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Original Paper

MICROBIOLOGICAL EXAMINATION OF CLINICAL MATERIAL IN HALITOSIS PATIENTS

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*Regarding oral biofilm aspects, there has been strong evidence for a microbiotic component in the aetiology of halitosis. Many oral microbiota have protheolytic and putative activity, but there have been no studies investigating the association of microbiota in oral biofilms with halitosis. The objective of this study was to determine species of oral microbiota in the periodontal area and dorsal part of tongue biofilm, and how their quantitative amounts differ in halitosis patients. The clinical bacterial material from halitosis patients (altogether 98 persons, volatile sulphur compounds (VSC) on average 380 ppb) was taken from periodontal pockets and the dorsal part of the tongue for microbiological diagnostics of anaerobic bacteria, using polymerase chain reaction (PCR) tests for the comparison of bacterial quantity. The study showed the primary aetiology factors of halitosis in Latvia, and offers possible versions of microbiological diagnostics of halitosis. Even though the examination of halitosis patients and determination of VSC using a halimeter is technically simpler and cheaper, the determination of aetiological factors and their combinations using microbiological examination of clinical material with PCR tests are more precise. A characteristic ecological niche of anaerobic bacteria is not only the anaerobic environment of periodontal pockets, but also the microbiota of the dorsal part of the tongue. Additionally, some anaerobic bacteria species (*Porohyromonas gingivalis, Tannerella forsythia, Treponema denticola, Prevotella intermedia*) in larger amounts are found on the microbiome of the tongue. Therefore, it is advisable to begin microbiological diagnostics in halitosis patients with quantitative diagnostics of* Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola *on the biofilm of the tongue coating.*

Keywords: *oral biofilm, halitosis, periodontal pockets, anaerobic bacteria, volatile sulphur compounds.*

INTRODUCTION

An increasing number of people in the world are paying more and more attention to the qualitative solutions of their health issues. Patients wish to receive qualitative treatment and a positive result. Nowadays, modern illness diagnostic methods and treatment possibilities are developing, which offer faster and more comfortable and qualitative results. It is very important to pay attention to the laboratory diagnostics of oral cavity pathologies. General oral biofilm studies are very important, as well as the diagnostics of various species. WHO data show that approximately half of people over the age of 65 have at least one pathology of the mucous membrane in the oral cavity (Vigild, 1987; Triantos, 2005; Wibert *et al*., 2020). Bad breath complaints are very common. Bad breath may be due to physiological and/or pathological causes of oral, systemic or nasopharyngeal origin, the principal cause of halitosis being of physiological origin involving the oral cavity (Espinosa *et al.,* 2003; Dundar and Kal, 2007; Dwivedi *et al.*, 2019). The diagnostics used in the prophylaxis and treatment methods of oral cavity illnesses like caries and various periodontic illnesses

still have achieved little in decreasing incidence (Boutaga *et al*., 2003; Thaweboon S. and Thaweboon B., 2011).

Oral cavity illnesses are directly associated with changes in the oral biofilm (Veloo *et al.,* 2011). Microorganisms found in great numbers in these biofilms are associated with the downgrading of molecular proteins in the inflamed focal points in the oral cavity, which starts with local disintegration of proteins in inflamed focal points in the oral cavity. If the inflammation is not treated, the spread of microorganisms may take place not only in the inflamed tissues, but also surrounding tissues with a suitable environment — an anaerobic environment (Balatsouras *et al*., 2004; Vellin *et al*., 2008). Deep anatomic formations, deep wrinkles on the tongue and plaque on the dorsal part of the tongue as well as chronic and inflamed focal points in the soft tissue of the oral cavity promote the uncontrolled avalanche-like spread of anaerobic bacteria. The oral microbes involved in VSC (volatile sulfur compounds) production have been identified as *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Porphyromonas endodontalis, Prevotella loeschii, Treponema denticola, Enterobacter cloacae*, and many others have been associated with the production of malodorous gas (Kamma *et al*., 2004; Kim and Amar, 2006; Sedlacek and Walker, 2007). Asaccharolytic species use proteins, peptides, or amino acids as their energy source, whereas some others, such as *Prevotella intermedia* or *Fusobacterium nucleatum* can use both carbohydrates and proteins (Del Pozo *et al.,* 2008; Gürlek *et al*., 2017).

The study aimed to determine the primary aetiological factors for halitosis in Latvia, giving possible options for microbiological diagnostics of halitosis. As part of the study, we plan to promote the laboratory study of the biofilm of the mouth and diagnostic methods with various oral cavity illnesses, ensuring timely, adequate and qualitative diagnostics of oral cavity illnesses, as well as to ensure technically wholesome and practically more easily usable diagnostic method application in the treatment process.

MATERIALS AND METHODS

Sample selection. A total of 258 randomly selected people who went to a dental clinic from 2003 until 2018 and complained about bad breath were asked to participate in the study "Bad Breath Diagnostics". A halitosis patient group (n = 98) was formed as part of the study, which included patients that had a distinctive tongue coating. They were selected randomly, including various age and sex groups. As part of the study we determined VSC production amount by a halimeter or portable sulphide monitor (Interscan Cooporation, Model RH-17E), and examined persons, whose VSC halimetric measurements were ≥ 100 ppb (parts per billion), or halitosis patients. This study did not have a control group. We planned to acquire clinical material for the comparative quantitative analyses from only those individuals whose VSC ≥ 100 ppb, and who had subjective complains for bad breath, to form a halitosis patient group with a high level of VSC. Patients were not included in the study if they had taken chronic anti-inflammatory drugs and antibiotics in the last three months, or had a history of any disease known to compromise their immune function. A total of 258 persons were examined with a halimeter, but only 98 of halitosis patients had a distinctive tongue coating. Therefore, a comparative diagnostic of anaerobic bacteria in periodontal pockets and on the biofilm of the dorsal part of the tongue was carried out on 98 halitosis patients. VSC halimetric measurements and clinical samples of both oral biofilms were obtained from all of these 98 halitosis patients. The average patient age was 51.2 years.

PCR test of bacterial material. The clinical bacterial material from halitosis patients (altogether 98, both sexes, VSC on average 380 ppb) was taken from periodontal pockets and the dorsal part of the tongue. Polymerase chain (PCR) was used to determine the bacterial community. This study did not have a control group. Clinical material from periodontal pockets was collected with five sterile paper points from the five deepest periodontal pockets of each halitosis patient and placed in one sterile Eppendorf test tube. Clinical material from the dorsal part of the tongue was taken with a sterile tongue cleaner and placed in a separate Eppendorf test tubes for each halitosis patients. For further bacterial quantitative examination we used PCR (micro-IDent®, Hain Lifescience, Germany) according to manufacturer instructions
(https://www.hain-lifescience.de/en/instructions-for-use.html; IFU-232-09 for combined identification of periopathogenic bacterial species micro-Ident). For this study we chose the main proteolytic pathogenic bacterial species of the periopathogens (Brogden and Guthmiller, 2002).

The micro-IDent® test is based on the DNA strip technology. The procedure is divided into three steps: DNA extraction from patient samples, two separate multiplex amplification reactions with biotinylated primers, and combined reverse hybridisation. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single stranded amplicons bind to the probes. Highly specific binding of complementary DNA strands is ensured by stringent conditions that result from the combination of buffer composition and specific temperature. Thus, the probes reliably discriminate the different sequences of the bacterial species. The streptavidin-conjugated alkaline phosphatase binds to the amplicons biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a coloured precipitate. A template ensures the interpretation of the banding pattern obtained (Santigli *et al*., 2016). The specificity of the micro-IDent® test is ensured by the accurate design of specific primers and probes, which considers, among others, homology comparisons of the sequences published in gene databases, and by stringent reaction conditions. The analytical specificity was determined with 32 DNA isolates from cultures or purified genomic DNA. The study included the following strains detectable with the micro-IDent®: 5 *Aggregatibacter actinomycetemcomitans*

strains (1 serotype a, 2 serotype b, 2 serotype c), *Porphyromonas gingivalis,* 2 *Prevotella intermedia* strains, and *Tannerella forsythia, Treponema denticola (*Leys *et al*., 1999).

For determination of analytical sensitivity, dilution series of plasmids containing the target sequences detectable with the micro-IDent® test were used. The following detection limits were determined: *Aggregatibacter actinomycetemcomitans* 50–100 genome equivalents/PCR or 1000–5000 genome equivalents per sample, all other species 500–1000 genome equivalents/PCR or 10 000–50 000 genome equivalents per sample.

Statistical analysis. Data acquired in the study was recorded in the study protocol specifically designed for this purpose, as well as in an electronic database. Qualitative and quantitative variable data acquired in the study were processed with descriptive statistical methods. When describing correlation scale variable distribution (central tendency, dispersion), we used the arithmetic mean and standard deviation. SPSS 17 for Windows (SPSS Chicago, IL) was used for statistical analysis.

RESULTS

Quantities of anaerobic bacteria from the gingival pockets and dorsal part of the tongue in halitosis patients differ. The amount of anaerobic bacteria in samples from the dorsal part of the tongue was significantly higher than that in periodontal pockets, however, not in the cases of all five species of anaerobic bacteria — *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola*.

Data on of *A. actinomycetemcomitans* bacterial concentration are shown in Figure 1. The most common concentration of *A. actinomycetemcomitans* in samples from the dorsal part of the tongue was 10^3 equivalent per sample, whereas in the periodontal pocket material the concentration was 10^4 equivalents per sample. Thus, *A. actinomycetemcomitans* had greater concentration in periodontal pockets rather than on tongue coating. Even with 10^1 equivalents per sample concentration of *A. actinomycetemcomitans* on tongue coating, the quantity of this microorganism in periodontal pockets was 10^4 equivalents per sample. Only in two of the examined persons, a 10^1 equivalent per sample *A. actinomycetemcomitans* concentration on tongue coating significantly differed from the bacterial concentration in periodontal pockets. Of these, one person had an *A. actinomycetemcomitans* concentration in the periodontal biofilm that was as low as in the biofilm of tongue coating (10^1 equivalent per sample), whereas another person had a significantly greater concentration of these bacteria in the periodontal film. At a concentration of 10^4 equivalents per sample of *A. actinomycetemcomitans* on tongue coating, the concentration of this microorganism in periodontal pockets was also 10^4 equivalents per sample; thus, the concentration of *A. actinomycetemcomitans* was similar.

Data on of *P. gingivalis* concentration are shown in Figure 2. The most common concentration of *P. gingivalis* in samples from the dorsal part of the tongue was 10^7 equivalents per sample, whereas in the clinical material of periodontal pockets the concentration was 10^5 equivalents per sample. In halitosis patients, the concentration of *P. gingivalis* was 10^6 equivalents per sample from the dorsal part of the tongue, and in in periodontal pocket samples — 10^5 equivalents per sample. At a concentration of 10^5 equivalents per sample of *P. gingivalis* on tongue coating, the concentration of this microorganism in periodontal pockets was also 10^5 equivalents per sample.

Data on of *T. forsythia* concentration are shown in Figure 3. A few samples had small (10^4 equivalents per sample) *T. forsythia* concentrations in both examined biofilms of the mouth. Larger concentrations (10^6 equivalents per sample

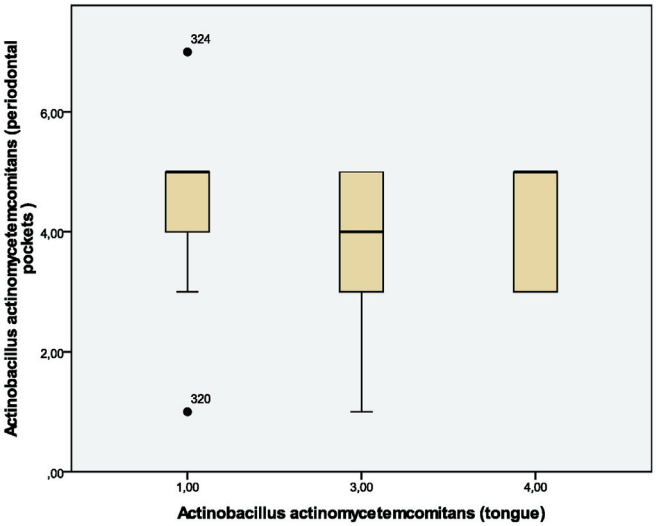

Fig. 1. Distribution of *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) equivalents per sample in periodontal pockets (vertically) and tongue (horizontally) biofilm polymerase chain (PCR) samples of halitosis patients (extremely high and low tongue concentration were not deleted).

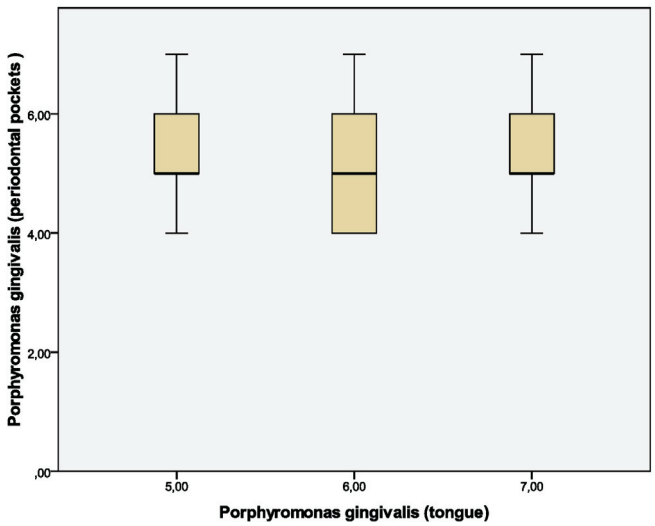

Fig. 2. Distribution of *Porphyromonas gingivalis* equivalents per sample in periodontal pockets (vertically) and tongue (horizontally) biofilm PCR samples of halitosis patients.

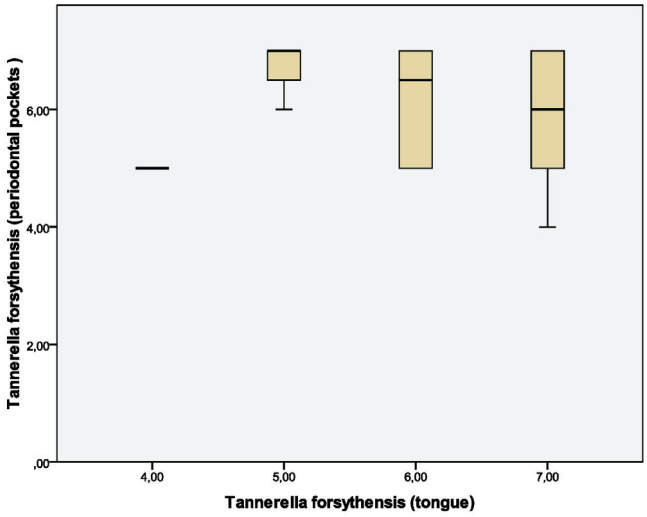

Fig. 3. Distribution of *Tannerella forsythia* (previously *Tannerella forsythensis*) equivalents per sample in periodontal pockets (vertically) and tongue (horizontally) biofilm PCR samples of halitosis patients.

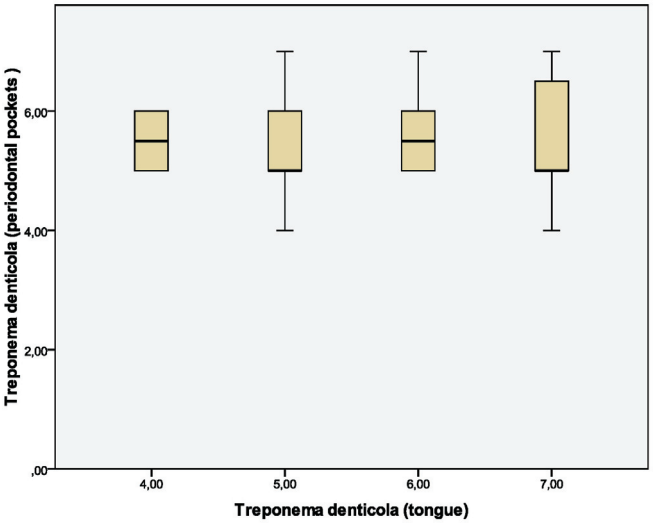

Fig. 4. Distribution of *Treponema denticola* equivalents per sample in periodontal pockets (vertically) and tongue (horizontally) biofilm PCR samples of halitosis patients.

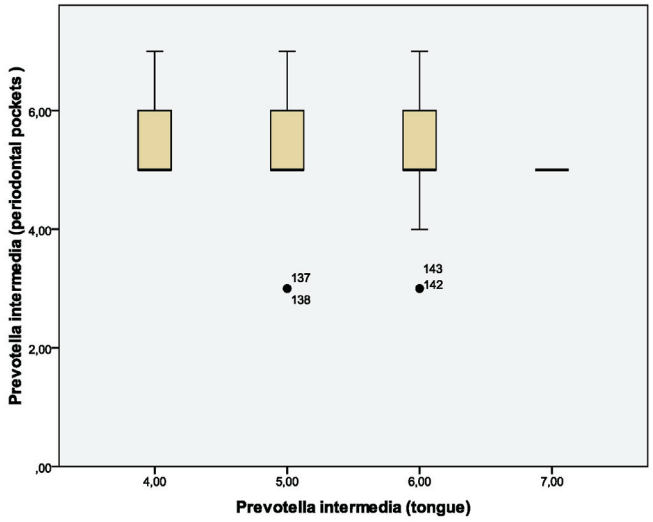

Fig. 5. Distribution of *Prevotella intermedia* equivalents per sample in periodontal pockets (vertically) and tongue (horizontally) biofilm PCR samples of halitosis patients (extremely low tongue concentrations were not deleted).

and 10^7 equivalents per sample) were more characteristic of this bacteria in both periodontal pockets and dorsal part tongue coating. The most common concentration of *T. forsythia* in samples from the dorsal part of the tongue was 10^7 equivalents per sample, whereas in the periodontal pockets the concentration was 10^6 equivalents per sample. In halitosis patients, when the concentration of *T. forsythia* was 10^6 equivalents per sample from the dorsal part of the tongue, in periodontal pocket samples the concentration was 10^6 equivalent per sample. For those patients whose *T. forsythia* concentrations in samples from the dorsal part of the tongue was 10^5 equivalents per sample, in the periodontal pocket material the concentration was 10^6 equivalents per sample.

Data on of *T. denticola* concentration are shown in Figure 4. The most common concentration of *T. denticola* in samples from the dorsal part of the tongue was 10^7 equivalents per sample, whereas in the periodontal pocket the concentration was 10^5 equivalents per sample. In halitosis patients, when the *T. denticola* concentration was 10^6 equivalents per sample from the dorsal part of the tongue, in the periodontal pocket, the concentration also was 10^5 equivalents per sample. For those patients whose *T. denticola* concentration in samples from the dorsal part of the tongue was 10^5 equivalents per sample, the concentration in periodontal pocket material was 10^5 equivalents per sample. In halitosis patients, when the concentration of *T. denticola* was 10^4 equivalents per sample from the dorsal part of the tongue samples, the concentration in periodontal pocket samples was 10^5 equivalent per sample. Generally, concentration of *T. denticola* in periodontal pockets was similar (about 10^5 equivalents per sample) at increasing bacterial concentration in clinical samples from the dorsal part of the tongue.

P. intermedia concentration in samples is shown in Figure 5. The most common concentration of *P. intermedia* in samples from the dorsal part of the tongue was 10^6 equivalents per sample, whereas in the periodontal pocket material — 10^4 equivalents per sample. In halitosis patients whose clinical material from the dorsal part of the tongue samples had a concentration of *P. intermedia* equivalent per sample, the concentration in periodontal pocket material was 10^4 equivalent per sample. For those patients whose *P. intermedia* concentration in samples from the dorsal part of the tongue was 10^4 equivalent per sample, the concentration in periodontal pocket samples was also 10^4 equivalent per sample. Thus, the concentration of *P. intermedia* in periodontal pockets was similar (10^4 equivalent per sample on average) at increasing bacterial concentration in clinical samples from the dorsal part of the tongue.

DISCUSSION

The severity of halitosis is affected from periodontal conditions, and also periodontal conditions are affected by halitosis. Besides periodontal conditions, untreated deep carious lesions also create a retention area for proteolytic bacterial

biofilm and may cause halitosis. Another important factor in halitosis is the flow of saliva. The amount of sulphur compounds is increased when salivary flow is low (Nieminen *et al*., 2018). Hundreds of oral bacteria may be attached to an epithelial cell desquamated from the tongue dorsum, whereas some bacteria are attached to each cell in other areas of oral mucosa. Only about ten proteolytic bacterial species are the main source of volatile sulphur compounds production (Dinesh *et al*., 2014). The presence of proteolytic bacteria and possibility of oral cell attachment are the determining factors in the aetiology of halitosis.

A. actinomycetemcomitans was diagnosed in greater proportion in periodontal pockets. Generally, all observed *A. actinomycetemcomitans* bacterial concentrations had significantly greater proportion in periodontal pockets, with 10 to 1000 times lower amounts on tongue coating. Thus, we can conclude that *A. actinomycetemcomitans* is more characteristic of biofilm of periodontal pockets, and to a lesser extent these glycolytic bacteria may be found on the dorsal part of the tongue. Other oral anaerobic bacteria can occur in greater concentration in the dorsal part of the tongue. In the studies carried out so far regarding the microbiological diagnosis of periodontal pockets, similar results have been found. In the present study, the concentration of *A. actinomycetemcomitans* in periodontal pockets was correlated with clinical parameters of periodontal pocket depth, and the mean concentration of *A. actinomycetemcomitans* was 10^4 equivalents per sample.

Even though it is technologically much simpler and cheaper to examine halitosis patients with the help of a halimeter, aetiological factors and their combinations are more easily determined using microbiological examination of the clinical material with PCR tests. A characteristic ecological niche of anaerobic bacteria is the biofilm of the dorsal part of the tongue and not just the environment of periodontal pockets. It should also be noted that some species of anaerobic bacteria are found in greater numbers on the biofilm of the tongue. It is very important to choose a diagnostically more sensitive spot for the collection of a clinical material in halitosis patients, as the highest concentration of protheolytic anaerobic bacteria are on the biofilm at the dorsal part of the tongue. Essentially, any oral site in which microbial accumulation and putrefaction can occur may be an origin for oral malodor (Krespi *et al*., 2006). VSC are produced by *P. gingivalis* from sulphur amino acids (Bollen and Beikler, 2012) in the artificial saliva. Some researchers have found that intraoral bacteria (*P. gingivalis, P. intermedia, T. forsythia*) metabolise desquamated epithelial cells and blood cells, leading to the production of VSC from cysteine and methionine and thus increasing the VSC values (Migliario and Rimondini, 2011).

CONCLUSIONS

Although even nowadays we still use organoleptic subjective diagnostic methods (e.g., "nose" method, palm smelling) in the diagnostics of halitosis, objective diagnostic methods — halimeter readings and quantitative examination of microbiological biofilm are being used more often. The quantitative determination of anaerobic bacteria with PCR is a precise, modern method, which is primarily used in the determination of microbiota of periodontal pockets prior to the specification of diagnostics and during treatment. In this study we carried out the comparison of quantitative diagnostics of five species of anaerobic bacteria in periodontal and tongue biofilm in halitosis patients with high halimetric measurements — 380 ppb. Only one oral bacteria, *A. actinomycetemcomitans,* occurred in greater amount in periodontal pockets than in tongue coating. The anaerobic bacteria species *P. gingivalis, P. intermedia, T. forsythia,* and *T. denticola* occurred in greater amounts on tongue coating of examined material of halitosis patients than in periodontal biofilm. Therefore, it is advisable to start the microbiological diagnostics in halitosis patients with quantitative diagnostics by PCR testing of *P. gingivalis, P. intermedia, T. forsythia, T. denticola* on the biofilm of the tongue coating.

ETHICS

The patients were selected for study according to the norms of the Ethical Committee of the Ministry of Welfare of Latvia (No. A-19, 10.08.2000). Prior to registration of halimetric measurements, patients were introduced with the study instructions regarding the history of their use in medicine.

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HALITOZES PACIENTU KLĪNISKĀ MATERIĀLA MIKROBIOLOĢISKA IZMEKLĒŠANA

Mutes biofilmu mikrobioloģiskajiem komponentiem ir būtiska loma disbiotisku attiecību veidošanā mutes dobumā, kas ir halitozes etioloģijas pamatā. Proteolītiskas īpašības piemīt daudziem orālajiem mikroorganismiem, bet nav pētījumu par noteiktu mutes biofilmu mikroorganismu saistību ar halitozi. Šī pētījuma ietvaros tika noteiktas baktērijas periodonta kabatu un mēles mugurējās daļas biofilmās, kā arī to skaitliskā daudzuma variācijas halitozes pacientiem. Halitozes pacientu klīniskais bakteriālais materiāls (kopumā 98 personas, gaistošo sēra savienojumu (GSS) daudzums vidēji 380 ppb) tika savākts no periodonta kabatām un mēles mugurējās daļas biofilmām anaerobo baktēriju kvantitatīvai mikrobioloģiskai diagnostikai ar polimerāzes ķēdes reakciju (PĶR). Pētījums uzrāda halitozes primāros etioloģiskos faktorus Latvijā un piedāvā iespējamos variantus halitozes mērķtiecīgai un efektīvai diagnostikai. Kaut arī halitozes pacientu izmeklēšana un GSS noteikšana ar halimetru ir tehniski vienkāršāka un lētāka, tomēr etioloģisko faktoru un to kombināciju noteikšana ir daudz precīzāka, izmantojot klīniskā materiāla mikrobioloģisku izmeklēšanu ar PĶR. Anaerobo baktēriju raksturīgā ekoloģiskā atrašanās vieta nav tikai periodonta kabatu vide, bet arī mēles mugurējās daļas biofilma. Pie tam dažas baktēriju sugas (*Porohyromonas gingivalis, Tannerella forsythia, Treponema denticola, Prevotella intermedia*) lielākā daudzumā var atrast tieši mēles biofilmu paraugos. Halitozes pacientu mikrobioloģisku diagnostiku ieteicams sākt ar *P. gingivalis, T. forsythia, T. denticola* un *P. intermedia* kvantitatīvu diagnostiku mēles mugurējās daļas biofilmā.