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Occurrence and serotype distribution of *Aggregatibacter actinomycetemcomitans* in subjects without periodontitis in Turkey

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Abstract

Objective—To determine the occurrence and serotype distribution of *Aggregatibacter actinomycetemcomitans* in subjects without periodontitis.

Design—Systemically healthy dental students without periodontitis (n=94), who had not used antibiotics within the last 3 months or received any form of periodontal therapy within the last 6 months, were included in the study. Pooled subgingival microbiological samples were collected from 4 first molars and 4 central incisors in each subject using sterile paper points. All samples were tested for the presence and the serotype of *A. actinomycetemcomitans* through PCR analysis of the 16S rRNA genes and the serotype-specific gene clusters in the DNA extracted from the samples.

Results—Of the 94 samples that were tested, 43 (46%) were positive for *A. actinomycetemcomitans*. No statistically significant differences in clinical parameters were found between subgingival sites with or without detectable *A. actinomycetemcomitans* (t-test, $P>0.01$). Among the 43 *A. actinomycetemcomitans*-positive samples, the serotype was identified in 21

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Author's contributions:

Başak Doğan and Casey Chen designed and implemented the research protocol, performed data analysis, and prepared the manuscript for submission. Jason Chen, and Jonathan Huang processed the DNA samples and performed PCR analysis. Sinem Yıldız Çiftlikli, examined the subjects and collected the samples. Tanju Kadir, and Anıl Kınacı Alnıak organized the study subjects, calculated clinical parameter of each subject and put these data into the computer.

Conflicts of interest

None

Ethical approval

The study design was approved by the Ethics Committee of Medical Faculty, Marmara University (MAR-YÇ-2009-0064).

samples. Fifteen were positive for *A. actinomycetemcomitans* serotype a, 1 for serotype b, 1 for serotype c, and 4 for serotype f, while serotypes d and e were not detected.

Conclusion—*A. actinomycetemcomitans* serotype a is the most commonly found serotype among Turkish dental students without periodontitis.

Keywords

Periodontitis; PCR; serotype; virulence

1. Introduction

Gram-negative, nonmotile, facultative *Aggregatibacter actinomycetemcomitans* is a major etiologic agent of aggressive periodontitis and an occasional cause of non-oral infections (Asikainen & Chen, 1999; Slots & Ting, 1999; van Winkelhoff & Slots, 1999). The natural population structure of the species is clonal and comprises genetically distinct strains distinguished by serotypes (Kaplan, Schreiner, Furgang, & Fine, 2002; Kilian, Frandsen, Haubek, & Poulsen, 2006).

The clonal population structure of bacterial species is a consequence of recombination barriers between clonal lineages. Over time, individual lineages may diverge and acquire different biological properties via gain and loss of genes and also exhibit heterogeneity in virulence. For example, serotype b strains have been found to be more frequently detected in periodontitis than in periodontal health (Asikainen, Lai, Alaluusua, & Slots, 1991). Presumably strains of serotype b may possess unique virulence determinants, and the detection of specific serotype b strains may be indicative of a greater risk for periodontal disease progression. However, the associations between specific serotypes and disease must be interpreted in the context of the distribution of serotypes in the general population. If serotype b is also found to be the dominant serotype among subjects without periodontitis, the association between serotype b and disease is merely an extraneous finding.

Indeed, the distribution patterns of different serotypes of *A. actinomycetemcomitans* vary among geographical locations and race/ethnicity of the subjects (Chen, Wang, & Chen, 2010; Fine et al., 2007; Mombelli, Gmur, Lang, Corbert, & Frey, 1999; Saarela et al., 1992; Yang, Huang, Chan, & Chou, 2005). More pertinent to this study, serotype c of *A. actinomycetemcomitans* has been reported to be associated with subjects with periodontitis in Turkey (Dogan et al., 2003). It was postulated that serotype c represents the dominant pathogenic clone of *A. actinomycetemcomitans* in the Turkish population. To further explore the implication of this finding, this study was undertaken to determine the occurrence and serotype distribution of *A. actinomycetemcomitans* in Turkish students with healthy periodontium.

2. Materials and Methods

2.1. Study Subjects

The study design was approved by the Ethics Committee of Medical Faculty, Marmara University (MAR-YÇ-2009-0064). Fig 1 provides an overview of the subject recruitment

process. The study subjects were recruited from dental students attending Marmara University, Faculty of Dentistry, Turkey. The students were informed that the participation in this study was voluntary. Full-mouth periodontal and panoramic radiographic examinations were carried out by a single examiner (S.Y.Ç.). For the assessments of periodontal health status, the plaque index (PI) (Silness & Loe, 1964), gingival index (GI) (Loe & Silness, 1963), bleeding on probing (BOP), probing pocket depth (PPD), and clinical attachment level (CAL) were recorded at six sites for each tooth, (except third molars), and the number of teeth was calculated. PPD and CAL were measured in millimeters using a manual periodontal probe (UNC15; Hu-Friedy, Chicago, IL, USA).

The disease diagnostic criteria were based on the recent case definition model issued by the Centers for Disease Control and Prevention in partnership with the American Academy of Periodontology (Eke, Page, Wei, Thornton-Evans, & Genco, 2012). The subjects without periodontitis were defined as (a) not having two or more interproximal sites with CAL ≥3 mm, (b) not having two or more interproximal sites with PPD ≥4 mm on the same tooth, or one site with PPD ≥5 mm, and (c) no evidence of bone loss.

The subjects were selected based on the following criteria: without periodontitis, free of any systemic diseases as determined by a medical status questionnaire and individual interviews, no antibiotic usage during the last 3 months, no periodontal treatment during the preceding 6 months, no use of any form of medication that could alter their systemic status, no pregnancy or lactation, no current orthodontic treatment, and willingness to participate in the study.

From 579 officially registered students (grades 1 to 5), 520 were initially examined. Two hundred eighty-four of the 520 students were determined to be without periodontitis. Of the 284 subjects, 94 students (65 females and 29 males) between 17 and 26 years of age fulfilled other criteria and were selected for the study.

2.2. Microbiological Samples

Subgingival microbiological samples were collected from all 94 participants with sterile paper points. In each of the patients, samples were collected from 8 teeth: 4 from the mesio-buccal subgingival sites of 4 first molars and the other 4 from the mesio-buccal subgingival sites of 4 central incisors. In the presence of any restorations or caries, samples were collected from the disto-buccal subgingival site of the same tooth or the mesio-buccal subgingival site of an adjacent tooth distal to the initial tooth chosen. Sampling was performed as previously described (Dogan et al., 2003). A sterile paper point (No. 30, MetaAbsorbent Paperpoints; MetaBiomed Co. Ltd., Chungbuk, Korea) was inserted into each subgingival site for 10 seconds. The paper points were then pooled into a single empty sterile eppendorf tube and immediately placed at 70°C, where they were preserved until sent to Division of Periodontology, Diagnostic Sciences and Dental Hygiene, Ostrow School of Dentistry by express mail delivery to be used for further analysis.

2.3. PCR analysis

Sterile paper points bearing subgingival samples were immersed in PBS and vortexed to dislodge the bacteria. After removing the paper points, the samples were centrifuged at

20,000 RPM for 5 min to pellet the bacteria, The DNA was then extracted with a QIAamp DNA Mini Kit (Qiagen Inc.) following the manufacturer's protocol. DNA concentration was determined with NanoPhotometer (Implemen).

Samples were tested for the presence of *A. actinomycetemcomitans* through PCR detection of the 16S rRNA gene as described previously (Ashimoto, Chen, Bakker, & Slots, 1996; Chen, Wang, et al., 2010). Serotype was determined based on detection of serotype-specific antigen gene clusters with a modification of the previously described protocols (Chen, Wang, et al., 2010; Kaplan et al., 2001; Suzuki, Nakano, Yoshida, Ikeda, & Koga, 2001). A summary of the primers and PCR thermocycler profiles is provided in Table 1. Briefly, amplification was performed in a 25 μL volume of PCR mixture containing 1x Longamp *Taq* reaction buffer, dNTPs at either 240 μM (for single PCR analysis) or 300 μM (for multiplex PCR analysis), 1 μL of LongAmp *Taq* DNA polymerase, 20 ng of template DNA, and a final concentration of 0.4 μM of each primer pair. For serotype analysis, each DNA sample was subject to three different PCR assays, consisting of a multiplex PCR assay for serotype a/e, a multiplex PCR assay for serotype b/c/f, and a single PCR assay for serotype c. Negative control (no DNA) and positive controls (DNA from *A. actinomycetemcomitans*) were included in the assays. The resultant amplicons were analyzed in 1% agarose gel. The positive controls were genomic DNA from sequenced strains D7S-1 (serotype a), HK1651 (serotype b), D11S-1 (serotype c), I63B (serotype d), SCC393 (serotype e), and D18-P1 (serotype f). Detection limit was determined based on PCR analysis of serially diluted DNA samples of the reference genomic DNA to attain the final amount/reaction of 40 ng, 4 ng, 0.4 ng, 0.004 ng, 0.0004 ng, 0.00004 ng, and 0.000004 ng for testing.

2.4. Statistical analysis

Student's t-test was performed for the CAL, GI, PI, % BOP and PD of *A. actinomycetemcomitans*-positive and *A. actinomycetemcomitans*-negative sites, and for the DNA concentrations of serotype-positive and serotype-negative samples (both positive for the detection of *A. actinomycetemcomitans*).

3. Results

A summary of the clinical parameters of the study subjects is provided in Table 2. In agreement with the diagnosis of non-disease, the subjects exhibited shallow pocket depths and minimal clinical attachment loss (whole mouth or sample sites). However there was a wide range of variation in PI, GI, and BOP.

The detection limit of *A. actinomycetemcomitans* was determined based on PCR analysis of serially diluted DNA from genomic DNA of the sequenced strains held in our lab (data not shown). The detection limit was found to be 0.04 pg of genomic DNA (equivalent to approximately 16 cells, based on the genome size of 2 Mb and 660 Dalton/base pair of double stranded DNA, and 6 copies of 16S rDNA gene/genome). Of the 94 samples that were tested, 43 (46%) were positive for *A. actinomycetemcomitans.* There were no statistically significant differences in PI, GI, PD, % BOP and CAL between *A. actinomycetemcomitans*-positive and *A. actinomycetemcomitans*-negative sites (t-test, $P>0.01$).

The detection limit for serotypes a, e and b was 4 pg of genomic DNA (1,600 cells), while that for serotypes c, f and d was 40 pg (16,000 cells) based on single copy of the serotype specific gene cluster/genome. Of the 43 samples that tested positive for *A. actinomycetemcomitans*, one sample's DNA concentration was found to be too low and the sample was excluded from serotype analysis. Among the remaining 42 *A. actinomycetemcomitans*-positive samples, the serotype was identified in 21cases. Serotype a was detected in 15 samples, serotype f in 4 samples, and serotype b and c each in one sample. Serotype d and e were not detected in the samples. The mean DNA concentration for serotype-positive and serotype-negative samples (both positive for *A. actinomycetemcomitans* by 16S rDNA analysis) was 13.4 and 12.5 ng/μl, respectively. No statistically significant differences were detected in the DNA concentrations between serotype-positive and serotype-negative samples (t-test, $P>0.01$).

4. Discussion

This study aimed to address two issues, the first being the frequency of *A. actinomycetemcomitans* colonization in periodontally healthy individuals and the second being the distribution pattern of different serotypes of *A. actinomycetemcomitans* among healthy subjects colonized by this organism. To address these issues, we examined by PCR the presence of distinct serotypes of *A. actinomycetemcomitans* in subgingival plaque of periodontally healthy subjects.

In this study the detection limit of *A. actinomycetemcomitans* was 0.04 pg of genomic DNA, similar to or better than the limit reported in a previous study (Ashimoto et al., 1996). The detection limits for serotypes varied from 4–40 pg of genomic DNA. To the best of our knowledge, this study is the first to examine the detection limits for serotype analysis of *A. actinomycetemcomitans* by PCR. PCR analysis of serotype-specific gene clusters was less sensitive than the detection of 16 S rRNA genes, in part due to the presence of 6 copies of rRNA genes in contrast to the single copy of the antigen-specific gene cluster in the genome of each strain (Chen, Kittichotirat, Chen, Downey, & Bumgarner, 2012; Chen, Kittichotirat, et al., 2010; Chen, Kittichotirat, Si, & Bumgarner, 2009; Kittichotirat, Bumgarner, Asikainen, & Chen, 2011).

There are a number of studies that have examined the distribution of distinct serotypes of *A. actinomycetemcomitans* in subgingival plaque. The study by Dogan et al. (Dogan et al., 2003) provides the most meaningful comparison to this study. The designs and the aims of the two studies are similar. Both studies recruited subjects from a Turkish population accessible through dental schools. The protocols for subgingival sampling and PCR detection of the organism were identical (Dogan et al., 2003). There were also several notable differences. The study by Dogan et al. (Dogan et al., 2003) included 20 subjects without periodontitis and 49 subjects with a diagnosis of aggressive periodontitis (localized and generalized) or chronic periodontitis. In contrast, the present study did not include subjects with periodontitis.

In the study by Dogan et al.(Dogan et al., 2003), the subgingival plaque was cultivated in order to identify *A. actinomycetemcomitans* for serotype analysis by immunodiffusion

assays whereas in our study serotype was determined via PCR analysis, which does not require the expression of the serotype antigens. In the present study, 46% of the healthy subjects harbored subgingival *A. actinomycetemcomitans*. The proportion of subjects colonized by *A. actinomycetemcomitans* appears to be higher than previously reported (Chen, Wang, et al., 2010; Slots & Ting, 1999), which may be in part due to different geographic locations and/or race/ethnicity of the study populations. In the study of a Turkish population, Dogan et al. (Dogan et al., 2003) found 30% of the healthy and 63% of the diseased subjects to be positive for *A. actinomycetemcomitans*. Therefore, we conclude that *A. actinomycetemcomitans* is a common oral bacterial species in the gingival crevice in subjects without periodontitis.

In this study *A. actinomycetemcomitans* serotype-positive samples were dominated by serotype a. Our finding is particularly interesting in comparison to the relatively low presence of serotype a in subgingival plaque samples from diseased subjects in the study by Dogan et al. (Dogan et al., 2003), which were instead dominated by serotype b, c and non-serotypeable isolates as determined by immunodiffusion assays. Due to the low amounts of available sample DNA, we were unable to repeat PCR analysis for all serotype-negative samples. Therefore it is possible that some of the serotype-negative samples harbored strains other than serotype a. With this caveat in mind, the results from our study and the study by Dogan et al. (Dogan et al., 2003) suggest that serotype a in the Turkish population is predominantly associated with non-diseased subjects. This observation is in contrast to the association between serotype a strains and periodontal disease progression found in the study by Hoglund et al. (Hoglund Aberg et al., 2013). It is unclear whether these *A. actinomycetemcomitans*-positive non-diseased subjects may eventually develop periodontitis.

There is a large body of evidence for the association of specific serotypes or genotypes of *A. actinomycetemcomitans* with periodontal health or disease (Asikainen, Chen, & Slots, 1995; DiRienzo & McKay, 1994; Paju, Carlson, Jousimies-Somer, & Asikainen, 2000). Strain-to-strain variation in the gene content of the bacterial genomes may offer a plausible explanation for such strain-to-strain variability in virulence and in disease association. Whole genome sequence analysis of *A. actinomycetemcomitans* has identified phylogenetically distinct groups of strains that my differ in gene content by as much as 20% of the genome (Kittichotirat et al., 2011). It remains to be investigated the possible heterogeneity in virulence among *A. actinomycetemcomitans* strains.

5. Conclusion

Serotype a is the dominant serotype of *A. actinomycetemcomitans* found in young Turkish dental students. The result is consistent with the hypothesis that the distribution patterns of *A. actinomycetemcomitans* serotypes among human subjects may vary according to geographic locations and periodontal status of the study subjects.

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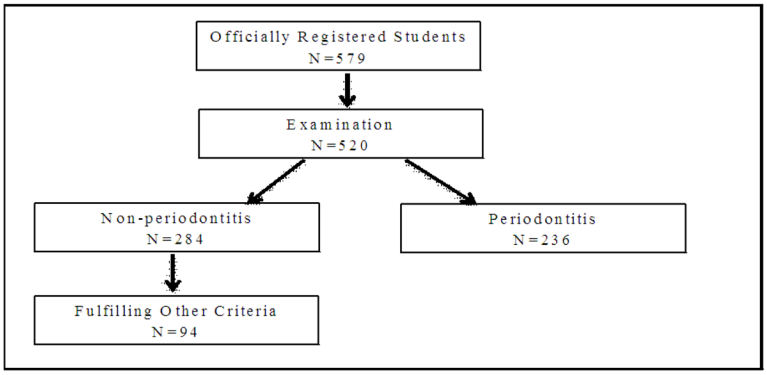

Fig 1.

A schematic presentation of the subjects included in the study. Microbial sampling and PCR analysis were performed only for the 94 students that fulfilled all inclusion criteria of the study.

Table 1

PCR primers and thermocycler profiles

Primers (5'–3')	Thermocycler profile
Detection of *Aa*	−94°C/3 min
TAG CCC TGG TGC CCG AAG C	−30 cycles of 94°C/30 s, 60°C/20 s and 65°C/30s
CATCGCTGGTTGGTTACCCTCTG	−65°C/10 min
Multiplex PCR for serotype a & e*	−94°C/2 min
a-specific	−30 cycles of 94°C/30 s, 55°C/30 s, 65°C/25 s
GCAATGATGTATTGTCTTCTTTTGGA	−65°C/10 min
CTTCAGTTGAATGGGGATTGACTAAAA	
e-specific	
CGTAAGCAGAAGAATAGTAAACGT	
AATAACGATGGCACATCAGACTTT	
Multiplex PCR for b, c and f*	−94°C/2 min
b-specific	30 cycles of 94°C/30 s, 48°C/30 s, 65°C/25 s
ARAAYTTYTCWTCGGGAATG	−65°C/10 min
TCTCCACCATTTTTGAGTGG	
c-specific	
ARAAYTTYTCWTCGGGAATG	
GAAACCACTTCTATTTCTCC	
f-specific	
ARAAYTTYTCWTCGGGAATG	
CCTTTATCAATCCAGACAGC	
d-specific PCR	−94°C/2 min
TTACCAGGTGTCTAGTCGGA	−30 cycles of 94°C/30 s, 55°C/30 s, 65°C/25 s
GGGTCCTGACAACATTGGAT	−65°C/10 min

*For multiplex PCR, each primer pair was prepared and combined into a final volume of 25 μl of reaction mixture.

Table 2

Clinical parameters of the whole mouth and sampled sites of the 94 non-periodontitis subjects.

	Whole Mouth	Sample sites
No. of teeth* (mean±SD)	27.52 ±1.08 (24–28)**	-
No. of teeth extracted* (mean±SD)	0.49±1.08 (0–4)	-
No. of decayed teeth (mean±SD)	3.72±3.40 (0–12)	-
No. of crowned teeth (mean±SD)	0.04±0.20 (0–1)	-
Plaque index (mean±SD)	1.12±0.34 (0.37–2.14)	1.23±0.45 (0.25–2.25)
Gingival index (mean±SD)	0.88±0.22 (0.10–1.32)	1.02±0.26 (0.25–1.50)
Probing pocket depth (mm, mean±SD)	2.14±0.27 (1.03–2.72)	2.31±0.38 (1.25–3.13)
Bleeding on probing (%, mean±SD)	18.24±14.44 (0–76)	26.99±21.80 (0–88)
Clinical attachment level (mm, mean±SD)	0.03±0.08 (0.00–0.58)	0.03±0.11 (0–0.50)

* Excluding third molars,

** Minimum - maximum