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Salivary infectious agents and periodontal disease status

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Background and Objectives: The potential of salivary microorganisms to diagnose periodontal disease and to guide periodontal treatment is a research topic of current interest. This study aimed to determine whether the salivary counts of periodontopathic microbes correlated with the periodontal pocket counts of the same infectious agents, and whether the salivary counts of the test infectious agents could distinguish among individuals with periodontal health and various types of periodontal disease.

Material and Methods: The study included 150 systemically healthy adults, of whom 37 were periodontally healthy, 31 had gingivitis, 46 had chronic periodontitis and 36 had aggressive periodontitis. Each study subject contributed microbial samples from the two deepest periodontal pockets of the dentition and from whole saliva. *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and Epstein–Barr virus were identified using the TaqMan real-time PCR methodology. Statistical analysis was performed using the Mann–Whitney *U*-test and the receiver operating characteristic statistics.

Results: C. rectus, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *T. forsythia* occurred with significantly higher copy-counts in salivary samples from patients with gingivitis, chronic periodontitis and aggressive periodontitis than from periodontally healthy individuals. *A. actinomycetemcomitans* only showed higher salivary copy-counts in subjects with aggressive periodontitis compared with subjects with healthy periodontium, and the salivary copy-counts of Epstein–Barr virus did not reveal any significant difference among the four subject groups studied. The diagnostic sensitivity for periodontitis was 89.19 for *P. gingivalis* and for *T. forsythia* and 86.49 for *P. intermedia*, with specificities ranging from 83.78 to 94.59. The optimal copy-counts per mL saliva for identifying periodontitis were 40,000 for *P. gingivalis*, 700,000 for *T. forsythia* and 910,000 for *P. intermedia*.

Conclusion: Salivary copy-counts of *P. gingivalis*, *T. forsythia* and *P. intermedia* appear to have the potential to identify the presence of periodontitis, whereas the salivary level of the other test infectious agents may possess little or no diagnostic utility. Longitudinal studies are warranted to determine the ability of salivary copy-counts of major periodontopathic bacteria to predict future periodontal breakdown.

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Highly sensitive and specific molecular detection methods have the potential to identify salivary molecules of diagnostic value (1). Salivary biomolecules can aid in the diagnosis of a variety of cancers, illicit and prescription drug use, hereditary disorders, hormonal irregularities, nicotine dependence and pathogenic viruses and bacteria (2,3).

A major advantage of salivary testing is the ease with which diagnostic samples can be collected by health professionals, by the individuals themselves or by parents for young children. Salivary sampling is painless and involves virtually no health or safety issues.

Salivary biomarkers for periodontal disease may assist in the assessment of the presence or the risk of destructive periodontal disease (4,5). Salivary testing for periodontopathic bacteria is premised on the idea that whole saliva and periodontal lesions tend to harbor similar relative levels of periodontal pathogens, that high salivary counts of periodontal pathogens imply presence or risk of periodontitis, and that a decrease in the salivary counts of periodontal pathogens can be used to assess the effectiveness of therapeutic intervention.

Few data exist on the utility of salivary microorganisms to identify the periodontal disease status. Patients with severe periodontal disease harbor elevated levels of periodontopathogens in saliva (6,7), which mirrors even higher pathogen counts in the periodontal pocket area (8). Umeda *et al.* (9) demonstrated a statistical relationship between the presence of periodontopathic *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Treponema denticola* in whole saliva and in periodontal pocket samples. The oral occurrence of *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* was not reliably detected by sampling either whole saliva or periodontal pockets (9), probably because the two species can persist in nonperiodontal sites, as best demonstrated in fully edentulous individuals (10,11).

Studies are needed to determine whether salivary microbial counts can be used to distinguish between individuals with healthy periodontium, gingivitis and periodontitis. Also, as Epstein–Barr virus appears to play an etiologic role in periodontitis (12,13), the salivary copy-counts of the virus may serve as an indicator of the periodontal disease status. The present study aimed to determine whether the salivary counts of six periodontopathic bacteria and Epstein–Barr virus correlated with the periodontal pocket counts of the same infectious agents, and whether the salivary counts of the test infectious agents could distinguish between individuals with periodontal health, gingivitis, chronic periodontitis and aggressive periodontitis.

Material and methods

A total of 150 subjects from Turkey (101 men and 49 women) took part in the study. Thirty-seven individuals were categorized as periodontally healthy, 31 as gingivitis patients, 46 as chronic periodontitis patients and 36 as aggressive periodontitis patients. All study subjects were systemically healthy, revealed normal salivary flow and had not received periodontal treatment or antibiotics for at least 6 mo prior to participating in the study. Each subject had a maximum of six teeth extracted other than third molars. The study was approved by the Institutional Internal Review and Ethics Board at the Gülhane Military Medical Academy.

The periodontal variables assessed included plaque index (14), gingival index (15), bleeding on probing, probing pocket depth and probing attachment loss. All probings were carried out using a Williams probe and were recorded at four sites (mesiofacial, midfacial, distofacial and midlingual) in each tooth.

Periodontally healthy subjects (33.1 ± 6.7 years of age) had no teeth with pocket depth exceeding 3 mm and no teeth with probing attachment loss or bleeding on probing. Gingivitis patients (30.9 ± 8.4 years of age) showed several teeth with bleeding on probing but did not exhibit teeth with pocket depths exceeding 3 mm and had no teeth with probing attachment loss. Chronic periodontitis patients (42.7 ± 8.2 years of age) had at least nine posterior teeth with 5–7 mm pocket depth and three teeth with 6 mm or more of probing attachment loss. Aggressive periodontitis patients (34.5 ± 7.3 years of age) exhibited probing attachment loss in excess of 5 mm on more than 14 teeth, with at least three teeth other than incisors or first molars.

Subgingival samples were collected from the two deepest pockets of the dentition. Prior to sampling, the sample sites were cleaned of supragingival plaque and saliva using sterile cotton pellets, and the sample teeth were isolated with cotton rolls and air dried. A sterile periodontal curette was inserted to the bottom of the periodontal pocket, and subgingival material was gently removed with a single stroke. The two pocket samples were pooled in an Eppendorf tube containing 500 μL of 10 mM Tris–HCl and 1 mM EDTA. Each study subject contributed a total of 4–5 mL of unstimulated saliva, which was collected in an empty glass test tube within 5 min. The subjects were instructed not to brush their teeth or eat for up to 1 h prior to sampling, which was performed at 8:30–9:30 AM. The subjects leaned head forward and kept the mouth slightly open with minimal head movement to allow passive drainage of the saliva into the test tube. All samples were stored at −80°C until processed.

TaqMan® real-time PCR assay was employed to determine the counts of the test infectious agents, using primers and techniques previously described (16). The infectious agents identified were *A. actinomycetemcomitans*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *P. gingivalis*, *P. intermedia*, *T. forsythia* and Epstein–Barr virus.

Statistical analysis was performed by using the SPSS 15.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA). After a logarithmic transformation of the microbial counts, the Mann–Whitney *U*-test was used to compare the microorganisms in subgingival sites and in saliva. The receiver operating characteristic (ROC) statistics determined the probability of a positive test, given disease (sensitivity) and the probability of a negative test, given no disease (specificity), and the ROC curve was used to identify an optimal cut-off point for the diagnostic test. Probability (p) values ≤ 0.05 were considered statistically significant.

Results

Figure 1 shows the average copy-counts of the seven infectious agents studied recovered from subgingival sites and

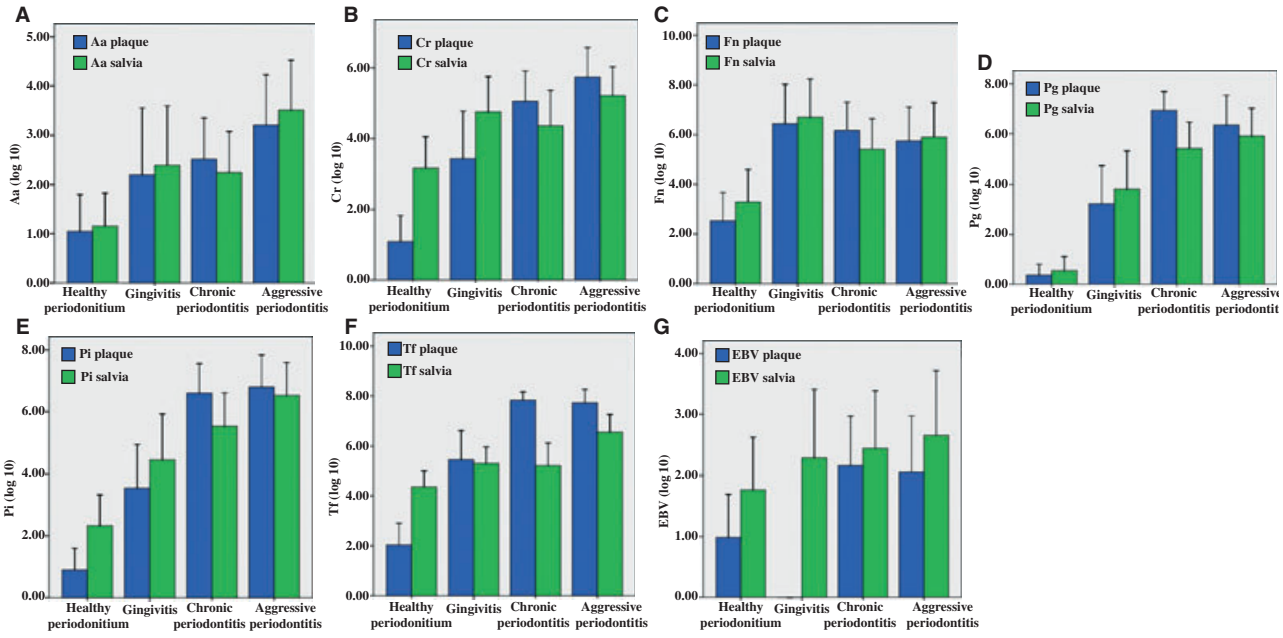

Fig. 1. (A) The average copy-counts of *Aggregatibacter actinomycetemcomitans* (Aa) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. No significant difference was found between subgingival plaque and salivary samples in any subject group. (B) The average copy-counts of *Campylobacter rectus* (Cr) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was a significant difference between subgingival plaque and salivary samples in healthy subjects ($p < 0.001$), and no significant difference between subgingival plaque and salivary samples in gingivitis patients ($p = 0.052$). (C) The average copy-counts of *Fusobacterium nucleatum* (Fn) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was no significant difference between subgingival plaque and salivary samples in any subject group. (D) The average copy-counts of *Porphyromonas gingivalis* (Pg) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was a significant difference between subgingival plaque and salivary samples in chronic periodontitis patients ($p = 0.003$). (E) The average copy-counts of *Prevotella intermedia* (Pi) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was a significant difference between subgingival plaque and salivary samples in healthy subjects ($p = 0.001$). (F) The average copy-counts of *Tannerella forsythia* (Tf) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was a significant difference between subgingival plaque and salivary samples in healthy subjects ($p < 0.001$), chronic periodontitis patients ($p < 0.001$) and aggressive periodontitis patients ($p = 0.002$). (G) The average copy-counts of Epstein–Barr virus (EBV) in subgingival plaque and in saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. There was a significant difference between subgingival plaque and salivary samples in healthy subjects ($p = 0.015$) and gingivitis patients ($p = 0.003$).

from saliva of subjects with a healthy periodontium, gingivitis, chronic periodontitis and aggressive periodontitis. The total copy-counts of *A. actinomycetemcomitans* (Fig. 1A) and *F. nucleatum* (Fig. 1C) showed no significant difference between the subgingival and salivary samples in the four periodontal study groups. *C. rectus* (Fig. 1B) and *P. intermedia* (Fig. 1E) demonstrated no difference in copy-counts between the subgingival and the salivary samples from individuals with gingivitis, chronic periodontitis and aggressive periodontitis, but both organisms showed higher copy-counts in the salivary samples than in the subgingival samples from periodontally healthy subjects ($p = 0.001$). *P. gingivalis* (Fig. 1D) showed similar copy-counts in subgingival samples and in salivary samples from healthy individuals and from patients with gingivitis and aggressive periodontitis, but exhibited higher copy-counts in subgingival samples than in salivary samples from chronic periodontitis patients ($p = 0.003$). *T. forsythia* (Fig. 1F) showed similar copy-counts in subgingival and salivary samples from gingivitis patients, but higher salivary copy-counts in periodontally healthy subjects ($p < 0.001$) and lower salivary copy-counts in chronic ($p < 0.001$) and in aggressive periodontitis patients ($p = 0.001$). Epstein–Barr virus (Fig. 1G) demonstrated similar copy-counts in subgingival and salivary samples from chronic and aggressive periodontitis patients, but higher copy-counts in the salivary samples from periodontally healthy individuals ($p = 0.015$) and from gingivitis patients ($p = 0.003$).

The highest absolute copy-counts were found for *P. intermedia*, *P. gingivalis* and *T. forsythia* in periodontitis patients, averaging between 10^6 and 10^8 copies per mL in subgingival and salivary samples (Fig. 1). Epstein–Barr virus in saliva from gingivitis and periodontitis patients exhibited average copy-counts between 10^2 and 10^3 / mL (Fig. 1G).

The salivary copy-counts of the seven infectious agents were compared between the periodontally healthy individuals on one side and each of the

three other subject groups on the other side. Significantly higher salivary copy-counts of *C. rectus*, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *T. forsythia* were found in patients with gingivitis, chronic periodontitis and aggressive periodontitis, whereas *A. actinomycetemcomitans* only showed higher salivary copy-counts in patients with aggressive periodontitis, and the salivary copy-counts of Epstein–Barr virus did not differ significantly among the four subject groups (data not presented). When comparing the salivary copy-counts of the gingivitis patients vs. the chronic and aggressive periodontitis patients, *P. gingivalis* showed significantly higher copy-counts in chronic periodontitis and in aggressive periodontitis patients, and *P. intermedia* and *T. forsythia* exhibited significantly higher copy-counts in aggressive periodontitis patients (data not presented).

Table 1 demonstrates the potential of salivary copy-counts of the test infectious agents to identify the periodontal disease status. For all six test bacteria, statistically higher copy-counts were found in the saliva from the combined group of chronic and aggressive periodontitis patients than in the saliva from the combined group of periodontally healthy subjects and gingivitis patients. The diagnostic sensitivity for periodontitis was 89.19 for *P. gingivalis* and for *T. forsythia* and 86.49 for *P. intermedia*, with specificities ranging from 83.78 to 94.59 (Table 1). The optimal copy-counts per mL saliva for identifying periodontitis were 40,000 for *P. gingivalis*, 700,000 for *T. forsythia* and 910,000 for *P. intermedia* (Table 1). None of the remaining test infectious agents exhibited high sensitivity and specificity values (Table 1). Also, combining the infectious agents and the subject groups in all possible variations yielded joint sensitivity and specificity values that were lower than those shown in Table 1 (data not presented).

Discussion

To the best of our knowledge, this is the first study to examine the ability of the salivary copy-counts of major periodontal pathogens to predict the periodontal disease status. To cover the full spectrum of the periodontal disease process, the subjects included in the study exhibited periodontal conditions ranging from periodontal health to advanced periodontitis. As no generally accepted 'gold standard' exists for distinguishing among various types of destructive periodontal disease (17), the clinical judgment of expert dentists formed the basis for the diagnoses of chronic periodontitis and aggressive periodontitis. The uncertainty in assessing the periodontal disease status may cause an error in disease classification, such as turning a true positive into a false positive, or a true negative into a false negative. Accidentally classifying false positives or false negatives as true values introduces 'noise' in the statistical test, resulting in a diminishment of the area under the ROC curve and an underestimation of the microbial–disease interrelationship. Also, as the type of periodontitis and the related infectious agents may differ in low- and high-income countries and within subpopulations of individual countries (18,19), the present cohort of patients from Turkey may not be representative of the population of individuals with periodontitis in various other countries.

The bacteria included in the study represented periodontopathogens of both major and moderate significance (20,21). Previous studies have identified cut-off points of subgingival counts of selective periodontopathic bacteria that were able to distinguish between disease-stable and disease-active periodontitis (22,23). The overall good agreement between subgingival and salivary bacterial counts found in this study then suggested the feasibility of using salivary microbial testing in the diagnosis of periodontal disease. It was noteworthy that species considered to be most periodontopathic exhibited both relatively low optimal salivary copy-counts and good sensitivity and specificity values in the prediction of periodontal disease. *P. gingivalis*, which may be the most pathogenic of the species studied (24), revealed an optimal copy-count as low as 40,000 and the best diagnostic performance of the test microorganisms. *T. forsythia*, another important periodontal pathogen (25), showed an optimal salivary copy-count of 700,000, and *P. intermedia*, which previously has been related to disease-active periodontitis (22), exhibited an optimal salivary copy-count of 910,000. *A. actinomycetemcomitans*, a major periodontopathogen of young individuals and of adults with refractory periodontitis (22,26), tended to show elevated salivary copy-counts in patients with aggressive periodontitis (Fig. 1A), but the organism did not demonstrate acceptable diagnostic performance in our cohort of adult individuals. In agreement with a previous study (27), the salivary copy-count of Epstein–Barr virus was not diagnostic of periodontal disease, probably because the salivary content of the virus originates not only from periodontal pockets but also from nondental oral sites, as evidenced by the high quantity of the Epstein–Barr virus DNA in the saliva of fully edentulous subjects (28).

Table 1. Comparison of salivary copy-counts of infectious agents in periodontitis patients (chronic + aggressive periodontitis patients) vs. nonperiodontitis patients (healthy subjects + gingivitis patients)

Infectious agent	Area under ROC curve*	Significance level	Criterion	Sensitivity	Specificity
A. actinomycetemcomitans	0.655	0.014	> 2,100	48.65	81.08
C. rectus	0.774	0.0001	> 700,000	59.46	91.89
F. nucleatum	0.720	0.0003	> 5,000,000	70.27	74.29
P. gingivalis	0.933	0.0001	> 40,000	89.19	94.59
P. intermedia	0.874	0.0001	> 910,000	86.49	83.78
T. forsythia	0.907	0.0001	> 700,000	89.19	86.49
Epstein–Barr virus	0.607	0.104	> 64,000	32.43	91.89

*ROC, receiver operatoring characteristic statistics.

The high sensitivity and specificity demonstrated here for three bacterial species suggests that salivary microbial diagnostics may have utility in a clinical setting as a guide to identify individuals with periodontitis. Quantification of these salivary bacteria and possibly other periodontal pathogens may someday be used to augment and perhaps supplant clinical examination in surveillance studies of periodontal disease. In the future, individuals may even submit salivary samples for the tentative diagnosis of periodontal disease. Salivary microbial analysis allows data collection in less time and without the expense and limitations associated with a clinical examination. However, the optimal copy-count thresholds presented here need to be validated prospectively, especially because of the inherent uncertainty in classifying patients with chronic/stable periodontitis vs. aggressive/advancing periodontitis. Important questions in need of clarification are the usefulness of salivary copy-counts of periodontopathic bacteria for an early diagnosis of periodontitis, as prognostic indicators of periodontal disease development and as markers of the effectiveness of periodontal therapy. Hopefully, a quantitative assay for infectious agents in saliva may prove to be helpful in periodontal epidemiological research and in the identification and management of populations at risk of destructive periodontal disease.

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