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Detection and quantification of four periodontal pathogens using digital droplet-PCR

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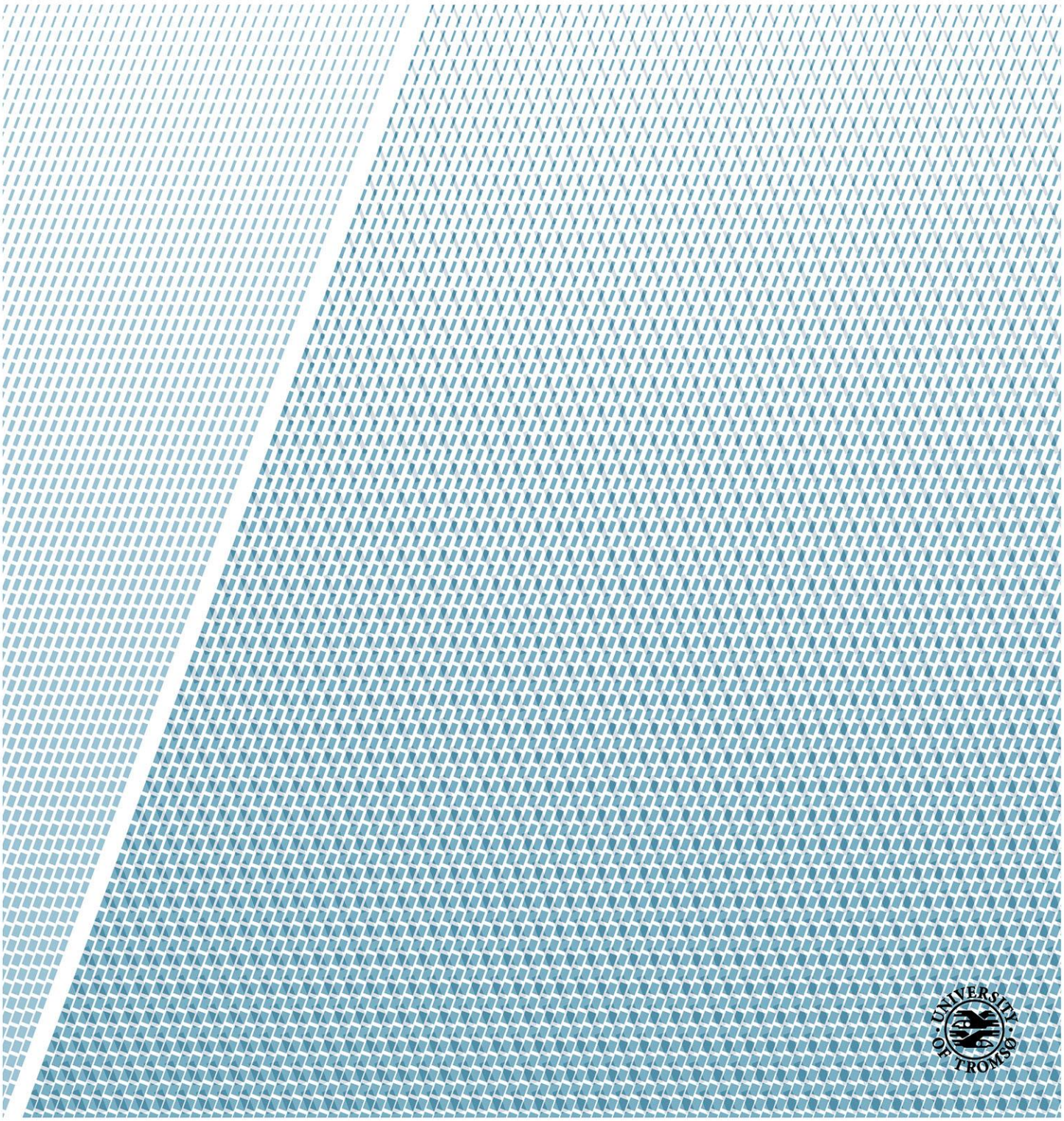

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ABSTRACT

In the treatment of periodontal disease, it can be of importance to know which bacteria are associated with the inflammatory response in individual patient. Microbiological testing is not common today as a routine test in periodontal treatment, often due to high costs and time consuming procedure. However, microbial diagnosis could improve the ability to identify patients at higher risk for developing periodontal disease, as well as monitor progression or remittance of diseases and, accordingly, choosing the appropriate course of treatment.

Aims: The aim of this study is to develop a quick molecular method for simultaneous detection (multiplexing) and absolute quantification of four periodontal pathogens associated with periodontal disease in the same clinical sample by digital droplet PCR. Such a method would make the use of microbiological testing more effective and less expensive, and could encourage dentists to choose microbiological testing as a useful tool for prevention, diagnosis and treatment of periodontal diseases.

Materials and method: Four designated periodontal pathogens were selected in this study. DNA was extracted from the four periodontal pathogens followed by amplification and quantification for performing a multiplex assay using droplet digital PCR (ddPCR).

Results: A 4-plex system to detect four different periodontal pathogens was successfully achieved. In this assay, four periodontal bacteria divided into two groups were labelled with two fluorophores (FAM and HEX). The amount of primer utilized in the assay was adjusted so that each bacterium could be distinguished from the others on the basis of the fluorescence intensity. The designed assay managed to detect and quantify the four bacteria and recognize them separately or in groups.

Conclusion: It seems the 4-plex assay developed herein is suitable for detection and quantification of periodontal pathogens, and the same assay can be used in other fields where accurate and reliable quantification and detection of multiple DNA-targets are needed. This 4-plex technique can make the workflow in detection and quantification of periodontal pathogens more effective by running just one sample in microbiological lab for several purposes.

Keywords: multiplexing, periodontitis, periodontal pathogens, digital droplet PCR

1. INTRODUCTION

Periodontal diseases are infectious diseases caused by bacteria, which may lead to harmful changes resulting in destruction of the supportive tissues of the teeth (1). Worldwide periodontal diseases represent a health problem. The prevalence of the disease is influenced by several factors, such as increasing age, geographical and socioeconomic conditions. A study between 2009 and 2010 revealed that approximately 47% of the population in United States of America had some form of periodontitis. Increasing age showed higher prevalence (2). A study done in Norway found that 24 % of investigated 35 years old had bone loss approximately (3).

1.1 Development of periodontal disease

Disturbance of the symbiosis between oral microflora and the host may induce the disease process. Common for most forms of periodontal diseases, is accumulation of periodontal bacteria that adhere to tooth surfaces. Local infection and inflammatory responses in the supportive tissues of the teeth is then triggered. The bacteria attached to tooth surface is organized as a dental biofilm.

An organic deposition of glycoproteins from the saliva and gingival exudate is established on the tooth surface within two hours of undisturbed biofilm accumulation. This is called the acquired pellicle, and pioneer bacteria such as streptococci are found at this stage. With further undisturbed biofilm accumulation, its composition becomes more complex, and accumulated microorganisms become pathogenic in the biofilm (4).

Gingivitis, a reversible form of gingival inflammation, is due to biofilm accumulation above the gingival line. With continuous irritation and inflammation that proceeds below the gingival line, the integrity of the junctional epithelium is, subsequently, damaged. Epithelial cells will degenerate and separate, and the attachment to the tooth breaks down. Ultimately, the tooth supporting tissues will be lost and tooth loss could be immanent (5).

1.2 Microbial complexes in subgingival biofilm

In 1998, Socransky *et al.* published a study that organized subgingival bacteria in chronic periodontitis as complexes. Bacteria in the so-called red and orange complex, respectively

strongly and moderately, are associated with chronic periodontitis.
Porphorymonas gingivalis, Treponema denticola and *Tannerella forsythia* belongs to the red complex, and these are usually found in severe form of periodontal disease, with clinical findings of deep pockets and bleeding on probing (BOP). In the orange complex, there are species like *Prevotella intermedia* and *Fusobacterium nucleatum*, with significant association with increased probing depth. Detection and identification of which organisms found in a periodontal pocket could provide essential information about the severity of the disease (6).

1.2.1 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a part of the normal oral microflora (7). *A. actinomycetemcomitans* is a gram-negative bacterium (8). The length of the genome (Accession no. CP001733) is 2.2 Mb and has a GC content of 44.67 % (9). More recent studies have shown that *A. actinomycetemcomitans* is highly associated with periodontal disease in adolescents (10, 11). A highly toxic clone of *A. actinomycetemcomitans* known as the JP2 clone is usually found in aggressive periodontitis (12).

1.2.2 *F. nucleatum*

F. nucleatum is a gram-negative, facultative anaerobe bacterium. The size of its genome (Accession no. NC_003454) is 2.17Mb (13). *F. nucleatum* is one of the largest microbes in the oral cavity with a size up to 2-4 μm (14). It is highly associated with the initiation and progression of periodontal disease (15), and it integrates into the biofilm after adherence and colonization of aerobic bacteria to the tooth enamel and root surface. It serves as a bridging organism between early colonizers as streptococci and actinomyces, and late colonizers as a variety of gram-negative bacteria, including those found in the red complex (16).

1.2.3 *P. gingivalis*

P. gingivalis belongs to the family of phorporymonadaceae (17). The genome of *P. gingivalis* (Accession no. CP007756) consists of a sequence of 2.3Mb (18). It is an asaccharolytic, gram-negative bacterium (19) and an non-motile obligate anaerobic rod that forms black-pigmented colonies on blood agar plates (20). *P. gingivalis* is associated with the onset of inflammation and tissue destruction during periodontitis. Development of a mixed biofilm, expansion into the gingival sulcus, and formation of a periodontal pocket (21).

1.2.4 *P. intermedia*

Bacteria in the genus *Prevotella* are anaerobe, non-motile, gram-negative coccoid rods (22), (23). Twelve different species in human microflora have been identified. Their habitat is mostly gingiva, oral mucosa, pharynx and in the vaginal flora. The genome of *P. intermedia* (Accession no. AP014597) consists of a sequence of 2,28 Mb (24). *P. intermedia* is considered to have an important role in development as well as progression of periodontal disease, both chronic and aggressive periodontitis (25, 26).

1.3 Virulence factors of the four investigated pathogens

The different periodontal pathogens possess mechanisms for evading the host response and cause tissue damage (5). *P. gingivalis* activates the kallikrein/kinin-pathway and increases the vascular permeability, which leads to swelling and redness in the gingiva. *P. gingivalis* also has the ability to activate thrombin and prothrombin that results in an increase in the propensity to bleed (27-29). Collagenase and proteases released are associated with the features of periodontitis – destruction of collagen and connective tissue matrix (30). An example is *F. nucleatum* that stimulates production of metalloproteinases, MMPs, involved in destruction of periodontal tissue (31). *P. gingivalis* produces proteases that are essential for its pathogenic ability; three cysteine proteases (rgpA, rgpB and Kgp) known as gingipains plays an important role in the pathogenesis and destruction of surrounding tissues (32, 33).

A genomic locus composed of 14 genes, tad locus, has been identified and found to be important in the pathogensis of *A. actinomycetemcomitans* for inducing bone loss (34). The production of leukotoxin, for example cytolethal distending toxin which is characteristic to *A. actinomycetemcomitans* (35), is considered as the most important virulence factor of this bacteria, and makes it capable to evade the immune system of the host by killing leukocytes (36). *P. intermedia* also has the ability to escape phagocytic activity and intracellular killing mechanisms by producing capsules with for example leukotoxin (22).

1.4 Polymerase chain reaction (PCR)

All bacteria have their own genome with genes that are conserved for many bacteria, like 16S rRNA (37), and genes that are specific for the particular bacterial species. To identify and detect a particular bacterium it is necessary to know which genes, or gene sequences, that are unique to that exact bacterium. PCR can amplify a specific DNA segment from only a small amount of starting material (38). It allows us to amplify DNA sequences for the purpose of identification and quantification.

1.4.1 Conventional PCR, Real-Time PCR and Digital PCR

During PCR, a number of heat and cool cycles are applied to allow heat-induced denaturation of the DNA double helix, annealing of primers and elongation of the PCR-product (38). DNA template strand, DNA polymerase, primers, probes and nucleotides are necessary components to do a PCR.

To amplify the gene sequence, two primers are needed; one forward primer and one reverse primer. Primers are short pieces of simple stranded DNA that is complementary to the target sequence. It is essential to have appropriate primers. One of the primers, forward primer, should anneal to the plus strand that is oriented in the 5'→3' direction. The other primer, reverse primer, should anneal to the minus strand oriented in the 3'→5' direction (38) and extension is done by adding nucleotides; adenine (A), thymine (T), guanine (G) and cytosine (C), the essential building blocks in new DNA.

In Real-Time PCR and in Droplet Digital PCR (ddPCR), a probe binds between the forward and the reverse primers and emits fluorescence, and makes it possible to quantify the DNA molecules. DNA polymerase, an enzyme such as Taq DNA, synthesizes new DNA strands that are complementary to the target sequence.

The PCR amplification cycles starts with an initial denaturation step at 94-98°C; that separates the DNA double helix into two complementary strands. Then the PCR initiates the first of a 3-step temperature cycle (38). Three different temperatures in the same cycle will accomplish three tasks: the first is denaturation of the template (in subsequent cycles also the denaturation of the amplicons) in 10-60 seconds. A step of 30 seconds allows optimal

annealing of primers/probe to the single stranded DNA in each end. This marks the starting point of the replication and enables the DNA polymerase to bind to the DNA-template. Further synthesizing give rise to new DNA called target sequence, in the third step.

The last step is elongation, and the optimal temperature may vary between different DNA polymerases. Taq DNA polymerase has an optimal elongation temperature at 70-80°C. The last step incorporates an extended period of elongation that allows synthesis of many incomplete amplicons to be completed. Termination of the thermal cycling happens by cooling the reaction mix down to 4°C and/or by adding EDTA to a final concentration of 10mM (38).

1.4.2 Digital Droplet PCR (ddPCR)

Digital droplet PCR (ddPCR) was introduced to provide high precision absolute quantification of target DNA for both research and clinical diagnostic applications(39-41). By counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions, ddPCR can measure absolute quantities.

ddPCR systems can use water-oil emulsion technology combined with microfluidics, or microchip technology. In the first mentioned technology the DNA samples are partitioned into approximately 20.000 droplets by using droplet generator. During the partitioning process, the target DNA and background DNA are randomly distributed into the droplets, with same size and volume that enables precise quantification of the target. In our thesis, the QX200 system from Bio-Rad is used as a representative of ddPCR that uses water-oil emulsion droplet technology combined with microfluidics.

In ddPCR, amplification is carried out within each droplet. The droplets are streamed in a single file on a QX200 droplet reader. This reader counts the fluorescent as either positive or negative droplets to quantify the target DNA (41). Prior to the generation of droplets, the ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labelled with FAM or HEX (or VIC) as reporter fluorophores, or an intercalating dye such as EvaGreen (41). The number of positive and negative droplets

is used to estimate the concentration of target DNA sequences and their Poisson-based 95% confidence intervals.

Compared to qPCR, ddPCR have several advantages. The method ddPCR operates with absolute quantification of DNA without need of endogenous controls or external calibrators. ddPCR, an end-point measurement, can provide an accurate quantification of target sequences at low concentrations. This rules out the need of standard curves for quantification of nucleic acids, and small variations in PCR efficiency between wells have no effect on the measured concentration. This is what makes the absolute quantification possible, and makes this technique ideal for measuring of target DNA, viral load analysis and quantification and microbes (41).

Especially important is that ddPCR is a high capasity assay with 15.000 – 20.000 PCR reactions per well. The partitioning of the samples allows sensitive, specific detection of single template molecules as well as precise quantification. It also reduces the effects of competition between targets, and therefore makes PCR amplification less sensitive to inhibition and considerably improves the discriminatory capacity of assays that differs only by a single nucleotide. The zone that separates the clusters of positive and negative droplet is well-defined. The ddPCR conducts thousands of PCR reactions per 20µl sample; and the position of the threshold do not significantly affect the concentration output, and the well-defined zone reduces the risk of bias by the small amount of droplets that do not reach the end point. Also, ddPCR makes multiplexing possible. This means that several genetic targets of interest can be detected at the same time (41).

1.4.3 Quantasoft™ Software

QuantaSoft™ software obtain data from the droplet reader on the number of positive and negative droplets for each fluorophore in every sample. The software uses a Poisson algorithm to calculate the start concentration of the target DNA molecule. The concentration is measured as units of copies per µl input.

The data from ddPCR can be viewed as a 1-D plot with each droplet plotted on the graph of fluorescence intensity versus droplet number. The ddPCR data from a duplex experiment where two targets are amplified by PCR can be viewed in a 2-D plot with channel 1 fluorescence (FAM) is plotted against channel 2 fluorescence (HEX or VIC) for each droplet (41).

1.4.4 Multiplexing

The principle of multiplexing is to use the differences in fluorescence amplitude signal to change the spatial positioning of the droplet clusters in the data plot made in QuantaSoft™ software. Varying the concentrations of probe assay results in shift of the end-point fluorescence amplitude. This shift makes it possible to include two or more assays in the HEX and/or FAM channels, and determine what concentrations will give good separation of the droplet clusters (41).

The platform QX 200 ddPCR system is generally used for detection of two fluorophores to make duplex reactions (42). However, the system has the ability to present higher multiplexing up to 10-plex. In our study, we utilize this feature to multiplex four different genetic targets, each represent a distinct oral bacteria. Multiplexing requires optimal calculations in primer concentration, since this affects the fluorescence of the amplicons. An optimal primer concentration will lead to a better separation of droplet clusters. The final challenge is to achieve distinct separation between the positive droplets. The goal with multiplexing is to detect several targets from one sample and easily distinguish between these. If there are more than one DNA target in a single positive droplet, the intensity of the fluorescent will then be increased and make it more complicated to distinguish the different targets (42).

1.5 The aim of our study

The aim with this study is to develop a ddPCR assay for simultaneous detection and absolute quantification of four oral bacteria that are associated with periodontal diseases in the same clinical sample at the same time, i.e. multiplexing. Such an assay would enable fast diagnosis of four oral bacteria that are implicated for periodontal diseases and may reduce costs to process clinical samples.

2. MATERIAL AND METHODS

2.1 Bacterial strains

Table 1: List of the bacterial strains used in this study

Bacterial strains:
P. intermedia ATCC 25611
P. gingivalis T22
F. nucleatum ATCC 25586
A. actinomycetemcomitans CCUG 56172

2.2 Cultivation

In order to obtain DNA from the four test species, the bacteria were cultivated on eight separate blood agar plates, two for each strain, and then placed at 37°C in an anaerobic condition using Anaerocult® A mini bags system (Merck Millipore). In brief, two bags of Microbiology Anaerocult® A mini bags wetted with 8 ml water each, and placed in an anaerobic jar, which were then sealed off, and placed in the incubator in 37°C overnight.

2.3 DNA-extraction

To perform ddPCR, access to bacterial DNA is essential. To obtain genomic DNA from the four bacteria (Table 1), DNA extraction was done. Two machines are necessary for this process: a centrifuge (Thermo Scientific MicroCL 21R), and QIAcube using an extraction kit (QIAamp DNA Mini Kit, QIAGEN).

The first step in the process is to transfer 1 mL phosphate buffer saline (PBS) by a pipette (Thermo Scientific) into 2 mL Eppendorf tubes (DNA LoBind tube). All tubes were marked in a systematic way to avoid any mislabelling. The bacteria are collected with an inoculating loop and transferred to the tube where they are suspended in PBS. The tubes are then placed into the centrifuge to pellet bacteria at 21,100xg and a temperature of 15°C for about 7 minutes. After pelleting the bacteria, the supernatant is discarded. The samples are now ready for DNA extraction in the QIAcube using QIAamp DNA Mini Kit according to manufacturer's instructions. When the extraction process is finished, the extracted DNA-were stored in a freezer (- 20°C) for further analysis.

2.4 Measurement of DNA concentration

Measurement of DNA concentration is essential for calculating the amount of DNA to be loaded in the ddPCR. This is mainly to make sure that the concentration is optimal to proceed with the ddPCR-procedure. DNA-concentrations were measured using Qubit ds DNA HS kit according to manufacturer's instructions.

2.5 Droplet digital PCR

2.5.1 Preparation of master mix and droplet generation

The preparation of a master mix for the ddPCR reaction, involves mixing super mix, molecular biology grade sterile water (Sigma ® Life Science), digestive enzyme and DNA sample altogether (Table 2). To make sure that the master mix is enough for all the ddPCR reactions, the master mix is calculated based on one to two additional DNA samples, for example when using eight samples for ddPCR reaction, the master mix is calculated for nine samples instead of eight. When testing the four bacteria separately, a different master mix was made for each bacterium. A total of 20 μL of each master mix was transferred to the middle wells in DG8 cartridges (Figure 1). Then 70 μL of droplet generation oil (Bio-Rad) was added for each ddPCR mixture in the same cartridge. The cartridge was then covered by a DG8-gasket, securely hooked on both ends of the holder before inserted into the QX200 droplet generator. The droplet generator generate partitions for each sample into 20.000 nanoliter-sized droplets. The DNA is randomly distributed into these droplets in this process. As a part of the optimization process, different amounts of DNA and primer/probes in the master mix were evaluated several times. When multiplexing of the four bacteria was done, only one master mix was made.

Figure 1: Illustrates the DG8-cartridge. The markings show where to place the oil and the sample, and where the droplets accumulate after using the droplet generator. Picture adopted from Droplet digital ™ PCR Applications Guide (Bio-Rad) (41).

Table 2: Example of preparing and calculating the mastermix. This table shows example of preparing the mastermix before running ddPCR of the four bacteria separately. To get the total of eight ddPCR mixtures (1 line in ddPCR) it was used two wells for each bacteria. However, the mastermix was calculated for 2,5 wells to have a little excess in case of wastage.

Mastermix	*A.actinomycetemcomitans*	*P. intermedia*	*P. gingivalis*	*F. nucleatum*
Supermix	2,5x 10µL	2,5x 10µL	2,5x 10µL	2,5x 10µL
Enzyme	2,5x 1µL	2,5x 1µL	2,5x 1µL	25 x 1µL
Sample	2,5x 1µL	2,5 x 1µL	2,5 x 1µL	25x 1µL
Primer	2,5 x 1µL	2,5 x 1µL	2,5 x 1µL	2,5 x 1µL
Water	2,5 x 8µL	2,5 x 8µL	2,5 x 8µL	2,5 x 8µL

2.5.2 Overview of primer/probe sequences and amplicon size of each bacteria

Table 3: The table shows the amplicon sequence of the four bacteria, including the attaching targets for primers and probe. Green: forward primer. Red: probe. Blue: reverse primer.

Bacteria	Accession number	Gene	Amplicon sequence	Function	Amplicon size
A. actinomycetemcomitans	CP001733	3-deoxy-D-manno-octulosonic acid transferase	ggcaaataaacgtgggtgacactctcgccgaaaaccgctttaacgcgatcggaaccggtgggcgtcatggtcgtcacggtgatc gccaaatgcgggtaatcttgctgaatgcgtttaattaacggcac ggcggcaatcacttcgcccacagacgcggcatgaataagcac gccgttgggtttcggtgcgggcaattcgccgtaaaacgcgtaa cgttcgc	Enzyme in lipopolysaccharide biosynthesis	219bp
P. intermedia	AP014597	16S ribosomal RNA	tggaccttccgtattaccgcggctgctggcacggaattagccggtccttattcgaagggtaaatgcaaaaaggcacg cgtgcctcactttactccccaacaaaagcagtttacaacccatagggccgtcaatcctgcacgctacttggctggt	Regulating gene expressin to impact bacterial metabolism, growth and virulence	152bp
P. gingivalis	CP007756	fimbrialprotein	acgaatcaaaggtggctaagttgaccgtaatggtttataatggagaacagcaggaagccatcaaatcagccgaaaatgcgacta	Facilitate host adhesion	84bp
F. nucleatum	NC_003454	23S ribosomal RNA	gatgaaccgcagaaggtgaaagtcctgtataagtaaatccttacacatataactttgctcccaagtaacatggaacacgaggaattctgtgtgaatcagtgaggacca	Catalyzes peptidebonds ynthesis	100bp

2.5.3 Multiplexing of three bacteria

The process started with optimizing the multiplexing parameters for three of the bacteria. These were *A. actinomycetemcomitans*, *P. intermedia* and *F. nucleatum*. We mixed a mutual master mix without the DNA calculated for nine samples. For enabling the QX200 to properly quantify the target bacteria, it is necessary that the loaded DNA concentration is optimized. To achieve that, the DNA concentration can be neither too high nor too low – as this will result in only positive or only negative droplets. By running ddPCR trials with different concentration of DNA in each well the optimal concentration was eventually found. We tested different values of DNA concentrations (Table 4). The mastermix was then distributed into eight wells, and then the same amount of DNA from each bacterium was added into the wells. Each well had a specific amount of DNA from each bacterium. The DNA was diluted from the original value of 100pg/µL to the values listed below "Total amount of DNA (pg)" in Table 4. To achieve this we used the method in Box 1.

Box 1. Calculation example of how to find the dilution factor from a DNA sample with a concentration of 100 pg/µL:

Calculation example:

There are three DNA samples under investigation, each with a concentration of 100 pg/µL. When the desired amount of DNA was determined, this amount was divided by the factor of three (the number of bacterial DNA samples) in order to get the DNA amount needed from each bacteria

100 pg/µL divided by three different bacteria equals 33 pg of each needed. The dilution factor: $\frac{\mathbf{100}}{\mathbf{33}} = \mathbf{3}$.

This means that relation between sample and water is 1:3. From 100 pg/µL of *F. nucleatum,* 10 µL was taken, and 20 µL water was added. This gave us the desired concentration of 33 pg/µL of that bacteria.

The same method was used for *A.actinomycetemcomitans* and *P. intermedia*. A total of 1 µL from each diluted sample was transferred to the master mix in well number one. The same approach was used for all the other values in "Total amount DNA (pg)".

Table 4: Calculation of DNA amount from each bacteria to get a particular total amount of DNA. The table shows the amounts of DNA in picogram that we used in our calculations and experiment.

Well	Total amount DNA (pg)	Amount DNA from each bacteria (pg/µL):
1	100pg	33pg/µL
2	75pg	25pg/µL
3	51pg	17pg/µL
4	51pg	17pg/µL
5	24pg	8pg/µL
6	24pg	8pg/µL
7	9pg	3pg/µL
8	9pg	3pg/µL

2.5.4 Multiplexing of four bacteria

The four different DNA samples had previous been diluted to a concentration of 100 pg/µL. By mixing them together, taking 20 µL of each bacterium, the concentration of each bacterial DNA in the final sample was eventually 25 pg/µL. The composition of the final master mix is seen in Table 5.

Table 5: Composition of the final master mix. Note: All components besides DNA was prepared for nine samples.

Final master mix content	Volume
Supermix	9 x 10 µL
Primer FAM for *P. gingivalis*	9 x 1,5 µL
Primer FAM for *F. nucleatum*	9 x 0,5 µL
Primer HEX for *P. intermedia*	9 x 0,5 µL
Primer HEX for *A. actinomycetemcomitans*	9 x 1,5 µL
DNA Sample	8 x 2 µL
Enzyme BSURI (Heal)	9 x 0,5 µL
Water	9 x 4,5 µL

2.5.5 Thermal cycler procedure

PCR amplification was accomplished by using the Thermal Cycler (C1000 Touch ™ Bio-Rad) with the amplification parameters presented in Box 2.

Box 2. Amplification parameters used in the PCR to amplify the genetic targets of the four bacteria
The stages of the Thermal Cycler. The table shows temperature, temperature acceleration and time for each step. As read from the table, step 2 and 3 are performed 40 times before the process continues**.**

The PCR-amplification steps:
Method: Calc. Lid: 105°C. Volume: 40 µL.
1. 95°, 10:00. Ramp 2,5°C/s.
2. 95°, 0:30. Ramp 2°C/s.
3. 58°C, 1:00.
4. Go to step 2, 39X.
5. 98°C, 10:00. Ramp 2°C/s.
6. 4°C, 0:00. Ramp 2°C/s.

2.5.6 Droplet reading

After completing the thermal cycler, the 96-well plate are placed into to the droplet reader (Droplet Reader QX200 ™ Bio-Rad), where each well was analysed for positive and negative droplets.

2.5.7 Data analysis

The data was directly transferred into the computer-based software Quantasoft ™, where positive and negative droplets were counted, and copy number of the target DNA was calculated by using the Poisson distribution. All of these procedures were performed for each of the bacteria separately for optimization, before the process of combining all of the four bacteria into one ddPCR reaction .

2.6 Quality assurance

To enable multiplexing it is essential that each bacteria has its individual primer that only detects the DNA sequence specific for that bacteria. In this process, it was therefore necessary to confirm that the primers/probes used did not target, or identify, any other bacteria than the one it was presumed. This was done by running ddPCR of each of the bacteria combined with the primers for the other bacteria. In another words the bacterial DNA together with the

wrong primer/probe combination should not give any amplification and to confirm that the primers did identify the intended DNA-target only.

2.6.1 Optimization

To be able to distinguish between the different bacteria in multiplexing it is essential to achieve good separation between the droplet clusters in QuantaSoft. Different fluorophores, FAM and HEX, were used to achieve this. Each fluorophore were used for two bacteria. To find the optimal conditions for the bacterial DNA in this trial the DNA concentration, temperature and primers were optimized.

2.6.2 Temperature

Temperature selection has a major influence on the quality of the results when performing any PCR reaction. Optimal temperature is essential to get the right results and good separation between the droplet clusters in the QuantaSoft 2-D plot analysis. The most critical stage is the annealing temperature in which the DNA primers are attaching to the template DNA. The optimal temperature was found by using a thermal cycler with the ability to use different temperatures on different lines of wells. Then, from the analysis, we were able to see which temperature that gives the best results, and used it for further analysis of the samples.

2.6.3 Designing primers

Designing of good primers is an important part of successful PCR amplification. There are several important criteria like melting temperature (T_m), base composition, length and content of GC. The T_m should be between 50-65°C and the content of GC should be between 50-60%. Sequences where G or C is repeated more than 3 times in a row should be avoided, and G and C should be placed at the 3' nucleotide if possible. Because the primers are used in pairs it is important to ensure that the paired primers do not show any significant complementarity between the 3' ends because this can result in primer dimers. Comprehensive formation of primer dimer may inhibit or decrease the amplification reaction.

3. RESULTS

3.1 Bacterial strains

All of the four bacteria species were successfully cultivated overnight anaerobically at 37°C, and had normal growth on blood agar plates.

3.2 Bacterial DNA extraction

DNA was successfully extracted from the four bacteria, and the amount of DNA was sufficient for running ddPCR experiments.

3.3 Measurement of DNA concentration

The DNA concentration of the four bacteria is presented in Table 6 and are given in ng/µL. It was decided to convert the DNA concentrations into picogram (1ng = 1000 pg). The samples therefore had to be diluted.

Table 6. Concentration of the extracted DNA (ng/µL) from the four bacteria.

Bacterium	Concentration	Dilution factor (to get 100pg)
F. nucleatum	6.68 ng/µL	66,8 (1µL DNA + 65,8 H_2O)
P. gingivalis	21.2 ng/µL	212
A .actinomycetemcomitans	72.0 ngµL	720
P. intermedia	21.6 ng/µL	216

Note: The DNA of *A. actinomycetemcomitans*, were initially too high to be measured by the machine. Therefore, the sample were diluted 1:10, and the DNA concentration were then measured to be 7,20 ng/uL. This means that the DNA concentration in the original sample is 72,0 ng/µL.

3.4 Digital Droplet PCR (ddPCR)

3.4.1 Quality assurance

To check the sensitivity of the primer/probe, each bacterium was detected and quantified separately using its primer/probe by ddPCR. The results obtained shows good sensitivity of the primers/probes (Figure 2-4).

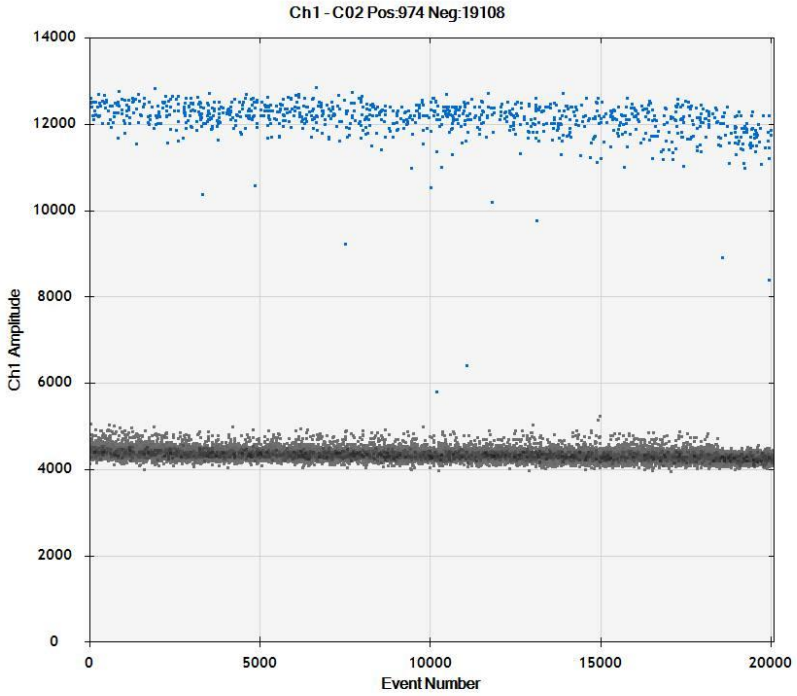

Figure 2: *A. actinomycetemcomitans* combined with its individual primer. The ddPCR shows identification of *A. actinomycetemcomitans* and good separation between positive and negative droplets.

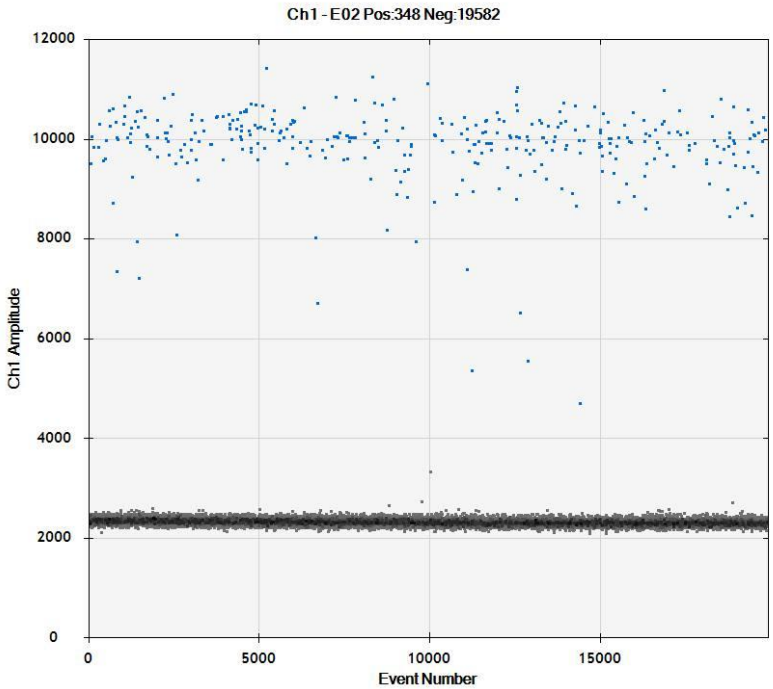

Figure 3: *P. Intermedia* combined with its individual primer. The ddPCR shows identification of P. intermedia and good separation between positive and negative droplets.

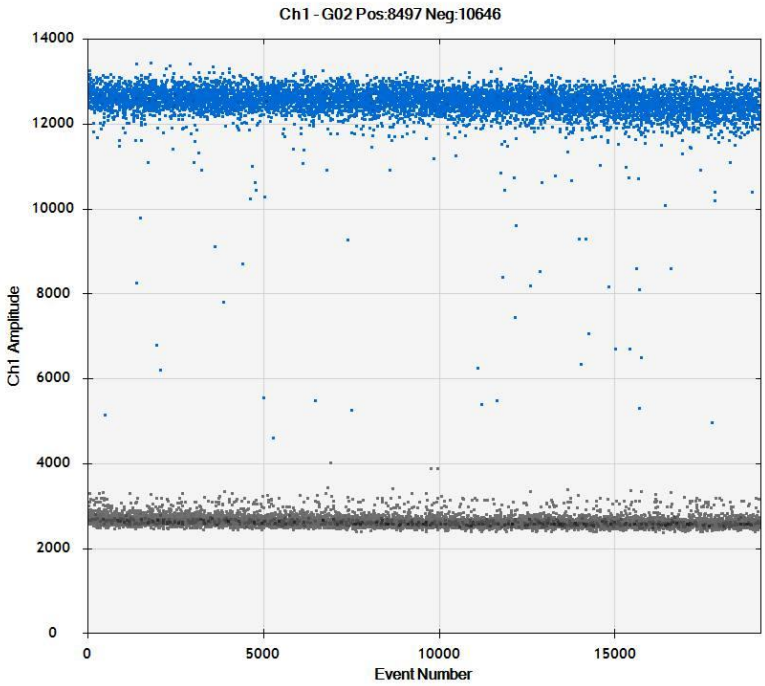

Figure 4: *F. nucleatum* combined with its individual primer. The ddPCR shows identification of *F. nucleatum* and good separation between positive and negative droplets.

When running ddPCR reaction for each individual bacterium with its specific primer/probe the sensitivity of the primers/probes was confirmed by high amplitude of positive droplets with good separation from the negative droplets (Figure 2-4).

The second step was to insure specificity of the primers/probes used in this study. In order to accomplish that, each bacterium was run in the ddPCR using primers of the three other bacteria to test specificity. It was confirmed that the four primers/probes are specific to their respective bacteria (i.e. the primer specific for DNA of *P. gingivalis* did not amplify *F. nucleatum, P. intermedia and A. actinomycetemcomitans*) (Figure 5).

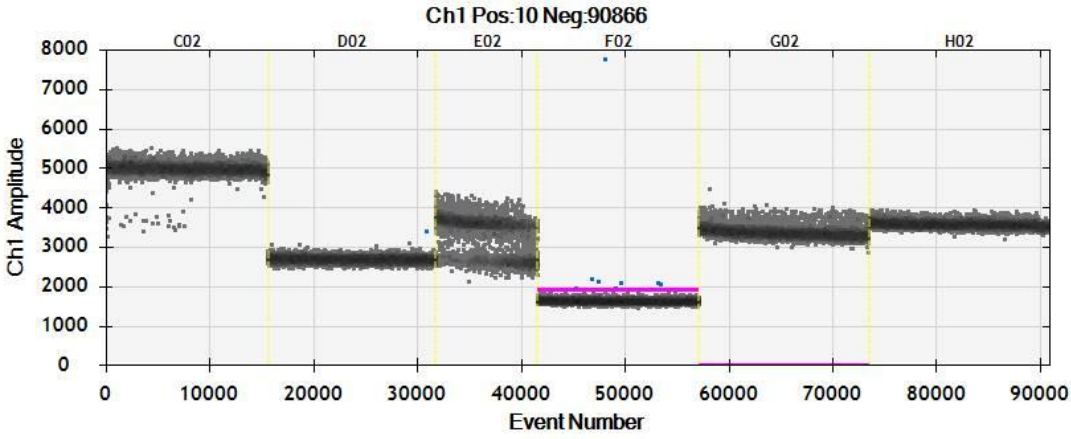

Figure 5: Crosschecking shows only negative droplets, and confirms that the individual primers do not identify faulty targets, in other words any of the other bacteria. It shows that the primers have high specificity. C02*: P. Intermedia* with primer for *A. actinomycetemcomitans.* D02: *P. Intermedia* with primer for *F. nucleatum*. E02: *A. actinomycetemcomitans* with primer for *P. intermedia*. F02: *A. actinomycetemcomitans* with primer for *F. nucleatum*. G02: *F. nucleatum* with primer for *P. intermedia*. H02*: F. nucleatum* with primer for *A. actinomycetemcomitans*.

3.5 Multiplexing

3.5.1 Detection of three bacteria

After confirming the specificity of the primers, the next step was to proceed with multiplexing experiments. Three of the bacteria were gathered in the same sample and master mix is prepared with all of the three primers, before ddPCR were carried through. The obtained result showed good separation between the positive droplets from each three species and the negative droplets. The three clusters representing the three bacterial species were identified in the 1-D plot in the QuantaSoft software (Figure 6).

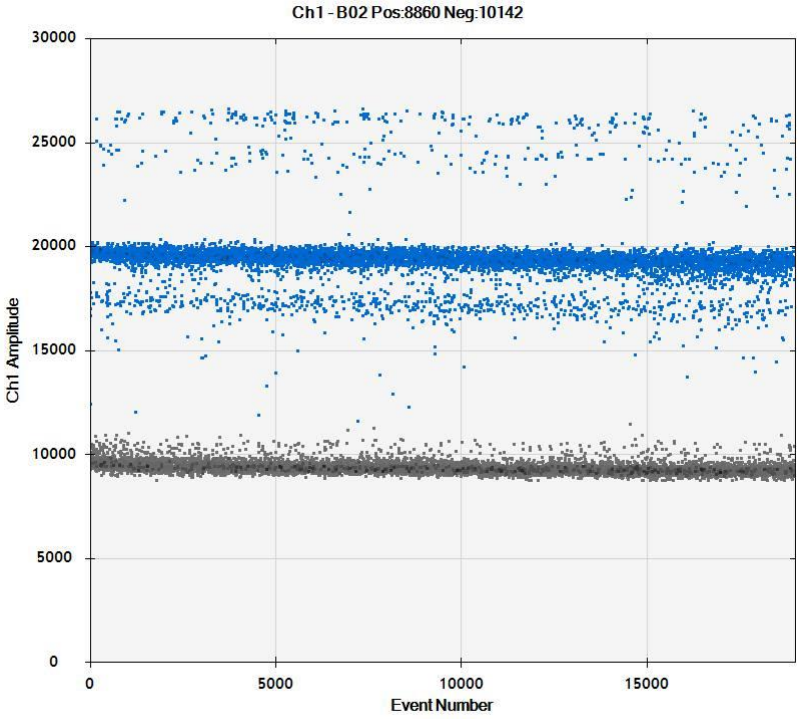

Figure 6: 1-D plot representing multiplexing by ddPCR of *A. actinomycetemcomitans, P. intermedia and F. nucleatum*.

3.5.2 Detection of four bacteria

After a successful multiplexing of the three bacteria, the next step was to proceed with multiplexing of the four bacteria altogether in the same sample. The sample with the mixed DNA from the four bacteria was then analysed by ddPCR. Analyses of the obtained results on QuantaSoft showed good separation between the different clusters, as well as between the positive and negative droplets. The different clusters representing the multiplexing of the DNA from four bacteria is presented in Figure 7.

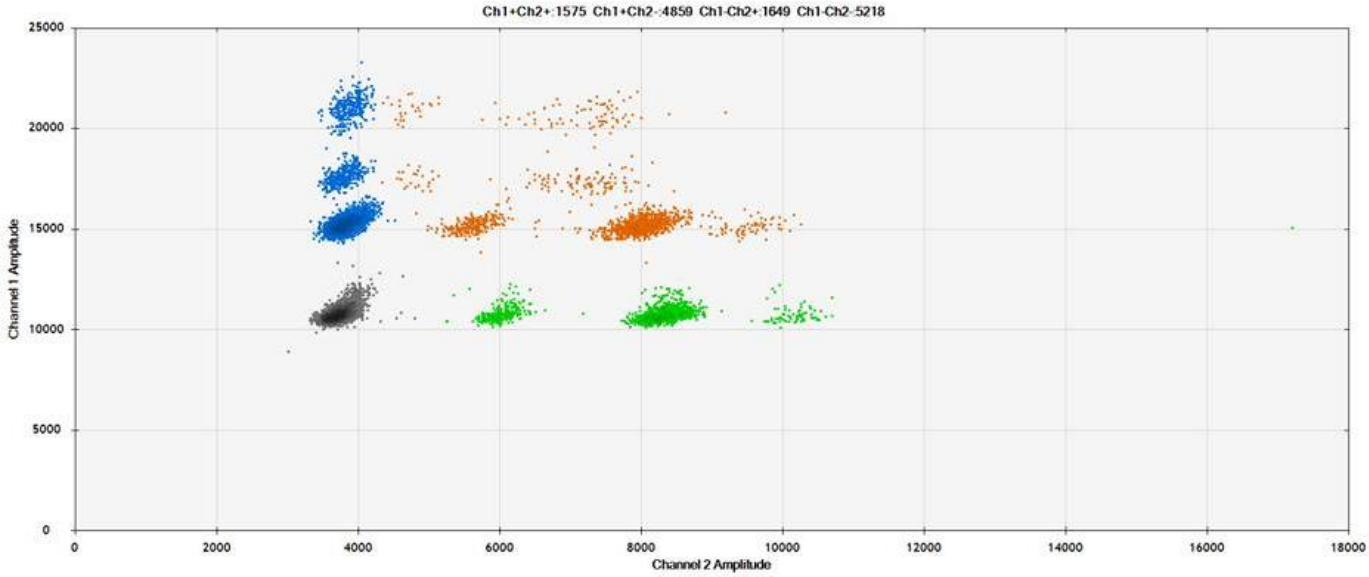

Figure 7: 2-D plot representing multiplexing four bacteria together. The blue clusters represents *P. gingivalis* and *F. nucleatum* detected by FAM and the green clusters represents *P. gingivalis* and *A. actinomycetemcomitans* detected by HEX. The primers with the highest concentration added gives higher amplitude of their target DNA, while the primers with lowest concentration gives a lower amplitude (see Figure 6). Orange clusters consists of DNA targets from both channels. The grey cluster represents the negative droplets.

Table 7: This table illustrates the results from Figure 7.

Channel 1 (FAM)	Signal from both *P.gingivalis* and *F. nucleatum*	Signal from *P. Gingivalis*, *F. nucleatum* and *P. intermedia*	Signal from *P. Gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans*	Signal from all four DNA targets
	P. gingivalis	Signal from *P. gingivalis* and *P. intermedia*	Signal from *P. Gingivalis* and *A. actinomycetemcomitans*	*Signal from P. Intermedia, A. actinomycetemcomitans and P. gingivalis*
	F. nucleatum	Signal from *F.nucleatum* and *P.intermedia*	Signal from *F. nucleatum* and *A. actinomycetemcomitans*	Signal *from P. Intermedia, A. actinomycetemcomitans* and *F. nucleatum*
	Negative droplets	Signal from *P. intermedia*	Signal from *A. actinomycetemcomitans*	Signal from both P. Intermedia and *A. actinomycetemcomitans*
	Channel 2 (HEX)			

3.6 Quantification of bacteria and reproducibility

The sample with all four bacteria, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *F. nucleatum* was tested several times. The results showed good sensitivity, specificity and reproducibility of the technique.

The absolute number of each genetic target per 20µL reaction mix were as follows:

Table 8: This table shows how many copies of the genetic target that was present in each PCR-well in our last experiment. The numbers/codes (i.e B01) in the left column indicates the name of the PCR-well.

	P. gingivalis	*F. nucleatum*	*A. actinomycetemcomitans*	*P. intermedia*
B01	8600	740	2340	488
C01	8580	760	2340	496
D01	8760	736	2380	540
E01	8700	692	2400	540
F01	8600	738	2480	486

The number of each bacterium per 20µL were as follows:

Table 9: This table show how many copies of the bacteria that were present in each PCR reaction in our last experiment. The numbers was calculated by dividing the number obtained in Table 8 by the copy number of genetic target in the genome of the bacteria. *F. nucleatum* has 5 genetic targets in the genome, while *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* have only 1 genetic target in their genome.

	P. gingivalis	*F. nucleatum*	*A. actinomycetemcomitans*	*P. intermedia*
B01	8600	148	2340	488
C01	8580	152	2340	496
D01	8760	147	2380	540
E01	8700	138	2400	540
F01	8600	148	2480	486

4. DISCUSSION

The aim of this study was to develop a method for simultaneous detection and absolute quantification of four oral bacteria associated with periodontal disease in the same clinical sample by ddPCR. Accomplishing this could make the workflow in detection of periodontal bacteria faster and more cost effective.

When the current study started in spring 2016, literature search for studies that are comparable to this one revealed only one study with multiplexing by ddPCR on a higher level than 3-plex (43). For BioRad QX200 system it is possible that a droplet contains more than one target molecule, which could further complicate the analysis due to the fact that several cluster may appear in the analyse-panel and their separation might not be as good as expected (44).

In 2011, Zhong *et al.* introduced a simple method for multiplexing (5-plex) using ddPCR (43). The idea was based on using different concentration for each fluorogenic probes tagged with the same colour. In doing so, it was possible to identify the different probes based on the intensity of the fluorescence in the 2-D plot. The result was an accurate and precise multiplexed measure of gene copy number across four different targets and a reference (43).

Dobnik *et al.* (2016) used BioRad's QX200 to develop a 4-plex system based on the principle with varying the concentration of primer and probe for two targets per fluorescence channel (42). The Bio-Rad QX ddPCR platform was not originally meant to be used for such high multiplexing, and the analyse software that was available when the trial was accomplished did not support analyse of this kind of experiments (42). Therefore Dobnik *et al*. developed their own web-tool to automate the analysis of the 4-plex results. Two 4-plex assays was developed for quantification of eight different DNA targets. In each assay two targets was labelled with one FAM and two with HEX (42). The ddPCR platforms, which include two fluorescence filters, support at least duplex reactions and with some development and optimization even higher multiplexing is possible. The study by Dobnik *et al* shows a development of a multiplex assay with ddPCR, but more important it represents the first thorough evaluation of several parameters in this kind of multiplexing using ddPCR and might be considered as a pilot study in this field.

Our study is quite similar to Dobnik *et al*. We used the same fluorophores, FAM and HEX, and the same method to separate between different DNA targets with the same fluorophore by taking advantage of the ability to detect different levels of fluorescence amplitude. However, in our study we used a newer version of the software, Quantasoft Analysis, that support multiplexing, even though the cluster selection had to be done manually to obtain quantitative data.

The process of optimizing variables and substantial factors, like temperature and concentrations were accomplished through several experiments. Sensitivity, specificity and reproducibility of the results were checked and confirmed throughout the experiments.

Microbial diagnosis could improve the ability to identify patients at risk for developing periodontal disease, as well as monitor progression of the disease, assist in periodontal diagnosis, and choice of optimal treatment. Periodontal diseases are a result of biofilm infections, and it could be of crucial importance to know which bacteria that are responsible for the inflammatory response in the individual patient. In most cases of patients with periodontal disease, conventional treatment with local mechanical debridement results in arrest of the disease progression processes. Microbial testing as a routine measure in today clinical practice is not very common. This is often due to high costs and the fact that it may be time consuming. However, in cases where patients do not respond well to the conventional treatment, microbiological testing should be indicated: such as patients with more severe forms of periodontal disease, such as aggressive and refractory periodontitis (45).

The interest of immunological and bacterial biomarkers in periodontal diseases have been in focus in the last two decades to develop a diagnostic tool, which sorts out patients with increased risk. The benefit of such a tool is to help in accurate assessment of the individual periodontal patient. More precise confirmation of the periodontal health on an individual level can give a clue in anticipating therapy outcome and effectiveness. In that way it may be possible to foresee eventually future severe progression of disease in an early stage and measure response among both hosts and treatment given (46).

P. gingivalis and *T. forsythia* is especially found to be crucial for progression of the disease (6). Microbial testing and detection of these bacteria could give information about the severity of periodontitis. *A. actinomycetemcomitans* is suspected to be the most probable causal factor for aggressive periodontitis in adolescents (47). Aggressive periodontitis needs more active treatment, often combined with antibiotic treatment. The unnecessary use of antibiotics gives concerns about development of antibiotic resistance, and a microbial diagnostic testing could be useful to make sure that only the true positive patients diagnosed with the presence of *A. actinomycetemcomitans* and aggressive periodontitis receives antibiotic treatment. *P. intermedia* are more frequent found in sites with compromised periodontium (48). *F. nucleatum* is one of the most common species in the oral microflora, both in healthy and diseased patients (49). Cell numbers of *P. gingivalis* is found to be significantly lower after periodontal treatment than before of a patient with periodontitis. Microbial testing before and after that shows this reduction indicates that treatment may be regarded as successful (50, 51).

Monitoring microbial status before and after treatment could give good information about the result of treatment, prognosis and possible recurrence of the disease. Detection of high number of periodontal pathogens in an assumed healthy periodontal pocket or saliva could predict that the patient may have a risk for developing a periodontal disease. A subgingival biofilm sample provides the most relevant information in a patient with specific periodontal locus, while when detecting and identifying a population of periodontal pathogens present in the oral cavity a saliva sample is more commonly used (52).

5. CONCLUSION

Using ddPCR for multiplexing of bacteria associated with periodontal disease is possible. Our results suggest that four different bacteria can be detected and separately recognized in one sample. We believe that this technique could make the workflow in detection of periodontal pathogens more effective by running just one reaction from the same sample, rather than detect and identify each pathogen separately. A more effective technique for detection of several pathogens from the same sample could lower the threshold for dentists to choose microbial testing as a useful tool for prevention, diagnosis and treatment of periodontal diseases.

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