Prevalence of six periodontal pathogens in subgingival samples of Italian patients with chronic periodontitis

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SUMMARY

The aim of this study was to investigate the subgingival prevalence of six periodontal pathogens in 352 Italian patients with chronic periodontitis. Possible correlations with clinical parameters, age, gender and smoking status were also investigated.
At first visit a pooled subgingival plaque sample was obtained for each subject by using the paper-point method. The samples were processed and analysed according to a commercially available quantitative real time polymerase chain reaction assay (Meridol® Perio Diagnostics, GABA International, Switzerland). *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), *Fusobacterium nucleatum* (Fn) and *Aggregatibacter actinomycetemcomitans* (Aa) were investigated.
Fn resulted the most frequently detected (95%) while Tf showed the highest load ($12x10^5$ cells/plaque sample). Aa was the less represented bacteria for load and presence. Bacterial load of Pg, Td, Tf and Fn showed a direct correlation to Bleeding On Probing (BOP) and presence of suppuration (p=0.0001). The bacterial load was always directly correlated to Probing Pocket Depth (PPD) (p=0.0001). Among the investigated variables, PPD resulted the most important risk indicator for periodontal pathogens. BOP appeared as a risk indicator for Td, Tf, Pg detection.
Few studies have described the microbiological pattern of chronic periodontal disease in the Italian population. Considering the different forms of periodontitis, similar investigations in other countries are needed to disclose any microbiological differences among populations, which may lead to more specific approaches to prevention and therapy.

KEY WORDS: Chronic periodontitis, Periodontal pathogens, Red complex, PCR, BOP, PPD.

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INTRODUCTION

Periodontitis is an inflammatory disease affecting the supporting tissues of the teeth. Due to its instauration a progressive destruction of the periodontal support is induced and, if untreated, it can ultimately lead to tooth loss (Kinane 2001).
Periodontitis is caused by microorganisms that grow on subgingival tooth surfaces, along with a host immune response. The bacterial role on onset and progression of periodontal diseases has been extensively documented (Curtis *et al.*, 2005; Van Winkelhoff and Boutaga 2005). In 1999 the American Academy of Periodontology proposed the last revised classification system for periodontal diseases identifying different forms of periodontitis (Armitage 1999). This system has lead to more focused investigations to comprehend the specificities of each form such as the microbiological aspects.

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A major contribution to modern periodontal microbiology comes from the 1998 Socransky *et al*. monumental study (Socransky *et al.,* 1998). Studying the relationships among subgingival bacteria from 13,261 plaque samples, the authors observed that species abundance clustered into major bacterial chromatic complexes.

A complex consisting of *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), and *Tannerella forsythia* (Tf) was strongly related to clinical parameters of periodontitis. Termed the Red Complex (RC), it has since been repeatedly associated with a specific form of periodontitis: chronic periodontitis (Haffajee *et al*. 1998; Papapanou *et al.,* 2002; Ximenez-Fyvie *et al.,* 2000).

An ecological succession of bacterial complexes involved in the initiation and progression of chronic periodontitis was also identified by Socransky *et al*. A bacterial consortium named Orange Complex (OC), including *Prevotella intermedia* (Pi) and *Fusobacterium nucleatum* (Fn), was indicated as the one preceding RC in colonization and proliferation (Socransky *et al.*, 1998).

Many clinical studies have been conducted worldwide providing evidence of associations between bacterial species and chronic periodontitis. Comparing data from different countries/populations it has become apparent that there are substantial differences in the composition of the subgingival microbiota (Haffajee *et al.,* 2004; Papapanou *et al.,* 2002; Sanz *et al.,* 2000; Yano-Higuchi *et al.,* 2000). Environmental, economic and genetic variables have been advocated to explain this observation (Mager *et al.,* 2003; Sirinian *et al.,* 2002; Van der Velden *et al.,* 2003). Owing to this, the study of the subgingival microbiota in a particular country becomes relevant not only to understand its implications in the pathogenesis of periodontal disease but also to identify its possible impact on treatment outcomes.

The aim of the present study was to investigate by means of real-time polymerase chain reaction (PCR) the prevalence and load of six periodontal pathogens on subgingival plaque samples from Italian chronic periodontal patients. Clinical parameters and their relations to microbial data were also investigated.

MATERIALS AND METHODS

Subjects, clinical examination and sample collection

This cross-sectional study was performed with samples from 352 consecutive patients with chronic periodontitis attending a periodontal office (LC) in Bologna, Italy.

Patients enrolled signed an informed consent form and the inclusion criteria were the following:

- diagnosis of chronic periodontal disease (Armitage 1999);
- presence of at least 12 teeth (except the third molars);
- no periodontal treatment in the last 6 months;
- absence of systemic diseases such as diabetes, arthritis, ulcerative colitis, Crohn's disease, HIV infection, cancer and heart disease;
- at least 3 months without systemic antibiotherapy;
- no anti-inflammatory drugs intake in the previous month;
- no ongoing pregnancy.

All patients were clinically and radiographically examined at first visit.

Plaque sampling was performed in a second session avoiding oral rinsing and before any periodontal procedure. Any bleeding, suppuration and PPD of the investigated sites were recorded at this time. Probing pocket depth (PPD) was always registered by means of a standard periodontal probe (Hu Friedy PCP11).

Five periodontal teeth (PPD >4 mm) were selected and at least one element for each quadrant was examined. Before sampling the supragingival plaque was carefully removed using a Gracey curette, the site was isolated with cotton roll and gently dried. For each site, a sterile paper cone was inserted into the bottom of the pocket and removed after 10 seconds. For each patient all paper cones were pooled in a sterile empty test tube and sent to the microbiological laboratory for examination.

Microbiological evaluations

The samples were sent to the GABA International GmbH laboratory (Munster, Germany). Samples were supplemented with reduced

Wilkins-Chalgren suspension and vortexed for 30 seconds to obtain a homogeneous suspension. 0.5 ml of the suspension was used for real-time PCR analysis. The cells were harvested by centrifugation (15,000 g at 4°C) for 10 min and immediately subjected to the automated process of the meridol® Perio Diagnostics (GABA International, Switzerland) analysis.
This method determines the total bacterial load present in the sample and quantifies six major periodontal pathogens with a sensitivity of 100 cells per type of pathogen: *Aggregatibacter actinomycetemcomitans* (Aa), *Fusobacterium nucleatum* (Fn), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia (*Pi), *Tannerella forsythia* (Tf) and *Treponema denticola* (Td). In brief, the bacterial genomic DNA was isolated and purified, than analyzed using oligonucleotid probe technology. Primers and probes were designed to match highly specifically to ribosomal DNA. The samples were also hybridized to a universal bacterial probe. This real-time PCR-based analysis was developed and validated by Carpegen GmbH as previously described (Jervoe-Storm *et al.*, 2005).
Following the previously cited Socransky principles the studied organisms were grouped into RC (*P. gingivalis, T. denticola, T. forsythia*) and OC (*P. intermedia, F. nucleatum*), while Aa was considered on its own, due to its independence from the bacterial complexes.

Statistical analysis

Prevalence and standard errors of the rates were computed. Rho Spearman correlation coefficient was used to evaluate the correlation between the bacterial cell count and the deepest value of probing depth, number of sites with bleeding on probing and number of sites with suppuration. χ^2 test was used for comparison of prevalence of the microorganisms between smokers and non-smokers. Each patient was identified by the five PPDs median value; the medians of each patient were synthesized using arithmetic mean.
Binary unconditional logistic regression (method backward) was used to explain which of the following variables (age, gender, smoking status, PPD, number of sites with BOP, number of sites with suppuration) was putatively associated with the presence of the microbial species; α was at 0.05; for multiple comparisons it was adjusted at 0.003.

RESULTS

The demographic and clinical characteristics of the 352 patients are shown in Table 1.
The six periodontal pathogens were all detected and at least one was found in each patient (Table 2): Fn and Aa were the most and the least prevalent microorganisms respectively. With regard to the median bacterial load, the microorganisms of the RC were the most abundant representing 44% of the total bacterial load of oral cavity and 66% of the total bacteria load of the 6 studied bacteria. Similar percentages were recorded for Pg (25%), Td (22%) and Tf (19%).
Bacterial load significantly increased by increasing mean probing depth of the five patient's sites; Spearman coefficient was the highest for Tf (r=0.52, p=0.0001) and the lowest for Aa (r=0.15, p=0.04). The bacterial load significantly increased by increasing the number of bleeding sites; Spearman coefficient varied from 0.14 for Pi (p=0.01) to 0.39 for Td (p=0.001).
With the exception of Aa and Pi, the bacterial load significantly increased by increasing the number of sites with suppuration; Spearman coefficient varied from 0.18 for Fn (p=0.001) to 0.26 for Td (p=0.001).
The prevalence of the six organisms was generally higher in smokers than non-smokers,

TABLE 1 - *Demographic and clinical characteristics of studied patients (N=352). SD: standard deviation; SE: standard error; PPD: pocket probing depth measured in mm; CAL: clinical attachment levels measured in mm; BOP: bleeding on probing.*

Characteristics	
Patients	352
% Females	58
Age (mean ± SD)	47±12
% Smokers	29
PPD (mean ± SD)	6±2
CAL (mean ± SD)	7±2
% sites with BOP (± SE)	31±0.01
% sites with suppuration (± SE)	0.04±0.02

TABLE 2 - *Number and prevalence of positive subjects for the examined microorganisms in the whole sample (N=352). Subjects with microbial load below the sensibility level of the test were considered negative. CI: confidence interval; IR: interquartile range; *: bacterial cells/plaque samples*

Microorganism		*Frequency of detection*		*Median bacterial load*		*Median bacterial load/ Median total bacterial load*	
		N (%)	*CI 95% (%)*	*(*10^5 cells)*	*IR*	*(%)*	*CI 95% (%)*
Red complex	Tf	306 (87)	85-89	12	57500-3550000	17	13-21
	Td	289 (82)	80-84	11	2525-5525000	15	11-19
	Pg	275 (78)	76-80	8.55	250-8125000	12	9-15
Orange complex	Fn	334 (95)	94-96	1.45	11000-700000	2	1-3
	Pi	232 (66)	63.5-68.5	1.07	0-2200000	2	1-3
	Aa	65 (18.5)	17-21	0	-	-	-

TABLE 3 - *Logistic regression on the microbial species of gender, age, smoking, BOP and PPD. Only variables with a significant contribute to the stepwise model are reported.*

Micro-organism	*PPD (mm)*		*Number of sites with BOP*		*Gender[a]*		*Smoke[b]*		*Age (years)*	
	OR	*CI 95%*	*OR*	*CI 95%*	*OR*	*CI 95%*	*OR*	*CI 95%*	*OR*	*CI 95%*
Tf	1.73	1.37-2.20	1.35	1.02-1.80	-	-	4.87	1.63-14.53	-	-
Td	1.44	1.19-1.74	1.50	1.16-1.93	-	-	2.44	1.14-5.24	-	-
Pg	1.40	1.17-1.67	1.34	1.09-1.65	1.67	1.02-2.75	-	-	1.03	1.01-1.06
Fn	2.02	1.43-2.86	-	-	-	-	-	-	-	-
Pi	1.32	1.16-1.52	-	-	-	-	-	-	-	-
Aa	-	-	1.27	1.10-1.47	-	-	-	-	-	-

OR: odds ratio; [a]reference category: female; [b]reference category: no smoker.

however this observation was statistically supported only for Td (89% vs 79%, p=0.04) and Tf (96% vs 83%, p=0.002). No significant differences in mean PPD, BOP and suppuration of the five sites were detected between smokers and non-smokers.

Results of logistic regression among periodontal pathogens and studied variables are reported in Table 3. Except for Aa, it was more likely to detect one of the examined species with increasing values of PPD. Smokers had 4.87 and 2.44 times more probability than non smokers to be positive for Tf and Td respectively. The probability to find members of the RC and Aa increased with the number of bleeding sites. Among the examined organisms, Pg was the only one significantly correlated to all variables studied, except for the smoking status.

DISCUSSION

For a long time it has been assumed that the mean subgingival microbial profiles in a given periodontal disease condition would be independent from the geographic area. However, the few reports that have compared dental plaque microbiota in individuals from different geographic locations have shown differences in microbial composition (Haffajee *et al.,* 2004; Papapanou *et al.,* 2002; Sanz *et al.,* 2000; Yano-Higuchi *et al.,* 2000). These studies were conducted in European countries, the USA, South America and recently in Asiatic populations, mainly in China and Japan. The results of these studies must be carefully interpreted for two main reasons: the different number of sample sites examined in each subject and the microbial technique employed. These two factors can strongly impact on the proportion of subjects positive for specific bacterial species.

Among the European studies, there are a few Italian reports evaluating the prevalence of some periodontal pathogens (Aimetti *et al.,* 2007; Santangelo *et al.,* 2004). The present data contribute to determine the epidemiological distribution of six main periodonto-pathogens (Pg, Tf, Td, Pi, Fn, Aa) detected by PCR in adult

Italian patients with chronic periodontal disease. In a total of 1760 sites from 352 subjects, the bacterial prevalence and load, the correlation with age, gender, smoking status, PPD, BOP and presence of suppuration were investigated.

The real-time PCR results show that at least one species of periodonto-pathogens was found in each patient.

The presence of Fn was assessed in 95% of patients which, is in line with reported prevalence rates (80%-100%) (Ali *et al*. 1994; Kamma *et al.,* 1994; Papapanou *et al.,* 1993; Sanz *et al.,* 2000). Published data (Paster *et al.,* 2001) have shown that Fn is one of the most abundant gram negative anaerobes in mature supragingival and subgingival plaques of both healthy subjects and patients with periodontitis. These results confirm its ubiquity.

The less prevalent microorganism was Aa, affecting only 65 patients with chronic periodontal disease (18.5%). This result agrees with a previous study conducted with Sri Lankan tea workers (Preus *et al.,* 1995), Brazilian (Cortelli *et al.,* 2005; Roman-Torres *et al.,* 2010) and Japanese populations (Yoshida *et al.,* 2003), and is close to the range defined by other authors (20%-40%) (Ali *et al.,* 1994; Ali *et al.,* 1996; Dahlen *et al.,* 1989; Kamma *et al.,* 1994; Papapanou *et al.,* 1993; Sanz *et al., 2*000). During the last two decades it has been shown that Aa can be regarded as a major pathogen in aggressive periodontal disease (Slots 1999; Slots *et al.,* 1990; van der Reijden *et al.,* 2008). Moreover, the species is represented by six serotypes (a-f) that have a different pathogenic activity in relation to the different amount of leukotoxin they produce. The highest leukotoxin-producing strain is uniquely associated with aggressive periodontitis (Mombelli *et al.,* 2002). The global distribution of the different Aa serotypes is not homogeneous, implying that the association between serotypes and periodontal status may depend on the geographical location and/or ethnicity of the study population (Fine *et al.,* 2007; van der Reijden *et al.,* 2008).

The high prevalence detected for Pg (78%) agrees with previous results obtained in Spanish (Sanz *et al.,* 2000), Kenyan (Dahlen *et al.,* 1989), Norwegian (Ali *et al., 1*994) and Romanian populations (Ali *et al.,* 1996). Among the examined organisms, Pg was the only bacterium significantly correlated to all variables studied except smoking status. Data from other studies are heterogeneous and some support the existence of differences in the prevalence of Pg between smokers and non-smokers (Haffajee and Socransky 2001); other authors found no significant differences (Apatzidou *et al.,* 2005; Decaillet *et al.,* 2012; Lie *et al.,* 1998). Dealing with clinical variables correlated to Pg, the present data are partially confirmed by the results of Mombelli *et al*. who found only a significant positive correlation between the number of deep sites (pocket depth >4 mm) and the prevalence of Pg positive sites (Mombelli *et al.,* 2000). Some authors suggest that Pg is associated with disease progression and the proportion of this species increases in deep pockets (Chen *et al*. 2010; Faveri *et al.,* 2009). A previous study by Preus *et al*. demonstrated that Pg load gradually increases from health sites to sites with gingivitis, to moderate periodontitis, up to severe periodontitis (Preus *et al.,* 1995).

The prevalence of Aa and Pg are in agreement with some recent studies that found an inverse relationship between the load of these two periodontal pathogens and a negative correlation between the proportions of Aa and the RC bacteria in patients with periodontitis (Chen *et al.,* 2010; Faveri *et al.,* 2009).

Tf has been evaluated in other studies (Ertugrul *et al.,* 2013; Herrera *et al.,* 2008; Kamma *et al.,* 1994; Thiha *et al.,* 2007; Tomita *et al.,* 2013) and the prevalence rate of 87% found is the highest after the Greek rate of 98.5% (Kamma *et al.,* 1994). Tf showed the highest correlation with PPD, its presence was associated with bleeding site and smoking status was a risk factor for its acquisition. These observations are in agreement with other published studies which found that smokers had significantly higher loads of, and were at greater risk of infection by Tf with respect to non-smokers (Yano-Higuchi *et al.,* 2000).

Pg and Tf co-infected 95% of the patients studied; this observation confirms previous data that investigated the ecological relationship between the two bacteria (Yano-Higuchi *et al.,* 2000). As suggested by Byrne *et al*., Tf may facilitate the emergence of Pg by providing an

environment favorable to its colonization and proliferation (Byrne *et al.*, 2009).
Td was strongly associated with smoking, supporting previous data by Haffajee *et al*. (Haffajee and Socransky 2001) and its load statistically correlated with the numbers of sites, BOP and suppuration.
The present study found that the proportion of the pathogenic cells examined out of the total cell count was between 2% (Fn and Pi) and 17% (Tf) and the load of all bacterial species (except Aa) was in direct relationship with the increment of PPD. In addition, the bacterial counts of the six examined species in the bleeding sites were significantly higher than those of sites without BOP. This parameter could predict the presence of the RC because of their significant association.
The sampling strategy of the present work was based on pooling of microbiota residing in different sites for each tooth. As demonstrated by Krigar *et al*., this sampling approach increases the bacterial counts per analysis compared to separate samples and thus may increase the probability of detecting existing pathogens (Krigar *et al.,* 2007). This study cannot elucidate if the pathogen distribution has any pathogenic or therapeutic implications. Longitudinal studies with sequential microbiological monitoring from different country/population are needed to disclose any clinically relevant differences.

CONCLUSIONS

The present survey evaluated 6 periodontal pathogens in a large cohort of chronic periodontal Italian subjects in relation to age, gender, smoking status and the main clinical parameters (PPD, BOP and suppuration).
The results demonstrate that the main putative periodontal pathogens are all detectable in Italian periodontal subjects. The low prevalence of Aa confirms its marginal role in chronic periodontitis.
Among the examined clinical parameters, PPD and BOP are the most important. In fact, the increase in PPD is directly correlated to the presence and load of all the bacterial species except Aa, while BOP could predict the RC.
Considering the different forms of periodontitis, similar investigations in other countries are needed to disclose any microbiological differences among populations, leading to more specific approaches in prevention and therapy.

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