Presence of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in Children with Clinical Signs of Periodontitis, Living in the Västerbotten County, Sweden

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ABSTRACT

This study has examined the presence of *A. actinomycetemcomitans* and *Porphyromonas gingivalis*, in 10 children and adolescents in Västerbotten County, who had been referred to the Department of Pediatric Dentistry due to suspected periodontal disease. Based on previous studies we hypothesized that the majority of the enrolled subjects would be hosts for *A. actinomycetemcomitans*. A second hypothesis was that a relationship between periodontal disease and heredity would be found.

The aim was to study to which extent the enrolled patients were infected with *A. actinomycetemcomitans* and to enhance the general knowledge of the bacterial composition in children/adolescents with clinical signs of periodontal disease. Another aim was to examine the nature of the disease in relation to heredity, oral habits and ethnicity. A third aim was to compare PCR and cultivations for detection of pathogens.

Sampling was made from pathological periodontal pockets by a dentist or a dental hygienist. Samples were sent to a laboratory for analysis by two different methods: cultivation and PCR.

Our results showed that the majority of the participants had non-European ethnicity, were positive for *A. actinomycetemcomitans* and 3 of 7 *A. actinomycetemcomitans*-positive subjects exhibited high leukotoxicity. No subject harbored the JP2 leukotoxin promoter genotype. Serotypes a-d and f, but not e, were detected.

We concluded that the majority of the patients were infected with *A. actinomycetemcomitans*, some of them had high leukotoxicity but none of them had the JP2-genotype. The culture based analysis was similar in terms of detecting *A. actinomycetemcomitans* compared to the PCR method.
INTRODUCTION

Periodontitis is a population disease that is expressed in two forms, a chronic and an aggressive one, of which chronic periodontitis (CP) is the most commonly occurring. CP is a frequent disease, affecting an older spectrum of individuals and the mild to moderate forms of CP have a prevalence of 13-57% (Rylev and Kilian, 2008). CP is expressed as general marginal bone loss in the dentition, and unlike aggressive periodontitis (AgP) there is no evidence of different prevalence of CP in different ethnic groups. AgP is a rare, rapidly progressing disease that can be expressed in two different forms, localized aggressive periodontitis (LAP) and general aggressive periodontitis (GAgP). It is often severe and characterized by its early onset. The prevalence of AgP among children/adolescents has been reported in numbers from 0.1% in Europe to 7.6% in Africa (Albandar and Tinico, 2002, Haubek et al., 1997).

The definition of AgP is based on rapid attachment loss and bone destruction, absence of systemic conditions related to periodontal disease and familial aggregation. Secondary features of AgP are plaque and calculus scores not corresponding to the severity of periodontal tissue destruction. The progression of attachment loss and bone loss may be self-arresting.

Etiology

A number of different studies have demonstrated a difference in the prevalence of AgP in different ethnic groups. For example, individuals that originate in West Africa and in Africa’s Mediterranean coast demonstrate a higher prevalence of AgP than other populations (Rylev and Kilian, 2008).

The etiology of AgP is multifactorial and not completely understood. Factors such as a genetic predisposition, a virulent microflora and deviations of the host response as well as environmental factors have been proposed as contributing factors. In aggressive periodontitis, bacterial infection evokes a local and systemic host inflammatory response. Susceptible individuals whose host response fails to efficiently control the virulent microflora and whose inflammatory responses are inefficient or excessive will suffer from disease initiation and eventually degeneration of periodontal tissues. Genetic factors increase the AgP susceptibility
(Lang and Lindhe, 2015) and expression of AgP is affected by environmental factors such as cigarette smoking, which has been shown in a large study (Schenkein et. al., 1995).

Microflora

The microflora in CP is similar to that of AgP in many ways and the dominating microbiota consists of gram-negative anaerobes such as *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* as well as the facultative anaerobe *Aggregatibacter actinomycetemcomitans*.

*P. gingivalis* has been associated with LAP and is thought to have a role in the disease progression (Gajardo et al., 2005). Hence, high presence of *P. gingivalis* in LAP has been demonstrated in Far East populations (Lang et al., 1999). The microbiological composition of pathological dental pockets has been studied among individuals with LAP and these studies demonstrate that *A. actinomycetemcomitans* is one of the bacterial species that is strongly associated with AP. *A. actinomycetemcomitans* occurs frequently and in high proportions in pathological periodontal pockets of young patients with periodontitis (Fine et al., 2006) and according to another study by Fine et al. there is also an association between *A. actinomycetemcomitans* and bone loss (Fine et al., 2007). Other pathogens that have been detected in lower numbers are *Parvimonas micra*, *Filifactor alocis*, *Tannerella spirochetes*, *Capnocytophaga spirochetes* and *Solobacterium moorei* (Shaddox, 2012).

*A. actinomycetemcomitans*

*A. actinomycetemcomitans* is a gram-negative bacterium with great genetic variations. It has the ability to penetrate the junctional epithelium and reach the connective tissue (Meyer et al. 1996). Several virulence factors of *A. actinomycetemcomitans* have been identified, such as lipopolysaccharides (LPS), bacteriocins, adhesins, leukotoxin (Ltx) and cytolethal distending toxin (CDT). Ltx has been thoroughly studied (Haubek and Johansson, 2014) and this toxin is able to kill immune cells in humans (Kelk et al., 2005). Various *A. actinomycetemcomitans* clones produce different amounts of leukotoxin and this has a direct relation to their virulence. A highly virulent clone of *A. actinomycetemcomitans* is the JP2 clone which has an increased expression of leukotoxin. The JP2 clone belongs to the serotype b strain (Haubek and Johansson, 2014) and occurs in high numbers in individuals of African origin (Haubek et al., 1997). There are also several prospective
studies from Morocco showing a strong correlation between the presence of the JP2 clone and development of aggressive periodontitis in young individuals (Haubek and Johansson, 2014). One of these studies has examined LAgP and GAgP and found a strong association with both of these forms of periodontitis and presence of JP2 clones in Moroccan young adults (Ennibi et al., 2012). A study from Ghana has shown that JP2 positive individuals have an increased risk of developing attachment loss >3 mm (Åberg et al., 2014a).

**Detection methods**

Different methods for detection are used for analysis of the microbiota in the pathological periodontal pockets. Examples of methods currently used are bacterial cultures, DNA hybridization, polymerase chain reaction (PCR), immunofluorescence and 16S rRNA sequencing (Rylev and Kilian, 2008). The different methods vary and each method has its advantages and limitations.

**Hypothesis**

The hypothesis of the study was that *A. actinomyctemcomitans* would be detected among the majority of the children and adolescents, 10-21 years of age, who had been referred to the Department of Pediatric Dentistry for investigation of suspected periodontal disease. A second hypothesis was that a relationship between periodontal disease and heredity would be found.

**Purpose of the study**

The primary aim was to study to which extent the enrolled patients were infected with *A. actinomyctemcomitans* and to enhance the general knowledge of the bacterial composition in children/adolescents with clinical signs of periodontal disease. An evaluation of the correlation between the presence of disease, ethnicity, oral hygiene and presence of periodontitis among parents/siblings was a second purpose of the study. Finally a third purpose was to compare PCR and cultivations for detection of pathogens in patients with AgP.
MATERIAL AND METHODS

Subjects

This student research project is based on 10 consecutive patients <25 years, with clinical signs of periodontitis, who had been referred to the Department of Pediatric Dentistry at Umeå University Hospital, Sweden, for clinical investigation and treatment during the period 2014-01 to 2015-06. Bacterial samples were taken from pathological periodontal pockets from the subjects for further microbiological analysis.

Questionnaire

Gathering of clinical parameters was obtained by questionnaires, which were handed out to the patients’ dentists’ at the Department of Pediatric Dentistry at Umeå University Hospital. The questionnaires included questions about health, gender, age, nationality, oral hygiene habits, presence of supra- and subgingival tartar, bleeding upon probing, pocket depth, and type of bone loss and presence of periodontal disease in the family. Data were analyzed by SPSS® (IBM®, Statistics data document, Versions 23).

Sampling

Bacterial samples were taken from teeth where pathological pockets (≥4mm) or bleeding were diagnosed after clinical examination. Pathological pockets with another etiology than periodontitis e.g. fractured roots and pockets with endodontic infection, were excluded. Before bacterial sampling, conducted by a dentist or dental hygienist at the Department of Pediatric Dentistry at Umeå University Hospital, the sampling sites were dried up with blaster and supragingival plaque was removed with a curette. A sterile paper strip was then placed in the deepest pocket in each patient and left for approximately 30 seconds to absorb gingival crevicular fluid. The paper strip was then transferred to a VMGAIII tube (Viable Medium of the Department of Bacteriology, University of Gothenburg, Anaerobic medium) and transported to the Clinical laboratory of Dental School in Umeå for the analyzes.

Quantification of A. actinomycetemcomitans and P. gingivalis by cultivation

Samples from periodontal pockets were transported in VMGAIII tubes and incubated at 37 °C for 2-3 min. The tubes were then mixed by vortex for 1 min, and serially diluted in a salt
buffer (Johansson et al., 2009). The TBV-plates (Trypticase Bacitracin Vancomycin), (Slots, 1982), were incubated at 37°C in 5% CO₂ for 3-4 days. The blood agar plates containing Colombia base, haemin (0.05 mg/ml), K-vitamin (0.01 mg/ml) and lysed horse blood (Hunt et al., 1985) were incubated at 37 °C in an anaerobic atmosphere for 7 days. The TBV-plates were used for quantification of \textit{A. actinomycetemcomitans}. In addition, isolates of this bacterium were collected from the TBV-plates. The blood agar plates were used for quantification of the total number of bacteria in the samples, and for quantification of \textit{P. gingivalis} (Lakio et al., 2002).

**Quantitification of \textit{A. actinomycetemcomitans} and \textit{P. gingivalis} by Q-PCR**

Samples from periodontal pockets were transferred to TE-buffer (Tris EDTA buffer, 10 µM/1µM pH 8.0). DNA was isolated by using GenElute Bacterial DNA kit (Sigma-Aldrich, St. Louise, MO, USA) according to the manufacturer's instruction. The reaction mixture contained 5µl Syber Green (KAPA, Biosystems), 4 µl template, 1 µl primer mix (5 µM each). The samples were run in the Q-PCR machine (Corbett Rotor-Gene 6000). Primers and PCR program are described by Kirakodu and co-workers (2008).

For quantification of \textit{A. actinomycetemcomitans} and \textit{P. gingivalis}, respectively, standard solutions containing DNA prepared from suspensions containing $10^8$-$10^{10}$cells/ml of the two bacterial species were used.

**Characterization of \textit{A. actinomycetemcomitans} by PCR**

For the PCR-based characterization DNA was prepared by treating bacterial suspension at 95 °C for 8 min and subsequently centrifuged at 12 000 x g for 10 min. The supernatant was used as template. For all PCR-based characterizations, PureTaq Ready-To-Go PCR (GE Healthcare; Buckinghamshire, UK) was used.

For the amplification a Ready-To-Go kit was used (Amersham Biosciences, Freiburg, Germany). For the serotyping the reaction mixture (25 µl) contained 5 µl template and a set of primers (a-e) (0.4 µM each) or serotype f (0.4 µM). Amplification was made in a PCR machine using program sero a-e, or sero f.

For the leukotoxin promoter typing the reaction mixture (25 µl) contained 5 µl template and 2 µl of primer mix (LTX34, 10 µM). Amplification was made in the PCR machine,
using program LTX34 (Brogan et al., 1994). Primers and the temperature profiles for the amplification of the various genes are summarized in Appendix 1b and 2 (Suzuki et al., 2001; Kaplan et al., 2001; Poulsen et al., 2003). The PCR products were separated in agarose (1.2 \%) gel electrophoresis in a Tris acetate buffer (40 mM; pH 8.3), containing 1mM EDTA.

**Determination of leukotoxic activity**

Leukotoxicity of the *A. actinomycetemcomitans*-isolates were determined as release of lactate dehydrogenase (LDH) from stimulated THP-1 cells. The following experiment was conducted. The sample mixtures were centrifuged and the supernatants were analyzed for LDH-activity (Wroblewski & LaDue, 1955), cells incubated in the absence of bacteria served as the negative control (20 \(\mu\)l RPMI-1640). The maximum LDH-activity was considered to be the one found in supernatants of cells treated with 0.1 \% Triton X-100 for 1 h. The relative release of LDH in the various samples is expressed in percent of the maximum activity. 28 ml phosphate buffer (0.1 M, pH 7.0), 1 ml Na-pyruvat (2.5 mg/ml) and 1 ml NADH (5 mg/ml) were mixed to obtain the substrate buffer. 180 \(\mu\)l of the substrate buffer was the added to 96-welled microtiter plates followed by 25 \(\mu\)l of the samples to each well. For the LDH-measurement the absorbance at 340 nm kinetic (2 min interval time) was read during 5 min. The isolates were distributed into three leukotoxicity groups; high leukotoxicity (≥ 60\% LDH activity), moderate (31-60 \%) and low leukotoxicity (0-30 \%). As a positive control a highly leukotoxic JP2 strain was used.

**Literature search**

Search for adequate literature and articles was made in PubMed, the National Library of Medicine, using the following MeSH terms: *Aggregatibacter actinomycetemcomitans*, aggressive periodontitis, infant, preschool, child, adolescent, leukotoxicity, JP2. Many references were also found through relevant articles recommended to us by our tutors.

**Ethical considerations**

Before starting the project, ethical permission was obtained from the local ethical committee of Institute of odontology, Umeå University. All the patients were referred to the Department of Pediatric dentistry for treatment and the clinical examinations done by patients' ordinary dentists was a routine. Thus no consent from the subjects was required.
The clinical parameters and analyzed bacterial samples were completely anonymous and could not be connected to a particular person when handled. Only the dentist and microbiologist in charge had access to personal data.

RESULTS

8 of the 10 participants had non-European ethnicity, of whom the majority (40%) had African background. 20% had Asian background and the same (20%) applies for the subjects with South American backgrounds. (Table 1b)

The daily oral habits were also investigated and the results demonstrated that the majority (90%) brushed their teeth twice a day with a manual toothbrush. Among the participants the majority (60%) answered that they had been consistent with their previous dental check-ups.

In the questionnaire, the subjects were also asked about their interdental cleaning (dental floss) habits. The results show that 40% of the subjects do not use any interdental cleaning tools, 30% practice interdental cleaning 1-3 times/week and 20% do it >3 times/week. One participant did not give any answer. Further clinical characteristics are summarized in Table 1a.

Result from the heredity inquiry exhibited that only 1 participant had knowledge of presence of periodontal disease in the family, this participant had both a mother and a sibling with periodontal disease manifestation.

Among all the participants, all except one subject showed bleeding upon probing (BOP) from the pathological periodontal pockets, where samples were taken. This divergent subject with a lack of BOP was also the only one suffering from a systemic disease, which was Down’s syndrome.

The screening for periodontal pathogens by the PCR method revealed that 9 of 18 samples were tested positive for *A. actinomycetemcomitans*, corresponding to 7 of 10 patients positive for *A. actinomycetemcomitans*. (Table 1a) The PCR-results were slightly lower than what detection of *A. actinomycetemcomitans* revealed using a cultivation method. The culture based quantification of *A. actinomycetemcomitans* detected 12 of 18 samples positive, which also corresponds to 7 of 10 patients.
For the *P. gingivalis* analysis, the Q-PCR results presented 8 samples positive, a number corresponding to 6 subjects. Using the cultivation method a slight difference could be seen, 6 samples and 5 subjects were tested positive for *P. gingivalis*. For detailed results, see table 2a and 2b.

None of the subjects in this study were tested positive for *A. actinomycetemcomitans* strains with the JP2 leukotoxin promoter type, see fig 1.

Among the 7 *A. actinomycetemcomitans* positive subjects, serotypes a-d and f, but not e were detected, see fig 2. None of the subjects with similar geographical backgrounds, harbored the same serotypes, see table 1b.

The leukotoxicity test of the *A. actinomycetemcomitans* isolates from patients who were positive for *A. actinomycetemcomitans* (7 subjects), 3 exhibited high leukotoxicity (≥ 60 %), 1 moderate (31-60 %) and 3 low leukotoxicity (0-30 %). The control isolate of the JP2-type indicated 64 % cell lysis.

**DISCUSSION**

The present study was undertaken to examine to which extent children and adolescents, with periodontal signs of periodontal disease in Västerbotten County were infected with *A. actinomycetemcomitans*. Our study demonstrated that the majority of the 10 included subjects were infected with *A. actinomycetemcomitans*. By using a combination of culture based analysis and PCR amplification for screening for periodontal pathogens, the two analyzing techniques could be compared to each other in sense of accuracy. The PCR-results showed a similar capacity at detecting *A. actinomycetemcomitans* compared to the culture based analysis. In addition to examining the ratio of *A. actinomycetemcomitans* infected subjects and comparing analyzing methods, this study also set out to evaluate a possible correlation between disease and heredity, ethnicity and oral hygiene.

The original hypothesis of the study was that *A. actinomycetemcomitans* would be detected among the majority of children and adolescents examined in this study. This hypothesis was confirmed by our results.

A pathological microflora associated with AgP includes key periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *T. denticola*. In this study we have
chosen to concentrate on *P. gingivalis* and *A. actinomycetemcomitans* because of their higher ratio in the pathological periodontal pockets of persons who are affected by AgP (Fine et al., 2006).

In the present study it was shown that the majority of the examined children/adolescents who had been referred to the Department of Pediatric Dentistry at Umeå University hospital because of suspected periodontal disease, harbored *A. actinomycetemcomitans* and almost half (43 %) of the subjects harbored highly leukotoxic strains of *A. actinomycetemcomitans*. This finding is interesting in view of the fact that none of the examined subjects harbored the JP2 genotype of *A. actinomycetemcomitans*. High leukotoxicity of *A. actinomycetemcomitans* has been suggested to be associated with LAP, which is mainly supported by studies of the JP2 genotype (Haubek et al., 2001).

In a previous study by Åberg et al., 2014b, a relation was found between high leukotoxic activity and *A. actinomycetemcomitans* of non JP2 genotype harboring individuals, thus giving the alternative conclusion that high leukotoxicity is not always bound to the presence of JP2 genotypes of *A. actinomycetemcomitans*. Åberg's study has highlighted that non JP2 genotypes of serotype b can also be highly leukotoxic, our findings further emphasize this theory. Compared analysis of the promoter sequence of the non JP2 to the JP2 leukotoxin promoter sequence could not be made, since none of the subjects in the present study harbored the JP2 genotypes. Studies have demonstrated that non JP2 highleukotoxic genotypes of *A. actinomycetemcomitans* are also associated to a higher prevalence of AgP (Åberg et al., 2014b).

In addition to these observations we discovered that two of the subjects were carriers of the rare serotype f strain of *A. actinomycetemcomitans*. There is evidence today that there is a correlation between serotype distribution of *A. actinomycetemcomitans* and ethnicity/geographic origin (Rylev and Kilian, 2008, Brigido et al., 2014). The serotypes a-d are most frequent while serotype e and f are rarely detected in most populations (Brigido et al., 2014). The two detected subjects with serotype f in the present study originated from Sierra Leone and Sweden, respectively. More studies need to be made on this subject, to be able to draw any conclusions on the possibility of a correlation between serotype f detection and ethnicity/geographic origin.
We also want to shed light on the fact that none of the subjects in the present study who shared geographical background demonstrated the same serotype of *A. actinomycetemcomitans*. This finding is interesting in light of the host-tropism theory that implies an adaptation of specific bacteria to certain hosts (Rylev and Kilian, 2008).

Regarding the ethnic distribution among the subjects in the present study, results have demonstrated that the majority of patients enrolled were of non-Swedish origin. In Sweden the prevalence of adolescents with periodontitis (or bone loss) is otherwise very low. One explanation to the high proportion of patients originating from other countries than Sweden might be that AgP is a disease with higher prevalence in other countries than Sweden.

There are several difficulties associated to studies on AgP, one being the difficulty to gather a sufficient number of patients given the rarity of the disease. Another difficulty when comparing different studies of AgP from different countries and ethnical groups is the lack of standardization of study design, the use of different inclusion criteria as well as periodontal disease definitions. Different distribution of the disease in different ethnic groups is another factor that makes studies made in different groups difficult to compare.

The comparability of the methods used to determine possible differences in the prevalence of oral bacteria is a critical factor in a study such as this one. The methods which are currently used all have their advantages and disadvantages. Selective growing conditions/media can make it difficult to compare studies using culture technique. Furthermore this analyzing technique is more quantitative in nature, due to the specificity restrictions of the method.

To be able to streamline work more rapidly, less time-consuming and large-scale methods are used, such as PCR amplification of the 16S rRNA gene and DNA hybridization techniques. These methods allow a quantitative and qualitative analysis of the bacterial composition. Furthermore, higher numbers of clinical samples can be analyzed than by use of the culture based methods. Among the advantages of PCR amplification of the 16S rRNA gene of periodontal bacteria, is the high sensitivity in detection, the assay speed and the multiple bacterial species that can be detected in the same batch (Lang et al., 2015). Further advantages of this method are the ability to detect none cultivable bacteria and the possibility to detect several subspecies of the same bacteria.
The present study analyzed a sum of 18 samples and the results by the two different methods (cultivation/PCR) coincided in the majority of these cases. This is a surprisingly positive outcome considering that we analyzed samples from the same site and not the same sample, with the two different methods.

In addition to clinical characteristics the present study investigated possible hereditary factors in the questionnaire. Our hypothesis was that we would find an outbreak of disease in family members related to the subjects. Previous studies have shown a relationship between presence of periodontal disease and presence of *A. actinomycetemcomitans* in family members of *A. actinomycetemcomitans* positive individuals with AgP (Rylev and Kilian, 2008). However, no such conclusion could be drawn in the present study due to the fact that only one of the dentists involved in the examinations answered the question regarding presence of disease among family members. However, the dentist who did fill in this information confirmed that both a sibling and the mother of the subject had periodontal disease.

Our results demonstrated that 90% of the subjects brushed their teeth twice a day, which is recommended as daily oral care to prevent caries and periodontal problems. Nevertheless, all of the included subjects exhibited signs of periodontal disease such as pathological periodontal pockets, BOP and bone defects. This is in accordance with AgP not demonstrating a strong relationship to poor oral hygiene standards. However, it is worth noting that toothbrushing twice a day is not equal to low PLI. Unfortunately, the question about PLI score was unanswered in the questionnaires in most of the cases and we were not able to do an optimal evaluation of the subjects’ oral hygiene. In a previous study it has also been demonstrated that there are no significant differences between children with juvenile periodontitis and a periodontally healthy control group with respect to dental plaque, gingival inflammation and calculus (Albandar, 1993). Absence or low scores of plaque and calculus is not always characteristic for subjects with AgP, as previously mentioned. However, this study failed to confirm this statement.

Similar to the routine cleaning habits twice a day, regular dental check-ups and interdental cleaning habits did not demonstrate any positive effect on the subjects’ periodontal conditions according to our study.

Since AgP is an uncommon disease and our study is limited to the county of Västerbotten, only 10 subjects are included in this examination. Even though our results are similar
to several previous studies, we are not able to draw any conclusions corroborated by statistical analysis due to the small number of subjects.

ACKNOWLEDGMENTS

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REFERENCES


**Table 1A.** Baseline characteristics of 10 children/adolescents with clinical signs of periodontal disease.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male (n = 6)</th>
<th>Female (n = 4)</th>
<th>All participants (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean)</td>
<td>16</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Sex (male/female, number of participants)</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>BOP (%. mean)*</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Presence of marginal bone loss (number of participants)</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Presence of pathological periodontal pockets 4-5mm (number of pockets)</td>
<td>52</td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>Presence of pathological periodontal pockets &gt;6mm (number of pockets)</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Presence of subgingival calculus (number of participants)</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> positive (number of participants)</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

*= BOP sites.
**Table 1B.** Ethnicity of the children/adolescents, presence of *A. actinomycetemcomitans* and characteristics of this bacterium.

<table>
<thead>
<tr>
<th>Patient code (n)</th>
<th>Ethnicity</th>
<th><em>A. actinomycetemcomitans</em> positive</th>
<th>Serotype</th>
<th>JP2</th>
<th>Leukotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ecuadore</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Colombia</td>
<td>+</td>
<td>d</td>
<td>nd</td>
<td>High</td>
</tr>
<tr>
<td>3.</td>
<td>Unknown</td>
<td>+</td>
<td>a</td>
<td>nd</td>
<td>Low</td>
</tr>
<tr>
<td>4.</td>
<td>Algeria</td>
<td>+</td>
<td>b</td>
<td>nd</td>
<td>High</td>
</tr>
<tr>
<td>5.</td>
<td>Cashmere</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Somalia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Sierra Leon</td>
<td>+</td>
<td>f</td>
<td>nd</td>
<td>Low</td>
</tr>
<tr>
<td>8.</td>
<td>Sweden</td>
<td>+</td>
<td>f</td>
<td>nd</td>
<td>Moderate</td>
</tr>
<tr>
<td>9.</td>
<td>Ethiopia</td>
<td>+</td>
<td>c</td>
<td>nd</td>
<td>High</td>
</tr>
<tr>
<td>10.</td>
<td>India</td>
<td>+</td>
<td>c</td>
<td>nd</td>
<td>Low</td>
</tr>
</tbody>
</table>

nd = not-detected
Table 2A. qPCR-based quantification of *Aggregatibacter actinomycetemcomitans* (A.a) and *Porphyromonas gingivalis* (P.g) (cell numbers/ml, thousand) in the samples from selected pockets.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Pocket</th>
<th>TBC*(per ml)</th>
<th>A.a (%)</th>
<th>P.g (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 74 dl</td>
<td>16,0</td>
<td>87,5</td>
<td>1,9</td>
<td></td>
</tr>
<tr>
<td>74+75+84</td>
<td>Nd*</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>2. 26 mb</td>
<td>14,0</td>
<td>12,0</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>6,2</td>
<td>10,2</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>3. 26 dl</td>
<td>4,3</td>
<td>0,4</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>16mb+26mb</td>
<td>Nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>4. 41</td>
<td>24,0</td>
<td>0,3</td>
<td>0,2</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>28,0</td>
<td>nd</td>
<td>3,9</td>
<td></td>
</tr>
<tr>
<td>5. 35 mb</td>
<td>1,0</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>6. 36 db</td>
<td>4,8</td>
<td>nd</td>
<td>7,7</td>
<td></td>
</tr>
<tr>
<td>7. 31 d</td>
<td>7,2</td>
<td>0,2</td>
<td>9,0</td>
<td></td>
</tr>
<tr>
<td>42 m</td>
<td>6,3</td>
<td>nd</td>
<td>1,6</td>
<td></td>
</tr>
<tr>
<td>8. 22 db</td>
<td>6,1</td>
<td>0,1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>32 db</td>
<td>19,0</td>
<td>1,3</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>9. 16 mb</td>
<td>5,6</td>
<td>nd</td>
<td>2,1</td>
<td></td>
</tr>
<tr>
<td>10. 16 mb</td>
<td>18,0</td>
<td>3,1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>36 mb</td>
<td>16,0</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>46 mb</td>
<td>1,8</td>
<td>nd</td>
<td>1,3</td>
<td></td>
</tr>
</tbody>
</table>

*nd = not-detected

# = total bacterial count
Table 2B. Culture-based quantification of *A. actinomycetemcomitans* and *P. gingivalis* (% of total viable count, million; TVC) in the samples from selected pockets.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Pocket</th>
<th>TVC*</th>
<th>A.a (%)</th>
<th>P.g (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>74 dl</td>
<td>0,5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>74+75+84</td>
<td>5,6</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2.</td>
<td>26 mb</td>
<td>0,7</td>
<td>7,3</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>0,1</td>
<td>13</td>
<td>nd</td>
</tr>
<tr>
<td>3.</td>
<td>26 dl</td>
<td>5,5</td>
<td>6,5</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>16mb+26mb</td>
<td>5,1</td>
<td>1,0</td>
<td>nd</td>
</tr>
<tr>
<td>4.</td>
<td>41</td>
<td>7,9</td>
<td>1,1</td>
<td>3,6</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>5,0</td>
<td>nd</td>
<td>2,0</td>
</tr>
<tr>
<td>5.</td>
<td>35 mb</td>
<td>0,8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>6.</td>
<td>36 db</td>
<td>0,5</td>
<td>nd</td>
<td>53,0</td>
</tr>
<tr>
<td>7.</td>
<td>31 d</td>
<td>22,0</td>
<td>5,0 x 10^{-3}</td>
<td>41,0</td>
</tr>
<tr>
<td></td>
<td>42 m</td>
<td>1,8</td>
<td>0,2</td>
<td>nd</td>
</tr>
<tr>
<td>8.</td>
<td>22 db</td>
<td>4,5</td>
<td>1,2</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>32 db</td>
<td>10,0</td>
<td>8,8</td>
<td>nd</td>
</tr>
<tr>
<td>9.</td>
<td>16 mb</td>
<td>4,6</td>
<td>3,0 x 10^{-2}</td>
<td>80,0</td>
</tr>
<tr>
<td>10.</td>
<td>16 mb</td>
<td>1,6</td>
<td>5,8</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>36 mb</td>
<td>24,0</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>46 mb</td>
<td>4,4</td>
<td>6,0 x 10^{-3}</td>
<td>73,0</td>
</tr>
</tbody>
</table>

*nd = not-detected
*TV* = total viable count
Fig 1. Leukotoxin promoter typing of *A. actinomycetemcomitans* isolated from the patients (P). MWS = molecular weight standard; JP2 = control strain.

Fig 2. Serotyping of *A. actinomycetemcomitans* (a-f) isolated from the patients (P). MWS = molecular weight standard.
APPENDIX

Appendix 1A. Primers for amplification of *P. gingivalis* genes, (Kirakodu et al., 2008).

<table>
<thead>
<tr>
<th><em>P. gingivalis</em>-primers</th>
<th>Total amount of bacteria (Universal primers), (Kirakodu et al., 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: AGGCAGCTTGCCATACTTGCG (108)</td>
<td>F: GATTAGATACCTGCTGATCCAC (116)</td>
</tr>
<tr>
<td>R: ACTGTAGCAACTACCCGATGT (109)</td>
<td>R: TACCTTGTTACGACTT (117)</td>
</tr>
</tbody>
</table>
### Appendix 1B. Primers for amplification of *A. actinomycetemcomitans* genes.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Primer Sequence</th>
<th>Amplicon length (bp)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GCA ATG ATG TAT TGT CTT CTT TTG GA CTT CAG TTG AAT GGG GAT TGA CTA CTA AAA C</td>
<td>428</td>
</tr>
<tr>
<td>B</td>
<td>CGG AAA TGG AAT GCT TGC CTG AGG AAG CCT AGC AAT</td>
<td>298</td>
</tr>
<tr>
<td>C</td>
<td>AAT GAC TGC TGT CGG AGT CGC TGA AGG TAA TGT CAG</td>
<td>559</td>
</tr>
<tr>
<td>D</td>
<td>TTA CCA GGT GTC TAG TCG GA GGC TCC TGA CAA CAT TGG AT</td>
<td>690</td>
</tr>
<tr>
<td>E</td>
<td>CGT AAG CAG AAG AAT AGT AAA CGA AAT AAC GAT GGC ACA TCA GAC TTT</td>
<td>211</td>
</tr>
<tr>
<td>F</td>
<td>AAA ATT TCT CAT CGG GAA TG CCT TTA TCA ATC CAG ACA GC</td>
<td>232</td>
</tr>
<tr>
<td>Leukotoxinpromotor</td>
<td>GCA GGA TCC ATA TTA AAT CTC CTT GT CCT TTA TCA ATC CAG ACA GC</td>
<td>1034</td>
</tr>
</tbody>
</table>

<sup>a</sup> base pair
Appendix 2. PCR temperature profiles for amplification of the *A. actinomycetemcomitans* strains.

<table>
<thead>
<tr>
<th></th>
<th>Serotype a-e promoter</th>
<th>Serotype f</th>
<th>Leukotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial denaturation</strong></td>
<td>96°C 2 min (1)</td>
<td>96°C 2 min (1)</td>
<td>94°C 1 min (1)</td>
</tr>
<tr>
<td><strong>Denaturation</strong></td>
<td>94°C 1 min (29)</td>
<td>94°C 1 min (29)</td>
<td>95°C 30 sek (34)</td>
</tr>
<tr>
<td><strong>Primer anneling</strong></td>
<td>54°C 1 min (29)</td>
<td>49°C 1 min (29)</td>
<td>55°C 30 sek (34)</td>
</tr>
<tr>
<td><strong>Extension step</strong></td>
<td>72°C 1 min (29)</td>
<td>72°C 1 min (29)</td>
<td>72°C 30 sek (34)</td>
</tr>
<tr>
<td><strong>Final step</strong></td>
<td>72°C 3 min (1)</td>
<td>72°C 3 min (1)</td>
<td>72°C 3 min (1)</td>
</tr>
</tbody>
</table>

\(a\) Number of cykles
Appendix 3. Enkät för provtagning – Aggressiv parodontit hos barn och ungdomar.

Födelsedata:  20____ - _____ - ____

Kön: 
- pojke ☐
- flicka ☐

Nationalitet:
- svensk ☐
- annan ☐

Vilken? ____________________________

Mor nationalitet
- svensk ☐
- annan ☐

Vilken? ____________________________

Far nationalitet
- svensk ☐
- annan ☐

Vilken? ____________________________

Fullt frisk
- ja ☐
- nej ☐

Mediciner
- ja ☐
- nej ☐

Om ja, vilka: ____________________________

Munhygien:

- Tandborstning
  - någon gång/vecka ☐
  - 1g/dag ☐
  - 2ggr/dag ☐

- El- tandborste ☐
- Manuell tandborste ☐
Approximal rengöring

- Ingen
- 1-3 gånger/vecka
- >3 gånger/vecka

Tidigare tandvård:

- Regelbundet
- Oregelbundet
- Aldrig varit hos tdl tidigare

Anhöriga med parodontit:

- Inga
- Moder
- Fader
- Syskon

Om ja, Kronisk- eller Aggressiv parodontit?

Kliniska parametrar

- BVS (%)
- PLI (%)

Antal patologiska fickor (st):

- 4-5mm
- >6mm

Vertikal bensänkning:

- Förekommer
- Förekommer ej
Pus:

- Förekommer
- Förekommer ej

Subgingival tandsten:

- Förekommer
- Förekommer ej

Supragingival tandsten:

- Förekommer
- Förekommer ej

**Bakteriologisk analys**

Tand/ficka/fickdjup: ________________________________