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ORIGINAL ARTICLE

Subgingival bacteria in Ghanaian adolescents with or without progression of attachment loss

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Objective: This study describes subgingival bacterial profiles associated with clinical periodontal status in Ghanaian adolescents with or without progression of attachment loss.

Materials and methods: Among 500 adolescents included in a cohort study, 397 returned 2 years later for a periodontal re-examination, including full-mouth CAL measurements. At follow-up, a subgroup of 98 adolescents was also subjected to bacterial sampling with paper points at four periodontal sites (mesial aspect of 11, 26, 31, and 46) and analyzed with the checkerboard DNA–DNA hybridization technique against DNA-probes from nine periodontitis-associated bacterial species.

Results: The 98 Ghanaian adolescents examined in the present study were similar to the entire group examined at the 2-year follow-up with respect to age, gender, and CAL ≥ 3 mm. A high detection frequency of *Fusobacterium nucleatum* and *Prevotella intermedia* (>99%) using checkerboard analysis was found, while for *Aggregatibacter actinomycetemcomitans* the detection frequency was <50%. A strong correlation was found at the individual level between the presence of *P. intermedia* and the total CAL change, and *P. intermedia* and *Porphyromonas gingivalis* were strongly correlated with a change in CAL and probing pocket depth (PPD) at the sampled sites. In a linear regression model, a significant discriminating factor for the total CAL change in the dentition during the 2-year follow-up period was obtained for *P. intermedia* and public school.

Conclusion: This study indicates that subgingival bacterial species other than *A. actinomycetemcomitans*, for example, *P. intermedia*, have a significant association with periodontal breakdown (change in CAL) in Ghanaian adolescents with progression of periodontal attachment loss.

Keywords: *subgingival bacteria*; *periodontitis*; *attachment loss*; *progression*; *adolescent*; *Ghana*

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Periodontitis is well-defined as an infectious disease of bacterial etiology deleteriously affecting the supporting tissues of the tooth to such an extent that it may lead to tooth loss (1, 2). Periodontitis is considered a polymicrobial infection, but several studies have indicated a major role of *Aggregatibacter actinomycetemcomitans* in adolescents in the disease previously called juvenile periodontitis (now aggressive periodontitis), characterized by a rapid loss of clinical attachment loss (CAL), especially around the permanent first molars and incisors (3, 4). In particular, this

association between *A. actinomycetemcomitans* and periodontitis has been strengthened by studies focusing on the presence of a highly virulent genotype of *A. actinomycetemcomitans*, named the JP2 clone, expressing a highly leukotoxic activity. A number of studies have strongly associated the JP2 clone with aggressive periodontitis in young individuals of Northern and Western African origin (5–9). Such studies have been carried out in order to understand the high prevalence of aggressive periodontitis in young individuals of these populations and to evaluate the role of the JP2 and non-JP2

genotypes of *A. actinomycetemcomitans* in the progression of periodontitis. In a recent study, Åberg et al. (10) associated the progression of CAL in a young Ghanaian population with the presence of the JP2 genotype of *A. actinomycetemcomitans*. In the present study, we also asked whether other bacterial species were commonly associated with periodontitis, for example, if bacterial species belonging to the red and orange complexes (11, 12) were present in these young individuals and to what extent they could be related to CAL.

The aim of the present study was to describe the subgingival bacterial profiles associated with the clinical periodontal status in Ghanaian adolescents with or without the progression of attachment loss.

Materials and methods

Study population

A total of 500 adolescents (mean age 13.2 years, SD: 1.5) were enrolled in the study undertaken in Ghana in 2009 (9). After 2 years, 397 (79.4%) were recruited for a periodontal re-examination (10). Concomitantly, we analyzed clinically and microbiologically a subpopulation consisting of 98 of these school children during 6 working days. Each day after the clinical registrations, 15–20 children were consecutively selected for the present study and microbiological sampling. Of these 98 adolescents from six different schools in the suburban area of Accra, Ghana, 52 (53.1%) were girls. Twelve of the adolescents (seven girls and five boys) were recruited from one private school, and the remaining 86 were from five public schools. Detailed information on the study population, demographics, and ethics, including signed consents by parents has been reported on previously (9, 10). Knowledge on the socioeconomic status, the medical health status, and the intake of antibiotics within the past 3 months before the examination are available from the studies by Åberg and co-workers (9, 10). If any children were medically diseased or had had received antibiotics during the past 3 months, they were excluded from the present study.

Clinical recordings

The adolescents were given a full-mouth re-examination, including recording of CAL and PPD in the year 2011, and subsequently the change of CAL from the year 2009 until 2011 was calculated. The clinical measurements were performed by the same periodontist (CHÅ) who performed the measurements at baseline (9) as well as in the total group of adolescents participating in the 2-year follow-up (10).

Microbiological analysis

Bacterial sampling was carried out using the paper point technique at four sites, that is, the mesial aspect of 46, 11,

26, and 31 as separate samples from each site. The paper points were transferred to Eppendorf tubes containing 100 µl TE-buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) to which 100 µl of 0.5 M NaOH was added, mixed, and kept cold for further transportation to the laboratory at the Department of Oral Microbiology, University of Gothenburg, Göteborg, Sweden. After reaching the laboratory, the samples were boiled and 800 µl 5 M ammonium acetate was added to each tube. The bacterial samples were analyzed with the checkerboard DNA–DNA hybridization method against 10 bacterial probes from species commonly associated with periodontitis (in Caucasians): *A. actinomycetemcomitans* (two probes, one against a non-JP2 genotype strain FDC Y4 and one against a JP2 genotype strain OMGSP47/12), the red complex bacteria (11) *Porphyromonas gingivalis* (OMGS 2860), *Tannerella forsythia* (strain ATCC 43037), *Treponema denticola* (strain OMGS 3271), and the orange complex bacteria (11) *Campylobacter rectus* (strain ATCC 33238), *Fusobacterium nucleatum* (strain ATCC 10953), *Prevotella intermedia* (strain ATCC 25611), and *Filifactor alocis* (CCUG 47790), as well as *Porphyromonas endodontalis* strain OMGS 1205 (13, 14). The probes were digoxigenin-labeled whole genomic probes prepared by random priming, using the high-prime labeling kit (Roche Diagnostics, Mannheim, Germany) from the strains of the panel. The hybrids formed between the bacterial DNA and the probes were detected by the application of an anti-digoxigenin antibody conjugated with alkaline phosphatase and incubated with a chemiluminescent substitute (CDP-Star, Roche Diagnostics). The evaluation of the signal was performed at a LumiImager workstation (Boehringer-Mannheim, Mannheim, Germany) by comparing the signals obtained with those of pooled standard samples containing 10^6 (high standard) or 10^5 (low standard) of each of the 10 bacterial species, and the amount (cell number) of each bacterial species in each sample was calculated. The sensitivity of the whole genomic probes constructed as above has been described previously and was set to 10^4 (15). The specificity (cross-hybridization) of each probe was checked separately against all 10 bacterial strains included in the panel.

Statistical analyses

The prevalence and the mean cell number were calculated for the bacterial species in each sample. Pearson's correlation coefficients were calculated for each bacterial species with regard to the difference in CAL between the years 2009 and 2011 at individual and at site level, as well as PPD at the sampled sites. Linear regression analysis was also applied stepwise, and a final model was constructed for the dependent variable CAL at the individual level. Variables included in the model were

Table 1. Characteristics of the 397 Ghanaian adolescents examined at the 2-year follow-up and those examined for the present study ($n = 98$)

Variable	All individuals examined at the 2-year follow-up, $n = 397$ (%)*	Individuals included in the present study, $n = 98$ (%)
Age (mean \pm SD)	15.0 (\pm 1.4)	15.3 (\pm 1.5)
Positive for non-JP2 genotypes	189 (47.6)	44 (44.9)
Positive for JP2 genotype	38 (9.5)	16 (16.3)
Antibiotics less than 3 months before examination	42 (10.6)	8 (8.2)
Gender (males)	181 (45.6)	46 (46.9)
School type (Private)	129 (32.5)	12 (12.2)
Presence of sites with CAL \geq 3 mm	156 (39.2)	47 (48.0)
Cigarette smoking	0 (0)	0 (0)
Diabetes	0 (0)	0 (0)

*From Åberg et al. (10).

age, sex, type of the school that the children attended, *P. gingivalis*, and *P. intermedia*.

Results

Table 1 shows the characteristics of the 397 Ghanaian adolescents, being the whole group examined at the 2-year follow-up in 2011(10), and those examined for the present study ($n = 98$). The subgroup used in the present study did not differ from the 2-year follow-up ($n = 397$), except for a lower number of children attending private schools (12.2% vs. 32.5%).

Table 2 shows the detection frequency of nine bacterial species at four sampled sites and at subject level (based on the presence in one or more out of a maximum of four sampled sites in a total of four samples). Overall, a high detection frequency of all investigated bacterial species was found. *F. nucleatum* and *P. intermedia* were found in almost all subjects ($>99\%$) and in addition found as the highest number of cells. Also, *C. rectus*, *F. alocis*, and *P. endodontalis* were detected quite frequently, while the

detection frequency of the three red complex bacteria, *P. gingivalis*, *T. forsythia*, and *T. denticola* was lower both at subject level and at site level in particular.

The DNA probe against the JP2 clone cross-reacted fully with the non-JP2 genotypes of *A. actinomycetemcomitans*. Further calculations on *A. actinomycetemcomitans* and clinical variables were based on the non-JP2 probe. The detection frequency of *A. actinomycetemcomitans* was $<50\%$ at both subject and site level.

The correlation between bacterial variables and the total change of CAL during the 2-year follow-up (over all sites measured), the change of CAL and of PPD at sampled sites, respectively, was calculated (Table 3). A strong correlation between *P. intermedia* and the total change of CAL during the 2 years over all sites measured was found ($p \leq 0.01$). *P. intermedia* and *P. gingivalis* also correlated strongly with the change of CAL ($p \leq 0.001$ and $p \leq 0.01$, respectively) and change of PPD ($p \leq 0.01$ and $p \leq 0.01$, respectively) of the sampled sites during the 2 years. All the other bacterial species showed no

Table 2. The prevalence of nine bacterial species in individuals and at sampled sites

Bacterial species	Prevalence of specific bacterial species at subject level* given in%, $n = 98$	Prevalence of specific bacterial species at site level given in%, ($n = 392$)	Mean number of cells $\times 10^4$ when present (\pm SD)
<i>A. actinomycetemcomitans</i>	49.6	45.3	1.1 (\pm 1.1)
<i>C. rectus</i>	89.8	84.2	3.1 (\pm 2.8)
<i>F. nucleatum</i>	100	84.8	14.4 (\pm 8.4)
<i>F. alocis</i>	89.8	79.4	6.8 (\pm 13.3)
<i>P. endodontalis</i>	95.9	86.0	11.5 (\pm 11.9)
<i>P. gingivalis</i>	87.8	59.1	8.5 (\pm 11.3)
<i>P. intermedia</i>	99.0	78.2	19.0 (\pm 16.7)
<i>T. denticola</i>	73.5	44.1	1.8 (\pm 1.8)
<i>T. forsythia</i>	78.6	48.4	3.0 (\pm 3.2)

*Based on the presence in at least one of four samples.

Table 3. Correlations coefficients (Pearson's) between bacterial variables and the total change in CAL*, change of CAL and change of PPD at the four sampled sites

Variable	CAL change* total	CAL change* sampled sites	Change of PPD sampled sites
<i>A. actinomycetemcomitans</i>	-0.028 ($p = 0.78$)	-0.032 ($p = 0.74$)	-0.013 ($p = 0.90$)
<i>C. rectus</i>	-0.01 ($p = 0.92$)	0.78 ($p = 0.44$)	0.027 ($p = 0.79$)
<i>F. alocis</i>	0.043 ($p = 0.67$)	0.019 ($p = 0.85$)	0.053 ($p = 0.60$)
<i>F. nucleatum</i>	0.004 ($p = 0.97$)	0.12 ($p = 0.22$)	0.07 ($p = 0.49$)
<i>P. endodontalis</i>	0.09 ($p = 0.36$)	0.058 ($p = 0.57$)	0.077 ($p = 0.45$)
<i>P. gingivalis</i>	0.19 ($p = 0.055$)	0.26 ($p \leq 0.01$)	0.29 ($p \leq 0.01$)
<i>P. intermedia</i>	0.29 ($p < 0.01$)	0.37 ($p \leq 0.001$)	0.30 ($p \leq 0.01$)
<i>T. denticola</i>	0.070 ($p = 0.49$)	0.050 ($p = 0.62$)	0.044 ($p = 0.67$)
<i>T. forsythia</i>	0.019 ($p = 0.85$)	0.06 ($p = 0.54$)	0.004 ($p = 0.97$)

*CAL difference between 2009 and 2011.

significant correlation with clinical parameters. Age and gender did not correlate with the clinical parameters (data not shown).

By use of the linear regression model, we wanted to identify explanatory factors for a difference in CAL at subject level between 2009 and 2011. The two bacterial species, *P. gingivalis* and *P. intermedia* were included in the model due to significant correlations with CAL in contrast to the other bacterial species mentioned in Table 3. *P. intermedia* ($p \leq 0.05$) and type of school ($p \leq 0.05$), but neither *P. gingivalis* nor sex and age came out as significant discriminating factors in the model.

Discussion

The present study's aim was to describe the subgingival bacterial profile using the checkerboard technique on samples from Ghanaian adolescents with a high frequency of periodontal attachment loss. A strong correlation was seen between *P. intermedia* and *P. gingivalis* with a retrospective loss of CAL as well as CAL at the sampled sites. *P. intermedia* turned out to be a significant factor in a regression model designed to find explanatory factors for CAL. Private schools seemed (although the limited number of adolescents) also to have a significant influence on CAL, while age and sex did not seem to influence CAL.

A. actinomycetemcomitans did not, to some surprise, show any association with CAL in the present study group using the 'checkerboard' DNA-DNA hybridization method. We recently reported that the presence of both the JP2 and non-JP2 genotypes of *A. actinomycetemcomitans* was strongly associated with the onset and progression of AL in the whole group of Ghanaian adolescents of which the present study group constitutes a subgroup (10). In the baseline study, *A. actinomycetemcomitans* was identified by polymerase chain reaction (PCR) as the only bacterial species studied. A distinction between non-JP2 and JP2 genotypes of *A. actinomycetemcomitans* was also made.

Strains were also isolated in the baseline study and cultured on selective media. Both PCR and selective culturing are sensitive methods detecting the target bacteria in much lower cell numbers than the checkerboard method for which an estimated detection limit of 10^4 cells has been calculated (15). The microbiological data of the progression study (10) were collected 2 years earlier at baseline, whereas the microbiological data for the checkerboard analysis were collected 2 years later, where the periodontal status had progressed in many individuals. It is our experience, also noticed in the literature, *A. actinomycetemcomitans* is generally present in low amounts in advanced periodontitis in adults using culture, checkerboard technique, and real-time PCR, presumably due to a change over time of the microbiota from being more aerobic and facultative anaerobic to becoming gradually more anaerobic (11, 16, 17). It should be pointed out that *A. actinomycetemcomitans* is a facultative bacteria that is probably competitive in the early phase of gingival crevice colonization/infection.

Based on a previous study (10), *A. actinomycetemcomitans* seems to be predictively related to the CAL, and in particular the JP2 genotype. Concomitantly, anaerobes are established in the subgingival plaque flora and may out-compete the facultative bacterial species as a result of the changing environment in the deepening gingival/periodontal pocket. The predominant bacteria (*P. intermedia* and *F. nucleatum*) in the present study are supporting this. *P. intermedia* constituted an important factor and correlated strongly with total CAL change and CAL and PPD change at sampled sites. This supports the fact that during the progression of periodontitis, the infection changes its character from being highly associated with *A. actinomycetemcomitans*, the JP2 genotype in particular, to a more anaerobic and polymicrobial type of infection. It is interesting to note that *P. intermedia* independently of other bacteria in the panel showed an association with CAL, which indicates that this species may have a certain impact on the progression.

It is suggested that such lesions may change its ecological character and the aggressive phase of the periodontitis turns into a more chronic type.

An interesting finding is the comparatively high proportion of anaerobes in the lesions. Several anaerobic bacterial species were present frequently in high numbers in samples from individuals with or without aggressive periodontitis. On the contrary, bacterial species, such as *P. gingivalis*, *T. forsythia*, and *T. denticola*, ('red complex species') showed a comparatively lower detection frequency and amounts. The participants in the present study were young (13–19 years of age), and it is claimed that these 'putative pathogens' or red complex bacteria do not occur in detectable amounts until after puberty in various populations (18–21). However, it is surprising that the red complex bacteria did not 'overgrow' the remaining parts of the flora, even in older individuals (17–19 years of age) or in those that showed CAL and deep periodontal pockets already at baseline. It is a possibility that the population of Ghana has a different microbiological profile compared to the profile in Caucasians or Asians.

The prevalence of the investigated species is similar to results reported in studies based on adults from China, Thailand, and Sweden 22–24 using the same checkerboard DNA–DNA hybridization technique, although the amount of these species was lower in the present study. Few studies have been performed trying to evaluate the prevalence of 'putative periodontopathogens' in populations of the sub-Saharan part of Africa (9). Most studies performed in other populations are done on adults, while studies on children are rare (25). Two other studies on aggressive periodontitis in young individuals but in other populations (Afro-Americans in Florida and from Brazil) have shown higher levels of anaerobes, for example, *P. gingivalis*, *F. alocis*, and *Parvimonas micra* (26, 27). The only known study in the African population is the study from Morocco based on young individuals with aggressive periodontitis (6, 8, 28). It was concluded that the JP2 clone of *A. actinomycetemcomitans* constitutes a minor proportion of the complex subgingival microbiota in patients with active disease. A recent study also emphasized the remarkably high concentrations of antibodies against the *A. actinomycetemcomitans* leukotoxin, which may indicate that the disease activity was terminated ('burned out') in some patients with aggressive periodontitis and that such individuals may develop immunity to aggressive periodontitis (29). However, it was disclosed that representative species from seven different phyla and the bacterial species (including *A. actinomycetemcomitans*) evaluated in that study probably constituted a minor part of the flora. The study also showed great subject heterogeneity between the proportions of clones within these seven phyla (27).

A correlation between the change of CAL and those subjects attending public schools compared to those attending private schools was found (9). Similar to children in public schools, the children from private schools were low in periodontopathogens (red complex), which indicates that as long as they are periodontally healthy they are having a low tendency for the colonization with these bacteria, including the detection of *A. actinomycetemcomitans* at a relatively low level. Children from private schools were too few in the present study to evaluate whether they are generally less colonized with the red complex bacteria and *A. actinomycetemcomitans* before periodontal attachment loss starts. It is not known which factors particularly predispose the public school children to appear with CAL at such a high rate in their adolescence. The same was found for the much greater cohort of 397 children published elsewhere (10). It may be related to some social or genetic factor, and the present study does not support that a microbiological factor would be involved.

Conclusions

This study indicates a close relation between the presence of anaerobic oral bacterial species and periodontal breakdown in Ghanaian adolescents, and furthermore indicates that some species, such as *P. intermedia*, may have a significant association with aggressive periodontitis in this young Ghanaian population. Once periodontitis has developed, the character of the periodontal infection may change over time from an infection primarily associated with *A. actinomycetemcomitans* into a more anaerobic polymicrobial type of infection.

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Conflict of interest and funding

The authors declare that they have no conflict of interest.

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