Serotyping *Aggregatibacter actinomycetemcomitans* by quantitative PCR – Method development

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Tiivistelmä – Referat – Abstract

Tutkielma perustuu hammaslääketieteen laitoksella tehtyyn menetelmän kehitystutkimukseen. Tutkimuksessa kehitettiin kvantitatiivisen polymeraasiketjureaktioon perustuva menetelmä tunnistaa *Aggregatibacter actinomycetemcomitans*in kuusi serotyyppiä (a-f). *A. actinomycetemcomitans* on oletettu patogeeni aggressiivisessa parodontiitissa ja sen roolia on tutkittu myös suuontelon ulkopuolisissa tulehduksissa. Tutkimus koostui seuraavista vaiheista: kullekin serotyypille spesifisen alukeparin suunnittelu, tehokkaan reaktio-olosuhteiden kehittäminen kokeiden kautta, alukkeiden spesifisyyden testaaminen puhdasviljellyillä bakteerikannoilla ja kliinisten näytteiden serotyyppaus suomalaisesta Parogene-aineistosta. Kehitellyllä menetelmällä identifioitiin 44:stä sylkinäytteestä *A. actinomycetemcomitans*in serotyyppi (näytteitä yhteensä 252).

Avainsanat – Nyckelord – Keywords

Aggregatibacter actinomycetemcomitans, periodontitis, serotyping, saliva, qPCR

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1 INTRODUCTION

Aggregatibacter actinomycetemcomitans is a bacterium linked to aggressive periodontitis and is considered a putative pathogen also in some systemic diseases. Seven serotypes (a-g) of *A. actinomycetemcomitans* have been so far identified. In this study, we have developed a methodology to identify these serotypes by using quantitative PCR qPCR) (the serotype g is excluded from the study).

Advanced methods to identify serotypes of putative pathogens should support research on periodontitis and role of oral microbes also in systemic diseases and distant infections outside oral cavity. QPCR is a relatively affordable and quick method for bacterial characterization.

The phases of method development are following: designing serotype specific primers, developing and testing an efficient and sensitive qPCR assay for the primers, cross checking the primers with control templates using cultivated bacteria from pure cultures and clinical saliva samples.

2 REVIEW OF THE LITERATURE

2.1 Periodontal disease

Gingivitis and periodontitis are diseases affecting the tissue surrounding the teeth. Both of the conditions are manifestations of an inflammation reaction caused by bacteria and the major difference between these conditions is in the severity and extent of the reaction. The inflammation reaction is caused by dysbiosis of the bacterial biofilm on dental surface.

In gingivitis, the symptoms are redness, swelling, tenderness and bleeding of the gingiva. In periodontitis, the inflammation leads to irreversible destruction of periodontal ligament, the clinical definition being detectable attachment loss.(1) The change in the periodontal tissue is manifested in deepening of the crevice between the gingiva and the tooth. In progressing periodontitis, the crevice deepens to form a periodontal pocket towards the root of the tooth. Eventually the disease can cause alveolar bone resorption and tooth loss.(2) In periodontitis, the gram-negative and anaerobic bacteria become more prevalent in oral microbiota compared to gingivitis with a gram-positive majority. Also,

the number of bacteria may rise from $1x10^3$ to $1x10^8$ in periodontal pocket.(2) Clinically the disease is diagnosed by measuring the probing depth, i.e. depth of periodontal pockets, bleeding of the gingiva, amount of plaque, calculus, and exudate. Also, radiography is essential in analysis of loss of alveolar bone.(2)

Periodontitis is classified to three different diseases or forms, chronic periodontitis, aggressive periodontitis, and periodontitis as a manifestation of a systemic disease. Difference between these diseases is made following the rate of progress of the disease, patterns of destruction, age of onset, clinical signs of inflammation, and amount of dental plaque and calculus. The symptoms of periodontitis may be localized or generalized, meaning that the periodontal attachment loss either affects only some of the teeth, or manifests itself in all teeth.(3) Localized aggressive periodontitis differs from the other forms in three different ways. It affects only the periodontal ligament around incisors and first molars and it is associated with thin dental plaque, in contrast to chronic and general aggressive periodontitis which are characterized by relatively thick plaque and calculus. The same applies to clinical signs of inflammation, which tend to be obvious in chronic and generalized aggressive periodontitis in comparison to localized aggressive periodontitis with relatively modest signs of clinical inflammation. Regarding the age of onset of periodontitis, it is accepted that aggressive periodontitis clearly affects younger people than chronic periodontitis. Localized aggressive periodontitis is considered to have its onset in puberty and generalized aggressive periodontitis affects usually patients under 30 years old.(4,5)

The classification of periodontitis presented above is based on work done by the International Workshop for Classification of Periodontal Diseases and Conditions and American Academy of Periodontology in 1999. Even though this classification is widely accepted, the understanding of the disease evolves, as does the classification.(6) It is important to note when assessing research data and findings on periodontitis that there is variation in the criteria that are used to describe study subjects as patients with periodontitis or healthy. Hence, the criteria on whether study subjects have periodontitis or not, needs to be stated when reported prevalence rates are referred to.

Periodontitis is a relatively common disease. Circa 64 per cent of Finnish population over 30 years old suffer from periodontitis in one form or another. This figure is based on the Health 2000 study conducted in 2000 and 2001. The criteria for periodontitis were at least

one tooth with a probing depth 4 mm or more. Applying the same criteria, 50% of over 30 years old in the United States and 54% of over 15 years old in England have periodontitis.(7)

In the United States it has been estimated that 21.8% of the adult population (aged between 30 to 90 years) has the mild form of the disease, and 12.6% moderate or severe form of the disease. In this case, study subject was considered to have periodontitis if there was at least one tooth with a probing depth minimum of 3 mm or a grade I furcation involvement. For moderate form of periodontitis the criteria were at least one tooth with probing depth of 5 mm or at least two teeth having a minimum probing depth of 4 mm.(8)

Despite the classification of periodontitis to different categories according to symptoms, further consensus building has been needed to standardize definitions. A study conducted in Brazil has demonstrated differences in prevalence rates due differing case definitions of periodontitis. Applying five different definitions, the researches ended up with prevalence rates for periodontitis ranging from 65.3% to 13.8%.(9)

To address the issue of differing case definitions, uniform definitions have been proposed both by 5th European Workshop in Periodontology and by Centers for Disease Control and Prevention and American Academy of Periodontology. The following Tables 1 and 2 summarize these two definitions.(10,11)

Table 1: Definitions of periodontitis according to the Centers for Disease Control and Prevention and American Academy of Periodontology

Case of periodontitis	Definition
No periodontitis	No evidence of mild, moderate, or severe periodontitis
Mild periodontitis	≥ 2 interproximal sites with clinical attachment loss ≥ 3 mm, and ≥ 2 interproximal sites with probing depth ≥ 4 mm (not on same tooth) or one site with probing depth ≥ 5mm
Moderate periodontitis	≥ 2 interproximal sites with clinical attachment loss ≥ 4 mm (not on same tooth), or ≥ 2 interproximal sites with probing depth ≥ 5 mm (not on same tooth)
Severe periodontitis	≥ 2 interproximal sites with clinical attachment loss ≥ 6 mm (not on same tooth) and ≥ 1 interproximal site with probing depth ≥ 5mm

Edited from Eke et al 2012.(11)

Table 2: Definitions of periodontitis according to the 5th European workshop in periodontology

Case of periodontitis	Definition
Sensitive case definition (inclusive of incipient cases)	Presence of proximal attachment loss of ≥ 3mm in ≥ 2 non-adjacent teeth.
Cases with substantial extent and severity	Presence of proximal attachment loss of ≥ 5mmin ≥ 30% of teeth present.

Edited from Tonetti et al 2005.(10)

2.1.1 Pathogenesis of periodontitis

There is a strong link between chronic periodontitis and gingivitis. Albeit gingivitis does not always result in chronic periodontitis, chronic periodontitis is nearly always preceded by gingivitis. However, the relationship between dental plaque, gingivitis, and severe forms of periodontitis is not that clear. There are reasons to suggest that certain subjects in a population are more susceptible to the disease.(12)

The inflammation reaction is the key in understanding the damage to the connective tissue and even possible resorption of alveolar bone. The destruction of connective tissue and resorption of alveolar bone are caused mainly by host immune reaction, including secretion of matrix metalloproteinases and increase in osteoclastogenesis.(13)

Periodontitis is caused by disturbed interaction between dental biofilm and periodontal tissue. This interaction has been recently described through polymicrobial synergy and dysbiosis model (PSD model). The central idea in PSD–model is that although there are species strongly linked with chronic periodontitis, such as *Porphyromonas gingivalis*, this species alone is not able to initiate tissue destructive inflammation. Rather, it is suggested that the periodontitis is a result of synergistic and dysbiotic microbiota. Species such as *P. gingivalis* do have a key role, but they are not able to initiate dysbiosis and an inflammation reaction without pathobionts, which are able to contribute to the inflammation when homeostasis has been disrupted.(14)

Methods based on sequencing of 16SRNA have on the one hand confirmed the earlier results that have linked *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* with periodontitis. On the other hand, these studies have given evidence on previously

unrecognized associations between periodontitis and bacteria including *Anaeroglobus geminatus, Eubacterium saphenum, Filifactor alocis*, *Porphymoronas endodontalis* and *Prevotella denticola*.(15)

While the connection between some identified bacteria and periodontitis has been established in several studies, there are still a number of non-identified bacteria to be found in periodontal pockets of inflamed tissue (16). Also, while the prevalence and especially the amount of e.g. *A. actinomycetemcomitans* (and other above mentioned species) in samples from patients suffering of aggressive localized periodontitis is significantly higher than in samples from healthy patients, no individual species has been pinpointed as the cause for any periodontal disease (16). Hence, while certain bacteria such as *A. actinomycetemcomitans* have been strongly linked with localized aggressive periodontitis, and others to chronic periodontitis, the presence of this bacterium in saliva or dental pockets do not serve as an indication of periodontitis. An exception for this is the highly leucotoxic JP2 clone of *A. actinomycetemcomitans*.(3)

Smokers have a higher risk to suffer from periodontitis and treatment fails more often with smokers than non-smokers. It is suggested that tobacco contributes to periodontitis by affecting vascular metabolism and inflammatory response.(17) Other environmental risk factors are stress and insufficient diet.(2)

2.1.2 Links to systemic health

Periodontitis has been linked to several systemic diseases. E.g., pulmonary disease, diabetes, stroke, and adverse pregnancy outcomes have been assumed to be linked with periodontitis, but research to establish the links is still underway.(2)

The assumption of periodontitis as a risk factor for atherosclerotic vascular diseases (ASVDs) is based on both indirect and direct linkages. The association between systemic inflammation markers and ASVDs, and association between periodontal disease and systemic inflammation markers are one indirect linkage. Another indirect linkage is hypothesized to be result from molecular mimicry, i.e. cross reactive antibodies linking periodontal inflammation to cardiovascular inflammation. By direct linkages it is referred to transient bacteremia and vascular infection by pathogens entering circulation in inflamed periodontal tissue. Evidence of this link has been studied by searching putative periodontal pathogens in atherosclerotic plaque. DNA of these pathogens has been found

in samples of atherosclerotic plaque, including DNA of *A. actinomycetemcomitans*. At the time any causal relationship between periodontitis and atherosclerotic vascular disease are not confirmed, albeit the association between these is confirmed.(18) A link has been found between presence of subgingival *A. actinomycetemcomitans* and coronary artery disease(19), and salivary levels of *A. actinomycetemcomitans* associate with acute coronary syndrome.(20)

2.2 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans[1] is a non-motile, facultatively anaerobic gram-negative rod. Its primary habitat is human dental surfaces.(22) *A. actinomycetemcomitans* is an oral commensal and also considered a putative pathogen (23). Infections caused by this species outside oral cavity are rare, the most common being endocarditis (24). It has also been reported as a causative agent of an infection leading to a brain abscess (25).

The prevalence of *A. actinomycetemcomitans* varies greatly between geographically and ethnically/racially distinct populations. In Asian populations, *A. actinomycetemcomitans* seems to be a common member of oral microbiota in healthy subjects. E.g. in Vietnamese children under 11 years old the isolation frequency has been 78%, whereas in Finnish children of the same age the frequency was 16%.(26) Also, in United States it has been indicated that *A. actinomycetemcomitans* is more common among Asian-American and Hispanic subjects than among Caucasian population.(27) In Finnish adults, the prevalence of *A. actinomycetemcomitans* in saliva samples of 1 294 over 29 years old subjects has been reported to be 20%.(28)

2.2.1 Virulence factors of *A. actinomycetemcomitans*

A. actinomycetemcomitans secretes a protein called leukotoxin (LtxA) that affects polymorphonuclear leukocytes, monocytes, lymphocytes, erythrocytes and endothelial cells in humans. The effect of leukotoxin on hosts' cells varies from membrane dysfuntion to induced apoptosis, depending on the cell. Polymorphonuclear leukocytes have been reported to release proteolytic enzymes and matrix metalloproteinase 8 by the exposure to LtxA. (29)

[1] Previous names and year of naming: *Bacterium actinomycetem comitans* in 1912, *Actinobacillus actinomycetemcomitans* in 1929, *Haemophilus actinomycetemcomitans* in 1985.(21)

Cytolethal distending toxin (CDT) inhibits cell division and causes apoptosis. Furthermore, CDT has an increasing effect on expression of cytokine RANKL (NF-κB ligand), which has a central role in bone resorption. However, the role of CDT in periodontal attachment loss has not been considered relevant in research concerning *A. actinomycetemcomitans*.(29)

2.2.2 Lipopolysaccharide and serotyping

A. actinomycetemcomitans, like other gram-negative bacteria, has an outer membrane which is largely constructed of lipopolysaccharide molecules. The lipopolysaccharide is comprised of O-antigen, core oligosaccharide, and lipid A (Figure 1). The presence or absence of O chains determines whether the LPS is considered rough or smooth. Since O antigen is exposed on the very outer surface of the bacterial cell it is a target for recognition by the host antibodies. The antigen binds to receptors in the host's cells resulting in an immune response.(30). The O-antigen is constructed of repetitive glycan regions containing a variety of monosaccharides (31). Like polysaccharides forming the bacterial capsule, the O-antigen protects the bacteria from phagocytosis.(30) It has not been shown that in case of *A. actinomycetemcomitans* the virulence would be determined by O-antigen (12). Also, *A. actinomycetemcomitans* strains lacking a serotypeable antigen has been identified (32). Serotyping methods have evolved from using immunodiffusion to identify antisera produced in rabbits (such as in serotyping the a, b and c serotypes of *A. actinomycetemcomitans* (33)) to advanced molecular biology methods such as polymerase chain reaction (PCR), gas chromatography and nuclear magnetic resonance (34).

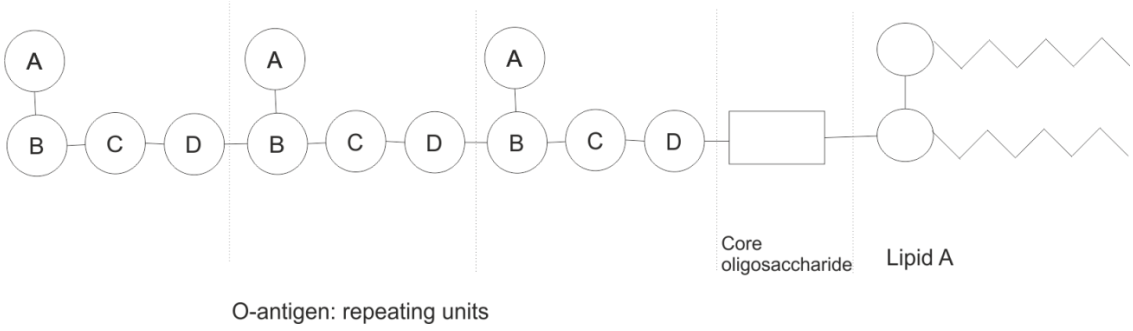

Figure 1: Simplified structure of lipopolysaccharide. The O-antigen is a glycan polymer and its structure varies between strains. The O-antigen is attached to core oligosaccharide. Lipid A is composed of glucosamine disaccharide and fatty acids that attach the molecule into the membrane.

Serotyping of gram-negative bacteria is mainly based on the variation of the O-antigen. There is generally high variability in the O-antigen structure in gram-negative bacteria.(31)

2.3 *A. actinomycetemcomitans* and periodontitis

As described above, periodontitis is a disease that is not specifically caused by any single bacteria. However, *A. actinomycetemcomitans* is linked to localized aggressive periodontitis in numerous studies. Research so far has not confirmed any central role for it in any other periodontal disease than localized aggressive periodontitis.(35)

Some genotypes of *A. actinomycetemcomitans* have shown significantly higher leukotoxicity than others. Especially the JP2 strain of serotype b has been linked to aggressive periodontitis among youth of Western African or Mediterranean origin. The JP2 clone is endemic for populations in North Western Africa.(29)

JP2 clone has a 530 bp deletion in the leukotoxin promoter region. Association between localised aggressive periodontitis and the JP2 clone of the b-serotype among Moroccan juveniles has been suggested by a two-year follow up study (36). In this study, subjects not having symptoms of periodontitis in the beginning of the study period had a significantly higher risk of developing localized aggressive periodontitis if they were carriers of the JP2 clone compared to those that either were *A. actinomycetemcomitans* positive without JP2 clone or were not carrying the species at all. Also subjects with non-JP2 clone *A. actinomycetemcomitans* had a higher risk to develop localized aggressive periodontitis compared to those free of the bacteria. The leukotoxicity of non-JP2 serotype b genotypes has also showed high variation and there are highly leukotoxic genotypes that do not have the deletion in the promoter region.(12,36,37)

2.3.1 Serotypes of *A. actinomycetemcomitans* and periodontitis

Serotypes of bacteria are considered to be of the same species, but differ from other strains of the same species due to the variance in cell surface antigens that produce different responses by the immune system. Altogether seven serotypes of *A. actinomycetemcomitans* have been identified (a-g) (34). A, b, and c were originally serotyped by Zambon et al (38,39). The serotypes of *A. actinomycetemcomitans* differ from each other due to distinct structures of their O-antigen components (21). The serotypes a, b, and c are globally most prevalent.(39)

A recent review concerning the role of different serotypes of *A. actinomycetemcomitans* in different forms of periodontitis and in different ethnic groups and geographical regions shows that there are differences in which serotype is dominant in healthy individuals and in patients with periodontitis depending on geographic location and ethnicity. However, there is no evidence to point out globally a single serotype that would be prevalent in samples from patients with periodontitis.(39)

2.4 Quantitative PCR method

PCR was developed in early 1980s. The method relies in use of DNA polymerase, an enzyme that is able to synthesize DNA from nucleotides copying the DNA sequence of the targeted DNA.

The PCR has three temperature phases, which are denaturation, annealing, and elongation. In denaturation temperature the hydrogen bonds between the bases of the DNA strands break and DNA is in single strand form. In annealing temperature, the hydrogen bonds are reformed between DNA bases which allows not only for the template strands to reform double strand but also for the primers to anneal to the target sequences. Elongation temperature is optimized for the used polymerase, which performs the actual replication of the amplicon.

QPCR is based on polymerase chain reaction, but differs from conventional PCR in that the accumulation of products can be measured for each cycle. The measurement on amplified DNA can be measured by using fluorescent methods that are either specific or non-specific for the targeted DNA sequence, called the amplicon. In both cases, the increased fluorescence in the reaction mixture reflects the amplification of DNA, as the concentration of double strand DNA increases from cycle to cycle.

The polymerase needs primers to start the replication of the target DNA. Primers are designed to match the targeted DNA sequence on both of the strands that are copied. Two primers are needed, forward and reverse, which limit the area of the DNA sequence that is targeted. In qPCR, the reaction components include the polymerase, a fluorescent dye, deoxyribose nucleotide triphosphates, $MgCl_2$ and components that from a buffer to optimize the pH for the reaction.(41) The function of $MgCl_2$ is to bring Mg^{2+} into reaction. Mg^{2+} has a catalytic role in polymerase chain reaction (42).

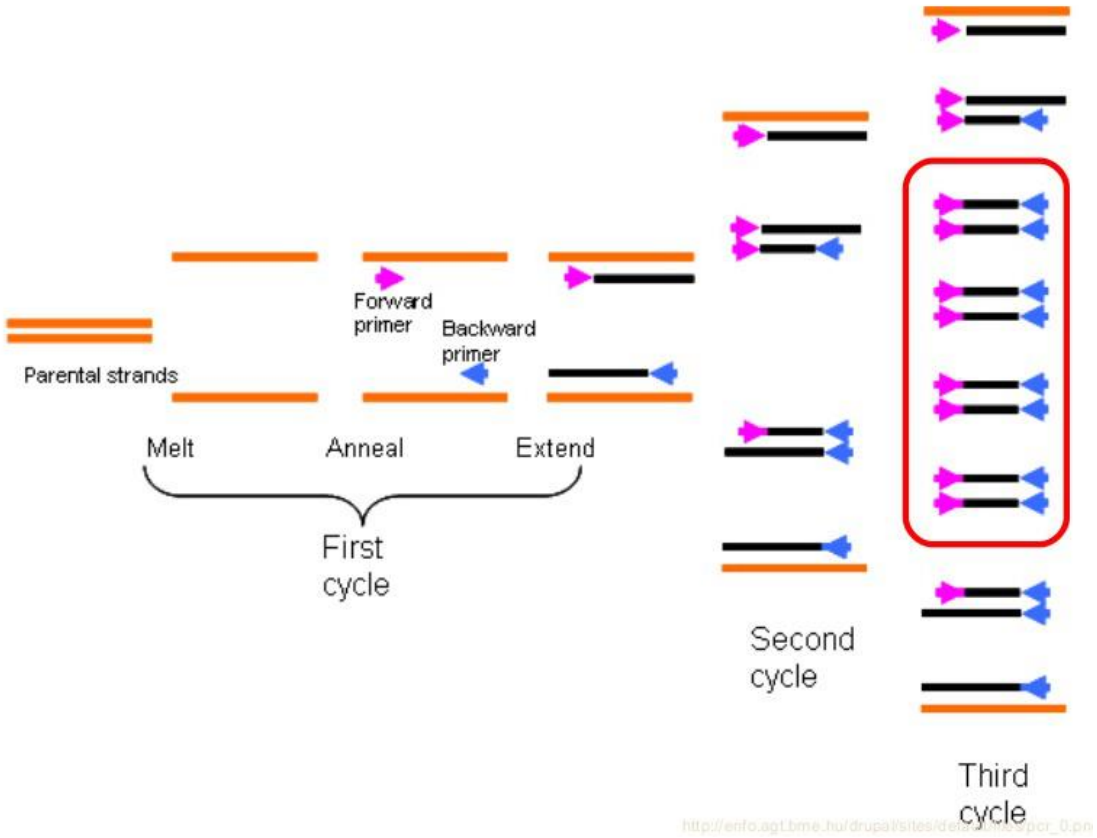

Figure 2: Exponential amplification through PCR. Each cycle of PCR produces new copies of the wanted gene, the amplicon. Picture from Slideshare.net (40)

2.4.1 Primer design

Different factors guide the primer design. On the one hand, the primer should be sensitive and specific to the sequence targeted. On the other hand, the primers and the produced amplicons should be functional from the PCR reaction efficiency point of view. Considering the reaction efficiency, the shorter the primer is, the more efficiently it anneals to the target DNA. However, there is a minimum primer length as very short primers are not specific.(43)

The primer design should follow the instructions provided by the equipment manufacturers. For Strategene 3005P the recommendation for the length of the primer is 15-30 base pairs. In addition to length, design of the primers should take into account the base composition so that the melting temperature of the primers is similar. Furthermore, the base composition should not allow folding of the primer on to itself.(44) The design of the 3' end of the primer is crucial, as the polymerase will start the elongation process from an annealed 3' end.(43)

The reaction spanned by the primers, i.e. amplicon length, should be between 100 and 300 base pairs for SYBR Green I when using Stratagene 3005P. This is longer than it is recommended for probe based methods, as the longer the amplicon is, the more SYBR Green dye molecules can bind to amplified product. However, if SYBR green is used having in mind future use of probes, the length should be kept closer to the minimum length.(44)

2.4.1 Controlling the results

The specificity of the primers can be controlled by analysing the dissociation curve. The dissociation curve shows the temperature in which the double strand DNA in the assay melt, i.e. the hydrogen bonds between the pairing strands break. The segment three in Figure 3 represents the phase where qPCR machinery measures the melting point. The dissociation curve should display only one peak to show that the DNA products have similar length and similar base pair composition. The longer a DNA double strand is, the higher the dissociation temperature as the amount and hence the strength of hydrogen bonds is bigger. The effect of base pair composition is due to the difference between number of hydrogen bonds between CG and AT bases.

3 AIMS OF THE STUDY

The study aims at developing a method to identify serotypes of *A. actinomycetemcomitans* by using quantitative PCR. The method development consist of following phases: Designing primers that are sensitive and specific for the target DNA, optimising the PCR reaction, testing the designed primers by using cultivated strains of the target species as templates and applying the method on clinical samples.

4 MATERIALS AND METHODS

4.1.1 QPCR with SYBR® Green

The commercial kit used in this study is Brilliant III ultra-fast SYBR® Green master mix. The fluorescent dye, SYBR® Green I, is a non-specific dye that binds to any double stranded DNA.(44,45) The qPCR instrument used is the Stratagene Mx3005P Real-Time QPCR System. The results were analysed with Strategene MxPro software (46). The reference dye used in this study is ROX by Agilent Technologies.(44)

The concentrations of different components for qPCR on Stratagene Mx3005P are given by the manufacturer of the reaction mixture.(45) The volumes are listed in Table 3.

The pipetting of different components was done in a laminar flow hood, which along with the necessary instruments were swiped with ethanol in the beginning of each session.

Table 3: Volumes of reaction components per sample.

	Volume (µl)
Brilliant III Ultra-Fast SYBR® Green Master Mix	10
F-primer 10 µM	0,4
R-primer 10 µM	0,4
ROX 1:500 dilution	0,3
H_2O	6,9
Template	2
Total	**20**

4.1.2 Reference strains

Forward and reverse primers acquired from Thermo Scientific were tested with reference strains representing six serotypes (a-f) of *A. actinomycetemcomitans*. The tests were done on both DNA that was purified from bacterial colonies and also on bacterial samples transferred directly from colonies on blood agar to purified water. The strains were grown on supplemented *Brucella* agar plates (containing 5% horse blood, hemin [5 µg/ml], vitamin K_1 [100 mg/ml], and *Brucella* agar) and incubated in an atmosphere of 5% CO_2 at 37°C for 3 days. The cultures were transferred into Todd-Hewitt broth (3% TH, 1% yeast extract), where they were further grown for 2 days under the conditions mentioned below. We confirmed the purity of the cultures by colony morphology and Gram staining. After removing the broth by centrifugation at 5,500 × *g* at room temperature for 15 min, the bacteria were washed with phosphate-buffered saline (PBS) (10 mM phosphate [pH 7.4], 150 mM NaCl) and DNA was extracted by phenol-chloroform method by Saija Perovuo.

Table 4: *A. actinomycetemcomitans* reference strains used in the study

Serotype	Strain
a	ATCC 29523
b	ATCC 43718
c	ATCC 33384
d	IDH 781
e	IDH 1705
f	CU 1000

ATCC, American Type Culture Collection; IDH, Institute of Dentistry, University of Helsinki.

The extracted DNA and non-purified bacterial samples were diluted in purified nuclease free H_2O. The same H_2O was used to dilute the reference dye and the reaction mixture for the samples.

4.1.3 Thermal profile setup

The thermal profile used in this study follow the recommendations from Agilent technologies for the used instrument Stratagene Mx3005P and is presented in Figure 3. The denaturation temperature was set to 95 °C. The recommended annealing temperature is 60 °C. The used Taq polymerase is designed to function optimally in the annealing temperature, thus the thermal profile had only two steps, the annealing, and elongation temperature both being 60 °C.

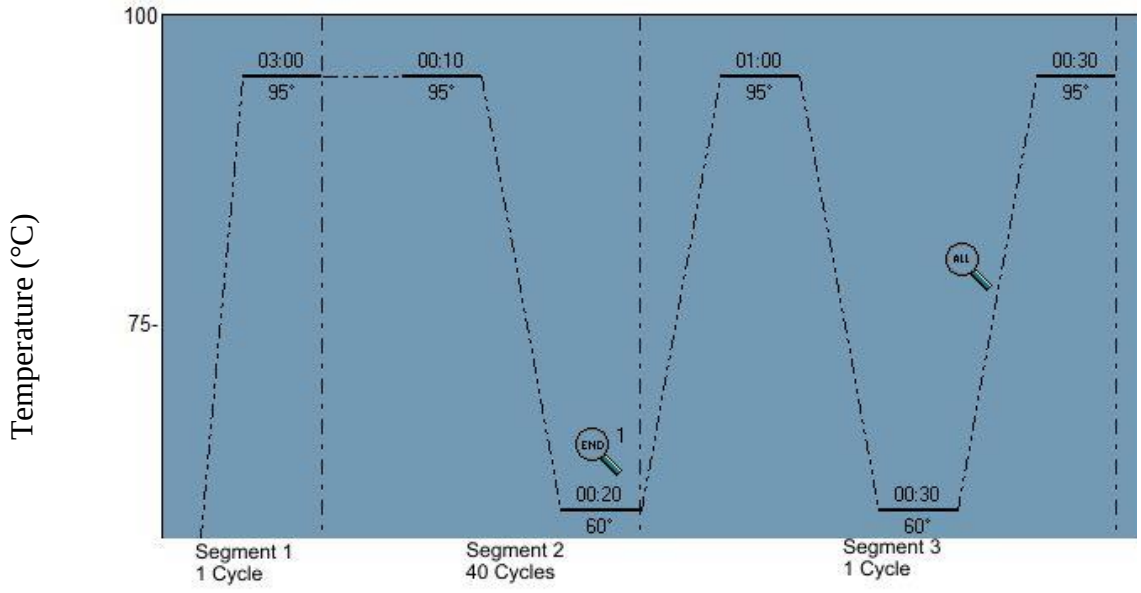

Figure 3: Standard thermal profile used in this study.

4.1.4 Primers

We designed the primers to match the target sequences Primer-BLAST developed at National Center for Biotechnology Information. The Primer-BLAST assists in primer sequence design following user given parameters for the target species and specificity checking utilising different genome databases.(47) The primers were designed by PhD Kati Hyvärinen with Primer-Blast and manufactured by Thermo Scientific.

The primer nucleotide sequence for each serotype is presented in Table 5. The primers were first dissolved in H_2O and then diluted to concentration of 10 µM. The primers were stored in temperature of -20 ˚C.

Table 5: The primer sequences used in the study for *A. actinomycetemcomitans*

Serotype	Sequence	
	Forward	Reverse
a	5'-ACTGGTGCATCAGGTCAAGA -3'	5'-TGAGCTGCTACGCCGATAAG-3'
b	5'-TCTCAATGTGACGCAGCTCT-3'	5'-TGTTCATCACGACAGGGCAA-3'
c	5'-TTTGGACCAACAGGGGTTGA-3'	5'-CAAAAGCACTCCGACAGCAG-3'
d	5'-TGCGGACAAGCGTAGTGAAT-3'	5'-CCATAGACCTATACCGCCTCC-3'
e	5'-TGGGCATTTATTCAGCCAGTCA-3'	5'-ACCATTCCTGCAATCAACCA-3'
f	5'-GGCGGAGATAGTTCTTGGCA-3'	5'-ACAACCCCTCCTACCTCACA-3'

4.1.5 Study population

The developed primers were tested on samples from a Finnish study population that participated in Parogene study (part of Corogene study) between years 2006 and 2008.(48) DNA extracts from saliva samples of the Parogene patients have been studied to find links between *A. actinomycetemcomitans* and coronary artery disease. In the current study, serotype-specific qPCR was applied to DNA extracted from saliva samples from patients (n=264). These patients were selected among the whole cohort, since their subgingival bacterial samples had been found positive for *A. actinomycetemcomitans*. Among these patients, 54 saliva samples had also been found *A. actinomycetemcomitans* positive by qPCR analysis.(19,20)

4.1.6 Plate setup

The study was conducted in two phases. Firstly, the primers were tested with standard controls using reference strains described above. Primers for each serotype were tested

with each reference strain and a non-template control was included. Secondly, the Parogene samples were tested with the most optimal primers. Wells with reference strains for each serotype were included as controls, as well as a non-template control well with purified H_2O.

5 RESULTS

5.1 Optimisation of primer concentration with Brilliant III Ultra-Fast SYBR Green Master Mix

The Brilliant IIII Ultra-Fast SYBR® Green Master Mix was first tested using primers non-specific for *A. actinomycetemcomitans* serotypes. These primers were originally developed in University of Helsinki to identify and quantify five different putative pathogens for periodontitis using Brilliant SYBR® qPCR Master Mix (49). Reference strain used in the test was ATCC 33384 (serotype c). The DNA concentration 5.26 ng/µl of template was measured with Nanodrop 1000. The template was diluted serially (1:10, 1:100, 1:1000 and 1:10 000) and assays with different primer concentrations were prepared and analysed in qPCR. Following primer concentrations combinations for forward and reverse primers were tested:

1) forward 200 nM and reverse 200 nM
2) forward 200 nM and reverse 400 nM
3) forward 400 nM and reverse 200 nM.

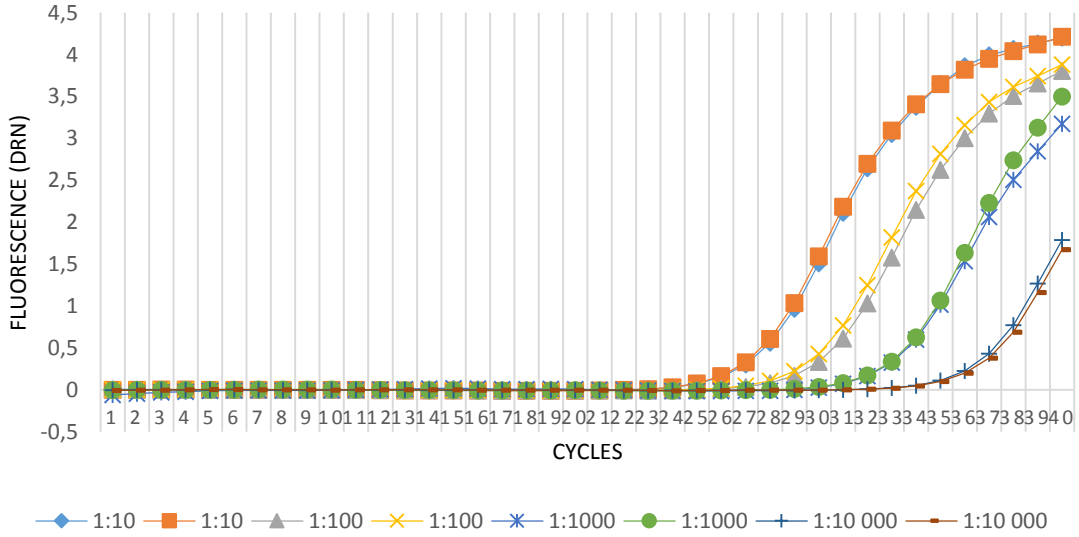

Figure 4: The amplification curves of dilution series for purified DNA-sample of c serotype of *A. actinomycetemcomitans* (ATCC 33384). The starting template concentration for dilution was 5.26 ng/µl. In this representative figure, the primer concentration is 200 nM for both forward and reverse primers.

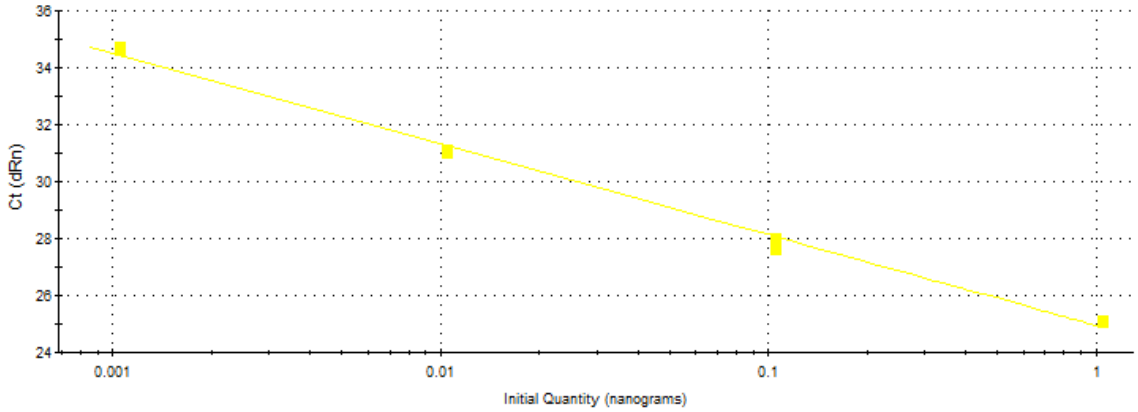

Figure 5: Standard curve for dilution series for purified DNA-sample of c serotype of *A. actinomycetemcomitans* (ATCC 33384). The starting template concentration for dilution was 5.26 ng/µl. Primer concentration is 200 nM for both forward and reverse primers.

Figures 4 and 5 show the results for the primer concentration combination (200 nM for both forward and reverse primers) that was proven to be most efficient for the master mix and qPCR machinery in use. The R square value for the curve is 0.995 and efficiency 105.7%. The respective values for combination number 2 (forward 200 nM and reverse 400 nM) were 0.985 and 121.2% and for combination number 3 (forward 400 nM and reverse 200 nM) 0.962 and 135.0%.

5.2 Sensitivity and specificity of the serotype-specific primers applied to control templates

The primers were cross checked against each serotype. The tested primers were not specific enough to allow for quantification of targeted serotypes. The figure 6 shows amplification plots for primers designed to be specific for serotype c as an example. The amplification of the targeted serotype starts at an earlier cycle than that of others, but the primers are not specific enough for the serotype c, as the amplification occurs also in control wells containing DNA from other serotypes. The cross check amplification plots for primers designed for the other serotypes were similar to those presented in Figure 6.

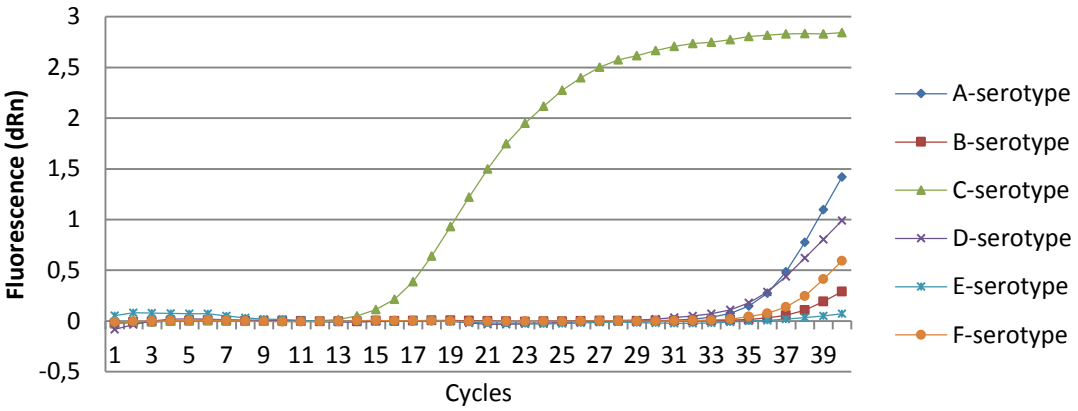

Figure 6: Primers designed for serotype c cross-reacted with other serotypes. The green line exceeding the threshold level on cycle 13 is the well including bacterial residues from cultivated c strand. The other wells where fluorescence has risen contain serotypes a, d, f, and b.

While the analysis of the amplification plots proved not to be a reliable method, analysis of dissociation curves were more useful to identify serotypes. The Figures 7–12 show the dissociation curves for respective primers cross tested with serotypes a–f.

Comparison of dissociation curves show that products in the targeted serotype specimen have a specific melting temperature that differs from that of the other serotypes. Furthermore, the considerably higher fluorescence values indicate that the amount of amplified DNA is much higher in the wells containing the targeted serotype.

The dissociation curves for each serotype:

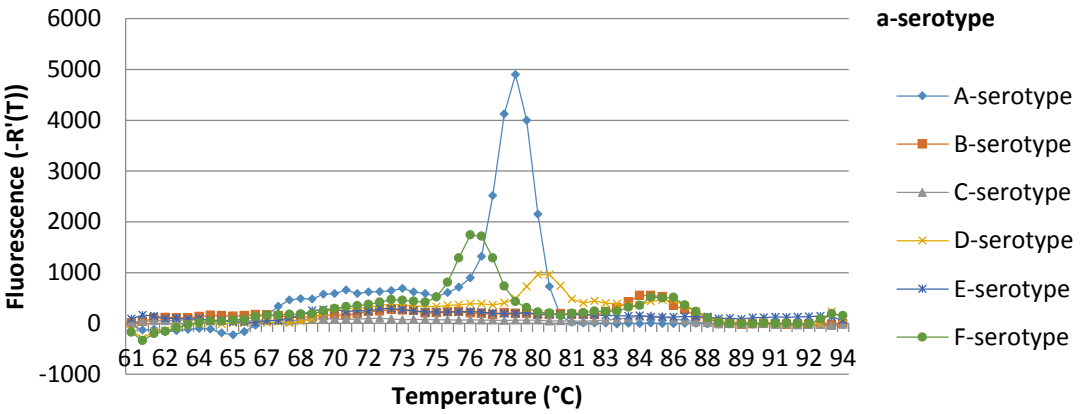

Figure 7: The dissociation curve of serotype a. The dissociation curve shows the products in templates containing a-serotype to dissociate in temperature of 78.6 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype a.

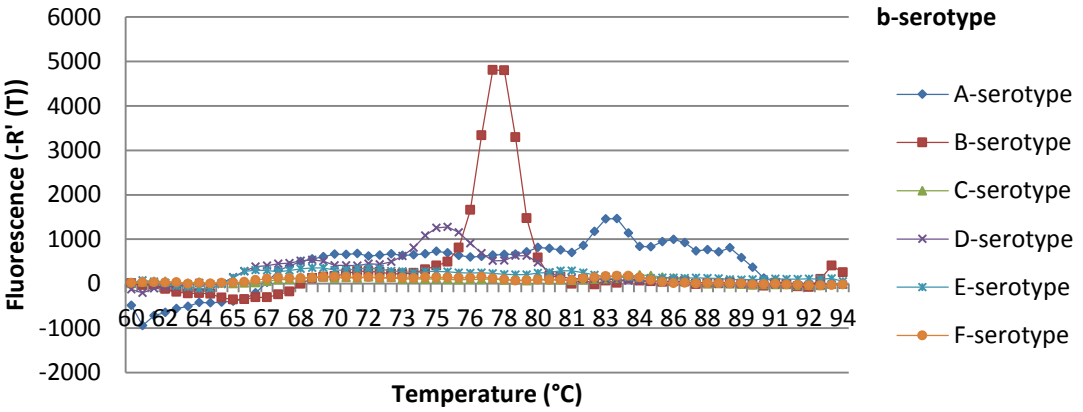

Figure 8: The dissociation curve shows the products in templates containing b-serotype to dissociate in temperature of 77.4 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype b.

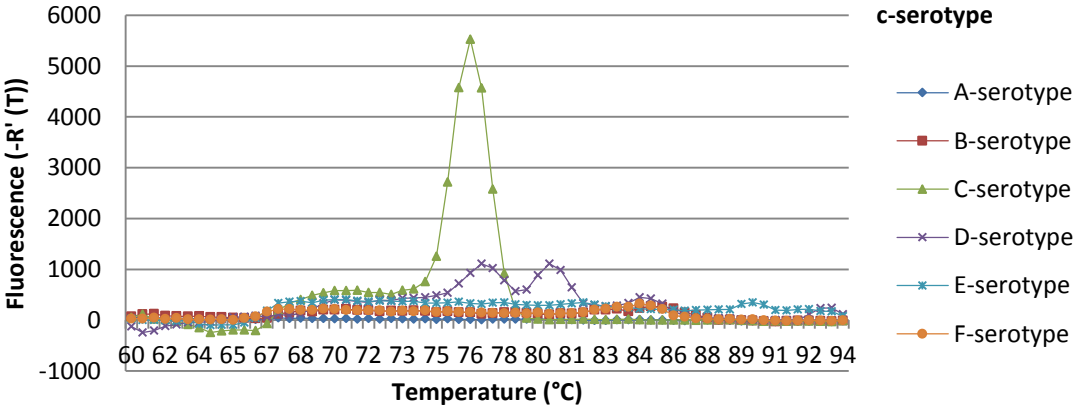

Figure 9: The dissociation curve shows the products in templates containing c-serotype to dissociate in temperature of 76.4 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype c.

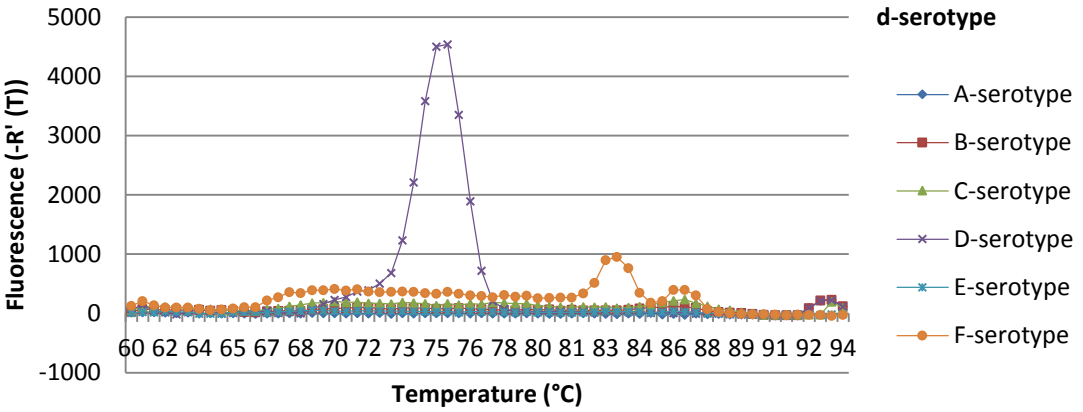

Figure 10: The dissociation curve shows the products in templates containing d-serotype to dissociate in temperature of 75.3 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype d.

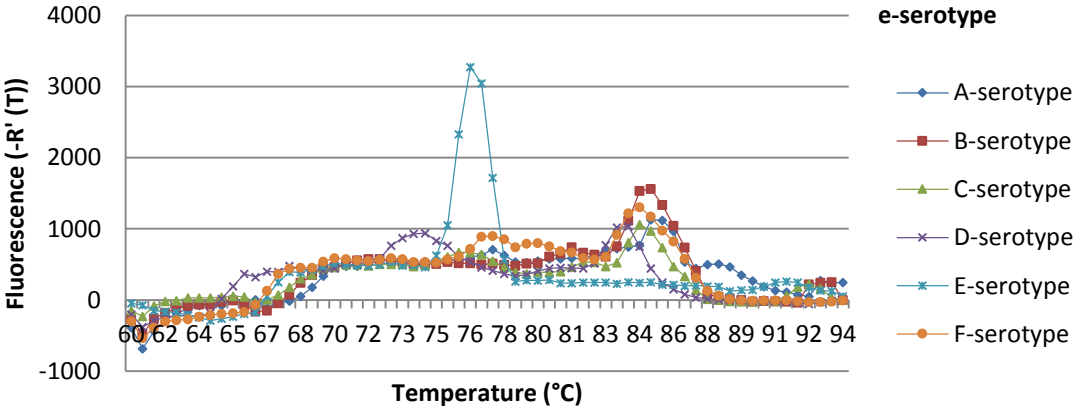

Figure 11: The dissociation curve shows the products in templates containing e-serotype to dissociate in temperature of 76.4 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype e.

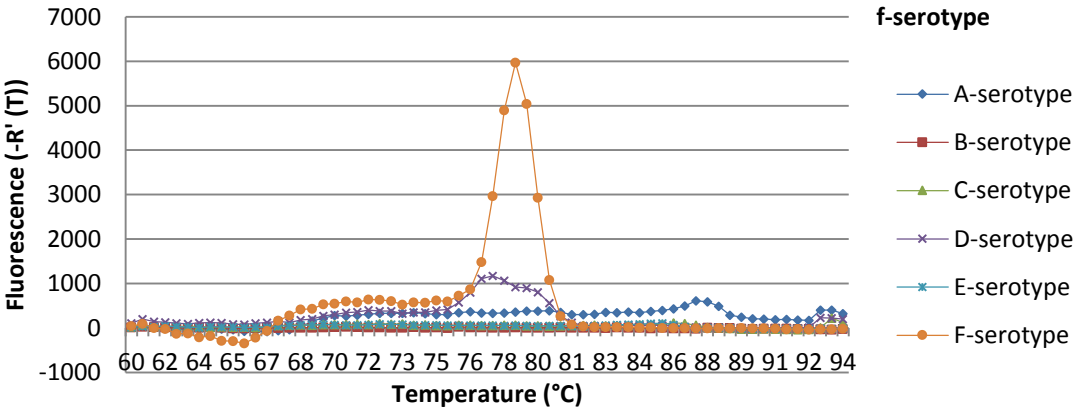

Figure 12: The dissociation curve shows the products in templates containing f-serotype to dissociate in temperature of 78.5 °C differing from products amplified from templates containing cultivated samples from other serotypes. The primers used in the assay were designed for serotype f.

5.3 The sensitivity and specificity of the primers applied to DNA extracts from saliva samples

DNA extracted from saliva samples from the Parogene population was tested with the serotype specific primers. By analysing the dissociation curves from 252 samples, the serotype was identified in 44 samples (17.5 % of the samples). Out of these 44 samples, 22 were found *A. actinomycetemcomitans* positive by Hyvärinen et al.(20) Figure 13 shows how serotype b positive saliva samples are identified with primers designed for serotype b. The highest peak is the control template (cultivated b-serotype). The dissociation curves show that seven wells out of 72 unknown samples are serotype b positive.

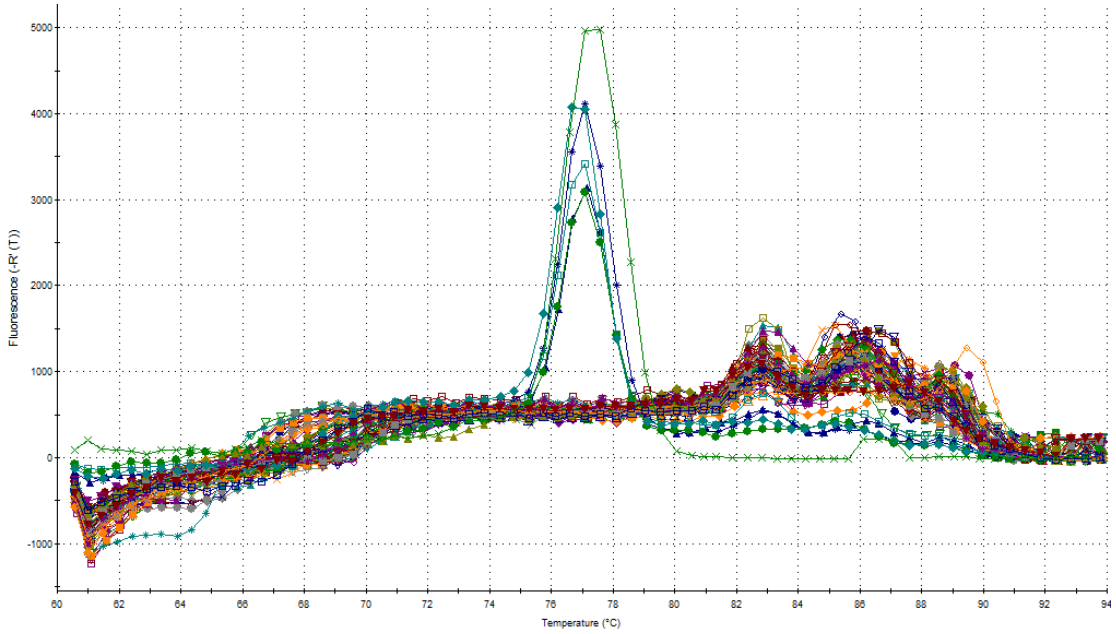

Figure 13: When using primers designed for b-serotype, the dissociation curve shows that seven samples (out of 72 unknown) from Parogene study are serotype b positive. The PCR products melt in the same temperature ranging between 76.7 and 77.6°C) as those for the control template containing a sample from b-serotype cultivation (the highest peak).

The figure 14 shows the distribution of serotype positive samples out of 252 tested samples. The most prevalent serotype was c, the other serotypes following in descending order b, a, e, and f.

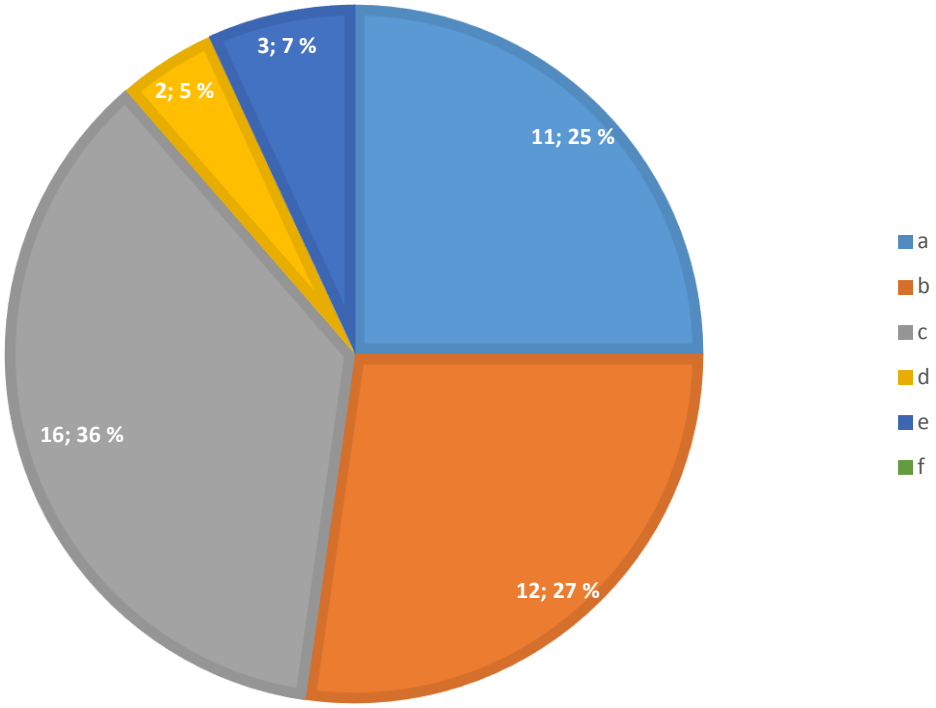

Figure 14: Serotype distribution of serotype positive samples (amount and percentage). Out of 252 tested samples the serotype could be identified in 44 samples.

6 DISCUSSION

In this study, a method to serotype *A. actinomycetemcomitans* bacteria by quantitative PCR was developed. Firstly, primer sets for each serotype (a–f) were planned with the help of genome database. Primer sequence was planned to correspond to LPS O-antigen, which is the bacterial structure that differentiates the serotypes from each other. Serotype g was excluded due to lack of the reference strain.

Secondly, the most efficient primer concentrations were found by experimenting with three different primer concentrations. The primers tested with Brilliant IIII Ultra-Fast SYBR® Green Master Mix using Stratagene Mx3005p qPCR machinery.

Thirdly, the primers were cross-checked against other serotypes of *A. actinomycetemcomitans* by using cultivated reference strains. In this process, the most specific and sensitive primers were chosen for the next phase. Fourthly, the developed primers were further tested on saliva samples from 252 *A. actinomycetemcomitans* positive individuals from the Finnish Parogene study.

Uniqueness of the DNA sequence that the primers are designed for is crucial for the specificity of quantitative-PCR. The results of the tests conducted in this study show that the developed primers are specific enough to identify different serotypes from each other, even though the specificity of the primers was not enough for quantification purposes. The identification of the serotypes was done based on the analysis of dissociation curves. The PCR products in the target serotype wells dissociated in a specific temperature making it possible to identify respective serotype from each other. Furthermore, the relative proportion of each serotype is not in contradiction with findings in population studies.

If developed further, the specificity of the primers should be increased and hence the study design redeveloped. The advantage of SYBR® Green I is that assay design is simpler and costs are lower compared to more specific methods. The obvious disadvantage of the SYBR® Green I is that the fluorescence levels are affected in the assay by the presence of any double strand DNA, whether it is DNA comprised of the target sequence (amplicon), primer-dimer or other nonspecific products containing a double strand of DNA.(50) The advantage of increased specificity is that is makes possible quantification of bacterial samples. Quantified information on samples increases possibilities to analyse research data.

A worthwhile option would be to use a probe-based method to separate different serotypes from each other. For example, Applied Biosystems' hydrolysis probe (trade mark *Taq*Man probe) is a DNA strand designed to match the amplified sequence between the forward and reverse primers. A reporter and a quencher molecule are attached to the probe. The presence of the quencher suppresses the fluorescence emitted by the reporter. When the polymerase reaches the sequence where the probe is attached, it cleaves the probe and releases the reporter dye. Consequently, the reporter and the quencher are separated and the signal emitted by the reporter dye increases. The method is specific as the fluorescence will increase only when a probe annealed to a specific target sequence is cleaved of when amplification is in the process.(50)

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