Prevalence of Aggregatibacter actinomycetemcomitans in Saliva from Children aged 7-9
- and evaluation of two different DNA extraction methods

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ABSTRACT

Periodontitis is an inflammatory disease caused by bacterial infection that can lead to loss of supporting tissues around the teeth. Studies show that different ethnic populations demonstrate major differences in prevalence of the disease and in which form the disease occur. The presence of the bacteria *Aggregatibacter actinomycetemcomitans* (A.a) is associated with the aggressive form of the disease, diagnosed primarily in young people.

The present study aims to describe the prevalence of A.a in children aged 7-9 years living in Sweden with different ethnic backgrounds and to evaluate two different ways of extracting bacterial DNA. The hypothesis was that prevalence of A.a would correlate with the origin of the subjects, thus anticipating a higher prevalence in subjects of African origin than those of other ethnicity.

Stimulated saliva samples from 85 children were studied. Two methods were used to extract DNA, manually and automatically. qPCR was used to investigate if the samples contained A.a.

The essential results showed that the highest prevalence of A.a was found in samples belonging to children with African origin. The manual method extracted DNA in a higher amount and from more samples compared to the automatic method.

Sweden is nowadays multicultural and the clinical issues change with the population. Other clinical questions needs to be answered and previous truths need to be reassessed, for example periodontal problems in younger individuals. In this study, the manual method of extracting DNA proved to be more sensitive than the automatic, though more studies need to be conducted to draw any conclusions.
INTRODUCTION

Diseases in the oral cavity are a major problem in the world, especially dental caries and periodontal diseases. The caries situation among children in Sweden is now better than it was a few decades ago. A lot of epidemiological data about this has been published. Periodontal diseases on the other hand are not as well investigated in young people (Johansson and Östberg, 2015). Sweden is today a multicultural population with a high diversity of ethnicities (Statistiska Centralbyrån, 2017). Studies show that different ethnic populations demonstrate major differences in the prevalence of the rapidly progressing form of periodontitis (Albandar and Tinoco, 2002). The first peak in immigration in Sweden was in the middle of the 20th century (Statistiska Centralbyrån, 2017). Because of the new heterogenic population, it is relevant to map out the prevalence of both the disease and its underlying factors.

The oral bacterium colonizes the oral cavity shortly after birth and the microbiome changes during the individuals’ lifetime (Xu et al., 2015). The oral cavity harbours particularly different surfaces. There is the regenerative and soft shedding surface of the mucosa and the non-regenerative non-shedding surface of the teeth. The non-shedding surface of the tooth and the gingival sulcus together makes a unique habitation for bacteria due to the fact that it is placed between the hard and soft tissue. Salivary defence mechanisms, pH, nutrients and susceptibility for colonization are a few of the factors that defines the nisch. Therefore, the subgingival ecosystem supports the growth of bacteria that through evolution has adapted to this type of environment (Asikainen and Chen, 1999).

Periodontitis is an inflammatory disease caused by a bacterial infection that leads to the loss of supporting tissues around the teeth. The most prominent factor associated with the aggressive form of the disease is the presence of the bacteria *Aggregatibacter actinomycetemcomitans* (A.a). It is hard to predict who has an increased risk to develop this type of periodontitis. A few studies have been conducted and the results indicate that important factors are microbial and/or host response. There are two forms of Aggressive periodontitis, the differences between them depends on which and how many teeth in the dentition that are affected. The localized form is especially associated
with the presence of A.a and the disease often affects young people. The relation concerning the microbiological flora and the disease is not completely understood. (Höglund Åberg et al., 2014).

A.a is a oral bacterium and is not part of the commensal microbiota of any other part of the human body. Furthermore, there is no evidence that A.a could exist in nature. A study by Asikainen and Chen described that A.a was the first to colonize a supragingival plaque surface when studying both humans and monkeys. This suggests that A.a is capable of colonizing clean and healthy tooth surfaces. It is well established that A.a is transmitted vertically through family members. This has been shown by examining the specific clones that the target carrier inhabits and comparing it to family members. Specific clones seem to remain within a certain lineage of hosts for centuries and the distribution of clones between ethnic groups have significant differences globally (Asikainen and Chen, 1999).

A.a has a lot of virulence factors that can promote the disease. One of the most prominent is leukotoxin. Leukotoxin can cause cell death of defensive cells. It can also activate release of proinflammatory cytokines and lysozymal cells. The leukotoxic activity is different from clone to clone. For example, the JP2-clone has a high leukotoxic activity and it is strongly associated with alveolar bone loss in young people. Studies indicate that the presence of both the JP2 and non-JP2 genotype is linked to people that has an African origin. One thing to remember, is that several studies show results indicating that even if you are a carrier of the bacteria you do not have to show signs of alveolar bone loss. There is, however a higher risk of developing the disease. (Höglund Åberg et al., 2012).

The greater quantity of studies shows prevalence in Aggressive periodontitis of <1 % when fractionated into a Caucasian ethnicity. However, other ethnic populations have shown striking differences. For example the prevalence of Aggressive periodontitis among African-Americans is 2.6 % and in the Moroccan population 7.6 % according to a systematic review by Albandar and Tinoco. The vast difference in prevalence between Caucasian and North-African ethnicities demonstrate that host-specific interactions could be the explanation. One difficulty when discussing epidemiology of periodontal disease and pathogenicity, is that the methods and measurements are not consistent
between the epidemiological studies published (Albandar and Tinoco, 2002). A study conducted in Örebro, Sweden, 1994, investigated children, 7-9 years of age, for signs of periodontal disease. The results showed that <0.5 % had a more severe form of periodontitis in the primary dentition (Sjödin and Matson, 1994). Furthermore, according to another Swedish study there is an association between A.a and periodontitis in young people. Reports show that A.a is a common phenomenon with periodontitis in the primary dentition (Sjödin et al., 1995).

There are different types of ways to study the oral microbiome, one of which is to extract the bacteria's DNA from for example saliva to demonstrate the prevalence of the bacteria. This method has been used for a long time and has evolved to more and more sophisticated systems. There are various specific kits for different types of biological samples on the market today. Due to the increased pressure of producing large quantities of results, automated extraction systems have been developed. These machines could, if proven to have validity, facilitate steps taken when extracting DNA. As we know of today, there are a few studies made with the aim of validating different methods of extracting DNA. Therefore, a method control will be performed in this study with the aim of evaluating the methods described above.

The object of this study is to describe the prevalence of A.a in children aged 7-9 years living in Sweden with different ethnic backgrounds and to evaluate two different ways of extracting DNA, manually and automated. In accordance with previous findings in the review by Albandar and Tinoco, our hypothesis is that the prevalence of A.a should correlate with the origin of the young subjects. The prevalence is estimated to be higher among the subjects of African origin and lower in those with an Non-African ethnicity. Our ambition is to investigate the prevalence of A.a to provide a new risk assessment for periodontitis among young children in Sweden. Another ambition is to see if the automated DNA extraction method can compete with the manual traditional method.
MATERIALS & METHODS

Study Design and Subjects
This project is a follow-up study to a project named DviTand (Gyll et al., 2017), which in turn is a follow-up study to another project, Divisum (Öhlund et al., 2013). The aim of the original study was to measure Vitamin D levels in blood of preschool children. 206 children aged 7-9 years participated. Divided into groups with fair and dark complexion, performed in two cities, Umeå and Malmö (Öhlund et al., 2013). The aim of DviTand was to investigate the correlation between various levels of vitamin D, antibacterial peptide LL-37 and dental caries in children. It included regular dental check-up, clinical photos and sampling of dental plaque and stimulated saliva. The children in these studies volunteered to participate after both verbal and written information and approval from the caretakers (appendix 1). In the present study saliva collected from the children participating in DviTand was used. All samples were de-coded before processing, and none of the samples could be traced to a specific child. Personal data of the participating children are kept under the Personal Data Act. Out of the 206 children, 85 participated in DviTand and also in the present study, 47 from Umeå and 38 from Malmö. The dropouts were mainly due to lack of time and that the families had moved from the area.

Saliva Collection
Routine methods were used for sampling of stimulated saliva. The children were asked to chew on a piece of paraffin and collect saliva in a test tube; at least 3 mL of saliva was needed. Prior to the sampling the children had been asked to avoid eating and drinking within two hours. The saliva samples were placed on ice and transported to the laboratory within two hours. The saliva was aliquoted and stored in -80° freezers until further analyses.

DNA Extraction
Two methods were used to extract DNA. The manual method, which is the routine method used, (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich, St. Louis, MO, USA) and an automated method (LIAISON® ixt, DiaSorin, Saluggia, VC, Italy) where
the Viral NA (nucleic acid) program was chosen for all the samples. For both methods, standard protocols described by the manufacturers were used.

**Manual Method**

Saliva samples were taken from the -80° freezer. Saliva was thawed and vortexed. Genomic DNA was extracted from the samples using the Gen Elute Bacterial Genomic DNA kit with addition of lysozyme and mutanolysin as described (Lif Holgerson *et al.*, 2011). To prepare the saliva samples, 20 µL of the Proteinase K solution was placed into a 1.5 mL microcentrifuge tube. 400 µL of the whole saliva sample + 600 µl M-dil (4.4 g NaCl, 0.42 g KCl, 1.0 g Na₂HPO₄ x 2H₂O, 1.0 g KH₂PO₄, 10.0 g C₃H₉Na₂O₇P x H₂O, 500 mL distilled water mixed with 0.1 g MgCl₂ x 6H₂O, 500 mL distilled water) was then added to the tube. To the saliva samples that were less than 400 µL, M-dil was added so that the total volume was 1000 µL. The solutions were then vortexed. To lyse the cells, 200 µL of the Lysis Solution C was added and then vortexed. The samples were then incubated for 10 min at the temperature 55°. 500 µL of the Column Preparation Solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at 13000 rpm for 1 min. The flow-through liquid was discarded. To prepare the samples for binding, 200 µL of ethanol (99.5 %) was added to the lysate and vortexed for 5-10 sec. To load the lysate, the entire contents of the tubes were transferred into the previously treated columns. The columns were then centrifuged at 13000 rpm for 1 min and placed in new 2 mL collection tubes. For the first wash, 500 µL of wash solution was added to the column and centrifuged for 1 min at 13000 rpm. The collection tube was discarded and the column was placed in a new 2 mL tube. For the second wash, 500 µL of wash solution was added to the column and centrifuged for 3 min at 13000 rpm to dry the column. The collection tubes containing the flow through liquid was discarded and the filters were placed in new 2 mL collection tubes and centrifuged for 1 min at 13000 rpm. The filters were then placed in to a new set of tubes and the previous tubes were discarded. To elute the DNA, 100 µL of the elution solution was pipetted directly into the centre of the column and then incubated for 5 min at room temperature. The columns were then centrifuged for 1 min at 13000 rpm.
Automated Method - Viral kit
To prepare the saliva samples in the machine LIAISON® ixt, 20 μL of the Proteinase K solution was placed into a 1.5 mL microcentrifuge tube. 400 μL of the whole saliva sample + 150 μl M-dil was then added to the tube. To the saliva samples that were less than 400 μL M-dil was added so that the total volume was 550 μL as recommended by the manufacturer. The solutions were then vortexed. The machine was loaded with pump-tip devices, reagent cartridges and sample cartridges. As recommended by the manufacturer when extracting bacterial DNA, the NA viral program was chosen and the DNA recovery time was 45 min.

DNA quality and quantity
DNA was evaluated using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) for both methods.

Bacterial Detection
To investigate if the samples contained DNA from A.a, qPCR Rotor gene 6000 was used in accordance with the manufacturer (Corbett, Milton, Cambridge, United Kingdom). To each test tubes 5 μl Kapa sybr fast qPCR MasterMix, 2 μM (1 μl) Primer specific gene 1TXA (1+2), (QF sequence CTaggtattgcgaaacaatttg, QR CCTGAAATTAAGCTGGTAATC), and 4 μL template 1 ng/μL was added. In the thermocycling program, the initial denaturation was 10 min at 95°C, after that 95°C, for 10 sec. Followed by 45 cycles + melt as followed: 5 sec at 55°C, for annealing and 10 sec at 72°C, for extension. Samples that gave weak or no results were re-runned to avoid false negative signals.

Statistical Analysis
The amount of bacteria was calculated using standard curves. Due to the fact that different kits were used, there are different standard curves for each method. The manual concentration was multiplied with 2.5 x 10^7, and the automatic with 1.86 x 10^7. Samples containing a concentration of bacteria 0.00001 ng/μL or less was considered as containing no bacteria. The cut-off values were therefore 250 bacteria’s in the manual method and 186 bacteria’s in the automatic method (appendix 2). Five samples were
excluded from the analysis due to lack of results. The data from the present study and the previous conducted studies within the projects (DviTand and Divisum) was placed in a database constructed in the statistical software IBM SPSS Statistics version 23 (IBM Corporation, Armonk, NY, USA). A p-value of <0.05 was considered as significant. Chi-square test was carried out to find correlations between the different variables.

**Ethical Considerations**

The original project, Divisum, was approved by the Regional Ethical Review board at Umeå University (Dnr: 2012-158-31M) as a multicenter study. The follow up project, DviTand was approved with an addendum to the original project, (Dnr: 2014-103-32M). This study has been approved from the local ethical committee of Institute of odontology, Umeå University. All samples have been decoded and the background data cannot be traced to a specific person at this point. The ethical problem could arise if the results point to a sample with a high prevalence of A.a. However, all the subjects in the study were examined and did not show any clinical signs of periodontal disease at the time. All the children are within the supervision of the public dental care system and will not be left without care if they were to exhibit clinical signs in the future.

**Literature Search**

To find literature, different methods were utilized. The PubMed database was used with the keywords “Aggressive Periodontitis/epidemiology”, “Periodontitis AND Aggregatetbacter actinomycetemcomitans AND Virulence factors”, “Tooth, Deciduous AND Alveolar Bone Loss”. These searches resulted in 128, 18 and 66 articles respectively. Other keywords were “Child” AND "Socioeconomic Factors" AND "Oral Health" and “review” was chosen as article type. This resulted in 16 articles. To find relevant articles, the abstracts were read. Articles were also collected and used from the reference list at the end of the chapter about Periodontal conditions (Sjödin B, Matsson L. 2009. 166-182). To find statistics about immigration in Sweden Statistiska Centralbyrån’s website was used (Statistiska Centralbyrån, 2017).
RESULTS

Data from 80 children was analysed, among these 33 samples were positive for A.a when using the manual extraction method, 41.3 %. There were no differences in distribution of gender between A.a positive and A.a negative children (table 1). The mean age for all children was 8.22 ± 0.69 (valid percent 96.3) and the age of A.a positive contra negative did not differ (table 1). None of the A.a positive children had been treated with antibiotics the last three months before sampling, but three of the children in the A.a negative group (table 1).

The participating children’s countries of origin were divided into areas in the original questionnaire (Divisum-study): Nordic, European, African, South American, Asian, Middle East and Australia/America. In the group of children that were A.a positive the majority was of Nordic origin (Sweden, Norway, Finland or Denmark).

In the group of children that were A.a positive in respect to the maternal country of origin, 51.5 % were Nordic, 30.3 % African, 12.1 % Asian and 6.1 % Middle eastern. In the group of children that were positive in respect to the paternal origin, 54.5 % were Nordic, 3 % European, 27.3 % African, 9.1 % Asian and 6.1 % Middle Eastern.

With respect to the educational level, 23 out of the 31 (two values missing) positive children had fathers with a higher education. Corresponding numbers for the mothers were 19 out of 33 children that were positive for A.a. Remaining samples that were positive for A.a had a mother or a father with secondary school as their highest education.

Out of the 80 samples, two children had African origin and both were positive for A.a. In respect to the mothers’ origin, 10 out of 15 samples (66.7 %) where the mother had African origin were positive for A.a. The corresponding number for fathers was 9 out of 17 (52.9 %). When using parental origin, which combines the mother and the father’s origin, 9 out of 13 samples (69.2 %) were positive in the African group, i.e. both parents originated from Africa (figure 1).
The highest amount of A.a was found in a sample who belonged to a child that was completely Nordic, in other words, both the mother, father and child had Nordic origin.

The manual DNA extraction detected that 33 out of 80 samples had prevalence of A.a while the automatic DNA extraction only detected eight samples as positive. Furthermore, the p-value was statistically significant (p<0.001), meaning there is a significant difference between the methods. The amount of bacteria that the methods extracted differed as well (table 2). Out of the 80 samples, only eight were positive in both extraction methods. Therefore, these are the only ones that can be analysed and compared. The automatic method extracted <0.5 % compared to the manual method in six out of the eight samples. The automatic method extracted at most 6.5 % of what the manual method extracted, see sample 2 (figure 2).

**DISCUSSION**

The aim of this study was to investigate the prevalence of A.a in children aged 7-9 years old and the correlation with their origin. As mentioned before, A.a is strongly associated with Aggressive periodontitis (Höglund Åberg et al., 2012). Not many studies have focused on examining the prevalence of A.a. The focus has mostly been on the prevalence of the disease Aggressive periodontitis. It is therefore difficult to compare the findings in this study to previous studies.

This is a descriptive study with 85 samples. Five of which were excluded due to the lack of results from one or both DNA extraction methods. Even though the remaining 80 samples are a good representation of the reality in Sweden, it would have been more statistically significant with more participants.

The results in this study showed that the highest prevalence of A.a was found among the children who had an African origin (figure 1). It is of importance to mention that the children of African origin in the present study was only two. But as seen in Figure 1, if the child had a mother or a father or both parents with African origin, the prevalence
was higher than those with parents that were from other parts of the world. It is impossible to predict if these children will develop alveolar bone loss. Though, previous studies show that the presence of the bacteria is associated with alveolar bone loss (Höglund Åberg et al., 2012). The systematic review by Albandar and Tinoco, analysed the prevalence of Aggressive periodontitis and found major differences between ethinical groups. The findings indicated that there were a much higher prevalence of the disease amongst African-Americans (2.6 %) and Moroccans (7.6 %) compared to Caucasians (<1 %). The present conducted study supports previous findings in the sense that the results show a similar pattern. It is hard to draw conclusions though, due to that studies have different focus points, inclusion criteria, methods and measurements.

One of the more unexpected findings in this study is that the prevalence of A.a was high in the Nordic group in the case of the child origin. We argue that most of these children have parents that are of Non-Nordic descent. Another unexpected finding was that the absolute highest amount of A.a was found in a sample who belonged to a child that was completely Nordic, in other words, both the mother, father and child had Nordic origin. We cannot exclude that the origin is completely Nordic but one explanation could be that the child’s grandparents are of Non-Nordic origin. The migration to Sweden started in the middle of the 20th century and the first immigrants might be grandparents today in 2017 (Statistiska Centralbyrån, 2017).

When analysing the material, on thing that stood out was the education level of the parents in respect to the prevalence of the bacteria. The results showed, somewhat surprising that the parents with a higher education level had more children that were positive for A.a than those with a lower education level. We know from previous studies that there is a strong correlation between socio-economy and oral health (Kumar et al., 2014). We could only speculate that the findings in this study are due to the bacteria’s ability to colonize a surface, independent of good oral health and more depending on genetic factors as inheritance.

The methods used in this study have strengths and weaknesses. The manual extraction method has many steps and takes more work hours to finalize while the automatic has
fewer steps, is easy to set up and can process 12 samples at a time. However, as shown in Table 2, the manual extraction method proved to yield far more DNA than the automatic (257 times more extracted DNA). The automatic method offered a variety of different kits optimized for different types of samples. The kit used in the present study was the viral NA extraction kit, which the manufacturer claims to be validated with respiratory swab samples and is meant for use in downstream Nucleic Acid Technology assays for detection. The automatic kit used was therefore not optimized for saliva samples, but recommended by the sales person for the machine. This is a source of error when comparing the two methods and could be the reason that the manual extraction method yielded more DNA and had a higher sensitivity. The kit for the automatic method did not contain lysozyme and mutanolysin whose task is to tear apart the bacteria so that the DNA can be copied. The result in this study was therefore based on the manual extraction method.

In a bigger perspective, it would be interesting to perform a follow up examination of all the children in this study to see if the participants that were positive for A.a will show signs of periodontal disease in the future. Only then could a conclusion about the prevalence of the bacteria and the disease be drawn. This future examination could indicate if the prevalence of the bacteria correlates to the risk of developing the disease. Sweden is now a more multicultural country than before (Statistiska Centralbyrån, 2017). There is a risk that the periodontal issues among young people in Sweden will increase and it is important for the clinician to be aware of the implication of this when examining children and adolescents. It is of paramount importance to have good prevention, care programs and guidelines. It is important to discover, diagnose and treat the disease as quickly as possible to generate the best results.

**Conclusion**

The highest prevalence of A.a was found among children with African origin or whose parents are of African origin. The manual method of extracting DNA proved to be more sensitive and yielded more DNA than the automatic. However, the recommended kit was not optimized for saliva samples, instead the kit was optimized for respiratory samples. If we could have had access to a kit optimized for oral saliva samples the
results might have been more comparable. The automatic could have clinical relevance but more studies need to be conducted to draw any conclusions.

Conflict of Interests
There were no conflict of interests and the study received no contributions from third parties.

ACKNOWLEDGEMENTS

We would like to acknowledge Pernilla Lif Holgerson, Agneta Rönnlund and Rolf Claesson who has been supporting and guided us through this project.
REFERENCES


Table 1.
Characteristics of manually extracted amount of A.a.

<table>
<thead>
<tr>
<th></th>
<th>A.a positive n=33</th>
<th>A.a negative n=47</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender (boys/girls, %)</strong></td>
<td>51.5/48.5</td>
<td>38.3/61.7</td>
<td>0.241</td>
</tr>
<tr>
<td><strong>Age (mean years, SD)</strong></td>
<td>8.3 ± 0.58</td>
<td>8.1 ± 0.74</td>
<td>0.539</td>
</tr>
<tr>
<td><strong>Antibiotics (yes, %, in the last 3 months before sampling)</strong></td>
<td>0.0</td>
<td>6.5</td>
<td>0.141</td>
</tr>
<tr>
<td><strong>Child origin</strong></td>
<td></td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>6.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Non-African (%)</td>
<td>93.9</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal origin</strong></td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>30.3</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>Non-African (%)</td>
<td>69.7</td>
<td>89.4</td>
<td></td>
</tr>
<tr>
<td><strong>Father origin</strong></td>
<td>0.270</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>27.3</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>Non-African (%)</td>
<td>72.7</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td><strong>Parental origin</strong></td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>27.3</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Non-African (%)</td>
<td>72.7</td>
<td>91.5</td>
<td></td>
</tr>
</tbody>
</table>

*P-value < 0.05 is set for a statistical significant difference.

** Two missing values (valid percent 97.5).
Table 2.
Comparison, manual and automatic DNA extraction.

<table>
<thead>
<tr>
<th></th>
<th>Manual DNA extraction</th>
<th>Automatic DNA extraction</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.a positive (%)</td>
<td>41.3</td>
<td>10.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Numbers of A.a (mean, SD)</td>
<td>1.15x10^5 ± 5.76x10^5</td>
<td>4.48x10^2 ± 3.21x10^3</td>
<td>N.A*</td>
</tr>
</tbody>
</table>

*Chi-2 test, p-value<0.05 is set for a statistical significant difference.
*Not available

Figure 1.

Correlation between A.a postive and origin (%)

Prevalence of A.a in percent if African or Non-African origin.
Figure 2.

Comparison between bacteria extracted in the eight samples that were positive for A.a in both manual and automatic extraction. Shown in amount of bacteria and percent of total amount of bacteria.
Appendix 1

Datum 20… - …… - ..... ....

Skriftligt samtycke till att delta i DviTAND (DvitaminTand) studien

Jag har i egenskap av målsman informerats om DviTAND-studien och jag har tagit del av och förstått den muntliga och skriftliga informationen. Jag har också förstått att mitt samtycke avser min son/dotter.


☐ Jag samtycker till att mitt barn deltar i DviTAND-studien

☐ Jag samtycker till att mitt barn deltar i DviTAND-studien utom momentet som avser …………………………………………………………………………………………………

☐ Jag samtycker till att kompletterande information kan inhämtas från mitt barns sjuk- och tandvårdsjournal.

Barnets namn:

Barnets födelsedatum:

Datum …………………

………………………………………………………………………………………………

………………………………………………………………………………………………

Förälders underskrift namnförtydligande telefon

Sign ansvarig tandläkare: …………………………………

LÄMNAS TILL: Pernilla Lif Holgerson
Projektledare, Institutionen för odontologi,
Umeå universitet, 901 87 Umeå pernilla.lif@odont.umu.se
Appendix 2

Calculations of the amount of bacteria from standard curves concentration.
Examples:

<table>
<thead>
<tr>
<th>Concentration (ng/µL)</th>
<th>Manual (amount)</th>
<th>Automatic (amount)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00001</td>
<td>250</td>
<td>186</td>
</tr>
<tr>
<td>0.0001</td>
<td>2500</td>
<td>1860</td>
</tr>
<tr>
<td>0.001</td>
<td>25000</td>
<td>18600</td>
</tr>
<tr>
<td>0.01</td>
<td>250000</td>
<td>186000</td>
</tr>
<tr>
<td>0.1</td>
<td>2500000</td>
<td>1860000</td>
</tr>
</tbody>
</table>