ASSOCIATION BETWEEN THE *NANOSYNBACTER LYTICUS* EPIBIOTIC BACTERIA AND INFLAMMATORY PERIODONTAL DISEASES

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The inflammatory periodontal disease pathogenesis is determined by microorganisms of the oral cavity. Along with the well-studied periodontopathogens, microbial agents of unproven clinical significance, including epibiotic bacteria, are found in individuals with gingivitis and periodontitis. The study aimed to determine the association between the N*anosynbacter lyticus* epibiont and inflammatory periodontal diseases. Conservative DNA sequences specific for the genera *Nanosynbacter*, *Schaalia* and the *Bacteria* domain were identified using PCR in 47 study participants (31 females and 16 males) aged 18–45 years. The results were expressed as indices determining the quantitative relationships between *N. lyticus* and *Schaalia spp*. (NS index), as well as between *N. lyticus* and representatives of the *Bacteria* domain (NB index). *Schaalia spp*. were not found in a large share (11/27, 40.7%) of patients with no periodontitis. All patients with moderate-to-severe periodontitis, as well as 75% of patients with mild periodontitis were carriers of *Schaalia spp*. All the *Schaalia*-positive samples from patients with periodontitis showed higher NS indices ($p < 0.05$) compared to *Schaalia*-positive samples from patients with no periodontitis: the median NS values were Me = 0.89 (0.79; 0.93) and Me = 0.63 (0.00; 0.73), respectively. The patients suffering from chronic generalized periodontitis had significantly higher NB indices (Me = 0.83 (0.79; 0.85)) ($p < 0.05$) compared to patients with no periodontitis, Me = 0.67 (0.00; 0.81).

Keywords: gingivitis, periodontitis, bacteria, epibionts, *Nanosynbacter lyticus*, *Schaalia odontolytica*

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ВЗАИМОСВЯЗЬ МЕЖДУ БАКТЕРИЯМИ-ЭПИБИОНТАМИ *NANOSYNBACTER LYTICUS* И ВОСПАЛИТЕЛЬНЫМИ ЗАБОЛЕВАНИЯМИ ПАРОДОНТА

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Патогенез воспалительных заболеваний пародонта детерминируется микроорганизмами полости рта. Кроме хорошо изученных пародонтопатогенов, при гингивите и пародонтите обнаруживаются микробные агенты с недоказанным клиническим значением, к числу которых принадлежат бактерии-эпибионты. Целью работы было определить взаимосвязь между присутствием эпибионта *Nanosynbacter lyticus* и воспалительными заболеваниями пародонта. У 47 участников исследования (среди них 31 женщина и 16 мужчин) в возрасте 18–45 лет с помощью ПЦР определяли наличие консервативных последовательностей ДНК, специфичных для родов N*anosynbacter, Schaalia*, а также домена *Bacteria*. Результаты выражали в виде индексов, определяющих количественные соотношения между *N. lyticus* и *Schaalia spp*. (индекс NS), а также между *N. lyticus* и представителями домена *Bacteria* (индекс NB). У значительной доли (11/27, 40,7%) пациентов без пародонтита *Schaalia spp*. не были обнаружены. Все пациенты со средней и тяжелой степенью пародонтита, а также 75% пациентов с легкой степенью пародонтита были носителями *Schaalia spp*. Для всех *Schaalia*-позитивных образцов от пациентов с пародонтитом индекс NS был более высоким ($p < 0,05$), чем у *Schaalia*-позитивных образцов без пародонтита: медианы показателя NS составляли соответственно Me = 0,89 (0,79; 0,93) и Me = 0,63 (0,00; 0,73). Индекс NB у пациентов, страдающих хроническим генерализованным пародонтитом (Me = 0,83 (0,79; 0,85)), был достоверно выше ($p < 0,05$), чем у пациентов без пародонтита, Me = 0,67 (0,00; 0,81).

Ключевые слова: гингивит, пародонтит, бактерии, эпибионты, *Nanosynbacter lyticus*, *Schaalia odontolytica*

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Inflammatory periodontal diseases are among the most common diseases of the oral cavity. Severe forms of periodontitis are found in more than 700 million people all over the world [1]. Despite the fact that periodontitis is considered to be a multifaceted process, the key role of microorganisms in the development of periodontitis is indisputable. Contemporary metagenomic studies show that periodontitis is not associated with the presence of several specific periodontal pathogens, it results from polymicrobial synergy of dozens of microbial species [2, 3].

To date, pathogenetic significance of many representatives of the *Bacteria* domain is poorly understood. Along with typical periodontopathogens, which include *Porphyromonas gingivalis*, *Prevotella spp*., *Treponema denticola*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Filifactor alocis*, *Peptostreptococcus spp*., etc. [4–7], many resident bacteria with poorly understood properties inhabit the oral cavity. The group of nonculturable bacteria, specifically epibionts living in symbiosis on the surface of other bacteria, is

of special interest. *Nanosynbacter lyticus* is a typical example of epibiotic bacterium of the oral cavity being an episymbiont of the *Schaalia* odontolytica periodontopathogen (formerly known as *Actinomyces odontolyticus*) [8]. Currently, *N. lyticus* is the only species of the genus *Nanosynbacter*, the presence of which in the oral cavity has been confirmed by certain studies. There are very conflicting reports concerning the role of *N. lyticus* in pathogenesis. According to some reports, the number of *N. lyticus* is positively correlated to inflammatory diseases of the oral cavity: periodontitis, pericoronitis [9, 10]. Other authors have discovered the opposite: the increase in the number of *N. lyticus* is associated with reduction of inflammation due to suppression of bacterial pathogens [11].

The study aimed to determine the association between the presence of *N. lyticus* bacteria and inflammatory periodontal diseases.

METHODS

The study involved 47 people (31 females and 16 males). Inclusion criteria: individuals of both genders, age 18–45 years; no history of dental treatment within at least six months. Exclusion criteria: taking antibiotics or using oral antiseptics within the last three months; pregnancy, postpartum period; age under 18 and over 45 years; acute inflammatory disorder; exacerbation of chronic somatic disorder, decompensated somatic disorder; cancer, refusal to participate in the study. General information about the patients is provided in Table 1. The informed consent to participation in the study was submitted by all subjects.

The clinical part of the study included collection of complaints, history taking, and oral cavity examination. Diseases of hard dental tissues and periodontal tissues were diagnosed based on ICD-10 (K05.31 — chronic periodontitis, K05.10 — chronic gingivitis, K02.1 — dental caries), as well as based on the Periodontal Disease Classification System of the Russian Dental Association.

Among surveyed individuals, chronic generalized catarrhal gingivitis (K05.10) was revealed in 21.3% of cases, mild chronic generalized periodontitis (K05.31) in 8.5% of cases, moderate chronic generalized periodontitis (K05.31) in 17% of cases, severe chronic generalized periodontitis (K05.31) in 17% of cases. The share of individuals having no inflammatory periodontal diseases in the entire surveyed population was 36.2%.

Subgingival samples were collected from the gingival sulcus or periodontal pocket for laboratory testing. The samples were transported to the laboratory for DNA extraction in a cold state within 6 h.

The samples were incubated with the lysozyme solution (Sisce Research Laborataries; India) with the final concentration of 1 mg/m at 37 °C for 60 min prior to DNA extraction [12]. Genomic DNA was extracted from the samples using the SKYAmp Micro DNA kit (SkyGene; Russia). The extraction quality control was ensured using the Equalbit 1x dsDNA HS Assay Kit (Vazyme; China) and the Fluo-200 fluorometer (Allsheng; China).

The extracted DNA samples were analyzed by real-time polymerase chain reaction (PCR). Conservative DNA sequences specific for the genera *Nanosynbacter*, *Schaalia* and the Bacteria domain were identified using three pairs of primers and three probes in different fluorescence channels (Table 2).

Nuclease-free Water (New England BioLabs; USA) was used as a negative control. The positive controls used were represented by the following: 1) artificially synthesized oligo-DNA identical to the fragment of the *Nanosynbacter* 23S rRNA (this group of bacteria was earlier referred to as Saccharibacteria or TM7) constructed based on the sequences from the GenBank database [13]; 2) DNA of bacteria of the genus *Schaalia spp*. (formerly known as *Actinomyces spp*.); 3) mixture of bacterial DNA from the *Staphylococcus aureus* ATCC 29213; *Pseudomonas aeruginosa* ATCC 27853; *Escherichia coli* ATCC 25922 cultures.

PCR mixture composition: 10 μL of BioMaster HS-qPCR (HS-Taq DNA polymerase, mixture of dNTP, PCR buffer, Mg^{2+}, and sterile water) (Biolabmix; Russia); 2 μL of each specific primer (5 μM), 1 μL of specific probe (5 μM), 5 μL 0.1 ng/μL. The PCR mixture was prepared in accordance with the manufacturer's instructions. Reaction protocol: 5 min activation at 95 °C, then 35 cycles 15 s each at 94 °C, 15 s at 62 °C and 20 s at 72 °C. The reaction was carried out using the DT Prime thermal cycler (DNA-Technology; Russia).

To estimate the amount of DNA of each studied species, we determined the threshold cycle (Cp) value. Then we used Microsoft Excel 2010 tools to calculate the conditional indicators reflecting quantitative ratios of: 1) representatives of *Nanosynbacter* and the genus *Schaalia* (NS index) based on Cp values; 2) bacteria of the genus Nanosynbacter and the *Bacteria* domain (NB index) based on Cp values. The NS and NB values were calculated using the following formulae:

$$NS = \frac{Cp^{-1}_{Nanosynbacter} * 100}{Cp^{-1}_{positive\ control\ Nanosynbacter}} / \frac{Cp^{-1}_{Schaalia} * 100}{Cp^{-1}_{positive\ control\ Schaalia}}$$

$$NB = \frac{Cp^{-1}_{Nanosynbacter} * 100}{Cp^{-1}_{positive\ control\ Nanosynbacter}} / \frac{Cp^{-1}_{Bacteria} * 100}{Cp^{-1}_{positive\ control\ Bacteria}}$$

Statistical analysis of the results was performed using IBM SPSS Statistics for Windows, version 27.0 (IBM Corp.; USA).

RESULTS

When assessing 47 samples, no correlations between the NS and NB indices and the patients' age and gender were revealed. No nucleotide sequences specific for the genus *Schaalia* were found in 12 samples out of 47 (25.5%). All the *Schaalia*-negative samples were obtained from patients having no signs of moderate-to-severe periodontitis; only one *Schaalia*-negative sample was obtained from the patient with mild periodontitis. *N. lyticus* were found in 7 *Schaalia*-negative samples out of 12.

No *Schaalia spp*. were found in a large share (11/27, 40.7%) of patients having no periodontitis. All patients (100%) with moderate-to-severe periodontitis, as well as 3 patients with mild periodontitis out of 4 (75%) were carriers of *Schaalia spp*.

Table 1. Gender and age distribution of subjects

Total surveyed population, individuals	*n* = 47	
Average age of surveyed individuals, years	34.2 (± 8.36) (min 18 – max 45)	
Gender distribution of surveyed population, individuals	Males *n* = 16	Females *n* = 31
Average age of surveyed individuals depending on gender, years	34.8 (± 9.41) (min 20 – max 45)	32.6 (± 8.54) (min 18 – max 43)

Table 2. Primers and probes used in the study

	Oligonucleotide	*Nanosynbacter*	*Schaalia spp.*	Total bacterial DNA
Forward primer		5'-GGCTTATAGCGCCCAATAG-3'	5'- GGTCTCTGGGCCGTTACTGA -3'	5'-TCCTACGGGAGGCAGCAGT-3'
	Reverse primer	5'-CGGATATAAACCGAACTGTC-3'	5'-CCCCCACACCTAGTGCCC -3'	5'-GGACTACCAGGGTATCTAATCC TGTT-3'
	Probe	(FAM) –5'-CATAGACGGCGCTGTTTGGCAC-3'-(RTQ1)	(FAM)-5'-CGTGGGGAGCGAACAGGATTAGATACC-3'-(TAMRA)	(ROX)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(RTQ2)
	Reference	[14]	[15]	[16]

The NS index reflecting the ratio of the genus *Nanosynbacter* representatives and *Schaalia spp*. was zero in 8 samples out of 47 (17%); none of these samples was obtained from patient with periodontitis. High NS indices were reported for all *Schaalia*-positive samples from patients with periodontitis (K05.31): Me = 0.89 (0.79; 0.93), which is significantly higher ($p < 0.05$) compared to other Schaalia-positive samples, for which Me = 0.63 (0.00; 0.73) is reported.

Among 10 samples obtained from patients with chronic generalized catarrhal gingivitis, three samples contained no genetic markers specific for the genus *Schaalia* and one sample contained no *Nanosynbacter*-specific markers. That is why no positive correlation between the NS index values and the diagnosis of chronic generalized catarrhal gingivitis (K05.10) was revealed ($p > 0.05$).

More interesting results were obtained when assessing the NB index (see Figure). The NB indices of patients suffering from chronic generalized periodontitis (K05.31) (Me = 0.83 (0.79; 0.85)) were significantly higher ($p < 0.05$), than that of patients with no periodontitis (Me = 0.67 (0.00; 0.81)). The samples from patients with mild periodontitis showed the NB index values (Me = 0.77 (0.38; 0.78)) that were not significantly different ($p > 0.05$) from the NB values reported for the samples from patients with no periodontitis, but were significantly lower ($p < 0.05$), than the NB values reported for the samples from patients with moderate-to-severe chronic generalized periodontitis (Me 0.85 (0.81; 0.85) and Me 0.84 (0.81; 0.88), respectively). The NB values of patients with moderate-to-severe periodontitis showed no significant differences ($p > 0.05$).

No significant correlations of the NB index with other groups of patients (gingivitis, patients with no inflammatory diseases of the oral cavity) were identified.

DISCUSSION

The first interesting observation made when assessing the results is related to discrepancies in the presence of *N. lyticus* and *Schaalia spp*. bacteria in the test samples. This proves that *S. odontolytica* is not the only host of *N. lyticus*. The fact of the *N. lyticus* symbiosis with representatives of other taxons, including *Actinomyces oris*, *Fusobacterium nucleatum*, was predicted earlier [17–19].

The second finding confirms a negative contribution of the *Schaalia spp.* bacteria to pathogenesis of moderate-to-severe periodontitis. Actually, 100% of patients with moderate-to-severe chronic generalized periodontitis (K05.31) were carriers of *Schaalia spp*., while a large share of patients (40.7%) with no periodontitis were not carriers of this group of bacteria. This finding complements the literature data suggesting that *S. odontolytica* (formerly known as *A. odontolyticus*) actively forms biofilms in periodontal pockets, but is not a significant periodontopathogen [20].

The most important finding of our study is related to positive correlation between the NS index (showing the ratio between representatives of *Nanosynbacter* and *Schaalia spp*.) and the severity of chronic generalized periodontitis. The *S. odontolytica* virulence increase under the influence of the increasing number of *N. lyticus* epibionts secured on these bacteria can be the most logical explanation of this fact. A similar observation was made by other researchers, who reported increased biofilm formation by *A. odontolyticus* (now named *S. odontolytica*) induced by epibionts via regulators of the quorum sensing system [21].

An observation related to no relationship between the number of *Nanosynbacter* and chronic generalized gingivitis can be considered a useful result.

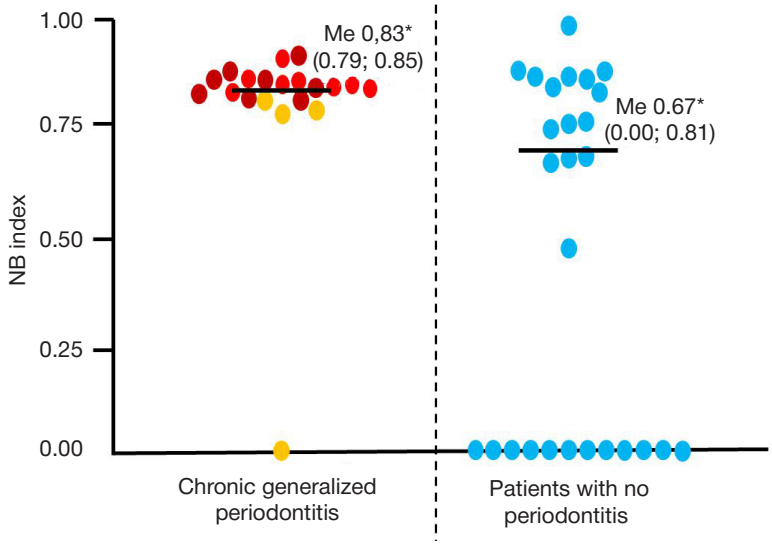

Fig. NB index showing the ratio of representatives of the genus *Nanosynbacter* and the *Bacteria* domain in patients with chronic generalized periodontitis and individuals with periodontitis. Mild periodontitis is highlighted in *orange*, moderate periodontitis is highlighted in *red*, severe form is highlighted in *maroon*. Me — median (lower and upper quartiles); * — significant differences between the values, $p < 0.05$

CONCLUSION

N. lyticus are likely to be symbionts of not only bacteria of the genus *Schaalia*, but also representatives of other taxons. Our findings foster a conversation about conducting a prospective study aimed to search for new *N. lyticus* hosts. Bacteria of the genus *Schaalia spp*. are involved in pathogenesis of chronic generalized forms of moderate-to-severe periodontitis. The NB index reflecting the ratio between representatives of the genus *Nanosynbacter* and the total number of bacteria increases in chronic generalized periodontitis, it can result from the increase in the virulence of pathogenetically significant bacteria under the influence of *N. lyticus* epibionts. The NS index reflecting the ratio between representatives of the genus *Nanosynbacter* and *Schaalia spp*. cannot be useful in terms of assessing the disease severity in patients with chronic generalized gingivitis.

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