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Java project on periodontal diseases: serotype distribution of *Aggregatibacter actinomycetemcomitans* and serotype dynamics over an 8-year period

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Abstract

Objective: To investigate the serotype distribution and stability of *Aggregatibacter actinomycetemcomitans* over an 8-year period in untreated Indonesian subjects.

Material and Methods: Clinical periodontal status and the presence of *A. actinomycetemcomitans* were established in 1994 and 2002 in 107 subjects from an Indonesian tea estate deprived from dental care. On an average, 3.6 isolates per patient were subcultured and serotyped using specific PCR reactions.

Results: In 1994, the predominant serotype was b (53.7%), whereas a and c occurred in 17.1% and 14.6% of the subjects, respectively. In 2002, a reduction in serotypes a (7.5%) and b (30.2%) occurred. Serotypes c and e increased in prevalence from 14.6% to 35.8% and 2.4% to 9.4%, respectively. Multiple serotypes were found in 12.2% in 1994 and 17% in 2002. From 24 subjects who were positive at both time points, 14 (58.3%) had the same serotype, whereas in 10 subjects (41.7%), a different serotype was found. Mean clinical attachment loss had increased from 0.74 mm in 1994 to 1.96 mm in 2002 but could not be related to subgingival presence of *A. actinomycetemcomitans*.

Conclusions: *A. actinomycetemcomitans* serotypes distribution in Indonesian young adults shifts from predominantly serotype b to a more equal prevalence of serotypes b and c. This shift suggests an opportunistic character of *A. actinomycetemcomitans*.

Key words: *A. actinomycetemcomitans*; periodontitis; serotyping

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To study bacterial spread in relation to virulence, it is of importance to focus onto one or more genes that encode for putative virulence factors such as toxins, outer membrane structures or whole pathogenicity islands (Franco et al. 1999, Kaplan et al. 2002). Some of the outer membrane structures can lead to a specific anti-inflammatory response. Such host responses are not always restricted to one unique bacterial strain but can also be induced by certain distinct groups of strains from one single species as in *Haemophilus influenzae* (Bajanca et al. 2004). One such intra-species division is called ''serotypes''. In some species, certain serotypes have enhanced virulence, e.g., in *Neisseria meningitidis* only certain serotypes are able to cause endemic periods of meningococcal disease (Caugant et al. 1990, Ashton et al. 1991). Also periodontal pathogens as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* have several serotypes that differ in virulence, of these the latter is the microorganism of interest here (Zambon et al. 1983, Van Winkelhoff et al. 1999). During the last two decades, it has been shown that *Aggregatibacter*

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actinomycetemcomitans can be regarded as a major pathogen in destructive periodontal diseases (Rodenburg et al. 1990, Ebersole et al. 1991, Slots & Ting 1999). The species is represented by six serotypes (a–f). Serotype b has been found more frequently and detected in higher numbers in active periodontitis lesions, whereas serotypes a and c have a stronger association with periodontal health (Zambon et al. 1983, Ebersole et al. 1991, Haffajee & Socransky 1994, Asikainen et al. 1995). Yang et al. (2004) found serotype b significantly more often in aggressive than in chronic periodontitis. They also found serotype b more frequently in periodontitis subjects under the age of 18 years (60.9%) in comparison to subjects older than 35 years (29%). The global distribution of the different *A. actinomycetemcomitans* serotypes is not homogeneous (Hölttä et al. 1994, Celenligil & Ebersole 1998), which implies that the association between serotype and periodontal status may be depending on the geographical location and/or ethnical status of the study population (Dahlén et al. 2002, Fine et al. 2007).

Recently, van der Velden et al. (2006) have been able to identify two risk factors for the onset of periodontitis, i.e. subgingival calculus and the subgingival presence of *A. actinomycetemcomitans*. In the present study, we aimed to investigate serotype distribution of *A. actinomycetemcomitans* in the study population, to study longitudinally changes in the serotype distribution in this cohort and to relate serotype shifts of *A. actinomycetemcomitans* to progression of periodontal disease.

Material and Methods

Patients and sampling

The study population of the ''Java project on periodontal diseases'' has been described in detail by Timmerman et al. (2001) and van der Velden et al. (2006). In brief, the subjects participating in a longitudinal, prospective study living in a village at the government-owned Malabar/Poerbasari tea estate, PTP XIII, on Western Java. Besides emergency dental treatment, no preventive or regular dental care programmes are available in that remote population. Emergency dental treatment was provided by a local general physician. In 1994, 158 (69 males, 89 females) subjects of the original population of 255 subjects were available for microbiological evaluation. In these subjects, the deepest bleeding site with the greatest amount of attachment loss in each quadrant of the dentition was selected and sampled with sterile paper points. In total, four sites per patient were sampled using two paper points per site. From the 158 subjects examined in 1994, 107 subjects were re-sampled at the same sites in 2002. Paper point samples were pooled per subject and collected in 2.0 ml reduced transport fluid (RTF; Syed & Loesche 1972). Samples were analysed for the presence and levels of *A. actinomycetemcomitans* by culture. Detection limit of *A. actinomycetemcomitans* was 20 cells per pooled sample. Plaque index, bleeding index, probing pocket depths and clinical attachment loss were determined as described previously (van der Velden et al. 2006). The research project has been approved by the Institutional Research Boards of the Academic Centre for Dentistry Amsterdam, the Netherlands, and the Dental School of the Padjadjaran State University, Bandung, Indonesia.

Culture

Samples were 10-fold serially diluted in RTF and aliquots of 100 μl were used to inoculate TSBV-agar plates for selective isolation and growth of *A. actinomycetemcomitans*. Plates were incubated for 5 days at 37°C in air with 5% CO_2. Plates were transferred from Indonesia to the Netherlands overnight in air+5% CO_2 and upon arrival further incubated for 3 days. A maximum of four isolates per subject were subcultured on 5% sheep blood agar (Oxoid no. 2, Basingstoke, UK). *A. actinomycetemcomitans* colonies were identified on the basis of the typical colony morphology with translucent, irregular edged often with a star-like inner structure and adherent to the agar, and a catalase-positive reaction upon exposure to 3% H_2O_2. All original samples as well as the purified and identified isolates from both 1994 and 2002 were stored at -80°C until further use.

Serotyping

Serotypes a–f were determined by PCR technique which is based on specific sequences from the gene clusters responsible for the distinct serotypes. The PCR reaction for serotypes a, b, c and e was performed as a multiplex PCR as described by Suzuki et al. (2001). When an isolate showed a negative result, a PCR was performed with serotype d (Suzuki et al. 2001) and serotype f-specific primers (Kaplan et al. 2002). Briefly, from a pure culture of *A. actinomyctemcomitans* on blood agar base, one to four colonies were taken using a sterile toothpick and heated in a microwave during 2 min. at full capacity. The lysate was suspended in a 25-μl volume of PCR mixture consisting of 10 mM of each deoxynucleoside triphosphate, 5 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 μl Taq-buffer (Promega, Thermophilic DNA Polymerase 10 × Reaction Buffer, Mg free) and 0.5 U Taq polymerase (Promega, PCR Core System I). Known *A. actinomycetemcomitans* serotypes were used as positive controls and involved: serotype a: ATCC 29523; serotype b: ATCC 29522; serotype c: NCTC 9710; serotype d: IDH781; serotype e: OMZ 534; serotype f: NL103.

LktC promoter deletion

In order to study the prevalence of high leukotoxin-producing *A. actinomycetemcomitans* clones, isolates were screened for the 530-bp deletion in the promoter region of the leukotoxin C gene by a separate PCR reaction (Haubek et al. 1996). The PCR results in a 1034-bp product in isolates with the complete promoter, whereas isolates with the 530-bp deletion show a PCR product of 504 bp. The molecular weights of the PCR products were determined by visualization and comparison with standard molecular weight markers using agarose gel electrophoresis.

Statistical analysis

Differences in clinical data over time were analysed using a paired-sample *t*-test. Differences in mean clinical attachment loss of the subjects grouped by colonization status of *A. actinomycetemcomitans* in 1994 and 2002 were analysed by ANOVA and a post-hoc Bonferroni comparison of means.

Results

From the total number of 158 patients in 1994, 65 (41.1%) were culture positive for *A. actinomycetemcomitans*. In 2002, 53 subjects out of a total of 107 subjects were *A. actinomycetemcomitans* positive (49.5%), including 35 new

A. actinomycetemcomitans-positive subjects. Twenty-four subjects were *A. actinomycetemcomitans* positive both in 1994 and 2002, whereas 17 had no longer detectable levels of *A. actinomycetemcomitans*. The mean age of the *A. actinomycetemcomitans*-positive subjects was 29.4 years in 1994 and 38.2 years in 2002. In this paper, all data presented are restricted to the 107 subjects for whom the clinical and microbiological data were available for both 1994 and 2002. The number of *A. actinomycetemcomitans* isolates per subject ranged from one to four with a mean number of 3.6 per subject.

LktC promoter deletion

The results of the PCR serotyping and the presence of a 530-bp deletion in the promoter region of the *LktC* gene of *A. actinomycetemcomitans* strains, isolated in 1994 and 2002 are shown in Table 1. All *A. actinomycetemcomitans* isolates in both 1994 and 2002 displayed a 1034-bp PCR product using the *LtxC* primers, indicating absence of the deletion in the promoter region of the *LtxC* gene. None of the strains belonged to the highly leukotoxic clone JP2.

Serotype distribution

In 1994, the predominant serotype was type b (53.7%), whereas serotypes a, c and e showed a distribution of 17.1%, 14.6% and 2.4%, respectively. Serotypes d and f were not detected. Multiple serotypes were found in 12.2% of the patients (Table 1). In 2002, a reduction in the prevalence of serotype b to 30.2% and serotype a to 7.5% was observed, whereas serotype c had increased in prevalence from 14.6% to 35.8%. The prevalence of serotype e had increased from 2.4% in 1994 to 9.4% in 2002. None of the isolated strains were non-typeable.

The study population contained 24 subjects who were *A. actinomycetemcomitans* culture positive both in 1994 as well as in 2002. Changes in the serotype distribution in these subjects are presented in Table 2. In these subjects, the same proportional changes in serotype distribution between 1994 and 2002 were observed as in the whole study population (Table 1). In 14 of the 24 subjects (58.3%), the same serotype was found in 1994 and 2002. In four of these subjects, an additional serotype was lost or gained. In 10 of these 24 subjects (41.7%), a complete change in serotype distribution was observed.

Table 1. Serotype distribution of *Aggregatibacter actinomycetemcomitans* in *A. actinomycetemcomitans*-positive Indonesian subjects

Serotype	*A. actinomycetemcomitans*-positive subjects	
	1994, % (*n*)	2002, % (*n*)
a	17.1 (7)	7.5 (4)
b	53.7 (22)	30.2 (16)
c	14.6 (6)	35.8 (19)
d	0	0
e	2.4 (1)	9.4 (5)
f	0	0
a+b	2.4 (1)	1.9 (1)
a+c	7.3 (3)	1.9 (1)
a+e		1.9 (1)
b+c	2.4 (1)	3.8 (2)
c+e		7.5 (4)
Multiple serotypes	12.2 (5)	17.0 (9)
LtxC deletion	0	0
Total	100 (41)	100 (53)

Table 2. Changes of serotype distribution of Indonesian subjects *Aggregatibacter actinomycetemcomitans* positive in both 1994 and 2002 ($n = 24$)

Stable distribution		Distribution with a received or lost serotype		Full changed serotype	
a → a	1*	ac → a	1	a → b	2
b → b	7	bc → b	1	b → ac	1
c → c	2	c → bc	1	b → c	3
$n = 10$		e → ec	1	b → e	3
		$n = 4$		ab → c	1
				$n = 10$	

*Numbers are representing the numbers of subjects carrying *A. actinomycetemcomitans* strains divided by serotype.

Clinical data

The mean clinical attachment loss increased from 0.74 to 1.96 mm between 1994 and 2002 (Table 3). This group was subdivided into four subgroups: subjects positive for *A. actinomycetemcomitans* in both 1994 and 2002 (subgroup 1, $n = 24$), subjects positive in 1994 but negative in 2002 (subgroup 2, $n = 29$), subjects negative in 1994 but positive in 2002 (subgroup 3, $n = 17$) and subjects negative at both time points (subgroup 4, $n = 37$). The mean attachment loss increased significantly in all subgroups between 1994 and 2002. The differences in mean attachment loss in subgroups 1–4 were 1.32, 1.38, 1.09 and 1.08 mm, respectively. However, these differences were not statistical significant between the four groups.

The mean probing pocket depth of all 107 subjects increased significantly from 3.31 to 3.57 mm between 1994 and 2002 ($p < 0.001$). Subgroups 1 and 2 consisting those subjects who were positive for *A. actinomycetemcomitans* in 2002 were responsible for this increase. All other clinical parameters remained unchanged, except for the plaque index in subgroup 2.

No relationship was found between the degree of periodontal destruction and the presence of a distinct serotype. There was no statistical difference in probing pocket depth or attachment loss comparing serotype b, *versus* the group of non-serotype b. In 1994, the probing pocket depths of *A. actinomycetemcomitans*-positive subjects were 3.34 mm for serotype b-positive subjects and 3.19 mm for non-serotype b subjects. For attachment loss, this was 0.74 and 0.79 mm, respectively. In 2002, the pocket depth values were 3.70 mm for serotype b-positive subjects and 3.72 mm for non-serotype b subjects. For attachment loss, these values were 2.28 and 2.05 mm, respectively.

Discussion

This study is part of the ''Java project on periodontal diseases'', which involves a longitudinal research project investigating risk factors for onset and progression of periodontitis in an untreated population. *A. actinomycetemcomitans* was found to be a significant risk factor for the onset of attachment loss of $\geqslant 2$ mm in this study population (odds ratio 4.3) in the period between 1987 and 2002 (van der Velden et al. 2006). Because of this observation it is of interest to investigate serotype distribution of this pathogen, as there are data available suggesting a relationship between the presence of periodontitis in young adults and the presence of a distinct serotype of *A. actinomycetemcomitans* (Zambon et al. 1983, Asikainen et al. 1991). *A. actinomycetemcomitans* isolates were available from 1994 and 2002 only, as the microbial analysis in 1987 was based on indirect immunofluorescence. Serotype distribution and serotype dynamics were therefore studied with bacterial isolates from 1994 and 2002 representing an 8-year period,

Table 3. Clinical parameters of the Indonesian subjects in 1994 and 2002, and subdivided by those positive for *A. actinomycetemcomitans* in both 1994 and 2002 (subgroup 1), those positive in 1994 but not in 2002 (subgroup 2), those negative in 1994 but positive in 2002 (subgroup 3), and those negative at both time points (subgroup 4)

All subjects ($N = 107$)	1994	2002	*p*-value
Plaque index (0–2)	1.16 (0.37)	1.08 (0.39)	0.05
Bleeding index (0–2)	1.21 (0.40)	1.28 (0.37)	0.08
Pocket depth (mm)	3.31 (0.55)	3.57 (0.57)	<0.001
Attachment loss (mm)	0.74 (0.50)	1.96 (1.10)	<0.001
Subgroup 1 Aa-positive 1994+2002 ($N = 24$)	1994	2002	*p*-value
Plaque index (0–2)	1.13 (0.32)	1.13 (0.40)	0.96
Bleeding index (0–2)	1.25 (0.43)	1.33 (0.32)	0.34
Pocket depth (mm)	3.21 (0.50)	3.59 (0.54)	0.001
Attachment loss (mm)	0.63 (0.45)	1.95 (1.12)	<0.001
Subgroup 2 Aa-negative in 1994; positive in 2002 ($N = 29$)	1994	2002	*p*-value
Plaque index (0–2)	1.29 (0.46)	1.06 (0.38)	0.001
Bleeding index (0–2)	1.24 (0.45)	1.41 (0.40)	0.029
Pocket depth (mm)	3.36 (0.78)	3.81 (0.81)	0.001
Attachment loss (mm)	0.90 (0.63)	2.28 (1.27)	<0.001
Subgroup 3 Aa-positive in 1994; negative in 2002 ($N = 17$)	1994	2002	*p*-value
Plaque index (0–2)	1.11 (0.31)	1.24 (0.40)	0.30
Bleeding index (0–2)	1.14 (0.38)	1.11 (0.37)	0.79
Pocket depth (mm)	3.38 (0.47)	3.48 (0.37)	0.12
Attachment loss (mm)	0.95 (0.51)	2.04 (1.10)	<0.001
Subgroup 4 Aa-negative in 1994; negative in 2002 ($N = 37$)	1994	2002	*p*-value
Plaque index (0–2)	1.11 (0.35)	1.00 (0.38)	0.10
Bleeding index (0–2)	1.19 (0.36)	1.22 (0.33)	0.69
Pocket depth (mm)	3.31 (0.39)	3.40 (0.33)	0.25
Attachment loss (mm)	0.59 (0.32)	1.67 (0.84)	<0.001

SD values are given in the parentheses.

in which study subjects had not received professional dental care.

In 1994, serotype b predominated in our study population (53.7%). Other studies have also shown a high prevalence of serotype b in young individuals with periodontitis (Zambon et al. 1983, Asikainen et al. 1991). In contrast, equal distribution of serotypes a, b and c was observed in Korean LJP patients (Chung et al. 1989) and in a study from Finland in which the distribution for serotypes a, b and c was 25%, 23% and 25%, respectively (Asikainen et al. 1995). Besides the serotypes a, b and c, we found a relatively high proportion of serotype e in the Indonesian population in 2002. This is in concordance with other studies from the Far East such as Japan (46.7%; Yoshida et al. 2003) and China (10.9%; Mombelli et al. 1999). In our study, the prevalence of serotype e was 18.6% and may be regarded high in comparison to Finland (6%; Saarela et al. 1992). In the present study, we observed a relatively high prevalence of subjects with multiple serotypes, both in 1994 and 2002 (12.2% and 17.0%, respectively). Other authors have also reported on this phenomenon in China (16.8%; Mombelli et al. 1999) and Japan (25%; Yoshida et al. 2003). In Finland, the prevalence of multiple clones varied between 5.5% (Saarela et al. 1992) and 7.0% (Asikainen et al. 1995). Therefore, carrier-ship of multiple *A. actinomycetemcomitans* serotypes seems more common in the Far East in comparison to Europe.

The serotype distribution in our study population had changed dynamically in the period between 1994 and 2002. We observed a decrease in detectable serotypes a and b positive subjects and a concomitant increase in serotypes c and e positive subjects. These shifts were observed in the whole group of *A. actinomycetemcomitans*-positive patients as well as in the 24 patients who were *A. actinomycetemcomitans* positive in both 1994 and 2002. Although we found serotype stability at both occasions in 58.3% of the subjects, complete change of serotype was observed in 41.7% of the subjects (Table 2). This serotype dynamics of *A. actinomycetemcomitans* under natural conditions, thus without any periodontal or antimicrobial interventions, has not been reported previously. Dynamics of *A. actinomycetemcomitans* colonization has been studied by others in American and Australian subjects with no or minimal disease and it was found that a significant proportion of the study populations can lose or gain this pathogen in periods ranging from 1 to 5 years without periodontal intervention (Lamell et al. 2000, Cullilan et al. 2003). This is in contrast to the observation of Saarela et al. (1992) who found no

shifts in serotype distribution in Finnish adult patients with periodontitis and periodontally healthy subjects during a 1–6-year period including periodontal treatment. This discrepancy might be caused by differences in periodontal status, oral hygiene level and age.

It is important to realize that there are some restrictions to the conclusions that can be drawn from our study. Finding the same serotype does not exclude the possibility of re-infection by a different genotype, which can only be established by more detailed DNA fingerprinting of the isolates. Also, not finding a certain serotype does not necessarily indicate the absence of a particular serotype, as it may be present under the level of detection by the used method. Our observations may also be restricted by the fact that we serotyped on an average 3.6 isolates per subject. Another important issue is the location of sampling. We have chosen to sample the deepest bleeding pocket of every quadrant. However, these were not the same in 1994 and 2002 per se. It is possible to fail isolating *A. actinomycetemcomitans* if this bacterium is not (longer) located in the deepest pocket but in shallow pockets only or on the mucous membranes. However, the most likely site to isolate *A. actinomycetemcomitans* is still in the most affected sites (Mombelli et al. 1994).

In the present study, we have not been able to show that subjects with subgingival serotype b had developed a worse periodontal condition in 2002 in comparison to subjects with non-serotype b *A. actinomycetemcomitans*. We observed that the mean increase in probing pocket depth between 1994 and 2002 was significantly greater in subjects culture positive in 2002 in comparison to subjects without detectable *A. actinomycetemcomitans* in 2002. This confirms that subgingival presence of *A. actinomycetemcomitans*, but not a specific serotype is associated with a higher degree of inflammation (Fine et al. 2007).

In conclusion, the distribution of serotypes of *A. actinomycetemcomitans* in Indonesia is predominantly serotype b in young adults and shifts to a more equal prevalence of b and c under natural conditions during an 8-year period. Serotype stability in this period of 8 years was observed in the majority of subjects but shifts in serotype distribution were significant. In our study, subjects with serotype b did not show more periodontal attachment loss than subjects with non-serotype b *A. actinomycetemcomitans*.

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Clinical Relevance

Scientific rationale for the study: To study the natural serotype stability of *A. actinomycetemcomitans* in subjects without periodontal care.

Principal findings: Changes in colonization of different *A. actinomycetemcomitans* serotypes was observed in a significant proportion of the study population.

Practical implications: Colonization of a given serotype of *A. actinomycetemcomitans* can be persistent for several years but infection with other serotypes can occur under natural conditions.