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Bacterial Markers of Periodontal Diseases and Their Practical Significance in Dentistry

A. B. Chukhlovin, A. M. Solovyova*, S. K. Matelo, I. V. Kobiyasova*, E. B. Morosova, A. V. Hokhlacheva, B. G. Teplyakov, K. A. Syssoev, V. E. Konstantinova**, L. N. Matelo**, Areg A. Totolian**

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The incidence and prevalence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythensis* in specimens of subgingival dental deposit were evaluated in 495 residents of St. Petersburg aged 6-82 years. The microorganisms were detected by gene-specific PCR of 16S rDNA. In accordance with age-specific increase in the incidence of gingival diseases, the percentage of samples containing *T. forsythensis* and *P. gingivalis* was significantly higher in adult and elderly patients in comparison with adolescents. The presence of *T. forsythensis* significantly correlated with the presence of gingivitis and dental deposit. In addition, the incidence of *T. forsythensis* was significantly higher in tobacco smokers. These results attest to a relationship between *T. forsythensis* infection and more frequent periodontal diseases associated with aging and tobacco smoking.

Key Words: *periodontium; microflora; deoxyribonucleic acid polymerase chain reaction; age; tobacco smoking*

More than 500 bacterial species are present in the oral cavity. These bacteria are often detected in the dental deposit and dentogingival pouches in periodontitis. Pronounced toxicity for the gingiva and its bone base has been demonstrated for many anaerobic bacteria, detected in the dental deposit: *A. actinomycetemcomitans*, *T. forsythensis*, *P. gingivalis*, *T. denticola*, and *S. noxia* [3,11]. These pathogens are hypothesized to be involved in the loss of the dentogingival adhesion and destruction of the underlying bone tissue. These bacteria are often detected in the subgingival tissues, particularly in aggressive course of the disease (up to 80-90% cases) in comparison with 10-30% in normal subjects [7]. However, the prognostic significance of periodontal microorganisms for various age groups and populations has never been precisely determined. A classical microbiological approach is the "golden standard" for these studies. However, gene-specific DNA diagnosis is more convenient for overall screening.

We evaluated the incidence of three species of the marker periodontal bacteria (*A. actinomycetemcomitans*, *T. forsythensis*, *P. gingivalis*) in a representative group of residents of the same region, classified by age, sex, and dental status.

MATERIALS AND METHODS

The study was carried out on a random sampling of 495 permanent residents of St. Petersburg, se-

I. P. Pavlov State Medical University, St. Petersburg; St. Petersburg Medical Academy of Postgraduate Education; Stomatological Initiative Company, Moscow. ***Address for correspondence:*** alexei.chukh@mail.ru. A. B. Chukhlovin

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lected at education institutions and treatment-and-prophylaxis centers. Each participant in the program gave informed consent to examination. Five age groups were represented in the sampling: 6-7, 12-13, 16, 17, 35-44, and over 65 years of age, with 1:1 male to female ratio in each group. Standard dental and oral examinations were carried out in all participants. The criteria for selection into the group for examination and methods for sample collection were described previously [1]. Periodontal bacteria were detected in the subgingival dental deposit. Biological material was put into 0.1 M EDTA and stored at -20°C until DNA extraction.

In order to isolate bacterial DNA in large series of samples, the cell precipitate was washed in standard TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), the cells were preincubated with proteinase K (0.3 mg/ml) for 20 min at 65°C, lyzed, DNA was purified on an adsorbent, and washed using a DNA-Sorb kit (Interlabservis Firm). DNA was dried on the adsorbent and eluted in 50 μl TE buffer.

Species-specific sense primers complementary to rRNA 16S gene were used for DNA diagnosis of the marker bacteria: 5'att ggg gtt tag ccc tgg tg 3' for *A. actinomycetemcomitans*; 5' tgt aga tga ctg atg gtg aaa acc 3' for *P. gingivalis*; 5' tac agg gga ata aaa tga gat acg 3' for *T. forsythensis*; and a common antisense primer 5' acg tca tcc cca cct tcc tc 3' [12]. The specificity of these primers was verified with DNA from pure cultures of the respective bacteria.

The reaction mixture for multiplex PCR contained 3 μl 5x buffer for PCR with 15 mM $MgCl_2$ (Amplisense), mixture of ATP, thymidine triphosphate, guanosine triphosphate, and citidine triphosphate (30 μmol each; Helicon), 0.15 μM each sense primer (specific), 0.3 μM antisense primer (Sintol), Taq-DNA-polymerase (Helicon; 0.25 U per sample), analyzed DNA (2.5 μl per sample) in a total volume of 15 μl. PCR was carried out in an ICycler amplifier (Bio-Rad) according to the protocol: 3 min at 95°C, 30 sec of primary denaturing at 95°C, 36 cycles of denaturing (30 sec at 95°C), annealing (40 sec at 61°C), elongation (50 sec at 72°C), and final elongation (1 min at 72°C).

PCR products were separated by electrophoresis in 1.5% agarose gel with ethidium bromide (0.5 μg/ml). The gels were examined and photographed on UV transilluminator TCP-20M (Vilber Lourmat) using a Canon Powershot digital camera.

Summary data were charted and classified using Excel software. The results were statistically processed using WinStat or Statistica 5.0 software. The significance of differences was evaluated using the χ^2 or Mann—Whitney nonparametric test. Coefficients of correlations and their significance were evaluated using Spearman's test.

RESULTS

Signs of periodontitis were detected in more than 61% examined subjects starting from the age of 16-17 years. The number of carious and filled teeth sharply increased (14.1±0.5) in the group of subjects aged 35-44 years, the incidence of stomatorrhagia reached 50%, the incidence of loose teeth in this group was 12%; in elderly and senile subjects the percentage of severe periodontitis reached 79.3± 3.5% and the number of lost teeth was 13.1±0.8, which was in line with age-specific dynamics.

Tobacco smoking was noted in 8% children aged 12-13 years and in 23% adolescents (16-17 years). The number of tobacco smokers was the greatest at the age of 35-44 years and decreased significantly in the group of subjects aged over 65 years (48 and 15%, respectively; the difference significant at $p<0.01$).

Evaluation of the incidence of microorganisms in different age groups showed low *A. actinomycetemcomitans* infection rate in the youngest children (6-7 years) and subsequent increase of the detection rate of this bacterium by the age of 12-13 years; the rate of *P. gingivalis* infection increased by 16-17 years (reaching about 40%; Table 1).

T. forsythensis was detected in youngest children more rarely than two other bacterial species, its incidence increasing with age, reaching the maximum in adults (35-44 years), which can reflect the relationship with augmenting severity of periodontal diseases at this age (Table 1).

Sex-specific differences were detected for some clinical parameters. Tobacco smoking was significantly more incident in men (26% *vs.* 12% in females; $p<0.0001$). The mean number of decayed teeth was also significantly higher in men (3.3 *vs.* 2.7; $p=0.004$). High incidence of caries in our sampling correlated with tobacco smoking ($r=0.159$; $p=0.01$), which was a possible explanation of the detected differences. On the other hand, no sex-specific differences in the incidence or severity of gingival and periodontal diseases were detected in the total sampling by the CPITN and Silness-Loe indexes. Comparison of the incidence of periodontal bacteria showed significantly higher incidence of *P. gingivalis* in men (40.1 *vs.* 30.6%; $p=0.03$).

The results of clinical examinations and PCR analysis were evaluated by analysis of correlations using Spearman's test in a united group of 300 adolescents and adults (mean age 42.6±24.3 years). The younger groups (under 12 years) were exclu-

TABLE 1. Age-Specific Differences in the Incidence of Subgibngival Pathogenic Bacteria, Manifest Dental and Gingival Diseases, and Tobacco Smoking

Parameters	Age groups, years					Total sample
	6-7	12-13	16-17	35-44	65-82	
Number of examinees	95	100	100	100	100	495
A. actinomycetemcomitans, % of total sample	29.5	40.0*	40.0	33.0	45.0	37.6
P. gingivalis, % of total sample	22.1	29.0	40.0*	39.0	46.0	35.4
T. forsythensis, %, of total sample	12.6	14.0	14.0**	31.0**	47.0**	23.8
Stomatorrhagia, %	0	0	0	50**	51	20.4
Loose teeth, %	0	0	0	12**	21	7
DFL index	5.7±0.3	3.8±0.3	4.9±0.32	15.3±0.5**	20.7±0.7**	10.1±0.8
Caries sum	3.8±0.3	2.5±0.3	2.1±0.2	4.7±0.4*	1.9±0.3**	3.0±0.2
Filling sum	2.0±0.3	1.3±0.2	2.6±0.3	9.4±0.5*	5.7±0.4*	4.2±0.2
Sum of lost teeth	0	0.03±0.03	0.1±0.04	1.2±0.2*	13.1±0.8**	2.9±0.3
Tobacco smoking by the moment of examination, %	0	8	23*	48**	15**	19

Note. *$p<0.05$; **$p<0.01$ compared to younger age group (according to χ^2 test).

ded from analysis because of low incidence of periodontal diseases. Analysis of correlations for the entire sampling showed that the incidence of caries (DFL index: number of decayed, filled, and lost teeth), gingivitis (Silness-Loe index), and periodontitis (CPITN 17/16/14), and dental deposit index correlated significantly with the presence of *T. forsythensis* in the gingival sulci (the respective coefficients of correlations were 0.219-0.255; $p<0.01$), while the presence of *A. actinomycetemcomitans* and *P. gingivalis* was not associated with dentogingival diseases. The correlation between the DFL index and the presence of *T. forsythensis* was highly significant, but both parameters increased with age and did not correlate.

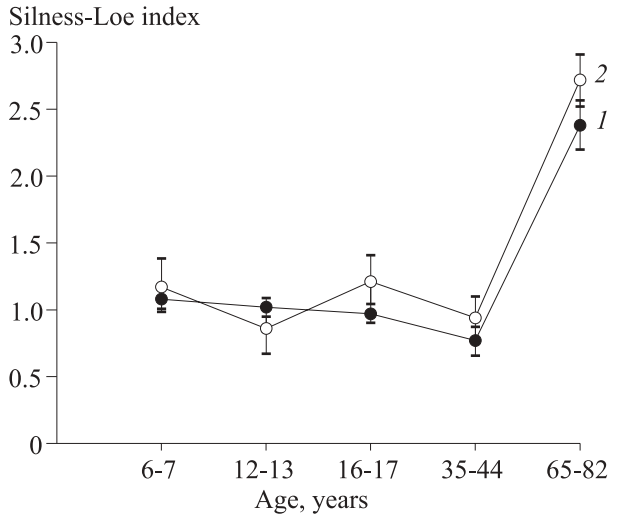

Fig. 1. Mean values of Silness-Loe gingivitis index in *T. forsythensis*-negative (*1*) and positive (*2*) groups. The difference between the groups for subjects aged over 16 years is significant at $p<0.002$.

These clinical microbiological relationships can reflect a pronounced association between the presence of a certain bacterium and combined gingival disease leading to dental deposit, disorders in the dentogingival adhesion, and development of chronic periodontitis.

The severity of gingival inflammation (according to Silness-Loe index) and dental deposit was significantly higher in *T. forsythensis*-positive subjects with the bacterium detected in the subgingival tissues (Figs. 1, 2). The corresponding differences were significant in the groups starting from the age of 16 years ($p<0.002$). These age groups are liable to the development of progressive gingivitis and periodontitis, and therefore the presence of *T. forsythensis* in the subgingival material is presumably a factor of high risk of periodontal and gingival diseases in adolescents and adult population of St. Petersburg.

Evaluation of the age-specific dynamics of *T. forsythensis* in the subgingival specimens from tobacco smokers and nonsmokers showed that the percentage of positive tests was significantly higher in tobacco smokers aged 16-44 years (36.6 *vs.* 4.7%, respectively; $n=200$; $p=0.0004$). This difference was not observed in the group aged over 65 years, presumably because of the leading role of other infectious and age-associated factors in the development of periodontal disease (Fig. 3).

Our results confirm high sensitivity of PCR of oral microorganism DNA used in epidemiological screening studies. A simple single-staged multiplex PCR (modified original method [12]) used in our study proved to be sufficiently sensitive for clinical biological comparison.

Age-specific dynamics of anaerobic gingival bacteria detected in our study is in line with modern theories on early colonization of the oral cavity by specific dental microflora and its multiplication with the development of dentogingival infections. Microorganisms colonize the oral cavity starting from the neonatal period [8]. *P. gingivalis* and *A. actinomycetemcomitans* were detected by PCR method in summary samples from the oral cavity of 37 and 48% of healthy children and adolescents irrespective of age (0-18 years), which indicates early colonization of the oral cavity.

High incidence of the marker microorganisms can vary greatly in different studies. In a study carried out in the USA in periodontitis patients, *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythensis* were detected in 19, 79, and 17% cases, respectively [10]. In Japan [2] *P. gingivalis* was detected in 87% patients with periodontitis and 37% controls. It is possible, that in different countries different bacteria play the leading role in the pathogenesis of periodontitis. Examinations of 70 young patients with periodontal infections detected *A. actinomycetemcomitans* in 40% specimens of dental deposit, the presence of this microorganism clearly correlating with stomatorrhagia and formation of dental deposit [9]. We detected a relationship between the presence of *T. forsythensis* and gingival disease and increase of dental deposit.

The cytotoxic effects of *A. actinomycetemcomitans* and *P. gingivalis* were described not once [5], while the possible pathogenetic role of *T. forsythensis* is less known. It was hypothesized that this bacterium is involved in the loss of the dentogingival adhesion [13]. It was shown that *T. forsythensis* exhibited a trend to predominant colonization of the foci of inflammation in comparison with normal sites [6]. The incidence of *T. forsythensis* in adolescents was reliably associated with loosening of the dentogingival contacts, which suggests an important role of this microorganism in juvenile periodontal disease [4]. Hence, our data on high gingivitis index and dental deposit formation in *T. forsythensis*-positive patients indicate an important role of this microorganism (as well as of *P. gingivalis*) in the development of periodontitis. On the other hand, it is unclear, whether bacterial invasion is a primary destructive factor or a result of periodontal inflammation [14].

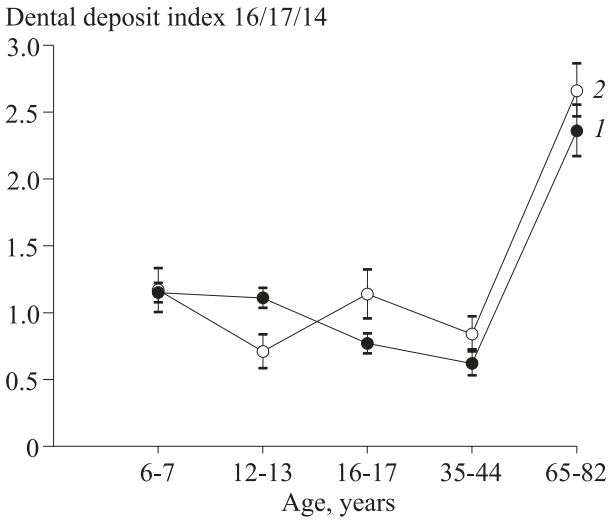

Fig. 2. Mean values of dental deposit index in *T. forsythensis*-negative (*1*) and positive (*2*) groups. The difference between the groups for subjects aged over 16 years is significant at $p<0.0002$.

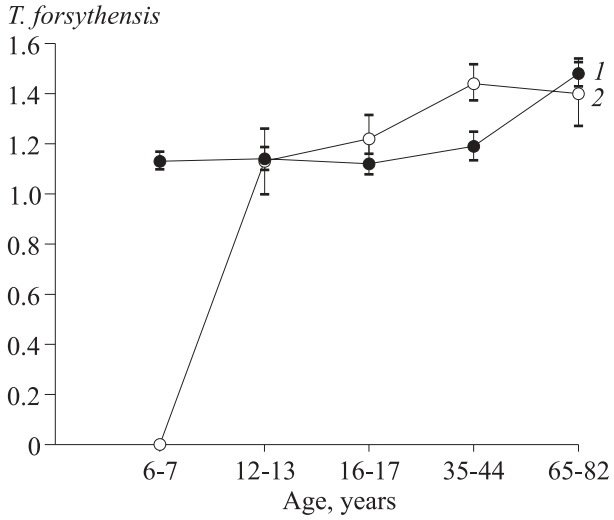

Fig. 3. Comparative incidence of *T. forsythensis* in the subgingival samples from nonsmokers (*1*) and tobacco smokers (*2*). The difference between these groups is significant at $p=0.0004$.

In addition, our study shows that tobacco smoking is associated with a high incidence of *T. forsythensis* in the dental deposit and a higher incidence of periodontal disease in young and adult people, which confirms the pathogenetic relationship between certain bacterial species and periodontal diseases, particularly in exposure to harmful factors.

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