QUANTITATIVE ANALYSIS OF PERIODONTAL PATHOGENS IN PERIODONTITIS AND GINGIVITIS

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Periodontal tissues surround the teeth and provide their attachment. Periodontal diseases include a mild and reversible form named gingivitis, and periodontitis that is the main cause of tooth loss in adults. Gingivitis, that affects gums and coronal junctional epithelium, as well as periodontitis, that is characterized by loss of connective tissue attachment, are caused by a persistent inflammatory response promoted by alteration of periodontal biofilm. The aim of the study was to test whether the prevalence or relative amount of each species was associated with a particular clinical condition. Periodontal evaluation of 539 unrelated patients was performed by the Periodontal Screening and Recording (PSR) system. Subgingival samples were obtained from the site with the worst PSR score. A selection of eleven bacterial species was evaluated by quantitative real time PCR. Some bacterial species were found to be associated with all phases of periodontal disease, such as *Tannerella forsythia, Treponema denticola,* and *Treponema lecithinolyticum,* while other species were more specifically associated with periodontitis, such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis*, or with gingivitis, such as *Capnocytophaga ochracea* and *Campylobacter rectus.* Quantitative and qualitative analyses helps to better understand the microbial changes associated with different stages of periodontal disease.

Periodontal tissues include four defined structures that constitute the support of teeth: gingiva, cementum, alveolar bone, and the periodontal ligament. Periodontal diseases are extremely prevalent worldwide, affecting roughly half of the adult population (1, 2). Gingivitis, the mildest form of periodontal disease, is a rapidly inducible and reversible inflammatory affection of the gingiva, mainly caused by accumulation of bacterial biofilm (3). The combination of bacterial infection and persistent inflammatory response can eventually induce the progressive destruction of the deeper periodontal tissues, a worse form of periodontal disease called periodontitis. Gingivitis and periodontitis can be considered a continuum of the same inflammatory process, although it is important to note that gingivitis lesions do not necessarily progress to periodontitis (4). Additional risk factors include genetic susceptibility, tobacco smoking, alcohol consumption, and systemic conditions such as diabetes, osteoporosis, malnutrition and stress (1, 5). Effective treatment of periodontal infections is important to reduce local inflammation and bacteremia. In addition, poor periodontal health

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appears to increase the risk of cardiovascular disease, pulmonary disease, preterm and low birth weight (6-8).

The present investigation compared the microbial colonization in different periodontal conditions. The oral cavity is populated by a large number of bacteria species that form polymicrobial communities attached to biotic or abiotic surfaces. The biofilm formed by the oral microbiota includes both symbiontic and potentially pathogenic species. The advent of periodontal diseases appears to be associated with a microbial shift, more commonly known as dysbiosis, that could be considered either a decrease in the number of beneficial symbionts and/or an increase in the number of pathogens (9). While the classic infection diseases are caused by a single exogenous species, periodontal diseases are considered to be the result of biofilm community changes involving a set of species that could either be endogenous and/or exogenous (10). In this complex scenario, the identification of the pathogenic species becomes a hard task. Indeed, as no single bacterial species was able to meet Kock's postulates, alternative and more flexible criteria of causality were suggested to unravel the etiology of periodontal diseases (9). Specifically, Hill's criteria that include biological plausibility, dose response, strength of association, specificity of association, consistency and temporality, should be all equally considered.

Among the over 700 bacterial species that have been catalogued in the oral cavity, we analyzed a selection of 11 species likely involved in periodontal diseases. A highly specific and quantitative approach was used to analyze their distribution in periodontal disease progression from healthy individuals to gingivitis and periodontitis patients. Selection included members of the red complex *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, widely regarded as major periodontal pathogens; members of the orange complex *Fusobacterium nucleatum*, *Campylobacter rectus*; as well as *Aggregatibacter actinomycetemcomitans*, *Atopobium rimae*, *Eubacterium saphenum*, *Porphyromonas endodontalis*, and *Treponema lecithinolyticum,* considered potentially pathogenic species (11, 12), and *Capnocytophaga ochracea* that was suggested as a diagnostic marker of periodontal health (12).

The purpose of the study was to investigate the bacterial population in subgingival biofilm in association with the degeneration of periodontum. The prevalence and quantitative data of 11 bacterial species were analyzed for correlation with occurrence of chronic periodontitis and gingivitis.

MATERIALS AND METHODS

Sampling

A total of 539 patients participated in the study. Patients were randomly selected over a period of 8 months according to the following criteria. A lower age limit of 20 years was fixed. All individuals had good general health; exclusion criteria included diabetes, hypertension, rheumatoid arthritis, depression-anxiety, and obesity; as well as noticeable exposure to risk factors, such as alcohol consumption and smoking. Patients had not taken antimicrobial or anti-inflammatory drugs or had periodontal treatment in the previous 2 months.

Case definition was performed according to the Periodontal Screening and Recording (PSR) system. Gingival bleeding and pocket probing depth were monitored by a specific WHO probe and scored using the basic method indicated by the WHO oral health survey (13), Basically, the mouth was divided into sextants for the PSR examination. The probe is walked circumferentially around each tooth in the sextant being examined. The clinician observed only the position of the color-coded reference marking in relation to the gingival margin and the presence of furcation invasion, mobility, mucogingival problems, or recession. Only the highest code obtained was recorded for each sextant in the mouth.

The American Dental Association and the American Academy of Periodontology suggest that all routine dental examinations include a screening examination using PSR to identify patients who need a comprehensive periodontal assessment. The results of this screening examination are used to separate patients into two broad categories: those who have periodontal health or gingivitis, and those who have periodontitis. These two categories were the main subject of the investigation, the control group of patients with low PSR in all sextants (PSR code 0, 1, or 2), and a second group of patients with evidences of periodontitis in one or more sextants (code 3, 4, or *). In addition, healthy patients with no periodontal disease (code 0) were compared to patients with plaque-associated gingivitis (code 1 or 2).

The study was approved by the Ethics Committee of the Ospedale di Circolo e Fondazione Macchi, Varese. Written informed consent was obtained from enrolled

individuals.

After the removal of supragingival biofilm, a sample of the periodontal pocket microbiota was taken from a single site using sterile paper probes, among sites having the highest score, or randomly in the case of healthy patients. Specimens were processed to extract and purify DNA using a method that includes two consecutive incubations with lysozyme and proteinase K, in order to ensure an indiscriminate Gram positive and negative bacterial lysis. Once extracted, DNA was purified through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA).

Quantification of 16S rRNA genes

Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount of 11 bacterial species.

The 845 sequences of 16S rRNA gene of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1) were aligned to find either a consensus sequence or less preserved spots, useful to optimize the specificity of primers and dual labelled hydrolysis probes. PCR oligonucleotide sequences were designed by Primer3web (http://primer3.ut.ee/) and Primer Express (Life Technologies) software. The specificity of PCR assays was also checked by Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Absolute quantification assays were performed using a 7500 Sequence Detection System (Applied Biosystems). The thermal cycle included 10 min incubation at 95°C to activate polymerase, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles. Each experiment included non-template controls to exclude reagents contamination and serial dilutions of the specific synthetic template (Eurofin MWG Operon, Ebersberg Germany). These positive controls were used to plot standard curves, i.e. threshold cycle values against the log of the copy number, that were used either to check amplification efficiency and for quantification of targets in each sample.

Statistical analysis

Data from quantitative PCR included the amount of each of the 11 investigated bacterial species and a measurement of the bacterial load from up to 539 patients. These amounts matched the number of DNA molecules detected in the real time PCR tubes that was directly related with the number of bacteria in the specimens. In order to enhance data analysis with noise removal, statistical analysis was performed on relative amounts, calculated as ratios between the amount of each species and the total bacterial load. This was able to reduce variability due to random factors such as specimen conservation, DNA extraction efficiency, and purification yield, as well as variability due to systematic factors, for instance the higher amount of bacteria expected in specimens from deeper pockets characterizing periodontitis.

It was verified that observed data, specifically the relative amounts and the absolute amounts of bacterial species, did not fit the Gaussian distribution, either as is, or after common transformations of data. For this reason, quantitative data analysis was performed with the non-parametric Mann-Whitney statistics.

The prevalence of each bacterial species in the patient groups was compared by Pearson's *Chi*-square test on 2x2 contingency tables.

RESULTS

Specimens of subgingival plaque from a total of 539 patients were investigated for the presence of eleven bacterial species by quantitative PCR.

The total amount of bacteria detected in periodontal pocket specimens was strongly dependent on patient diagnosis; indeed it increased progressively from healthy (PSR code 0), through gingivitis (PSR code 1, 2), to periodontitis (PSR code 3, 4, or *) (Kruskal-Wallis test, P value$<10^{-11}$). In order to contrast the effect of this confounding variable, statistical tests to evaluate pathogen involvement in periodontal disease were performed by comparing the relative amount of each species (i.e. ratio of specific pathogen load to total microbial load), instead of the crude measured amount.

The relative amount of eleven bacterial species was investigated to test for association with periodontitis. Periodontitis patients (PSR code 3, 4, or *) compared to unaffected controls (PSR code 0, 1, and 2) showed higher levels of 6 species of pathogens with strong statistical support: *E. saphenum, P. endodontalis, P. gingivalis, T. forsythia, T. denticola, T. lecithinolyticum* (Table I). Another two species, *C. rectus* and *C. ochracea*, showed borderline levels of significance. In particular, *C. ochracea* appeared to be less abundant in periodontitis.

The direct observation of data indicated that only a fraction of samples showed detectable amounts of specific pathogens. For this reason, in order to further detect the role of each bacterial species in periodontitis we analyzed the prevalence of bacterial species among patient groups. Table IV reports the observed prevalence of the investigated bacterial species and their association with periodontitis. The lowest prevalence was observed for *A.*

Table I. *Relative amounts for bacterial species detected in periodontal pockets of periodontitis patients and controls. Controls included both healthy and gingivitis patients.*

Species	Group	Relative amount, %			Mann-Whitney test	
		Quartiles				
		0.25	Median	0.75	U	P value
A. actinomycetemcomitans	control	0.00	0.00	0.00	33901	0.647
	periodontitis	0.00	0.00	0.00		
A. rimae	control	0.00	0.02	0.22	33312	0.557
	periodontitis	0.00	0.02	0.21		
C. rectus	control	0.33	1.32	3.40	27859	0.034
	periodontitis	0.20	0.90	2.43		
C. ochracea	control	0.00	0.00	1.56	30650	0.038
	periodontitis	0.00	0.34	1.46		
E. saphenum	control	0.00	0.00	0.00	30183	0.002
	periodontitis	0.00	0.00	0.02		
F. nucleatum	control	0.48	2.40	4.95	29582	0.332
	periodontitis	0.69	2.10	5.55		
P. endodontalis	control	0.00	0.00	0.53	26524	<0.001
	periodontitis	0.00	0.15	1.94		
P. gingivalis	control	0.00	0.00	0.08	22897	<0.001
	periodontitis	0.00	0.15	6.11		
T. forsythia	control	0.00	0.00	0.36	25022	<0.001
	periodontitis	0.00	0.12	2.44		
T. denticola	control	0.00	0.00	0.61	26321	<0.001
	periodontitis	0.00	0.33	2.02		
T. lecithinolyticum	control	0.00	0.00	0.21	29239	0.002
	periodontitis	0.00	0.04	0.48		

Table II. *Occurrence of bacterial species in periodontitis patients and controls. Controls include both healthy and gingivitis patients.*

Specie	Group	Negative	Positive	Prevalence	P value	OR (95% C.I.)
A. actinomycetemcomitans	control	302	31	0.09	ref.	ref.
	periodontitis	189	17	0.08	0.675	0.9 (0.5-1.6)
A. rimae	control	141	192	0.58	ref.	ref.
	periodontitis	96	110	0.53	0.330	0.8 (0.6-1.2)
C. rectus	control	162	161	0.50	ref.	ref.
	periodontitis	68	125	0.65	0.001	1.9 (1.3-2.7)
C. ochracea	control	27	306	0.92	ref.	ref.
	periodontitis	19	187	0.91	0.650	0.9 (0.5-1.6)
E. saphenum	control	269	64	0.19	ref.	ref.
	periodontitis	144	62	0.30	0.004	1.8 (1.2-2.7)
F. nucleatum	control	56	253	0.82	ref.	ref.
	periodontitis	15	168	0.92	0.002	2.5 (1.4-4.5)
P. endodontalis	control	174	159	0.48	ref.	ref.
	periodontitis	66	140	0.68	<0.001	2.3 (1.6-3.3)
P. gingivalis	control	221	112	0.34	ref.	ref.
	periodontitis	76	130	0.63	<0.001	3.4 (2.3-4.9)
T. forsythia	control	173	160	0.48	ref.	ref.
	periodontitis	53	153	0.74	<0.001	3.1 (2.1-4.6)
T. denticola	control	191	142	0.43	ref.	ref.
	periodontitis	84	122	0.59	<0.001	2.0 (1.4-2.8)
T. lecithinolyticum	control	194	139	0.42	ref.	ref.
	periodontitis	86	120	0.58	<0.001	1.9 (1.4-2.8)

Table III. *Relative amounts for bacterial species detected in periodontal pockets of gingivitis patients and healthy controls.*

Species	Group	Relative amount, %			Mann-Whitney test	
		Quartiles				
		0.25	Median	0.75	U	P value
A. actinomycetemcomitans	healthy	0.00	0.00	0.00	12635.5	0.581
	gingivitis	0.00	0.00	0.00		
A. rimae	healthy	0.00	0.00	0.09	11153.5	0.035
	gingivitis	0.00	0.00	0.08		
C. rectus	healthy	0.00	0.00	0.40	9932	0.003
	gingivitis	0.00	0.01	1.00		
C. ochracea	healthy	0.00	0.00	0.17	10384	0.003
	gingivitis	0.00	0.00	0.46		
E. saphenum	healthy	0.00	0.00	0.00	12402	0.420
	gingivitis	0.00	0.00	0.00		
F. nucleatum	healthy	0.00	0.00	0.14	9967.5	0.006
	gingivitis	0.00	0.03	0.26		
P. endodontalis	healthy	0.00	0.00	0.21	11763.5	0.158
	gingivitis	0.00	0.00	0.90		
P. gingivalis	healthy	0.00	0.00	0.11	12773.5	0.891
	gingivitis	0.00	0.00	0.38		
T. forsythia	healthy	0.10	0.71	3.20	11230	0.037
	gingivitis	0.51	1.64	3.56		
T. denticola	healthy	0.06	1.80	3.60	11274	0.036
	gingivitis	0.81	2.70	5.40		
T. lecithinolyticum	healthy	0.00	0.00	0.70	10958	0.012
	gingivitis	0.00	0.18	1.89		

Table IV. *Occurrence of bacterial species in gingivitis patients and healthy controls.*

Specie	Group	Negative	Positive	Prevalence	P value	OR (95% C.I.)
A. actinomycetemcomitans	healthy	109	13	0.11	ref.	ref.
	gingivitis	193	18	0.09	0.520	0.8 (0.4-1.7)
A. rimae	healthy	62	60	0.49	ref.	ref.
	gingivitis	79	132	0.63	0.017	1.7 (1.1-2.7)
C. rectus	healthy	74	46	0.38	ref.	ref.
	gingivitis	88	115	0.57	0.001	2.1 (1.3-3.3)
C. ochracea	healthy	19	103	0.84	ref.	ref.
	gingivitis	8	203	0.96	<0.001	4.7 (2.0-1.1)
E. saphenum	healthy	96	26	0.21	ref.	ref.
	gingivitis	173	38	0.18	0.461	0.8 (0.5-1.4)
F. nucleatum	healthy	29	87	0.75	ref.	ref.
	gingivitis	27	166	0.86	0.013	2.1 (1.2-3.7)
P. endodontalis	healthy	67	55	0.45	ref.	ref.
	gingivitis	107	104	0.49	0.459	1.2 (0.8-1.9)
P. gingivalis	healthy	81	41	0.34	ref.	ref.
	gingivitis	140	71	0.34	0.994	1.0 (0.6-1.6)
T. forsythia	healthy	71	51	0.42	ref.	ref.
	gingivitis	102	109	0.52	0.083	1.5 (0.9-2.3)
T. denticola	healthy	77	45	0.37	ref.	ref.
	gingivitis	114	97	0.46	0.106	1.5 (0.9-2.3)
T. lecithinolyticum	healthy	82	40	0.33	ref.	ref.
	gingivitis	112	99	0.47	0.012	1.8 (1.1-2.9)

actinomycetemcomitans, while the bacteria widely found in periodontal pockets were *C. ochracea* and *F. nucleatum*. Eight species displayed strong evidence of association with periodontitis, with odds ratios varying from 1.9 (95% C.I. 1.3-2.7) of *C. rectus* to 3.4 (95% C.I. 2.3-4.9) of *P. gingivalis*. Interestingly, the two statistical approaches, the quantitative analysis of relative amounts and the prevalence analysis, produced conflicting results for *F. nucleatum* and *C. ochracea*.

In the second part of the study we investigated the relative amounts and the prevalence of the 11 bacterial species in patients with gingivitis (PSR code 1 and 2) compared with healthy patients (PSR code 0). Quantitative analysis indicated that *C. rectus, C. ochracea,* and *F. nucleatum* were strongly associated with gingivitis, but also *A. rimae, T. forsythia, T. denticola, and T. lecithinolyticum* with a lower statistical support. The analysis of prevalence indicated a strong association between *C. ochracea* and gingivitis, and the odds ratio was 4.7 (95% C.I. 2.0-11). A significant increase of prevalence in gingivitis was observed also for *A. rimae, C. rectus, F. nucleatum, and T. lecithinolyticum.*

DISCUSSION

The use of molecular methods has become a valid alternative to bacterial cultivation in periodontal plaque composition. Real time PCR, in respect to endpoint PCR and other techniques, such as dot blot hybridization, offers several advantages including higher sensitivity, higher specificity (especially when a sequence specific dual-labelled probe is used for detection), and quantitative data production. Moreover, in recent years the cost of this technique has become more affordable.

Quantification of bacterial species by real time PCR requires a careful selection of target sequence to maximize specie-specificity. The optimal target is a single sequence having a balanced level of conservation, sufficiently different to confer the ability to discriminate among related species, but at the same time conserved in the different strains. We decided to target the 16S rRNA gene because its sequence is extremely well characterized across many known and unknown bacterial species, thus ensuring more confidence in assay design. A potentially adverse effect of this target is the reduction of bacteria quantification accuracy, because the numbers of 16S rRNA genes vary from one to 15 copies in different microbial genomes (14). However, no bias was actually introduced in the study, because the main aim of the investigation was to compare infection levels among groups of patients. Indeed, the error was systematic and equally affected the quantification of all samples. On the contrary, a multiple gene copy number can be a benefit because it boosts the sensitivity of the assay.

Another aspect that characterized our approach was the evaluation of the multiplicity of infection of each species; we compared proportions of bacteria - i.e. relative amounts calculated as a fraction of total bacterial load - instead of crude observed amounts. This approach focused more precisely on the differences of bacterial composition associated with periodontal disease, because it can balance the non-random bacterial load variability in specimens from patients with different diagnosis. Indeed, bacterial load is expected to increase along with periodontal disease severity, as a function of periodontal pocket depth increment.

The present cross sectional investigation analyzed the prevalence and the relative amount of eleven selected bacterial species in periodontal disease. First, we investigated which pathogens were involved in periodontitis and which ones initiate their settlement in gingivitis. Not surprisingly, the strongest levels of association with periodontitis were found for the three species that constitute the red complex (15). In fact, the nonparametric test showed that the relative amounts of *P. gingivalis, T forsythia*, and *T. denticola* were higher in periodontitis patients with respect to controls, that included healthy and gingivitis subjects. The prevalence of these species among periodontitis patients was significantly higher compared with patients without periodontitis. The calculated odds ratios were 3.4 (95% C.I. 2.3-4.9), 3.1 (95% C.I. 2.1-4.6), and 2.0 (95% C.I. 1.4-2.8), for *P. gingivalis, T. forsythia*, and *T. denticola,* respectively. Additional species that showed association with periodontitis in our study were *E. saphenum, P. endodontalis* and *T. lecithinolyticum*. This confirmed the preliminary report by Kumar and colleagues that compared the prevalence of 39 bacterial species in 66 healthy and 66 periodontitis patients (11). Indeed, prevalence of

E. saphenum, P. endodontalis and *T. lecithinolyticum* was significantly higher in periodontitis and further support was obtained by means of quantitative data analysis. Weak levels of association with periodontitis were found for *C. rectus, C. ochracea* and *F. nucleatum* in either quantitative or prevalence analyses. Data suggested instead that these three species were strongly related to gingivitis. This was particularly evident for *C. ochracea;* indeed, the comparison of data in Tables I-IV indicates that both prevalence and relative amounts were the highest in gingivitis.

Among the six species that showed association with periodontitis, *T. forsythia*, *T. denticola,* and *T. lecithinolyticum* also appeared to be related to gingivitis. Indeed, relative amounts and prevalence of gingivitis patients appeared intermediate with respect to groups of healthy and periodontitis patients. Notably, the other three species, *E. saphenum, P. endodontalis, and P. gingivalis,* showed a specific and strong association with periodontitis and no involvement in gingivitis.

It was previously observed that *A. rimae* was more prevalent in healthy subjects than in periodontitis patients, supporting *A. rimae* as a possible healthy marker candidate (11). Our results did not corroborate this preliminary result, because *A. rimae* was almost equally represented in all three patient conditions, with the exception of a slightly higher prevalence among gingivitis patients.

One of the most intensively investigated bacterial species in periodontal pathology has been *A. actinomycetemcomitans* (16). The most convincing correlation between the presence of A. actinomycetemcomitans and periodontal disease was found with localized aggressive periodontitis (17). No evidence of association between infection of *A. actinomycetemcomitans* and gingivitis, nor chronic periodontitis was found in our study. This result agreed with the evidence that in the recent literature no cohort investigation supported a role for *A. actinomycetemcomitans* in the pathogenesis of periodontitis in adults (18). In the present study it proved to be the less prevalent species, and indeed was found in only about 10% of cases. A systematic review of the literature reported that the prevalence of *A. actinomycetemcomitans* fluctuated among studies; it was observed that the high level of variation between studies possibly depended on the detection techniques, or on characteristics of the selected cohort, such as age, ethnicity, and social status (19). Indeed, prevalence was generally higher in young patients and in populations from developing countries, while our cohort consisted of Italian adult patients.

The method used to evaluate periodontal disease could be considered a potential weakness of this study. The PSR is a rapid and non-invasive measure of periodontal status, useful for case definition. However, it cannot be considered a comprehensive periodontal examination able to produce a precise diagnosis. PSR may underdiagnose periodontal conditions, i.e. disregarding clinical attachment levels it could not detect periodontitis in patients who had successful periodontal treatment. Nonetheless, PSR demonstrated a high predictive potential compared to current periodontal disease definitions as outlined by the 1999 International Workshop Classification of Periodontal Disease (20, 21).

It was observed that intra-species genetic heterogeneity can modulate bacterial pathogenicity in periodontitis (22). This could partially explain the relatively high prevalence of periodontal pathogens in healthy individuals. The identification of specific variations responsible for the elevated level of pathogenicity, as well as the development of specific diagnostic tests, could be the next challenge in periodontitis research.

Considering the high prevalence of periodontal disease and the negative consequence on human health, the advent of a specific and sensitive diagnostic test able to improve diagnosis based on physical examination is very attractive. Indeed, a diagnostic test monitoring the microbial species responsible for periodontal disease progression could help to define a more accurate prognosis and patient-tailored therapeutic strategies.

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