Department of Clinical Dentistry

Faculty of Health Sciences

Detection and association of β-lactam resistance genes in *Fusobacterium nucleatum* and *Prevotella intermedia*

Investigation of cfxA in saliva samples

Jakobsen, Sofie and Knedahl, Ingeborg.

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Supervisor: Mohammed Al-Haroni
BDS, PhD, FHEA Associate Professor

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Abstract

Background: Antibiotic resistance is an ever-increasing problem worldwide, caused by unnecessary and excess use of antibiotics. The problem has engaged more attention and research the recent years.

Aims: The aim of this study is to detect levels of the antimicrobial resistant gene *cfxA* in saliva samples, and to investigate the linkage between this gene and the two chosen bacteria; *Prevotella intermedia* and *Fusobactierium nucleatum*. The saliva is collected from experienced dentists and dental hygienists, to investigate the presence of the *cfxA* gene compared to inexperienced 1st and 5th year dental students.

Material and method: Saliva was collected from a total of 20 experienced dentists/ dental hygienists at "Universitetstannklinikken" (UTK) and "Tannhelsetjenestens kompetansesenter for Nord-Norge" (TkNN). DNA was extracted, followed by amplification and quantification of the *cfxA* gene using ddPCR. In addition to saliva collection, a questionnaire was used to report the demographic data, attitudes and hygiene practices in order to study any correlation between levels of the *cfxA* gene and personal characteristics, e.g. habits, knowledge and commitment to hygiene procedures in the clinic.

Results: We detected the resistance gene *cfxA* in all the participants, but could not detect any significant linkage between the *cfxA* gene and the two bacteria, *P. intermedia and F. nucleatum*. The results report no significant difference level of the *cfxA* gene between the experienced group and previously reported results from dental students. In addition, no positive correlation was found between the level of the *cfxA* gene and previous use of antibiotics.

Conclusion: The study reports higher median values of the *cfxA* gene in the experienced participants compared to dental students. Also, a higher percentage of the experienced participants had high levels of the *cfxA* gene compared to dental students. The small sample size in this study is a possible reason for the lack of statistical significance, and it would be interesting to investigate the same topic in bigger sample size in future studies. The use of a different methodology could possibly detect linkage between the *cfxA* gene and the two chosen bacteria.

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1 Introduction

1.1 Antibiotics and resistance

Over the past 20 years, the rise in antibiotic resistant genes carriage in virtually every species of bacteria, including oral bacteria. The presence of antibiotic resistance genes in oral bacteria has become of great interest clinically in the last decade, and has attracted more research the recent years. Several antibiotic resistance genes have been found in bacteria residing in the oral cavity.

The more misuse of antibiotics leads to higher prevalence of antibiotic resistant genes among oral bacteria. On the other hand, development of new antibiotics is not progressing in a speed high enough to overcome the problem of antibiotic resistance. Therefore, it is important to stop the excessive use of antibiotics and prevent the spreading of antibiotic resistant bacteria.

1.2 β-lactam antibiotics

Among the most documented genes in the oral cavity are those responsible for the resistance toward β-lactam antibiotics (1). For example, the genes *cfxA*, *blaTEM and* blaZ (2) have been detected in oral bacteria. Phenoxymethylpenicillin, a member of the β-lactam antibiotics, are the most prescribed antibiotic in dental practice in Norway (3).

β-lactam antibiotics are a broad category of antibiotics constituting many families of antibiotics. This includes penicillins, cephalosporins, cephamycins, carbapenems, conobactams and β-lactamase inhibitors. By the definition, all β-lactam antibiotics have a β-lactam ring in their molecular structure.

Initially, the β-lactam antibiotics acted mainly on Gram-positive bacteria, but in the later years there has been developed broad-spectrum β-lactam antibiotics active against various gram-negative organisms. This has made this group of antibiotics more useful. This means that there are both broad and narrow spectre β-lactam antibiotics, where the broad spectres also are efficient against the gram-negative bacteria.

1.2.1   Molecule structure of β-lactam antibiotics

This big group of antibiotic has a great variation in structure, apart from the common β-lactam ring. A lactam is a cyclic amid and the β-lactam ring is a four-membered amid. It is named as such because the nitrogen atom is attached to the β-carbon atom relative to the carbonyl.
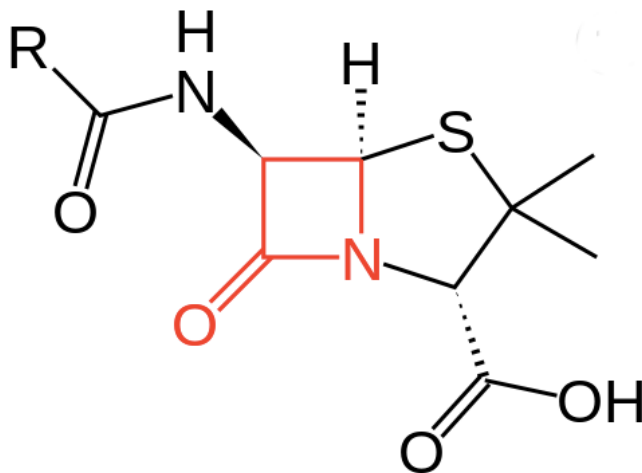
Figure 1. *General structure of penicillin with β-lactam ring marked with red. Photo adopted from khanacademy.org.*

1.2.2   Mechanism of action of β-lactam antibiotics

Nearly all of the β-lactam antibiotics work by inhibiting the cell wall synthesis in bacteria. The peptidoglycan layer is an important feature in bacterial cell walls, especially in gram-positive bacteria, where the peptidoglycan layer is the outermost and main component of the cell wall.

In action, the β-lactam ring opens and attach irreversibly to the enzyme that is incorporated in the bacterial cell membrane, and is a part of the third stage in the cell wall synthesis. These enzymes contains penicillin binding protein (PBP) which are the structure the β-lactam antibiotic binds to (4).

The PBP´s function is to catalyse the final crosslinking (transpeptidation) of the peptidoglycan. The binding of this protein thereby inhibits the synthesis of the peptidoglycan layer in the bacterial cell wall, and indirectly makes β-lactam antibiotics bactericidal (5).

1.3   Mechanism of β-lactam resistance

The most effective way of resistance towards β-lactam antibiotics is the production of β-lactamases, enzymes that hydrolyses the β-lactam ring. To overcome this problem, β-lactam antibiotics are often given with β-lactamase inhibitors.

Another method is to change the proteins to which the β-lactam antibiotics bind. β-lactam antibiotics cannot bind as effectively to these altered PBP's, and, as a result, the β-lactam antibiotics are less effective at disrupting cell wall synthesis. This includes methicillin-resistant Staphylococcusaureus (MRSA) and penicillin-resistant Streptococcus pneumoniae (6).

1.3.1   *CfxA* gene

 Most bacteria involved in dentoalveolar infections are highly sensitive to penicillins, but some exhibit resistance against these agents through the production of β-lactamase enzymes. This includes *Prevotella intermedia* and *Fusobactierium nucleatum*. The production is in turn associated with the expression of the *cfxA* gene, among others. *CfxA* gene therefor encodes for β-lactamase enzymes, i.e. class A β-lactamases. The *cfxA* gene is among the most b-lactamase producing gene in oral bacteria (7). This is why *cfxA* is the target gene.

The *cfxA* gene has previously been identified in *P. intermedia (8)*, while the β-lactamase gene *FUS-1* has been reported in *F. nucleatum* (9). Even though the latter bacteria is not yet linked to the *cfxA* gene, the possibility is not excluded.

1.4   Oral bacteria

The oral cavity has a very diverse microflora, with over seven hundred different bacteria detected. Additionally, there is many more yet to be identified. The oral cavity provides comfortable and favourable living conditions for the majority of the bacteria, partly due to frequent nutrition supply (10).

Bacteria play an important part in the immune system, but can under some circumstances be harmful. Bacteria found in the oral cavity play a critical role in most periodontal diseases. They organize themselves in a biofilm, and the pathogenesis is complex and multifactorial (10). However, not all of these bacteria play a significant role in the aetiology of periodontal diseases (11).

1.5   Oral biofilm

Together with bacteria, saliva poses the main component in oral microbiology. The salivary biofilm plays many roles in the maintenance of oral health, i.e. protection, lubrication, hydration and remineralisation (12). Proper hygiene helps to maintain a healthy oral flora, and prevents harmful effects and disease transmission. Because the bacteria within the oral biofilm have close proximity and stable structural properties, it seems to be the perfect environment for horizontal gene transfer (13). This can lead to the spread of antibiotic resistance genes amongst the biofilm inhabitants.

1.6   Periodontitis

Periodontitis is an inflammatory disease in the tissue surrounding teeth, caused by dental plaque, calculus and specific microorganisms. These microorganisms are able to colonize at a unique location. Factors like pH, redox potential, oxygen tension, and antagonism/ synergism between microorganisms is of importance. Defence mechanisms of the host will interfere with these factors, but some bacteria will be able to escape from the host response. Host defence mechanisms that may interfere will include saliva flow, gingival cervical fluid and good oral hygiene.

As a response to this bacterial infection, neutrophils and other defence mechanisms will use different strategies to try and kill these invading pathogens (14). Although the cause of this disease is the infection, the host immune response is important for the progression. After continuous challenge by different bacteria colonizing, the defence mechanisms of the host may result in a breakdown (10). Inflammation is the result of the host immune response. Because of the unique location of this disease, it will cause attachment loss, and if not treated, eventually tooth loss.

Bacteria alone cannot induce destructive periodontal disease (10). Risk factors as bad oral hygiene, smoking tobacco, stress, HIV infection and malnutrition also contribute to this disease.

1.6.1   Microbial complexes (red/ orange complex)

Bacteria of the oral cavity are organized in different complexes (10). Species of the red and orange complex are common in subjects with periodontitis (15). *P. intermedia* and *F. nucleatum* belongs to the orange complex. They both have moderate evidence of being periodontopathogenic microorganisms (14), and are usual members of the normal oral flora, though in low numbers (10).

1.6.2   Prevotella intermedia

P. intermedia is a gram-negative, anaerobe bacterium. They are a normal part of the oral flora, but can cause gingivitis and other types of oral lesions. *Prevotella spp.* often becomes resistant to antibiotics, especially tetracycline and penicillin (16,17).

1.6.3   Fusobacterium nucleatum

F. nucleatum is a gram-negative anaerobe bacterium, which we can find in the oral cavity among other places. It is often connected to invasive infections throughout the body. In the oral cavity it grows between gums and teeth, and is actively involved with the disease periodontitis (18).

1.7   Cross infection

Cross infection is the transfer of pathogenic microorganisms between people and equipment. As a dental worker it is important to be aware of this and know how to eliminate or at least minimise this. Transmission of dental infection can occur through infected air droplets, blood, saliva and instruments contaminated with secretions (19). Routines at the clinic have to be safe enough for both patient and clinician, and should not depend on weather the patient is a possible germ carrier or not (20). Increased rates of antibiotic resistance world wide has made it necessary to have proper guidelines, so in 2015 all the Norwegian odontology institutions got common guidelines for infection control. It is important that every clinician has knowledge about disinfection and good hygiene.

The standard precaution is to prevent microorganisms from being spread between patient and clinician, and between patients. The meaning is to reduce or to lower the risk of cross infection. Important elements is hand wash, barriers, discard sharps, cleaning and garbage sorting. A good standard protocol for hygiene is very important.

1.8   Aims of study

It is hypothesized that *P. intermedia* and *F. nucleatum* are mostly associated with the *cfxA* gene. These two bacteria, belonging to the orange complex, have been reported resistant to a panel of antibiotics, including β-lactam antibiotics.

The aim was also to investigate whether the prevalence of *cfxA* gene is higher with more experience in the clinic compared to a previously reported prevalence among dental students. Therefore the hypothesis was that experienced dentists/ dental hygienists (for convenience hereinafter referred to as experienced clinicians) have higher prevalence of the *cfxA* gene in their saliva than 1st and 5th year dental students. The previous study at "Institutt for klinisk odontologi" (IKO), has already suggested no significant difference between the 1st and 5th year dental students (21).

2   Materials and methods

Experienced clinicians were invited to participate the study. After the permissions was received, those who fulfilled the criteria where asked to donate saliva samples, and to complete a questionnaire about hygiene practices at the clinic.

Data obtained from 1st year dental/ medical students and 5th year dental students was already collected in a previous study at IKO at The Artic University of Norway (21). Medical students were included as they have the same curriculum as dental students in their first year of education.

2.1   Study population

2.1.1   Collecting saliva

Saliva samples were collected from experienced clinicians employed at "Universitetstannklinikken" (UTK) and "Tannhelsetjenestens kompetansesenter for Nord-Norge" (TkNN).

2.1.2   Consent

The subjects were asked to sign a consent form, containing information about the study aim, and how the saliva sample would be collected and used. They were informed that the collected data would be treated confidentially. See appendix I.

2.1.3   Saliva collection

Samples were collected from participants who have had more than 3 years clinical experience. All study subjects had general good health. The exclusion criteria were use of any antibiotic therapy within the last three months.

Each subject who donated saliva used a test for stimulated saliva secretion. The subjects where informed not to eat, drink or use any nicotine within the last hour before the sampling. The participants were sitting in an upright position. The subjects were relaxed during the procedure.

To stimulate saliva secretion the subjects were given a piece of paraffin wax to chew on for approximately 30 seconds before the collection. The subjects were spitting saliva frequently into a collecting tube for five minutes. The saliva samples was then stored in -80°C freezer for further analysis.

2.1.4   Questionnaire

Every subject was asked to fill out a form that included questions about their general health, use of antibiotics, oral hygiene and routines, hygiene practices at the clinic and attitudes related to infection control. Also knowledge about disinfection of instruments and other equipment, and the use of protecting supplements to prevent infection was included. See Appendix II.

2.2   Laboratory analysis

2.2.1   Bacterial DNA extraction

Bacterial DNA was extracted using QIAcube extraction. 800 µl of saliva were transferred to Eppendorf tubes and mixed with an equal amount of PBS (Phosphate Buffered Salin). The mixture was centrifuged for 10 minutes at max speed to pellet the bacteria. After this centrifuge, the excess liquid was removed. Automated DNA extraction in QIACube was done with QIAamp Mini Kit according to protocols and standard procedures.

2.2.2 Agarose gel-electrophoresis

To confirm the success of the DNA extraction, agarose gel-electrophoresis was used. Visualization of the extracted bacterial DNA from the saliva samples was done in 1 % agarose gel. The gel was prepared by dissolving agarose powder (Amresco®, VWR) in TAE (Tris-acetat-EDTA) buffer. The nucleic acid stain GelRed^{TM} Nucleic Acid (Biotium) was used to stain the DNA. GelRed^{TM} is a fluorophore that binds DNA, and when exposed to UV-light it will fluoresce. 10 µl extracted DNA was mixed with 2 µ log 6x Gel Loading Dye Blue (New England BioLabs, UK). Then 10 µl of this mixture was loaded on the gel. 10 µl of 1 kb DNA Ladder (New England BioLabs, UK) was loaded in a separate lane in the gel to act as a molecular weight reference for DNA size. The ladder contains bands ranging from 0,5 to 10 kb. The agarose gel was run at 120V for about 50 minutes, and then visualized with UV-trans-illuminator in the gel documentation system ChemiDoc^{TM} Touch Imaging System (Bio-Rad).

2.2.3 Measurement of extracted DNA concentration

Qubit 3.0 Fluorometric (ThermoFisher Scientific) was used to measure the concentration of DNA after extraction. It was done according to the manufacturer manufacturer's protocol. Qubit® working solution was made by mixing 1µl Qubit® ds DNA HS reagent with 199 µl Qubit® ds DNA HS buffer. 10 µl of DNA was extracted and mixed with 190 µl of the working solution in small tubes. Each tube got vortexed and incubated for 2 minutes prior to the Qubit Fluorometric.

2.2.4   Droplet Digital PCR (ddPCR)

All extracted DNA samples was diluted to approximately 100 pg before the prevalence of antibiotic resistant genes was tested by droplet digital PCR (QX200 ddPCR system, Bio-Rad). To achieve 20 µl of ddPCR mixture, 1 µl of diluted DNA was mixed with 10 µl of supermix, 1 µl primer/ probe and 8 µl of sterile water (Sigma ® Life Science). For the droplet generation a total of 8 different ddPCR mixures was transferred to middle rows of a DG8™ cartridge for a QX200 droplet generator. 70 µl of droplet generation oil for probes (Bio-Rad) was added for each ddPCR mixture in the same cartridge. Each DG8 cartridge generates 8 wells of droplets, representing 8 different ddPCR mixtures. This will make approximately 20 000 nanoliter-sized droplets which distribute the target and background DNA randomly into the droplets during the partitioning process (22). After the generation of droplets, 40 µl of each PCR sample was then transferred into a 96-well microliter plate and sealed with a perusable foil using a plate sealer (PX1TM PCR Plate Sealer, Bio-Rad) at 180°C. A Thermal Cycler (C1000 Touch ™ Bio-Rad) allows the PCR amplification, which happens within each droplet. Following the DNA amplification protocol, the PCR samples was preheated for enzyme activation at 95°C for 10 minutes, then amplificated in following three steps: denaturation at 95°C for 30 s, annealing and extension at 58°C for 1 min. A total of 40 cycles were performed before enzyme deactivation at 98°C for 10 minutes. Finally the plate containing the droplets was transferred to a droplet reader (Droplet Reader QX200™ Bio-Rad), where each droplet was and analysed individually. The generated data were transferred directly into the QuantaSoftTM software where the positive and negative droplet was counted and copy number of the target calculated using Poisson distribution.

Table 1. *Presents the test species, sequences 5´-3´ of forward primer (F), reverse primer (R) and the probe, target gene and amplicon size.*

Test species	Sequences 5´-3	Target gene	Amplicon size
Fusobacterium nucleatum	F-primer: cgcagaaggtgaaagtcctgtat R-primer: tggtcctcactgattcacacaga Probe: FAM-ctttgctcccaagtaacatg gaacacga-BHQ	23S rRNA	101 bp
Prevotella intermedia	F-primer: accagccaagtagcgtgca R-primer: tggaccttccgtattaccgc Probe: FAM-attaaggaccggctaattccgtgccag-BHQ	16S rRNA	153 bp

Table 2. *Presents the genetic targets, sequences 5´-3´ of forward primer (F), reverse primer (R) and the probe, function and amplicon size.*

Genes	Sequences 5´-3	Function	Amplicon size
CfxA	F-primer: tgaccataacaaggctta R-primer: gtttgtttactgaaggtct Probe: HEX-taactatacatctcctcttggtgctg-BHQ	Destruction of β-lactam antibiotics	81 bp
16S rRNA	F-primer: aaactcaaaggaattgacgggg R-primer: agtccgcaacgagcgcaa Probe: FAM-ctgtgtcagctcgtgtcgtga-BHQ	Subunit of bacterial ribosome	203 bp

2.2.5   ddPCR Data Analysis

The data obtained from the droplet reader was analysed using the QuantaSoft^{TM} software. A two-colour detection system measured the number of positive and negative droplets for each fluorophore (probe) in each sample. There are two channels in the QX200 to detect flourophors, i.e. FAM and HEX. This way *P. intermedia* and *F. nucleatum* was detected, respectively. The number and intensity of positive and negative droplets at shown in the 1-D and 2-D plots. Poisson algorithm was used to report the concentration of each genetic target as copies/µl of the final 1 x ddPCR reaction (5).

2.3   Statistical analysis

The data from both the ddPCR the questionnaire were analysed by SPSS Statistical software v24.0 for any significant difference between the study subjects using the nonparametric Mann-Whitney U Test.

2.3.1   Variables retrieved from the questionnaire

Variables for descriptive statistics were retrieved from the questionnaire. A questionnaire from a previous study done at IKO (21) was used.

2.3.3   Variables retrieved from laboratory work

From the laboratory work, following figures was retrieved:

(1) Absolute number of *cfxA* gene copies in each sample, reported as gene copy number per 1 nanogram of sample analysed.

(2) Absolute number of *16S rRNA* gene in sample, reported as gene copy number per 1 nanogram of sample analysed.

(3) Concentration of DNA in each sample, reported as ng/mL of sample analysed

The results were directly transferred from the Quantasoft™ software output to the SPSS file. The outcome variables to report was:

(4) Relative copy number of *cfxA* gene, reported as number of genes per 10^6 copies of *16S rRNA* genes.

(5) Relative copy number of *cfxA* gene, reported as number of genes per 1 nanogram of total DNA analysed.

The laboratory results (1) and (2) were adjusted for dilution of the original sample of extracted DNA. They were therefore multiplied by the dilution factor in order to refer to the initial DNA sample concentration.

To compute the outcome variable (4) the following formula was used:

$$\text{Relative copy number of resistance genes}^1 = \frac{\text{absolute number of cfxA gene copies\textasciicircum2}}{\text{absolute number of 16S rRNA gene\textasciicircum3}}$$

The reason for computing the new (4) variable is that the outcome will represent the number of copies of *cfxA* genes attributed to the bacterial community in the mouth. In contrast, the outcome variable (5) shows number of copies of resistance genes attributed to all DNA extracted from saliva. Variable (5) may therefore include DNA originating from viruses, fungi etc. as well as bacteria.

To compute the outcome variable (5) the following formula was used:

$$\text{Relative copy number of resistant gene}^4 = \frac{\text{absolute number of cfxA gene copies\textasciicircum5}}{\text{DNA consentration\textasciicircum6}}$$

Any differences in copy numbers of *cfxA* genes between the 1st and 5th year students, and experienced clinicians were compared using the Mann-Whitney U Test.

3   Results

3.1   Saliva collection

25 experienced clinicians were asked to be a part of the project. Two were excluded because of use of antibiotics within the last three months, and some didn't want to participate in the project. The saliva samples were collected from 21 subjects. All of the samples had volumes above the threshold, which was set to 5 ml, and they were considered to have a normal secretion (1.00 – 3.00 ml/min). Later it was found that one of the participants had used antibiotics within the last three months, and was therefore excluded after the samples were collected.

3.2   Questionnaire

The questionnaire was used to determine the age of each subject, their health issues or diseases and the use of antibiotics within the last three months. It was also used to investigate knowledge and attitudes among the experienced clinicians in terms of personal oral hygiene, routines at the clinic and the procedures for cleaning various instruments.

The age and general health for each subject varied a lot. The mean age of the subjects was 44 years. There were 70% females and 30% males. All of the participants considered themselves at good or very good general health.

Table 3. *List of answers to the questions included in the questionnaire.*

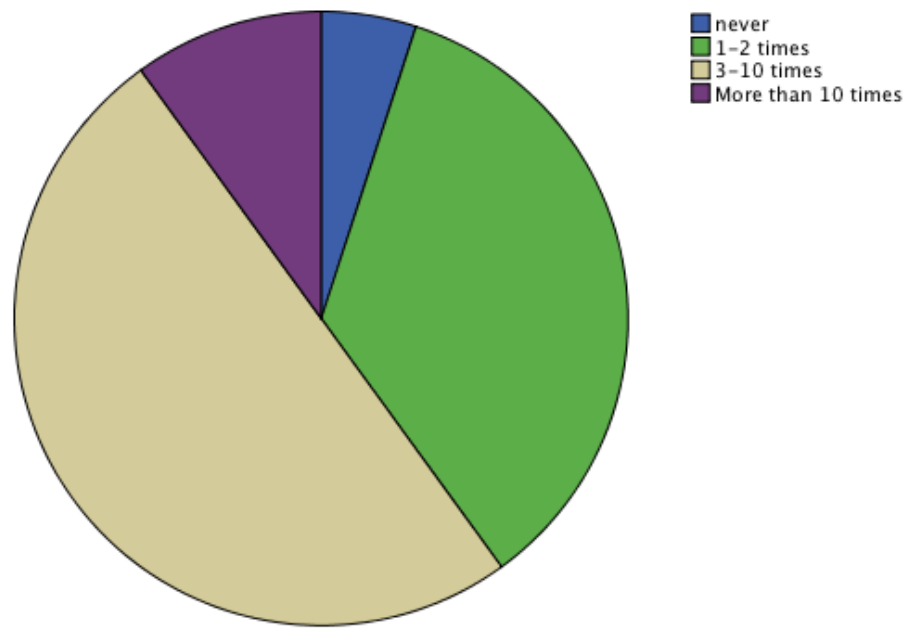|		Experienced clinicians (N=20)
Gender	Females	14 (70,0%)
	Males	7 (30,0%)
Dental health	Very good	11 (55,0%)
	Good	9 (45,0%)
	Neither bad or good	0 (0%)
	Not entirely good	0 (0%)
	Bad	0 (0%)
General health	Very good	11 (55,0%)
	Good	9 (45,0%)
	Neither bad or good	0 (0%)
	Not entirely good	0 (0%)
	Bad	0 (0%)
Frequency of sickness last 2 years	Never	2 (10,0%)
	Seldom	14 (70,0%)
	Occasionally	4 (20,0%)
	Often	0 (0%)
Satisfaction with teeth appearance	Very satisfied	9 (45,0%)
	Fairly satisfied	11 (55,0%)
	Rather dissatisfied	0 (0%)
Medicines daily	None	0 (0%)
	Oral contraceptives	2 (10%)
	Antihistamines	0 (0%)
	Immune-modulators	0 (0%)
	Thyroid hormones	0 (0%)
	Adrenomimetics	0 (0%)
	More than one drug	0 (0%)
Total amount of antibiotic courses in life	Never	1 (5,0%)
	1-2 courses	7 (35,0%)
	3-10 courses	10 (50,0%)
	More than 10 courses	2 (10,0%)

Figure 2. *Reported distribution of antibiotic courses taken throughout life by the experienced clinicians.*

3.3   Bacterial DNA extraction

The volume of saliva used to extract the DNA from was 800μl.

3.4   Gel-electrophoresis

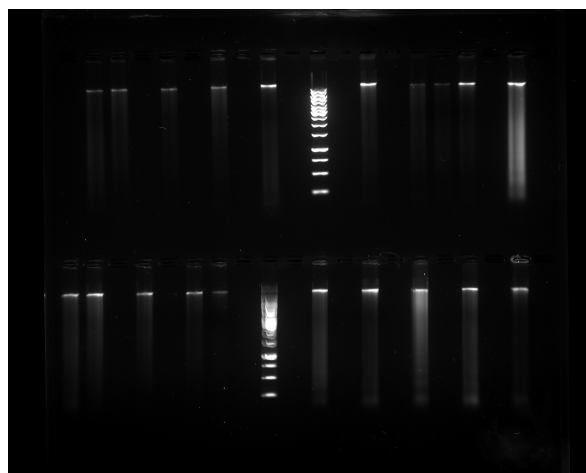The presence of DNA and its molecular size was verified by gel electrophoresis.

Figure 3. *Agarose gel electrophoresis showing DNA yield after DNA extraction*

3.5   Measurement of DNA concentration

The DNA concentrations obtained from the samples of the experienced clinicians are presented in the table below.

Table 4. *Concentrations of extracted DNA in ng/µL obtained from saliva samples of experienced dentists.*

Sample ID	Consentration ng/µL	Sample ID	Consentration ng/µL
D1	1,92	D11	2,38
D2	2,18	D12	3,00
D3	4,68	D13	2,68
D4	6,44	D14	1,54
D5	3,36	D15	1,55
D6	12,00	D16	2,98
D7	6,78	D17	2,86
D8	6,00	D18	2,76
D9	2,28	D20	2,12
D10	1,02	D21	2,40

(Sample D19 were excluded from the analysis because of recent use of antibiotics within the last three months)

3.6   Droplet Digital PCR (ddPCR)

The Quantasoft software analysed the data obtained from the droplet reader, and while the positive droplets (e.g contained at least one copy of the target DNA molecule) exhibit increased fluorescence, the negative do not. Figure 4 and 5 below represents 1-D plots of positive and negative separation of *cfxA* gene of one DNA sample. The positive droplets are located above the pink threshold line, while negative located below. The threshold line was changed for some of the samples to ensure satisfying separation, thus the different amplitude intensity.
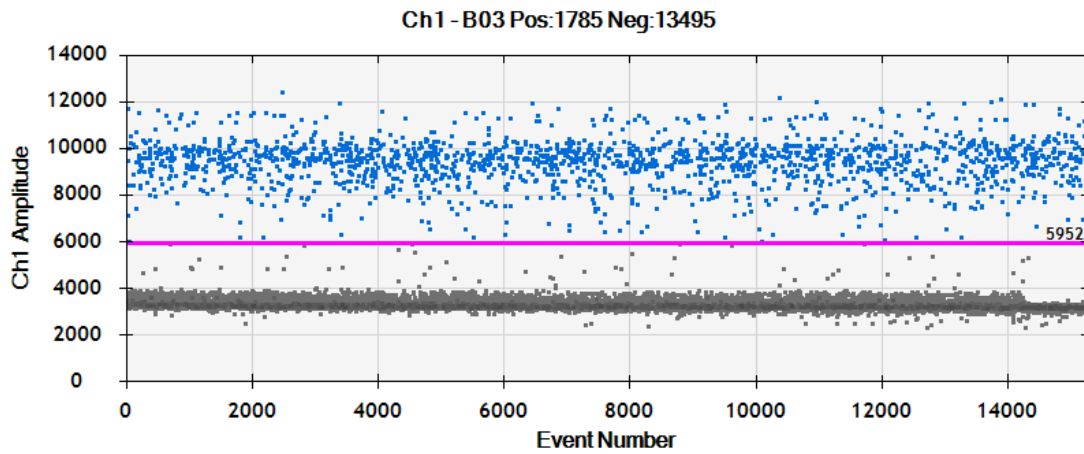
Figure 4. *1-D plot of a sample tested for the presence and levels of a gene 16S rRNA by the use of FAM-tagged probe. The plot illustrates the positive droplets located above the purple threshold line, and the negative droplets below.*
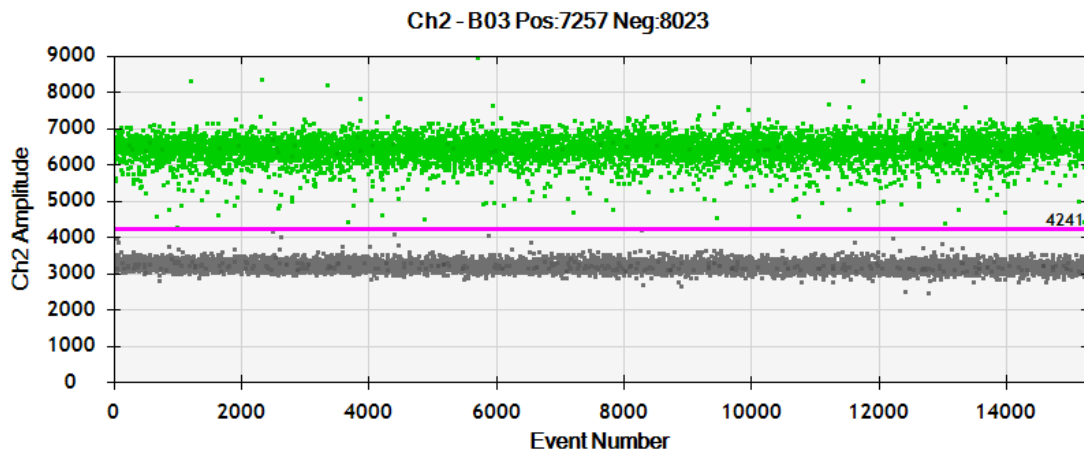

Figure 5. *1-D plot of a sample tested for the presence and levels of a gene cfxA by the use of a HEX-tagged probe. The plot illustrates the positive droplets located above the purple threshold line, and the negative droplets below.*
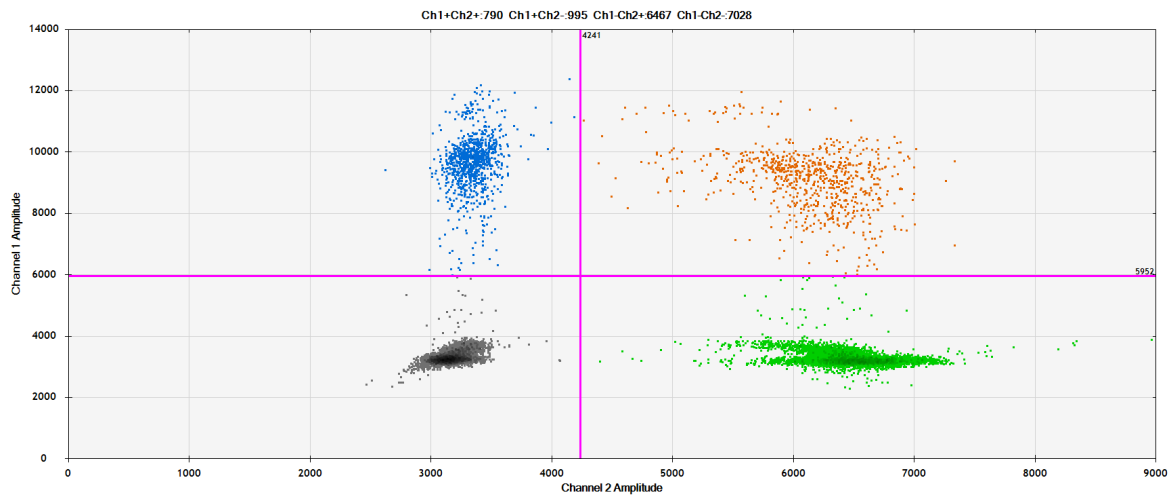
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Figure 6. *2-D plot with droplet clustered at 4 different groups. Upper left representing FAM positive and HEX negative droplets, upper right double positive droplets, lower left double negative droplete, lower right HEX positive and FAM negative droplets.*

A 2-D plot is another way to illustrate the obtained data from the ddPCR. As figure 6 shows, the droplets are plotted in 4 clusters, illustrating FAM positive and HEX negative, FAM positive and HEX positive (double positive) HEX positive and FAM negative, and HEX negative and FAM negative (double negative).

The upper right cluster demonstrates droplets containing double positive, i.e. two target genes in the same droplet. In this study it of interest to detect double positive droplets for the *cfxA* gene and *P. intermedia/ F. nucleatum*, and thus linkage between these, but it could not be found.

3.7   Data Analysis

Data obtained from the ddPCR experiment for the 20 subjects are presented in table 2. The data is presented as the level of *cfxA* genes detected per 1 ng of DNA obtained from saliva. It also reports the level of *cfxA* gene among the tested samples in relation to the total bacterial population using *16S rRNA* gene as a measurement unit for counting bacterial species present in saliva. The latter gives greater information about the presence of *cfxA* gene in bacteria as it exclude any *cfxA* gene detected from fungi, virus etc. The statistical analysis reveals no significant difference in the presence of *cfxA* gene between experienced clinicians and students ($P > 0,05$).

Table 5. *Median values of cfxA gene detected in the study populations expressed as either cfxA gene per one nanogram of DNA or cfxA gene per 10^6 copies of 16S rRNA.*

Sample population	Experienced clinicians (N=20)
cfxA/ng[1] DNA	13095.000
(max[2], min[3], SD[4])	168900.000, 290.000, 44552.639
cfxA/ 10^6 16S rRNA	19548.850
(max[2], min[3], SD[4])	104706.800, 1219.900, 30668.338

1: Nanogram; 2; Maximum; 3: Minimum; 4: Standard deviation

CfxA gene was detected in all participants. The values of copy number per 10^6 copies of *16S rRNA* gene was categorised as low, medium or high presence of *cfxA* gene. Samples containing copy number between 1 and 9999 were classified as low, between 10000 and 29999 as medium, and ≥30000 as high. According to this classification, the distribution of the *cfxA* gene in the low, medium and high groups were 6 (30%), 6 (30%) and 8 (40%), respectively.

Table 6. *Percentage of samples categorised as low, medium and high presence of cfxA gene per 10^6 copies of 16S rRNA.*

Categories	Low (1-9999 copies)	Medium (10000-29999 copies)	High (≥30000 copies)
cfxA	6 (30%)	6 (30%)	8 (40%)

The use of more than 10 courses of antibiotics throughout life didn't show a significant association with detected *cfxA* gene copies in the DNA analysed. Also, there was not found any significant association between the use of more than 10 courses of antibiotics throughout life and the level of *cfxA* gene per 10^6 copies of *16S rRNA*

4   Discussion

The aims of this study were to detect levels of antimicrobial resistant gene *cfxA* collected from saliva samples, and to investigate the linkage between the gene and the two chosen bacteria, *P. intermedia* and *F. nucleatum*. In addition, it was also aimed to compare the levels of the *cfxA* gene within the group of experienced clinicians, and to compare them with 1st and 5th year dental students reported previously.

As previously mentioned, resistance to penicillins is expressed through the production of β-lactamase enzymes produced by the *cfxA* gene. *CfxA* is one of the most reported genes in oral bacteria to confer resistance to β-lactam antibiotics and this is why *cfxA* is chosen in this study. There are 6 different types of the *cfxA* gene (22, 23), and the primer used in this study was designed to target all. Every copy of *cfxA* gene represents one resistant gene. The *16S rRNA* gene copy number was used as a representative of total bacterial level in the saliva samples. The gene has its disadvantages, as it can appear more than once in bacteria, e.g. in *P. intermedia* it appears 4 times. For simplicity, it's commonly known that one copy of *16S rRNA* gene is considered to represent one bacterium when working with samples with mixed bacteria.

The hypothesis that *P. intermedia* and *F. nucleatum* are mostly associated with β-lactamase resistant genes could not be ascertained. It was found high levels of the *cfxA* gene (40% of subjects had 30000 copies or more), but no linkage between the *cfxA* gene and the two bacteria *P. intermedia* and *F. nucleatum* was detected. This does not mean there is no association, only that it couldn't be proved in this study with the current experimental design. To find a linkage between the *cfxA* gene and bacteria in question, the two target genes have to be sufficiently close in the DNA sequence (see figure 7). In addition, for the primer to get access to the target gene, the enzyme BsuRI was used to cut the genomic DNA in our experiments and the probability for the two target genes to be in the same cut piece is lower the bigger the distance between them.

Bacteria also contain smaller circular DNA molecules, called plasmids. It's a possibility that the *cfxA* gene is located in the bacterial plasmid instead of the bacterial chromosome. This means that the two target genes already will be separated before the enzyme cuts the bacterial DNA, and thus it is impossible to find linkage with the experimental design used in this study.
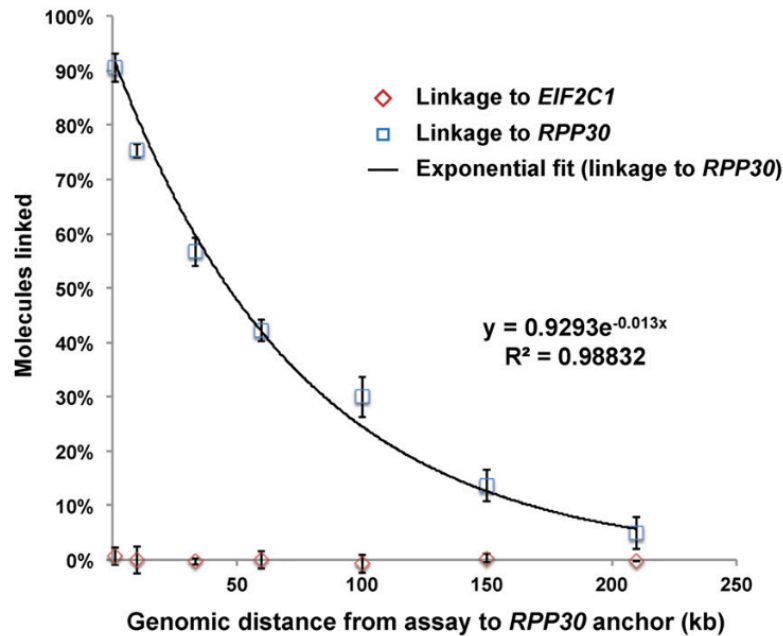
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Figure 7. The percentage of linked molecules at each genomic distance is shown as a function of distance. Adopted from Regan et al 2015.

The resistant gene *cfxA* was present in 100% of the samples. This could be explained by the high use of β-lactam antibiotics in the society. In Norway, penicillins are the first choice when treating bacterial infections that requires antibiotics (3). Accumulation of resistant genes in the oral cavity is multifactorial. In addition to repeated antibiotic courses due to infections, other risk factors include antibiotics misuse, cross infection, and traveling overseas.

The other hypothesis was that we would find a significant difference in *cfxA* gene levels between experienced clinicians and 1st and 5th year dental students. The thought was that experienced clinicians are more exposed to patients, and therefore they are more prone to cross contamination of pathogens with resistant genes. It's worth to comment that 40 % of the experienced clinicians had samples categorised as high presence of *cfxA* gene per 10^6 copies of *16s rRNA,* compared to the inexperienced students that had 18% of the samples categorised as high. In addition, the median value of *cfxA* per ng of DNA and *cfxA* per 10^6 copies of *16S rRNA* in the experienced participants samples was almost the double of that found in student samples. However, this study did not find a significant difference between the experienced clinicians and students (P>0,05). One reason for this could be that the sample size was too small to detect any significant differences between the two groups. Limited time and access to participants determined the final sample size.

The use of antibiotics among the participants was also investigated. It was thought that the higher levels of *cfxA* gene would be associated with more use of antibiotics. Up to 50% of the participants have had 3-10 antibiotic courses, and 10% more then 10 courses. There was no correlation between detected levels of *cfxA* gene and more use of antibiotics, therefore we cannot conclude that the use of antibiotics will affect the levels of *cfxA* gene.

In future studies it would be interesting to look at other methods to find linkage between the *cfxA* gene and any given bacteria. In our study the genomic DNA was most probably chopped up during extraction procedure before the digital droplet. This will complicate the probability to find any linkage, as small pieces of bacterial DNA is distributed in multiple droplets before ddPCR. One possible approach could be that the ddPCR is performed without genomic DNA extraction but rather with intact cells that could be lysed inside the droplet to release DNA. In this way the whole bacterial DNA will be secured in the same droplet, and if linkage is present, it would probably be revealed easily.

There is a lack in the literature of studies with similar methodology as our study. There is no data available in the literature about the linkage between the *cfxA* gene and the two chosen bacteria in mixed bacterial population. When comparing our result with other studies we could not find anything that associates *F. nucleatum* with the *cfxA* gene. However, *P. intermedia* has been reported to be associated with the *cfxA* gene multiple times (24, 25). To the best of our knowledge, there is no other study that investigates the levels of *cfxA* gene from saliva samples among experienced dental clinicians. Therefore, it would be interesting to design a study that includes a bigger sample size to see if there are any significant differences in the prevalence of *cfxA* gene collected from saliva between experienced clinicians and an inexperienced subject group.

5 Conclusion

This is the second study in Norway that investigates the presence and levels of antibiotic resistance genes, namely *cfxA,* in saliva samples. This is a follow-up study to a previous master thesis from the IKO at UiT, The Artic University of Norway. This study investigated two specific bacteria in relation to the *cfxA* gene, in contrast to unspecific bacteria in the aforementioned study.

In this study we investigated the presence and association between the antibiotic resistance gene *cfxA,* and the two chosen bacteria, *P. intermedia* and *F. nucleatum.* In addition, experienced clinicians were compared with 1st and 5th year students for the *cfxA* gene levels.

This study could not conclude that *P. intermedia* and *F. nucleatum* are mostly associated with the *cfxA* resistance gene. There was not found any linkage between the two bacteria and *cfxA* gene.

High levels of *cfxA* gene was detected among the experienced clinicians when compared with 1st and 5th year students, however, this was not found to be statistically significant, therefore we cannot conclude that working in the dental clinic, exposed to patients, is a risk factor for the transmission of bacteria with *cfxA* gene.

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Appendix I

Utbredelse av antimikrobielle resistensgener i spytt blant tannlegestudenter og tannleger

Dette er et spørsmål til deg om å delta i et forskningsprosjekt for å studere forekomst av antimikrobielle resistensgener i spytt, samt å vurdere sammenhengen mellom utbredelsen av disse genene og hygienevaner og tannleger/tannlegestudenters holdninger til bruk av smittevernutstyr. Bakterier og resistens-gener utveksles mellom ulike miljøer. Det er ukjent hvor hyppig resistente mikrober forekommer i befolkningen generelt og hvilke faktorer som bidrar til å utvikle resistens. Til tross for gode hygienevaner, kan tannleger/ tannlege-studenter ha en økt smitterisiko fordi de arbeider tett på mennesker og bruker utstyr som kan spre mikrober – for eksempel air-rotor. En problemstilling i studien er: Er utbredelsen av resistensgener avhengig av hvor lenge tannlegen/tannlegestudenten har vært i klinisk praksis?

Personene som blir inviterte til å delta i studien må ha generelt god helse og ikke ha tatt antibiotika de siste 3 månedene. Din medvirkning vil ta omtrent 20 min (informasjon om studien, spyttprøve og spørreskjema).

Forskningsansvarlig skal være instituttleder ved Institutt for klinisk odontologi (IKO) UiT, Claes-Göran Crossner. Prosjektleder er Mohammed Al-Haroni, tannlege og førsteamanuensis ved IKO, UiT.

HVA INNEBÆRER PROSJEKTET?

Vi vil samle inn spytt fra 1. års tannlegestudenter som enda ikke har behandlet pasienter og fra 5. års studenter/ instruktørtannleger ved IKO med klinisk erfaring. Du plasseres i et lyst rom i en stol med rett rygg. Du skal ikke ha spist, drukket, røykt eller hatt noe som helst i munnen den siste timen før undersøkelsen, og du skal ikke ha tatt andre medikamenter enn de «vanlige» kvelden før eller samme dag som undersøkelsen finner sted. Vi vil notere alle medikamentene som du vanligvis bruker. Du vil sitte stille i ca. 5 min. før prøven tas. I denne tiden skal du fortrinnsvis ikke snakke, men konsentrere deg om å roe ned kroppen. Eventuelle tannproteser beholdes i munnen. Parafinvoks (smakløs «kloss») tygges i 30 sek. slik at den blir myk. Spyttet svelges før testen begynner. Deretter må du IKKE svelge mens du samler spytt. Du skal tygge under hele testen (5 min), som om du spiser mat, litt på hver side. Du skal spytte regelmessig i et oppsamlingsbeger med glasstrakt. Deltakeren vil få sitt resultat umiddelbart. Dersom verdien er utenfor normalområdet, vil du bli oppmuntret til å ta kontakt med tannlege.

Referanseverdier for stimulert helsaliva	
0,70 – 0,99 ml/minutt	Lav sekresjon
1,00 – 3,00 ml/minutt	Normal sekresjon

Denne måten å samle inn spytt fra en pasient er den normale måten å samle inn spytt i en klinisk situasjon.

I prosjektet vil vi innhente og registrere opplysninger om deg. Kun informasjon som du selv gir til oss gjennom spørreskjema om personalia, tannhelseerfaring, holdninger og kunnskap, vil bli benyttet i studien.

MULIGE FORDELER OG ULEMPER

Deltakerne vil få individuell tilbakemelding om sin spyttproduksjon og informasjon om antimikrobiell resistens status. Det er så og si ingen ubehag, risiko eller kjente bivirkninger knyttet til å avgi en spyttprøve.

Appendix I

Det er frivillig å delta i prosjektet. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke. Dersom du trekker deg fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke deg eller har spørsmål til prosjektet, kan du kontakte Førsteamanuensis Mohammed Al-Haroni, tlf. 77649151, e-mail: mohammed.al-haroni@uit.no

HVA SKJER MED INFORMASJONEN OM DEG?

Informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Du har rett til innsyn i hvilke opplysninger som er registrert om deg og rett til å få korrigert eventuelle feil i de opplysningene som er registrert.

Kun deltakere fra 1. kull og instruktørtannleger ved IKO sine navn vil bli noterte, for at vi skal kunne følge opp informasjon fra disse personene etter 5 år. Alle deltakere vil tildeles en ID-kode. Denne koden knytter deg til dine opplysninger, og eventuelt til ditt navn. Koblingslisten mellom navn og kode oppbevares separat. I analysefasen vil alle opplysningene bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger.

Prosjektleder har ansvar for den daglige driften av forskningsprosjektet og at opplysninger om deg blir behandlet på en sikker måte. All informasjon om deg vil bli anonymisert og slettet senest fem år etter prosjektslutt. Studien avsluttes i november/desember 2020.

HVA SKJER MED PRØVER SOM BLIR TATT AV DEG?

Spyttprøven som tas av deg skal oppbevares i en forskningsbiobank under navnet *Saliva-Oral Ecology-IKO*. Biobank-ansvarlig skal være Instituttleder ved Institutt for klinisk odontologi (IKO), UiT; Claes-Göran Crossner. Vi vil kun studere DNA fra de bakteriene vi finner i spyttet ditt. Humant DNA fra dine celler vil hverken bli studert eller lagret i Biobanken.

Biobanken opphører etter prosjektslutt. Studien avsluttes etter at vi har samlet inn og analysert spytt for 2. gang; i november/desember 2020.

HVA SLAGS INFORMASJON KAN DE GENETISKE UNDERSØKELSENE I PROSJEKTET GI?

Formålet med Biobanken er å oppbevare innsamlede spytt-prøver og DNA fra de bakteriene vi finner i spyttet ditt, for å studere hvorvidt spytt-bakteriene dine er resistente mot antibiotika og for at vi skal kunne sammenligne resultatet med prøver som blir tatt fra tilsvarende grupper av tannlegestudenter og instruktørtannleger om 5 år.

- Tilbakemelding til deltager
  Dersom du ønske det vil Prosjektleder gi deg en individuell, muntlig tilbakemelding om hvorvidt bakteriene vi finner i spyttet ditt er resistente mot antibiotika, etter at de genetiske analysene er gjennomført.
- Tilfeldige funn
  Prosjektet skal ikke studere DNA i de andre cellene dine.

FORSIKRING

Som for all behandling av helse- og tannhelsepersonell i Norge er deltakerne i studien dekket av Norsk Pasientskadeerstatning (NPE).

Appendix I

SAMTYKKE TIL DELTAKELSE I PROSJEKTET

JEG ER VILLIG TIL Å DELTA I PROSJEKTET

Sted og dato   Deltakers signatur

                                                    Deltakers navn med trykte bokstaver

Appendix II

SPØRRESKJEMA

UTBREDELSE AV ANTIMIKROBIELLE RESISTENSGENER I SPYTT I EN TANNLEGESTUDENT POPULASJON

GENERELL INFORMASJON

1. Kjønn?

1 ☐ Kvinne
2 ☐ Mann

2. Når er du født?

Årstall:

3. Hvilket studieår/arbeidsfunksjon er du i?

1 ☐ 1. studieår (odontologi)
2 ☐ 5. studieår (odontologi)
3 ☐ Klinisk veileder (studentklinikken, IKO)

4. Hvordan er din generelle helsetilstand nå?

1 ☐ Svært god
2 ☐ God
3 ☐ Verken god eller dårlig
4 ☐ Ikke helt god
5 ☐ Dårlig

5. De siste to årene – har du ofte vært syk?

1 ☐ Aldri syk
2 ☐ En sjelden gang
3 ☐ En gang i mellom
4 ☐ Ofte syk
5 ☐ Flere ganger alvorlig syk

6. Hvordan er din tannhelse nå?

1 ☐ Svært god
2 ☐ God
3 ☐ Verken god eller dårlig
4 ☐ Ikke helt god
5 ☐ Dårlig

7. Er du fornøyd med utseende til tennene dine?

1 ☐ Svært fornøyd
2 ☐ Noen lunde fornøyd
3 ☐ Temmelig misfornøyd
4 ☐ Svært misfornøyd

8. Bruker du medikamenter/medisiner daglig?
   I tilfelle ja, hvilke?

9. Har du tatt antibiotika i løpet av de siste 3 månedene?

1 ☐ Ja
2 ☐ Nei
3 ☐ Usikker

10. Hvor mange ganger gjennom livet har du tatt en antibiotika-kur?

1 ☐ Aldri
2 ☐ 1-2 ganger
3 ☐ 3-10 ganger
4 ☐ Mer enn 10 ganger

11. I hvilken grad er du plaget med:
(*1:aldri ---------- 4:svært mye*)

	(1)	(2)	(3)	(4)
Føler du at du ofte er tørr i munnen?	☐	☐	☐	☐
Føler du deg tørr i munnen når du spiser?	☐	☐	☐	☐
Har du ofte problemer med kjeveleddet?	☐	☐	☐	☐
Smaker ofte maten lite?	☐	☐	☐	☐

1

Appendix II

*12. De 5 påstandene nedenfor refererer til hvordan du har følt deg i løpet av **de siste 2 ukene**.*
(Sett en ring ved hver påstand – rundt det tallet som passer best for deg)

		Hele tiden	Ofte	Mer enn halve tiden	Mindre enn halve tiden	Sjelden	Aldri
A	Jeg føler meg trist og nedfor	1	2	3	4	5	6
B	Jeg føler meg rolig og avslappet	1	2	3	4	5	6
C	Jeg føler meg energisk, sprek og aktiv	1	2	3	4	5	6
D	Jeg våkner opp og føler jeg meg frisk og uthvilt	1	2	3	4	5	6
E	Hverdagen min er full av ting som interesserer meg	1	2	3	4	5	6

15. Har du en kronisk sykdom som innebærer at du jevnlig må ha medikamentell behandling?
(Antibiotika eller annen medisin)

1 ☐ Ja
2 ☐ Nei
3 ☐ Usikker

16. Røyker du? Om ja, hvor ofte?

1 ☐ Røyker hver dag
2 ☐ Røyker av og til
3 ☐ Røyker aldri

17. Hvor lenge har du røykt?

1 ☐ Jeg røyker ikke
2 ☐ Mindre enn i 3 år
3 ☐ I 3 eller flere år

18. Snuser du? Om ja, hvor mye?

1 ☐ Snuser hver dag
2 ☐ Snuser av og til
3 ☐ Snuser aldri

19. Hvor lenge har du snust?

1 ☐ Jeg snuser ikke
2 ☐ Mindre enn i 3 år
3 ☐ I 3 eller flere år

20. Hvor mange sigaretter/snus porsjoner per dag?

Antall sigaretter:

Antall snusporsjoner:

PERSONLIG HYGIENE

21. Hvor ofte pusser du tennene dine?

1 ☐ Morgen og kveld
2 ☐ En gang per dag
3 ☐ En gang i blant

22. Hvor ofte bruker du tanntråd/tannstikker?

1 ☐ Etter hver tannpuss
2 ☐ En gang per dag
3 ☐ En gang per uke
4 ☐ Sjeldnere
5 ☐ Aldri

23. Hvor ofte vasker du hendene dine (i klinikken)?
(Merk: her kan du gi flere svar)

1 ☐ Etter telefonbruk
2 ☐ Etter å ha skrevet journal
3 ☐ Etter å ha tatt røntgenbilde
4 ☐ Etter hver pasient
5 ☐ Før hver pasient
6 ☐ Prøver å begrense antall håndvask

Appendix II

24. Anser du at tannleger er under større risiko for smittespredning enn «folk flest»?

1 ☐ Ja, mer enn de fleste
2 ☐ Som «folk flest»
3 ☐ Nei, mindre enn de fleste
4 ☐ Vanskelig å besvare

25. Hvem er mest utsatt for smitte på et tannlegekontor?

1 ☐ Tannlegen
2 ☐ Pasienten
3 ☐ Tannhelsesekretæren
4 ☐ Vanskelig å besvare

26. Kjenner du til/har hørt om tilfeller der en pasient har blitt smittet etter et tannlegebesøk?

1 ☐ Ja
2 ☐ Nei

27. Kjenner du til/har hørt om tilfeller der tannlegen har blitt smittet på tannlegekontoret?

1 ☐ Ja
2 ☐ Nei

28. Tenker du at tannlegen kan beskytte seg mot smitte?

1 ☐ I svært stor grad
2 ☐ I stor grad
3 ☐ Usikker
4 ☐ I liten grad
5 ☐ Overhode ikke

29. Hva er det viktigste enkelt-tiltaket mot smitte?

1 ☐ Unngå nærkontakt med pasient (dråpesmitte)
2 ☐ God håndhygiene
3 ☐ Engangsartiklene
4 ☐ God rengjøring av utstyr
5 ☐ Sikre rutinger for å deponere klinisk avfall
6 ☐ Vanskelig å besvare

30. På hvilken måte kan tannlegen beskytte seg når han/hun har en «vanlig» pasient i stolen? (Merk: her kan du gi flere svar)

1 ☐ Sprite alle arbeidsflater mellom hver pasient
2 ☐ Godt såpe-håndvask mellom hver pasient
3 ☐ Godt håndvask med sprit mellom hver pasient
4 ☐ Ved å bruke engangsartikler som munnbind, hansker, kofferdam, plastfolie etc.
5 ☐ Ved å avstå fra å bruke f.eks. «air-rotor»
6 ☐ Ved å bruke spesielt egnet arbeidstøy
7 ☐ Ved å bruke øyebeskyttelse/ visir
8 ☐ Ved å bruke papirservietter og plast-hetter
9 ☐ Ved å alltid bruke assistent ved stolen
10 ☐ Ved å alltid spyle igjennom vann i treveis-sprøyten og drikkevannslangen før neste pasient
11 ☐ Ved å alltid la pasienten skylle munnen i ca. 1 minutt med munnskyllevæske
12 ☐ Ved å ikke berøre pasienten uten verneutstyr
13 ☐ På annen måte
14 ☐ Tannlegen kan ikke beskytte seg mot smitte
15 ☐ Vanskelig å besvare

31. Kan tannlegen eliminere smitterisiko ved å følge «hygieneveilederen»?

1 ☐ Ja
2 ☐ Nei
3 ☐ Vanskelig å besvare

32. Hva er viktige barrierer for god hygiene på tannklinikken?

1 ☐ Glemsomhet
2 ☐ Behandlingen tar lengre tid
3 ☐ Ubekvemme arbeidsforhold
4 ☐ Manglende kunnskap om smittevern
5 ☐ Pasienten forteller ikke om mulige smitte