bacteria and erythrocytes, detected mainly in dental pockets) were identified as *Entamoeba gingivalis* (Fig. 3).

In the contrast-phase microscope (100 × and 400 ×), in the samples deriving from oral cavity of several young and mid–age persons, some rounded or polygonal in shape forms were found. The double-walled objects, ~10–20 μm, were identified as cysts of protozoans – amphizoic *Acanthamoeba* spp. (Fig. 4). Some of these facultative parasites were alive in physiological salt solution; after excystation, they were transforming into trophozoites.

Prevalences of different protozoans found in mouth microbiome are presented in Table 1.

Of the total number of 15 oral microorganisms compared, their prevalence was seen in 13 cases greater in the group of middle-age patients than in the young group, in 1 case greater in the young group, and in one case equal in both groups (Tab. 1). However, in all cases, the p-values obtained by Fisher's exact test and t-test were greater than 0.05, and were considered as not significant.

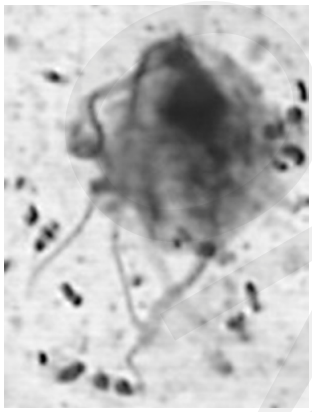

Figure 1. Micrograph of live Trichomonas tenax among bacteria detected in a swab from the periodontium

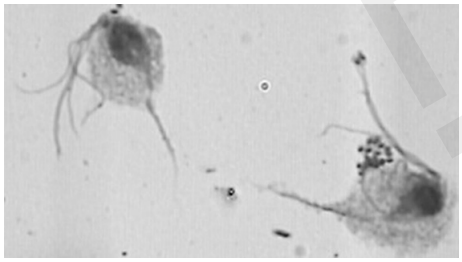

Figure 2. Trichomonas tenax trophozoites in saliva; axostyle, nucleus and flagella are visible

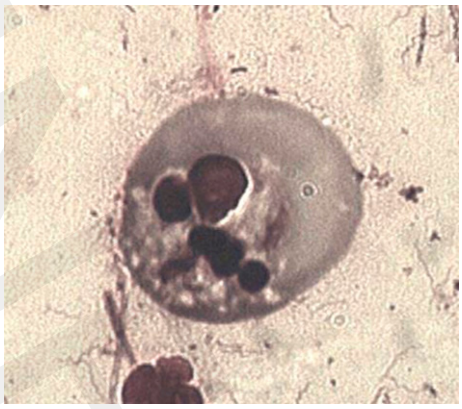

Figure 3. Micrograph of Entamoeba gingivalis with vacuoles; dental pocket swab; Giemsa stained

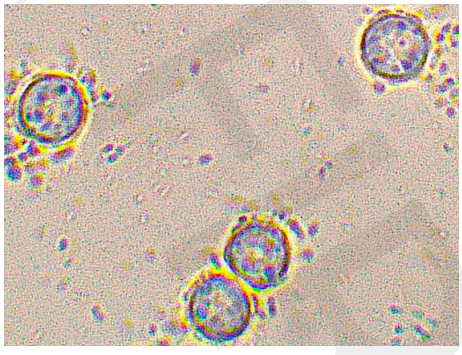

Figure 4. Micrograph of double-walled cysts of protozoans Acanthamoeba spp. detected in periodontal swab; cultured in BSC axenic medium

DISCUSSION

In the presented study, the oral cavities of Polish patients from different populations were assessed in terms of microbiome component diversity.

To date, the relationship between intraoral microbiota not belonging to the resident species, colonizing the human organism with various local and systemic disorders, has not received enough attention and has remained a rare subject of research. Simultaneously, some studies revealed that various metabolic disabilities had a different influence on oral cavity status, including species diversity of the mouth microbiome [14, 19, 23, 25–28].

In the present study, results of microscopic examinations of samples obtained from oral cavity swabs and *in vitro* tests, showed that in the superficial layer of periodontal biofilm and in the dental pockets of the patient groups analyzed, various microbiota belonging to different genera, species and strains of bacteria, fungi and protozoans, were detected. Special emphasis should be placed on the clear diversity in species composition of the oral cavity microbiome. Valuable data were obtained on some microorganisms – co-occurring in the polymicrobial communities – infectious species, potential factors of serious human health risk [14, 15, 20].

According to literature [25–28], the technological development and large-scale genome analysis increase possibilities for development of the knowledge about microorganisms that can colonize the human oral cavity. 'Studies involving the oral microbiota metagenomic projects like the HMP' (Human Microbiome Project), 'have confirmed that the oral cavity is one of the most taxonomically diverse body sites....', as has been emphasized by Lunsford et al. [29] from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA.

In the current study, different microorganisms were identified as the components of mouth microbiome present in the oral cavity, with some species posing potential risk factor of human infections. Among the microorganisms assessed in this study, there occurred *Acanthamoeba* spp., free-living amoebae, common in natural and in man-made habitats worldwide [24–28]. The protists occur in air, soil, dust and are found on the surface of equipment in the dental unit as well as in aquatic habitats, such as tap water, and a chlorinated swimming pool. These amoebae complete their life cycle in external environments without entering humans. However, in some circumstances the free-living amoebae are able to enter the human organism, multiply therein and exist as parasites. The *Acanthamoeba* pathogenic strains present a serious risk for human health as the causative agents of the *Acanthamoeba* keratitis. This vision-threatening corneal infection is non-opportunistic disease that affects immune-competent persons [21, 24–30].

In the presented study, Gram-positive bacteria *Enterococcus faecalis, E. faecium*, *Staphylococcus epidermidis, S. aureus,* as well as the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa,* occurred between microbiota of oral cavities in both groups of patients.

Gram-positive faecal enterococci *E. faecalis* and *E. faecium* may act as opportunistic agents of serious life-threatening infections especially dangerous for immunologically-compromised and elderly persons. Certain strains of the bacteria were frequently isolated in nosocomial diseases, e.g. pneumonia and urinary tract infections.

S. *aureus* strains, especially methicillin-resistant strains (MRSA) participating in the biofilm formation, disseminated to different distant sites, may cause severe complications resulting in difficult to treat diseases, e.g., those associated with surgical procedures, pneumonia and endocarditis. Some *S. aureus* strains are present in the human environment, which facilitates the asymptomatic carrying the vestibule of the nose or in the throat; bacteria spread easily from the place of colonization in the mouth, causing abscesses, very serious systemic infections, endodontic infections, osteonecrosis of the jaw, and infection of salivary glands.

Escherichia coli enteropathogenic, enterotoxic and enterohaemorrhagic strains are the causative agents of nosocomial infections and sepsis. *Pseudomonas aeruginosa* are known for their epidemiological role; the bacteria are particularly dangerous for immunocompromised persons, and have also been detected in the oral cavities of patients with systemic diseases.

Yeast-like fungi of the *Candida albicans* group are widespread in the human environment; the most-common species are *C. albicans*, *C. glabrata*, *C. tropicalis*. Different problems are mentioned as influencing the invasions, among others, the impaired immunological defence mechanisms [4, 6, 14, 22]. The fungi may infect human host tissues and organs via the skin, damaged mucous membranes, inhalation or ingestion, and co-occur in oral microbiome. Components of the fungal cell wall and products released by cells can inhibit an immune response, which is a serious health risk, even in people with an efficient immune system. It has been recently considered that yeast-like fungi are particularly prone to produce biofilms and that this ability is an important virulence factor of these pathogens. Improper oral cavity hygiene, micro-damage, presence of foreign bodies, and acrylic dental prosthesis attached to the mucous membrane and affecting its surface, are mentioned as the factors that favour infection of the oral cavity by the fungi, and the development of candidosis.

The oral protozoans *Trichomonas tenax* and *Entamoeba gingivalis* do not belong to the resident microorganisms [4, 14, 26, 27] and were rarely taken into account in clinical investigations. Nevertheless, they can have a pathogenic impact on oral structures. The species are more frequently detected in older persons, colonizing the oral cavity and may result in periodontitis. Flagellate *T. tenax* was detected in sinusitis, in the contents of lung abscesses, pleural effusion, and in people with systemic diseases undergoing immunosuppression. Among others, the fibronectin-like protein and collagenolytic effect of trichomonads, as well as oral amoeba activity in pathogenic dental pockets and inflammatory gingivitis, are underscored.

Amphizoic amoebae *Acanthamoeba* spp. cysts, which pose a threat to the human health, were cultured *in vitro* under axenic condition with the use of the Bacto Casitone, Difco, BSC [14, 26]. The protozoans concomitant with oral cavity microbiota showed the ability to transform into live trophozoite forms. It is known that facultative parasitic *Acanthamoeba* spp. tolerate various conditions in natural and man-made environments [14, 26, 30]. They grow in different culture media, e.g., non-nutrient agar plates seeded with *Escherichia coli*, the most commonly used medium peptone–yeast extract–glucose medium PYG, Bacto Casitone, Difco. The current study used mainly BSC – axenic medium that does not contain any external live food organisms suitable for the *in vitro* cultivation of *Acanthamoeba* strains.

CONCLUSIONS

In this interdisciplinary, qualitative study the composition of intraoral microbiome of overall healthy treated conservatively Polish patients was assessed in terms of threats to human health with infectious factors. The material from dental plaques, periodontium and dental pockets was examined directly microscopically and with use of appropriate selective culture media by *in vitro*_techniques. Different microorganisms have been detected and identified in the material from oral cavities colonized by polymicrobial communities.

The opportunistic and pathogenic bacteria and fungi, the parasitic oral protists and amphizoic amoebic strains – facultative parasites, with their endosymbionts all detected in the present study – are able to disseminate from the oral cavity to neighbouring structures and other organs. For this reason, it should be taken into account that components of the mouth microbiome may pose a clinical important threat for human health as the reservoir of potentially infectious agents. The results of this study show the need to expand knowledge on the diversity of human oral microbiome and a greater awareness of the importance of this medical problem.

Early identification of microorganisms occurring in the oral cavity, preventive elimination of concomitant potentially infectious strains, and the monitoring of microbiome composition during treatment are highly recommended to avoid severe health deterioration. Further quality research on the intraoral microbiome species that may pose severe local/general clinical diseases is needed to reduce the risk to human health.

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