Antibiotic resistance in healthcare settings:
prevalence and levels of tet(M) resistance gene in saliva of dental students

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Abstract

Rationale: Antibiotic resistance compromises achievements of modern medicine and is a major threat to the society. The oral cavity has the distinct microbial community that serves as a reservoir for determinants of antibiotic resistance. Tet(M) gene is abundant in bacteria residing in the oral cavity and is often identified in oral specimens. Absolute quantification of resistance genes copies allows quantitative studying the factors possibly associated with these numbers. Bacterial aerosol at the workplace may pose occupational risk. Exposure to aerosolized bacteria may contribute to increased resistance genes copy numbers in oral bacteria of dental personnel.

Objective: To investigate the prevalence and levels of tet(M) resistance gene in saliva samples collected from two observational groups and any associated factors that might influence the levels of tet(M).

Methods: Saliva samples were collected from 83 healthy dental students of Institute of clinical dentistry, UiT, Norway, in the period from 1 June to 27 November 2015. The study participants were grouped by their study year: 41 newly recruited students and 42 senior students. The latter were exposed to dental office environment as a part of the practice skill exercises. The quantification of tet(M) gene copies in DNA extracted from the saliva samples was done using droplet digital PCR (ddPCR) methodology. Self-administered questionnaires were distributed in order to obtain demographic and health-related variables. To find out whether the two observational groups had different number of tet(M) gene copies, Mann-Whitney-Wilcoxon test was used. Pearson's correlation coefficient and the linear regression analysis were used to reveal any association between antibiotic consumption and tet(M) gene copy numbers found in the samples.

Results: Each saliva sample was positive for tet(M) gene (100% prevalence). The number of tet(M) gene copies was not significantly different between the two groups with and without exposure to
dental office environment. The history of antibiotic courses taken in the past was not correlated with tet(M) gene copy number.

**Conclusion:** exposure to dental office environment does not influence significantly the tet(M) gene copy number in saliva samples in the study population. The number of antibiotic courses did not seem to be associated with the tet(M) copy numbers.
Abbreviations

AR  antibiotic resistance
ARGs  antibiotic resistance genes
CFU  colony-forming unit
CVR  covariance ratio
ddPCR  droplet digital polymerase chain reaction
DNA  deoxyribonucleic acid
DUWS  from dental unit water system
GP  general practitioner
HGT  horizontal gene transfer
HVE  high-volume evacuator
IKO  Institute of Clinical Dentistry
MIC  minimum inhibitory concentration
μL  microliter
MRSA  methicillin resistant *Staphylococcus aureus*
MSIS  Surveillance System of Communicable Diseases
ng  nanogram
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NorPD</td>
<td>Norwegian prescription database</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Q</td>
<td>question</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence unit</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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1 Introduction

1.1 Antibiotic resistance - general terms

1.1.1 Antibiotic resistance (AR)

The threatening status of antibiotic resistance has been recognised and raised highly on the agenda among international health authorities. Still, the current epidemiologic figures are immense. The latest WHO report reveals that the prevalence of antibiotic resistance in some clinically significant bacteria may exceed 50% in many countries of the WHO regions (1). Recent surveys put the annual death toll attributed to antibiotic resistance at 23,000 lives for the US. The situation is even worse in the EU countries. Around 25,000 people die every year as a consequence of untreated infections (2, 3). Aside from human costs, AR entails huge economic losses. The direct healthcare costs and the cost of productivity losses in the US are estimated at $ 20-35 billion and $ 35 billion per year, respectively (4, 5). Yet these figures are likely to be underestimated. According to Smith and Coast, there are certain limitations in health economic studies of antibiotic resistance (5). The authors have estimated that a hip-replacement surgery without antibiotic prophylaxis might lead to two times higher probability of postoperative infection development. The consequent mortality was calculated to be 30% higher under the designed scenario (5). Such economic modelling is difficult to apply to the diversity of medical cases where antibiotics are involved (5). Branches of medicine such as surgery, obstetrics, and oncology are dependent upon the effectiveness of antibiotic treatment (5-7) and it is difficult to estimate the actual cost of antibiotic loss (5). Nonetheless, the current knowledge of the economic losses caused by antibiotic resistance should indicate how big the problem already is.

Apart from the current state of bacterial resistance to antibiotics, the prospect of the post-antibiotic era may imply dramatic changes for society. Even though much effort has been done in tackling the issue,
it is widely accepted that the achievements are not enough to restrain the spread and maintenance of antibiotic resistance rates. In a number of publications the post-antibiotic era is regarded as nothing but an unavoidable reality (1, 6, 8). This literally means our society may roll back to the times where a minor skin injury could lead to deadly complications. Public health bodies appeal to fight AR immediately (1, 2). The passive acceptance of the problem can compromise the status of modern medicine - inaction may become a critical mistake for the well-being of societies (1, 5, 6, 8).

In essence, AR is the ability of bacteria to survive the antibiotic treatment. The property of pathogenic bacteria to resist antibiotic therapy became evident soon after the invention of antibiotics. An enzyme capable of undermining the effect of penicillin was discovered even before the introduction of the drug to a wide human use (6). Apart from developing adaptation to one type of antibiotic agents, bacteria have managed to upgrade their protective mechanism(s) so that they could survive treatment with several antibiotics (6). Recent findings have shown the existence of bacterial strains which are totally drug resistant, even to drugs of last resort (6, 9).

While the "antibiotic resistance" term comprises the resistance of bacteria to antibiotics, antimicrobial resistance refers to the resistance of a number of microorganisms, such as bacteria, fungi, viruses, and parasites, to antimicrobial agents (1). In the present work, it has been chosen to use the term "antibiotic resistance" given that the study deals with tet(M)-mediated bacterial resistance to tetracycline. Further on, all antibacterial and antimicrobial agents are referred as "antibiotics".

The resistant bacterial strains pose a major concern for clinicians, patients and public health authorities as they become increasingly observed both in hospital and community settings. Ineffective antibacterial treatment due to AR demands alternative therapeutic options, frequently more expensive (and not readily available). Failures of a first-line treatment also lead to higher rates of mortality and
increase in days of stay under treatment (1). Not only have these pernicious bacterial properties compromised the success of infectious diseases' treatment, but the outcomes of some modern surgical procedures, such as organ transplantation or implantation of medical devices, have become more uncertain (6, 7). Anti-cancer therapy may in some cases do more harm than good if antibiotics have no effect (7).

1.1.2 Antibiotic resistance classification.

There are several types of antibiotic resistance described in literature. Primarily, resistance can be either intrinsic or acquired (6, 7, 10). In addition, some bacteria are phenotypically refractory to antibiotic challenge. This type of resistance, known as non-inherited, is caused by structural and physiological adaptations of bacterial populations to antibiotic's action (11). This phenomenon occurs, for example, due to ability of bacteria to form biofilm - a bacterial community residing on solid surfaces of bodily tissues, implanted devices, etc. (11). The dental biofilm is an example of such communities (12). Biofilms may contain several bacterial species, the interaction of which results in altered properties allowing to withstand exposure to antibiotics (11).

It is well accepted that the acquired resistance is primarily responsible for maintenance and expansion of antibiotic resistance at levels concerning public health authorities worldwide (6, 7, 10). Apart from the abovementioned classification, which is based on the mechanisms of antibiotic resistance, AR can be either clinical or and microbiological. The clinical AR refers to the condition where an adequate antibiotic therapy against an infectious disease, in terms of dosage and schedule of administration, does not cure a patient (13). In contrast, the microbiological antibiotic resistance refers to the presence of mechanisms conferring the resistance in bacterial isolates tested in the laboratory (13). However, the detection of the resistance mechanisms among bacteria in the laboratory may or may not correlate with clinical resistance (14).
1.1.3 Genetic determinants of antibiotic resistance

According to S.B. Levy, the development of antibiotic resistance principally can be expected where the co-occurrence of two main factors is presented: an antibiotic and the genetic prerequisites within the bacterial genome, namely, the antibiotic resistance genes (ARGs) (8). Interactions of these two elements are extensively investigated and, to date, it is widely accepted that bacteria either can acquire the drug resistance through transfer of genes of resistance or through mutation in the bacterial genome (6-8). The resistance genes do not travel alone - they are disseminated via one of the mobile genetic elements: plasmid, transposon, integron, naked DNA, and bacteriophages (8). All these means of resistance gene transmission can be united under the term of horizontal gene transfer (HGT) (10). Mutations and HGT are considered to be the main genetic mechanisms responsible for ARGs dissemination (10, 15).

Mutation of bacterial DNA is the alteration within the bacterial genome so that new properties are acquired (10). The mutant bacterial strains spread the altered genetic information through the passage of genes to its offspring, i.e. vertical gene transfer (VGT) (16).

Mutations to resist antibiotics can be exemplified by a study of a Mycobacterium tuberculosis strain where 29 mutations have been observed during prolonged treatment with use of a combination of antibiotics (6). The susceptible M. tuberculosis strain evolved to multidrug resistance strains in order to survive the treatment. Such a rapid reaction against its extinction may be explained by the presence of genes that have potential to be altered/mutated, proto-resistant genes (15), and selection pressure exposed to the bacteria by applying antibiotics (14). This kind of selection follows the same Darwinian principle where the fittest organisms proliferate, with an amendment, it is a human-made scenario (6). HGT is considered the most contributing factor to the dissemination of antibiotic resistance globally (10). While the vertical gene transfer is responsible for passing parental genes to offspring, HGT is a sophisticated mechanism of transferring genetic information that makes bacteria able to obtain genes within a single generation. When the resistance genes are acquired, bacteria are able to recombine
them in order to obtain new properties such as resistance or/and virulence (17). The passage of the genes is not constrained within certain genera of bacteria - it has been documented that it may occur between different species (12, 17). HGT and mutations, the two main mechanisms of acquired resistance occurrence, have evolved due to high selection pressure by contemporary antibiotics applications (6, 15).

The origins of the resistant genes are not entirely understood. It has been hypothesized that ARGs originate either from bacteria of normal flora or from environmental bacterial populations (6, 7). The concept of antibiotic resistome was proposed by Davies (6) and Wright (15). The resistome is the collection of those genes that are responsible for conferring antibiotic resistance in bacteria (15). It is not surprising to know that microbial world is full of rivalries for existence. The bacterial production of toxic compounds to kill other bacteria was estimated to occur as early as 3.4 billion years ago (18). The development of resistance to bacterial toxins has occurred as a natural response in order to survive in the aggressive environment (15). Some unspecific bacterial defensive mechanisms such as reduction of concentration of toxins in the cell and prevention of toxins entering the cell existed even before discovery of antibiotics (6, 15).

According to Davies, the emergence of resistance to specific toxins might be brought about by anthropogenic activity. The hypothesis states that ARGs development may be a co-evolutionary response of environmental bacteria confronting a variety of pollutants which has saturated our planet since the beginning of industrial era (6). Still, the origin of ARGs remains unclear (7, 10).

1.1.4 Factors that promote antibiotic resistance

A number of studies and surveillance records have reported that the overall amount of antibiotics consumed is positively correlated with the incidence of antibiotic resistance (8, 10, 14, 19, 20).

According to Canton and colleagues, the results of the ecological studies found the reciprocal
correlation: those countries with high levels of resistance have higher levels of antibiotic consumption (14).

In the ecological study by Bronzwaer et al., the resistance of *Streptococcus pneumoniae* to β-lactam antibiotics was positively correlated with consumption of the antibiotics in 11 European countries. *S. pneumoniae* was chosen as an indicator of susceptibility to penicillin because of its clinical relevance and evidence of increasing rates of its drug resistance. The outpatient sales of antibiotics were used as a representation of drug consumption (20). In the study by Mackenzie et al., the research team found a positive association between the total use of antibiotics in 128 European hospitals and the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) (21). In the systematic review by Bell et al., the authors showed the development of antibiotic resistance at both individual and community level was significantly associated with consumption of antibiotics. The authors included results from a variety of study designs such as cross-sectional, case-control, ecological, cohort, and experimental studies (19).

The role of mankind in promoting antibiotic resistance is beyond doubts. It is difficult to estimate quantitatively amounts of drugs produced by pharmaceutical companies as disclosure of this information often lies outside the companies' area of concern. It is assumed that during the period from the discovery of antibiotics to the present day the pharmaceutical industry released immense amounts of these drugs at GP's disposal and for other purposes (6).

In general, the application of antibiotics for nontherapeutic purposes is observed in aquaculture, veterinary, cattle and research industry (6). The use of antibiotics for growth promotion in the cattle industry promotes resistance at clinically important levels (8, 14). This type of antibiotic application is considered a factor aggravating the present state of the resistance problem and has been banned in Europe (8). Yet it is debatable whether compliance to this ban is universally achieved. Many developing countries have poor control over antibiotics usage which can be seen as an opportunity for pharmaceutical industry. The consequence is the distribution of low-quality drugs into markets of
developing countries that plays a role in dissemination of the resistance (6). In light of this, the resistance issue is seen as a problem that is fuelled by social inequalities. Taken together, anthropogenic activities are predominantly responsible for selection pressure for ARGs to be omnipresent in the environment (6, 10, 14, 15).

1.2 Antibiotic resistance in Norway

1.2.1 Status of antibiotic resistance

Although the resistance to antibiotics in Norway is estimated as being low compared to other EU countries, some infectious diseases have become difficult to treat because of emerging antibiotic resistance bacteria (22). The prevalence of antibiotic resistance in clinical isolates from Norwegian hospitals vary across the types of bacterial isolates. So, the Norwegian Surveillance System of Communicable Diseases (MSIS) reported the raise in proportion of nonsusceptible *Escherichia coli* to fluoroquinolones from 9.1% in 2011 to 11.7% in 2013. This increase was found to be correlated with prescribing behaviour regarding this antibiotic (22). In 2013, MSIS detected 22% increase in total MRSA events compared to 2012. The resistance to gentamycin and ampicillin in a sample of 81 clinical isolates of *Enterococcus faecium* was found to be 46.8% and 97.5%, respectively (22).

To our knowledge, a number of studies have been done to examine antibacterial resistance in oral environment of Norwegian population. In the study by Al-Haroni et al., the samples of dental biofilm were collected from 21 dental patient volunteers attending two dental offices in Bergen. A comparison group consisted of 34 dental patients visiting dental clinics in Sana'a, Yemen. The examination of the Norwegian participants showed that 7.9% and 11.3% of cultivable bacterial strains collected from the dental biofilm were resistant to ampicillin and metronidazole, respectively. These figures were lower in comparison to those of the Yemeni observation group (23).
In the study by Handal et al., bacterial isolates were obtained from dental biofilm of 25 patients with refractory marginal periodontitis. The patients were referred from a dental clinic in Oslo after being examined by a specialist in periodontics. The conventional treatment of the periodontitis was not successful prior to enrolment to the study. The age of participants ranged from 39 to 66 years. The results showed that 68% of patients had bacteria resistant to β-lactam antibiotics in their dental biofilm (24).

The available data on antibiotic resistance in Norway points to the effectiveness of policies aimed to contain the issue. Even though there are some changes in figures of antibiotic resistance, overall, the level the resistance is sustainable and few countries do better (22).

1.2.2 Consumption of antibiotics

The Norwegian prescription database (NorPD) was introduced in 2004. It has enabled to link the prescription of drugs, including antibiotics, sold in pharmacies to the individual personal number of residents of Norway (25). This database covers the whole country population providing high-quality statistics at individual and population levels and thus overcoming the disadvantages attributed to self-reported drug consumption.

Overall antibiotic consumption in Norway is considered to be stable over years and less than in other European countries (26). In 2012 Norwegian total sales of systemic antibiotics tallied 17.4 defined daily doses (DDD) per 1000 inhabitants per day. The two most frequently prescribed antibiotics are penicillin and tetracycline (27). Since NORpd allows to correspond drug prescriptions with age and gender of patients, it was possible to evaluate differences in the prescribing pattern. In the study by Blix et al., it has been found that females aged 19 to 35 years consume twice as high amount of all systemic antibiotics than males matched by age. The consumption of tetracycline however was estimated to be higher in males (37% of all prescribed systemic antibiotics) in the age group of 15-34 years than in females (23% of all prescribed systemic antibiotics). The same study showed high
antibiotic users were adolescent males and males older than 75 years. Interestingly, tetracycline was the most prescribed drug for the adolescent males (28).

1.3 Oral cavity and antibiotic resistance

1.3.1 Oral cavity as a reservoir for antibiotic resistance

The oral cavity is the entrance to the gastrointestinal tract and the respiratory system. The mouth as an organ is responsible for some essential functions such as food chewing, swallow, speech, and breathing. They make the oral cavity a unique ecological niche colonized by a multitude of microorganisms - bacteria, viruses, fungi etc. The composition of oral bacterial inhabitants is complex with around 1000 distinct species (29, 30). Oral bacterial communities are dynamic and comprised of different species depending on the distinct features of the habitat microenvironment. For example, bacterial composition and diversity in dental plaque is different from that of saliva or tongue (29).

Microbes of the normal microflora, or alternatively commensals, are generally considered as "bacterial strains deemed not actively responsible for a pathogenic process..."(31). Their presence is commonly perceived as of benefit to the human host. Gut microflora and skin microflora are the examples (31). An individual is estimated to accommodate \(3.9 \times 10^{23}\) commensals on average. This figure is much less for pathogens (31). According to Marshall et al., commensal organisms might represent a sufficient reservoir of antibiotic resistance. The authors point to the growing evidence that commensals are capable to transfer the resistance genes to clinically significant pathogens (31). Still, the findings are modest because previous works were focused mainly on studying pathogens (1, 31). As stated by Sommer et al., the normal human microflora might interact with bacterial pathogens at the site of infection. The authors emphasized the possibility of passing the genetic traits conferring resistance to antibiotics to initially susceptible pathogens (32).
It is noteworthy that oral bacteria can acquire genetic fragments conferring resistance from other transient bacteria (12). Therefore, oral bacteria could exchange genetic material with intestinal bacterial community and bacteria found in food and drinks (17). Olsen and co-workers consider the oral microbial community as being a reservoir for dissemination of ARGs that enriches from the environmental reservoir (resistome) (17).

A recent review has shown that ARGs found in oral cavity predominantly confer resistance to β-lactams, macrolides, and tetracyclines (33). However, the authors emphasized these genes do not directly correspond to presence, or magnitude of antibiotic resistance in the samples. Instead it was suggested to consider the identified genes as a potential to confer clinically significant antibiotic resistance (33).

Nevertheless, the data on ARGs prevalence in the mouth are alarming. A report by Lancaster et al. discovered that prevalence of tetracycline resistance bacteria was 97.9% in in a group of 47 presumably healthy children aged 4-6 (34). Another study of international European group of volunteers comprised of 21 healthy adults showed the tetracycline resistance genes were present in each saliva sample (35). The same study demonstrated high prevalence of erythromycin resistance genes. Participants from all countries were positive for the presence of erythromycin resistance gene except those from England (35). A study of saliva collected from 52 Japanese healthy volunteers illustrated the presence of resistant genes to β-lactams in all samples (36). Taken together, the aforementioned findings describe the high prevalence of resistant bacteria in oral cavity across several countries. These findings may have serious clinical implications as mentioned antibiotics are used for treatment of a variety of infectious disorders such as pneumonia, cholera and some sexually transmitted diseases (35). The possible shift from mere presence of the resistance genes to clinical antibiotic resistance may constitute a public health challenge. Besides public health implications, the presence of antibiotic resistant bacteria in oral cavity cause difficulties of dental diseases treatment
The oral bacteria are responsible for a number of oral disorders such as dental caries and its complications, periodontal pathology (29, 37). If not being effectively treated, dental diseases eventually lead to teeth loss, which ultimately may contribute to the loss of gastrointestinal tract functionality, appearance, and speech alterations. The oral bacteria can be also found at other sites of human body. Although their causative role in infectious diseases is under debates, the oral pathogens were identified in infected myocardial tissues, acute infections of liver and brain (29).

1.3.2 Identification of antibiotic resistance in oral cavity

In oral microbiology, biological samples such as saliva, dental plaque, and mucosa swabs are conventionally examined in the laboratory to identify antibiotic resistance bacteria (33). Saliva is a biofluid that is easily accessible for sample collection. The process is quick, cheap and non-invasive. Saliva has a diagnostic value as it contains a multitude of bacterial species: those of residents or transient oral microflora, and commensals or pathogenic bacteria (29). However, it should be noted that bacterial composition of saliva is distinct in comparison to other bacterial communities residing, for instance, in dental biofilms. The differences are seen both in taxonomy of the bacterial communities and the proportions of the taxa (29). In this respect saliva may be thought of as a "representative" of all bacterial communities colonizing the mouth as the biofluid bathes nearly all surfaces of the oral cavity.

Among the diversity of the oral bacteria, it is estimated that only half can be cultivated (29). This means that some 500 bacterial species found in the mouth cannot grow under laboratory conditions and, thus, the investigation of resistance genes present in cultivable species from saliva is limited to those bacteria that are cultivable. Until recently, a few studies explored the proportion of those samples that exhibit clinical antibiotic resistance (34, 36, 37). The presence of clinical antibiotic resistance in a bacterial isolate is conventionally identified through determining the minimum inhibitory concentration (MIC) value for bacterial isolates found in clinical samples. The MICs are
commonly defined in the laboratory in order to provide information on what concentration of antibiotics is enough for successful treatment of a given bacterial infection in clinical settings (14). In spite of huge clinical importance, this approach is not able to monitor the potential of ARGs in non-cultivable bacteria. In the light of the fact that oral bacteria can easily transfer and exchange the resistance genes through HGT mechanisms (12, 17), the need in examining bacterial DNA along with bacterial colonies should be considered. Scientific progress allowed the use of new technologies of molecular detection and quantification of molecules of interests such as ARGs. The droplet digital PCR (ddPCR) is an example of these molecular techniques (38).

The ddPCR detects and quantifies copy number of molecules of the gene(s) of interest with high precision. The unique feature of the method is that a DNA sample is partitioned up to 20,000 droplets prior to polymerase chain reaction. After the procedure, the droplet reader device calculates the amount of DNA target copies. The outcome of this measurement is presented as a number of target gene copies (38). The previous works that aimed to quantify absolute numbers of bacterial gene copies in a given biological sample used normalization of the gene quantification results (39), which is important for the comparison of gene copies numbers across several samples. The reason behind is that DNA extraction procedure yields unknown proportion of bacterial DNA in the analyzed samples. Normalization is also used to reduce the effect of PCR efficiency variability. The normalization procedure accounts for impact of DNA that comes from viruses, fungi, protozoan, and human cells. Conventionally, a "housekeeping" gene such as 16S ribosomal RNA (16S rRNA) gene can be chosen for the purpose of quantification of copy number of certain bacterial genes (40-42). Another method, normalization by total DNA concentration, has been described in works aimed to quantify gene copy numbers (43).

Interestingly, in studies exploring distribution of the resistance genes in oral cavity the question of quantity of the genes was not addressed. As stated by Moraes et al., the majority of publications aimed
to investigate the prevalence of clinical antibiotic resistance across samples found in the oral cavity (33).

1.3.3 Factors that promote antibiotic resistance in oral cavity

Humans encounter resistance genes as early as at the very first seconds of life - during delivery (44). Furthermore, the mode of delivery is known to be an important factor determining the profile of resistance genes in the oral bacteria. The study by Allicea-Serrano and colleagues has identified tet(M) and tet(O) resistance genes in oral samples collected from those babies who were delivered vaginally. The babies who were delivered by Caesarean section, in contrast, were positive for presence of tet(W) and tet(O) bacterial resistance genes (44).

It has been suggested that antibiotic therapy for treatment of both systemic and local infectious diseases may lead to the selection of resistant bacterial strains and their transient elevation in the mouth (45, 46). However, the proportion of antibiotic resistant strains found to return to the baseline level after some time after start of the therapy. These findings do not explore the quantitative changes of the resistance genes in oral cavity. Genetic material carried by bacteria, no matter live or dead cell, is readily transferrable to commensals or other bacterial species (12). In theory, the interaction of oral bacteria and antibiotic agents cannot be averted - the stable levels of antibiotic concentrations in saliva during therapy with antibiotics (47), and the variety of microorganisms in the mouth create comfortable conditions for selection of the fittest microbes and, consequently, dissemination of resistance genes occurs (6).

An alternative way to affect the variation of resistance genes in the mouth is through consumption of food and drinks which could contain ARGs (17). Use of antibiotics as growth promoters in food industry may generate a linkage between environmental reservoir of antibiotic resistance and the reservoir represented by human microflora (10, 32). The genes that confer resistance to antibiotics have been identified in commercially available food and drinking water (48, 49).
In addition to environmental exposures, some therapeutic practices such as former traditional use of amalgam fillings in dental treatment may have contributed to selection of mercury-resistant bacteria and promotion of antibiotic resistant oral strains (50).

1.3.4 Antibiotic resistance as an occupational risk in dental office

A number of studies sought to elucidate the occupational risk in dental practice with regard to microbial contamination. One of the mechanisms to pose a risk of pathogens transmission to medical personnel is through the exposure to bioaerosol that is present in the dental office environment (51, 52). A contemporary dental unit consists of a set of handpieces including a high-speed air-rotor handpiece (turbine), a low-speed micro engine, an ultrasonic scaler, and an air-water handpiece, at least (51). The handpieces are used in the way the bioaerosol and splatter are formed. Bioaerosols are the suspension of particles less than 50 micrometres in diameter that can stay airborne for a long period of time (51). Splatter is referred to as particles larger than 50 micrometres in diameter (52). The substance consists of air, water/saliva, particles of dental restorations, enamel and carious tissue fragments. The area of splatter dispersion is estimated as up to 120 cm from the site of dental operation (51).

The composition of bioaerosol is complex and may include water particles generated by use of handpieces and biofluids derived from the oral cavity (51-53).

The water from dental unit water system (DUWS) has been extensively investigated as a part of the infection control program in dental offices. A worrisome fact is that the water from DUWS is found to be contaminated by microorganisms despite the adherence to guidelines for maintenance and disinfection of the DUWS in dental practice (53).

In the review by Walker and Marsh, the presence of microorganisms in DUWS is explained by the formation of microbial biofilms on the inner surface of the tubing. The authors propose the biofilms
are a reservoir for pathogens and, possibly, the resistance genes associated with them. The biofilms of the tubing are found to be recalcitrant towards disinfection procedures. The elimination of the microbial aggregates is difficult even with use of effective commercial agents (53).

On the other hand, the dental bioaerosol is constituted from the particles that derives from the oral fluids. The latter is an environment harboring a variety of microorganism such as bacteria, viruses, fungi, etc. (29, 52). A dentist equipped with the handpieces mobilizes dental debris, saliva, dental plaque through use of high-speed bur rotation or ultrasound vibrations (51, 52). This biological mixture is aerosolized and, coupled with droplets from DUWS, theoretically contaminates air and surfaces in the radius of up to two metres with the centre at the operating site (54).

A number of studies aimed to measure bacterial load in the dental aerosol (55, 56). The researchers used culture-based methods where the samples of air in dental office were collected and cultured on a nutrient medium for further assessment. The bacterial quantity was estimated in colony-forming units (CFU) per cubic meter of space (52). This method provides a good representation of bacterial load for comparison of various techniques in dental practice, e.g. ultrasonic scaling versus air-rotor drilling.

Still, there may be an underestimation of total bacterial load in the dental aerosol (52, 56). The reasons for that are as follows: some bacteria cannot be cultured at all, the nutrient medium favours growth of limited types of bacteria, and bacteria are prone to disruption of the cell due to aerosolization (56, 57). Despite possible limitations of methodology, Dutil and co-workers estimated concentration of bacteria in aerosol as much as 2.8 x 10^3 CFU per cubic meter (56). On a contrary, the research group of Bennett discovered this concentration to be 7.0 x 10^3 CFU per cubic meter at its peaks (55). Dutil et al. suggested that the possible reason for such discrepancy in numbers is due to impact of oral hygiene condition on the concentration of bacteria in aerosol. The volunteers in his study had no oral pathology and received only hygienic dental cleaning (56). These findings indicate that there is an abundance of
living bacteria in the dental aerosol and more bacteria may be aerosolized when treating patients with poor oral health state.

Possibly downplayed bacterial concentration figures may disguise the prevalence of the resistance bacteria in the dental aerosol. A recent *in vitro* study showed that the cellular membrane of some bacterial cells is damaged during aerosolization. The disruption of bacterial cell structures invokes the release of free bacterial DNA as a part of aerosol composition (57). The results of the study suggest that detection of potentially dangerous DNA in the dental aerosol is complicated. A few studies have been done aiming to estimate the prevalence of the resistance genes in aerosols in healthcare settings. The report presented by Gilbert *et al.* describes the presence of tetracycline and erythromycin resistance genes in air samples obtained from wards of a pulmonary care unit (58). A study of bioaerosol samples collected from air of vacant wards in a hospital showed up to 70% of the cultured bacteria were resistant to β-lactam antibiotics (59). Prevalence of antibacterial resistance in dental bioaerosol or quantification of ARGs are currently not described in literature.

The small size of the particles in the bioaerosol poses a special risk onto dental team. The smallest particles less than 5 micrometres are able to reach bronchi and alveoli, which makes the aerosol a vector in infections transmission (51, 52, 56). Dental professionals use personal protective equipment such as gloves, gowns, goggles, visors, masks to protect themselves from the bacterial contamination as postulated in the guidelines (60). Regardless these precautions, the dental bioaerosol particles are able to go around the protection contacting with eyes, skin, oral mucosa and respiratory system (51, 52). Additionally, the contact with the aerosol is possible even when the treatment is done. The bioaerosol remains persistent in dental office air for up to 30 minutes after the end of a dental procedure (55). This implies that dental staff must not remove the protective means when communicating with patient past procedure.
Inhalation of airborne bacteria in the dental office is a challenge for both dental team and patients. Patients may be exposed to aerosolized microbes from DUWS and from own oral cavity (51). A face mask protects dental staff from inhalation of the aerosol only if it is worn and fitted properly (56).

Experts in the field of occupational health encourage dental team members to reduce the volume of the dental aerosol before it is gone from the operational site in the mouth (51, 52). A technical solution for it is the use of high-volume evacuator (HVE). Commonly, it is an air-sucking tube with the opening of 8 millimetres at minimum. The device evacuates up to 30 cubic metres of air per minute from the operation site (52). This advantage, however, is possible only when a dentist cooperates with a dental assistant. This working technique is not universally implemented. The example is a dental hygienists who mainly work alone (52). Another way to minimize bioaerosol formation is use of rubber dam. This device isolates teeth from the oral environment and prevents saliva bathing the operation site. Even though this method is highly effective in reduction of aerosol, it cannot be applied in certain clinical situations of dental treatment (52). The reduction of airborne bacteria in the air of dental office is also achieved by use of air-ventilation systems, maintenance and cleaning of DUWS, and compliance to the general infectious control recommendations (51).

Although the combination of the protective measures minimize the risks of exposure to bioaerosol in dental office (51, 52), it does not mean the risk is completely eliminated (52). Considering the limitations in methodology of the bacterial load estimates in the aerosol and the scarcity of literature about the bacterial resistance in the dental aerosol, the occupational risk in dentistry may be not fully recognized yet.

1.3.5 Tetracycline resistance

One of the most relevant forms of resistance in dental practice is tetracycline resistance (33, 37). Today, tetracycline is one of the most prescribed antibiotics in Scandinavian countries (27). It is the second most prescribed antibiotic for systemic use in Norway (22).
Tetracycline is effective against infections caused by a variety of gram-positive and gram-negative bacteria (61). The wide usage of tetracycline as a human and animal therapeutic predetermined an extensive dispersion of the antibiotic in the environment (62).

The resistance to tetracycline in bacteria is primarily due to acquisition of new accessory genes. The genes are often associated with the mobile genetic elements such as plasmids and transposons (61). The tetracycline resistance genes, *tet* genes, encode proteins (I) to reduce concentration of antibacterial agents in the bacterial cell through activation of efflux-pumps, (II) to protect the bacterial ribosome from its inhibition by tetracycline, (III) to activate enzymes of tetracycline molecule modification/inactivation, and (IV) to induce unknown mechanism of the resistance (61-63). More than 1190 *tet* genes have been identified and classified into 41 resistance determinant groups (63).

The most abundant tetracycline resistance determinant in the oral environment is *tet(M)* (12, 35, 62, 64). The gene encodes proteins of ribosomal protection against inhibitory properties of tetracycline molecules (63). The high prevalence of *tet(M)* in the oral cavity can be explained by its location on the Tn916-like mobile genetic element which, in turn, is found in many oral bacteria. This mobile genetic element is also capable of being spread among different oral bacterial genera (12, 17, 35). In addition, there is an experimental evidence that Tn916-like transposons can be exchanged between the dental biofilm bacterial community and other members of oral microbiome (65). Commensals carrying *tet* genes can be virtually found in oral cavity of nearly everyone (62). Such a dramatic presence of *tet* genes may be a consequence of the huge selective pressure exerted by the introduction of tetracycline in 1953 (62).

The screening of both commensal flora and oral pathogens for the presence of *tet(M)* should be helpful for understanding of the prospective of clinically significance of their presence (31)
1.4 Aim of the study

It is known that bacteria and resistance genes are being exchanged between different communities, individuals, and bacterial populations. Despite probably good hygiene habits, dentists/dental students might have an increased risk of contamination because they work closely with infected people and use equipment that can help microbes to spread in the working environment such as handpiece. In other words, are the prevalence and levels of tet(M) found in saliva associated with how long the dentist / dental student has been working in the clinic? The hypothesis of this study is that there is an association between tet(M) in saliva and exposure to dental office environment.

1.4.1 Specific objectives

(1) To detect the tet(M) gene and quantify the number of tet(M) gene copies in the bacterial DNA extracted from saliva samples.

(2) To evaluate whether the level of tet(M) gene in saliva is different between 1st- and 5th-year dental students.

(3) To explore any possible association between detected tet(M) gene copy numbers and total number of antibiotics' courses taken previously in life accounting for possible confounders.

1.5 Implications of findings

Since our method is based on absolute quantification of tet(M) gene copy numbers in samples from healthy subjects, the results will reflect the levels of the resistance genes found in bacterial DNA at individual level. The study hence will use a novel approach where samples collected from participants are compared on the basis of absolute numbers. It is noteworthy that the number of ARGs may not reflect the clinical antibiotic resistance (13, 66). We aim to study the presence of antibiotic resistance in healthy subjects for better understanding of factors that can possibly influence it. The knowledge of these factors should help in getting more insights into the AR problem, especially on how often
healthy individuals carry ARGs and for how long they persist without any selection pressure at the individual level.

The comparison of the two observational groups, one that have been exposed to dental clinical settings and the other that have not, will provide an insight of how the antibiotic resistance is influenced by dentistry as an occupation. Our results could reveal also how effective personal protection equipment (PPE) used in dentistry prevent transmission of ARGs in dental offices. Our study in this sense can be viewed as a pilot one for the assessment of possible risks in catching ARGs via aerosolized bacteria exposure in dental offices, and, not least, for establishing droplet digital PCR-based method of quantification of *tet(M)* gene in saliva samples.
2 Materials and Methods

2.1 Study design

This is a comparative cross-sectional study that uses both descriptive and analytic approaches. Demographic characteristics and information about health parameters were obtained by a structured questionnaire composed of 32 questions, see Appendix 1. Laboratory analysis was done to reveal the presence and levels of the genetic targets of interests in the saliva samples. The data collection was carried out from 1st of June 2015 to 27th of November 2015. The saliva samples were collected from participants at the Department of Clinical Dentistry (IKO), University of Tromsø. The participants filled in the questionnaire immediately after saliva sampling.

2.2 Study participants

A total of 97 dental students of the Department of Clinical Dentistry (IKO), University of Tromsø were invited to the study of whom 47 and 50 were from the 1st year and 5th year of study, respectively. The invitation letters (see Appendix 2) were sent out through the student's e-mail service provided by the university. The invited students were presumably healthy.

The 1st-year students were new to the IKO and have not started to take any sessions in the dental clinics or treat any patient at this stage of their education. The 5th-year students were introduced to dental practice during the 6th semester of their study (3rd-year) studying at the IKO, and have spent approximately 1.5 years providing dental treatments to patients.

The participants were recruited to the study if fulfilled the following inclusion criteria: (1) being a dental student either in the 1st- or 5th-year of study at IKO, (2) absence of any systemic health problems, (3) and no antibiotic was taken in the last 3 months prior to study. Accordingly, the exclusion criteria were: (1) presence of a systemic disease, (2) a continuous need for medication
known to alter the composition of oral bacteria, and (3) use of any antibiotic during the past 3 month.

All participants provided both verbal and written consent to participate in the study prior to saliva sampling and filling in the questionnaire. The study was ethically approved by the Regional Committee for Medical and Health Research Ethics, North Norway (Ref.nr. 2015/1048).

2.3 Sample collection

The participants were informed that they should have avoided smoking the last hour before saliva collection session. Consumption of food and drinks were not advised one hour prior to saliva sampling. Those participants with orthodontic appliances were asked to keep them in the mouth during the procedure. In the beginning, the participants were asked to sit quietly in a dental chair for 5 minutes to relax. Then, they were given a sterile paraffin wax to chew for approximately 30-60 seconds. The first portion of stimulated saliva was swallowed. The saliva expectorated in the next 3 minutes was then collected into a disposal sterile container (approximately 5 ml), coded with a unique identification number, and then stored at -80°C for further analysis at the laboratory. The unique identification number was used to match the laboratory results with the questionnaire data.

2.4 Laboratory methods

2.4.1 Saliva samples processing and DNA extraction

Approximately 800 µL of saliva were diluted with 200 µL of phosphate buffered saline (PBS) and then centrifuged at 21,000 G for 5 min to pellet bacterial cells. The supernatant was discarded and the pellet was washed three times in 50 µL PBS. The pelleted cells were then transferred to QIAcube robotic workstation (QIAGEN) for DNA extraction using QIAamp mini kit (QIAGEN). The final DNA sample was eluted in 50 µL of TE buffer.
2.4.2 Total DNA quantification

The concentration of total DNA was quantified using Qubit® 3.0 fluorometer according to the manufacturer’s instructions (ThermoScientific). In brief, the Qubit® 3.0 was calibrated using the Standard #1 and #2 supplied with the starter kit. To prepare the assay tubes, 198 µL of the Qubit working solution and 2 µL of a DNA sample were used. The DNA concentration from the samples was measured in ng/µL.

2.4.3 Assessment of extracted DNA by agarose gel electrophoresis

The extracted DNA from the saliva samples was evaluated by gel electrophoresis. A total of 10 µL of the eluted DNA was run in 1% agarose gel. Electrophoresis was performed at 95 V for 1 hour. GelRed stained was used to visualize DNA in the gels using ChemiDoc Touch Imaging System (Bio-Rad).

2.4.4 Droplet Digital PCR

The presence and copy number of tet(M) and 16S rRNA genes were measured by QX200™ Droplet Digital™ PCR system (Bio-Rad). The PCR reaction mix was prepared as follows: (1) 10 µL of 2X ddPCR super mix for probes (no dUTP), (2) 1 µL of custom target primers/probe for tet(M) labelled with FAM, (3) 1 µL of custom target primers/probe for 16S rRNA labelled with HEX to measure total bacteria (4) 7 µL nuclease-free water (5) 1 µL of suitable amount of diluted DNA template (1:500). The DNA extracted from Bacillus subtilis was used as a positive control for tet(M) and 16S rRNA genes.

The prepared 20 µL reaction mix for the ddPCR was loaded to the DG8 Cartridge (Bio-Rad) and droplets were generated by QX200™ Droplet Generator (Bio-Rad). The prepared ddPCR reaction mix (40 µL) containing the droplets was then transferred to 96-well PCR plate (Eppendorf) for DNA amplification. The plate was sealed with Pierceable Foil Heat Seal (Bio-Rad) and placed into C1000 Touch Thermo Cycler (Bio-Rad). The amplification parameters are shown in Appendix 3. The 96-well
PCR plate was then placed in the QX200™ Droplet Reader (Bio-Rad) for reading the positive and negative droplets and, accordingly, absolute quantification of the target molecules is calculated using QuantaSoft software (version 1.3.2.0, Bio-rad). The QuantaSoft software package was also used to analyse the data. The concentration of tet(M) and 16S rRNA genes, were set as number of gene copies per 1 µL of loaded sample. The threshold value to distinguish positive and negative droplets was set manually at 2200 of relative fluorescence units (RFUs) for tet(M) and at 4000 RFUs for 16S rRNA. The threshold was implemented universally to all samples in order to ensure comparability between samples.

2.5 Variables

2.5.1 Variables retrieved from the questionnaire

Variables for descriptive statistics were retrieved from the questionnaire. They were: gender, year of birth, year of study, general health state, oral health state, a total of antibiotic-courses taken, presence of a chronic disease, smoking status, smoking duration, snuff user status, snuff use duration, count of daily cigarettes/snuff portions, frequency of teeth brushing, frequency of interdental cleaning appliances use, hand washing after phone usage in dental clinic, hand washing after filling in ambulatory medical card in dental clinic, hand washing after performing X-ray examination in dental clinic, hand washing after each patient in dental clinic, hand washing before each patient in dental, propensity to minimize hand washing in dental clinic, age.

The age was computed as follows:

age = 2015 – year of birth

Initially nominal variable retrieved from question 8 in the questionnaire was converted into a categorical variable comprised of the following categories: 0 – none, 1 – antihistamines, 2 – oral
contraceptives, 3 – drugs for treatment of colitis, 4 – thyroid hormones, 5 – immune-modulators, 6 – several drug entities.

The participants reported a commercially available mark of drug for open-ended question 8 in the questionnaire, if applicable. Among these answers were following pharmaceutical names: "Levaxin", "Mercilon", "Microgynon", "Cerazette", "Loette", "Colazide", "Grazax", "Aerius", "Zyrtec", and "Zetirizine". In addition some of participants provided answers containing application of a therapeutic agent, e.g. "antihistaminer","p-piller", without their commercial names. We converted previously nominal variable into category variable sorting the drugs according to pharmaceutical registry (67) with following categories: (I) thyroid hormones, (II) drugs for colitis treatment, (III) oral contraceptives, (IV) immune-modulators, (V) antihistamines.

2.5.2 Variables retrieved from laboratory work

The variables retrieved from the laboratory work were: (1) absolute copy number of \textit{tet(M)} gene in the sample, which was reported as gene copy number per 1µL of the DNA used in the ddPCR mix, (2) absolute number of 16S rRNA gene in the sample, which was reported as gene copy number per 1µL of the DNA used in the ddPCR mix, (3) the concentration of DNA samples, which was reported as nanograms of DNA per 1 microliter of the DNA sample. The outcome variables were then calculated and reported as follows: (4) copy number of \textit{tet(M)} gene per 1 million copies of 16S rRNA gene, (5) copy number of \textit{tet(M)} gene per 1 nanogram of extracted DNA. The laboratory results (1) and (2) were adjusted for dilution of the original DNA sample. The initial DNA sample had a 500-fold dilution prior to ddPCR. Therefore, (1) and (2) from QuantaSoft readings were multiplied by 500 in order to refer to the initial DNA sample concentration. To compute the outcome variable (4) the following formula was used:
relative copy number of $tet(M)$ gene$^1$ = \frac{\text{absolute copy number of } tet(M) \text{ gene}}{\text{absolute number of 16S rRNA gene}} \times 10^6

The reason for computing the new (4) variable is that the outcome represents the number of $tet(M)$ gene copies normalized to 1 million copies of $16S \text{ rRNA}$ gene. In contrast, the outcome variable (5) describes the copy number of $tet(M)$ gene per 1 ng of extracted DNA from the saliva samples. The latter could include DNA molecules from bacteria, viruses, fungi, and human cells.

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

relative copy number of $tet(M)$ gene$^2$ = \frac{\text{absolute copy number of } tet(M) \text{ gene}}{\text{DNA concentration}}

In order to perform parametric statistical tests without violating the assumptions, the outcome variables (4) and (5) were undergone log10 transformation for further statistical analysis. As a result, two new variables were used for analysis, namely (4.1) and (5.1), denoting log10 transformed variables (4) and (5), respectively.

2.5.3 The levels of measurement

The binary variables were as follows: gender, year of study, presence of a chronic disease, smoking status, snuff user status, hand washing after phone usage in dental clinic, hand washing past recording the ambulatory medical card in dental clinic, hand washing after X-ray examination in dental clinic, hand washing after each patient in dental clinic, hand washing before each patient in dental, minimize hand washing in dental clinic. Question number 13 was used to retrieve information whether a participant has a chronic disease or not. It was categorised as a binary variable because there were no participants who had chosen "not sure" on this question. Question number 14 was used to retrieve information about participants’ smoking habits and it was categorised as a binary variable because there were no participants who had chosen "I smoke every day" on this question. Consequently, the

---

$^1$ number of $tet(M)$ gene per 1 million copies of 16S rRNA gene

$^2$ number of $tet(M)$ gene per 1 ng of total DNA analysed
binary variable was coded as "0" for non-smokers and "1" for those who had chosen occasional smoking.

The categorical variables were as follows: general health state, oral health state, a total of antibiotic-courses taken, smoking duration, snuff use duration, frequency of teeth brushing, frequency of usage interdental cleaning appliances.

The continuous variables were as follows: all the laboratory variables and age.

2.5.4 Dummy variables

Initially categorical variable retrieved from question 10 (Q10) in the questionnaire had the following possible answers: 1 – never, 2 – 1-2 courses throughout life, 3 – 3-10 courses throughout life, 4 – more than 10 courses. Consequently, this variable was used as a set of dummies in the linear regression analysis with "never" as a reference group.

Question number 11 in the questionnaire was split into four categorical variables with 4 possible categories: "never", "occasionally", "often", "very often". For the linear regression analysis the categorical variables were converted to a set of dummy variables with "never" being the reference.

Question number 12 in the questionnaire was split into five categorical variables with 5 possible categories to choose. These were "the whole 2 weeks", "often", "more than 7 days", "less than 7 days", "seldom", "never". For the statement (A) "I felt myself sad and depressed", the direction of scale was inverted so that "never" had score "1", seldom - score "2" and so forth. For the linear regression analysis the categorical variable were converted to a set of dummy variables with "never" as the reference. The statement (E) was converted to the set of dummies with "the whole two weeks" as the reference without change in the direction of the scale.
Initially categorical variable retrieved from question 15 in the questionnaire had 3 possible categories to choose. These were: "I do not smoke", "less than 3 years", "more than 3 years". For inclusion the variable to the regression model, a set of dummies was created with "I do not smoke" as the reference.

2.5.5 Data sources/measurement

All the variables acquired from the laboratory results and the questionnaire were comparable across the study group as the procedure of data collection was consistent for each participant. Even so, the variables obtained from questions 21-32 had missing values for the group of 1st-year students because the questions were irrelevant for their position. That is why statistical models with these variables were analyzed only for the group of 5th-year dental students.

2.6 Statistical analysis

A statistical software, IBM SPSS Statistics Version 22 and RStudio Version 0.99.879, were used for the statistical analysis and data visualization. R-packages Rcmdr, ggplot2, Hmisc, boot, and car were used in the statistical analysis. Histogram plots, Q-Q plots, the values of skewness and kurtosis, and Shapiro-Wilk test were used for the assessment of normality of distribution for the laboratory analysis outcome, i.e. variables (4) and (5). The assessment of normality of the outcome distribution was done to choose appropriate statistical tests in further analysis.

To compare difference in copy numbers of tet(M) gene between the 1st- and the 5th-year dental students the Wilcoxon-Mann-Whitney test was used. It is an assumption-free test which is conventionally applied to compare two independent groups when data are not normally distributed (or violate other assumptions) (68). Computation of the effect sizes had the following R-script:

```
effect_size<-function(wilcox_model, N){z<-qnorm(wilcox_model$p.value/2);
r<-z/sqrt(N); cat(wilcox_model$data.name, "Effect size, r = ",r)}
effect_size(wilcox_model, 83) (68)
```
Prior to deciding whether the linear regression is an appropriate analytical tool to use, we performed correlational analysis between the level of \textit{tet}(M) gene identified and consumption of antibiotics throughout life. If at least one of these binary variables had p-value less than 0.05 of its Pearson $r$ coefficient, the set of dummies with "none" as the reference group was used in the linear regression analysis. The regression model was adjusted for age and gender. The assessment of the regression model was performed as suggested by Field \textit{et al.} (68). For the assessment of influential cases in the model, the following statistical methods were performed: hat values, covariance ratios, and Cook's distance. To evaluate applicability of the regression model above the drawn sample, assumption of independence, assumption of no multicollinearity, and values of variance inflation factor were assessed. The loss of predictive power of the model, also known as shrinkage, was estimated by using adjusted $R^2$ of the adjusted regression model. The $\alpha$-level was set at 0.05.
3 Results

3.1 Questionnaire results

A total of 97 participants were examined for eligibility to be included in the study. Of the 97 participants who provided informed consent to enter the study, 14 were excluded from the study because of previous use of antibiotics in the last 3 months prior to the study. Forty-one and forty-two students comprised the 1st- and the 5th-year dental student groups, respectively. The response rate was 100%. The demographic characteristics of the participants are shown in Table 1.

The mean age of the participants was 23.9 years (SD=3.74). The 5th-year dental students (mean age = 26.2, SD=2.9) were significantly older than 1st-year dental students (mean age = 21.6, SD=2.9), p-value <0.001. The proportion of those students considering own dental health as "very good" was significantly higher in the 5th-year students group, 17 out of 42 participants, than in the counterpart group, 5 out of 41 participants (p-value<0.05). Most of the students considered their dental health as being of a good state, 50 out of 83 participants. Significantly more 1st-year students, 8 out of 41, reported that the status of their dental health "neither good nor bad, (p-value<0.05). This proportion was 2 out of 42 in the 5th-year students. Only one participant in the 1st-year students group evaluated his/her dental health as of being "not entirely good".

The majority of respondents reported no use of any medications on a regular basis 52 out of 83. Use of oral contraceptives was as twice as high in the 5th-year females, 14 out of 42, compared to females in the 1st-year group, 6 out of 41 (p-value<0.05). While the 1st-year students have not been consuming any thyroid hormones, adrenomimetic, or a combination of several medicines, the consumption of these drugs was reported by two, one, and three 5th-year students, respectively (p-value<0.05). Table 1 summarizes all the answers obtained from the questionnaire regarding the two observational groups.
<table>
<thead>
<tr>
<th></th>
<th>1&lt;sup&gt;st&lt;/sup&gt;-year students, N=41</th>
<th>5&lt;sup&gt;th&lt;/sup&gt;-year students, N=42</th>
<th>Total, N=83</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>30 (73.2%)</td>
<td>33 (78.6%)</td>
<td>63 (75.9%)</td>
</tr>
<tr>
<td>males</td>
<td>11 (26.8%)</td>
<td>9 (21.4%)</td>
<td>20 (24.1%)</td>
</tr>
<tr>
<td><strong>Dental health</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very good</td>
<td>5 (12.2%)</td>
<td>17 (40.5%)</td>
<td>22 (26.5%)</td>
</tr>
<tr>
<td>Good</td>
<td>27 (65.9%)</td>
<td>23 (54.8%)</td>
<td>50 (60.2%)</td>
</tr>
<tr>
<td>Neither good nor bad</td>
<td>8 (19.5%)</td>
<td>2 (4.8%)</td>
<td>10 (12%)</td>
</tr>
<tr>
<td>Not entirely good</td>
<td>1 (2.4%)</td>
<td>0 (0%)</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td><strong>General health</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very good</td>
<td>22 (53.7%)</td>
<td>24 (57.1%)</td>
<td>46 (55.4%)</td>
</tr>
<tr>
<td>Good</td>
<td>17 (41.5%)</td>
<td>17 (40.5%)</td>
<td>34 (41.0%)</td>
</tr>
<tr>
<td>Neither good nor bad</td>
<td>2 (4.9%)</td>
<td>1 (2.4%)</td>
<td>3 (3.6%)</td>
</tr>
<tr>
<td><strong>Frequency of sickness last 2 years</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3 (7.3%)</td>
<td>5 (11.9%)</td>
<td>8 (9.6%)</td>
</tr>
<tr>
<td>Seldom</td>
<td>22 (53.7%)</td>
<td>25 (59.5%)</td>
<td>47 (56.6%)</td>
</tr>
<tr>
<td>Occasionally</td>
<td>12 (29.3%)</td>
<td>12 (28.6%)</td>
<td>24 (28.9%)</td>
</tr>
<tr>
<td>Often</td>
<td>4 (9.8%)</td>
<td>0 (0%)</td>
<td>4 (4.8%)</td>
</tr>
<tr>
<td><strong>Satisfaction with teeth appearance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very satisfied</td>
<td>17 (41.5%)</td>
<td>17 (40.5%)</td>
<td>34 (41.0%)</td>
</tr>
<tr>
<td>Fairly satisfied</td>
<td>22 (53.7%)</td>
<td>24 (57.1%)</td>
<td>46 (55.4%)</td>
</tr>
<tr>
<td>Rather dissatisfied</td>
<td>2 (4.9%)</td>
<td>1 (2.4%)</td>
<td>3 (3.6%)</td>
</tr>
<tr>
<td><strong>Medicines used daily</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>31 (75.6%)</td>
<td>21 (50.0%)</td>
<td>52 (62.7%)</td>
</tr>
<tr>
<td>Oral contraceptives</td>
<td>6 (14.6%)</td>
<td>14 (33.3%)</td>
<td>20 (24.1%)</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>3 (7.3%)</td>
<td>1 (2.4%)</td>
<td>4 (4.8%)</td>
</tr>
<tr>
<td>Immune-modulators</td>
<td>1 (2.4%)</td>
<td>0 (0%)</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>Thyroid hormones</td>
<td>0 (0%)</td>
<td>2 (4.8%)</td>
<td>2 (2.4%)</td>
</tr>
<tr>
<td>Adrenomimetics</td>
<td>0 (0%)</td>
<td>1 (2.4%)</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>More than 1 drug</td>
<td>0 (0%)</td>
<td>3 (7.1%)</td>
<td>3 (3.6%)</td>
</tr>
<tr>
<td><strong>Total amount of antibiotic courses in life</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>10 (24.4%)</td>
<td>1 (2.4%)</td>
<td>11 (13.3%)</td>
</tr>
<tr>
<td>1-2 courses</td>
<td>13 (31.7%)</td>
<td>20 (47.6%)</td>
<td>33 (39.8%)</td>
</tr>
<tr>
<td>3-10 courses</td>
<td>15 (36.6%)</td>
<td>15 (35.7%)</td>
<td>30 (36.1%)</td>
</tr>
<tr>
<td>More than 10 courses</td>
<td>3 (7.3%)</td>
<td>6 (14.3%)</td>
<td>9 (10.8%)</td>
</tr>
</tbody>
</table>
3.2 Laboratory results

The presence of \textit{tet}(\textit{M}) gene copies in the saliva obtained from dental students was assessed by droplet digital PCR. The median of \textit{tet}(\textit{M}) gene copies per 1 million copies of bacterial \textit{16S rRNA} gene was 6452 with interquartile range of 7486. The distribution of \textit{tet}(\textit{M}) gene copy numbers were positively skewed. The majority of participants were at the lower end of distribution. The range was from 814 to 66,062 of \textit{tet}(\textit{M}) gene copies per 1 million copies of bacterial \textit{16S rRNA} genes (Figure 1).

![Graph showing the distribution of tet(M) gene copies per 1 million of 16S rRNA genes among the whole study group (n=83).](image)

\textbf{Figure 1.} The distribution of \textit{tet}(\textit{M}) gene copies per 1 million of \textit{16S rRNA} genes among the whole study group (n=83).

On the other hand, the median of \textit{tet}(\textit{M}) gene copy numbers per 1ng of DNA obtained from saliva samples was 4250 with the interquartile range of 6509. The range of counts was from 468 to 50901 \textit{tet}(\textit{M}) gene copies per 1 ng of DNA. The distribution was also positively skewed with the majority of participants having lower counts of \textit{tet}(\textit{M}) gene (Figure 2).
3.2.1 Assessment of normality of data distribution.

1) Visual assessment of figure 1 and figure 2 suggested that there was a positive skewedness of the data. The majority of participants had low copy numbers of tet(M) gene per 1 million copies of 16S rRNA gene or/and per 1ng of DNA.

2) The Q-Q plots shown in both figures 3 and 4 represent the distribution of our data scores that deviated from the straight diagonal line of scores expected if the data would have been normally distributed.
3) Values of skewness (2.64) and kurtosis (8.51) for the outcome variable "tet(M) gene copies per 1 million copies of 16S rRNA gene" indicate that the data were not normally distributed. Values of skewedness (3.42) and kurtosis (14.48) for the outcome variable "tet(M) gene copies per 1 ng of DNA" indicate that the distribution of the scores were not normally distributed.
4) Shapiro-Wilk test was used to test the normal distribution of data of related to the variable "tet(M) gene copies per 1 million copies of 16S rRNA gene", and to the variable "tet(M) gene copies per 1 ng of DNA". The two variables were significantly not normally distributed with W = 0.706 (p-value <0.001) and W = 0.632 (p-value <0.001), respectively.

3.3 Comparison between two groups

The copy number of tet(M) gene per 1 million copies of 16S rRNA gene in saliva of the 1\textsuperscript{st}-year dental students (median = 6886.5, interquartile range (IQR) = 4967.3) did not significantly differ from the correspondent number of the 5\textsuperscript{th}-year dental students (median = 6237.0, IQR = 9877.9; Wilcoxon rank-sum test: W = 833, p-value = 0.917, r = -0.0114) (Figure 5).

![Figure 5](image)

\textbf{Figure 5.} Comparison of copy number of tet(M) gene per 1 million copies of 16S rRNA gene between 1\textsuperscript{st}- and 5\textsuperscript{th}-year students. The black bold line inside boxes is median. The grey boxes represent the middle 50\% of tet(M) gene distribution. The upper and lower whiskers represent upper 25\% and lower 25\% of tet(M) gene distribution. The dots are outliers of tet(M) gene distribution.

The copy number of tet(M) gene per 1 ng of DNA obtained from saliva samples of the 1\textsuperscript{st}-year dental students (median = 4667, IQR = 7324) did not significantly differ from the correspondent number of
the 5th-year dental students (median = 3987, IQR = 4883; Wilcoxon rank-sum test: W = 852.5, p-value = 0.942, r = -0.008) (Figure 6).

![Figure 6](image-url)

**Figure 6.** Comparison of copy number of tet(M) gene per 1 ng of DNA between 1st- and 5th-year students. The black bold line inside boxes is median. The grey boxes represent the middle 50% of tet(M) gene distribution. The upper and lower whiskers represent upper 25% and lower 25% of tet(M) gene distribution. The dots are outliers of tet(M) gene distribution.

### 3.4 Analysis of correlations and the multiple linear regression

The consumption of 3-10 courses of antibiotics throughout life was positively correlated with the log10 counts of tet(M) gene per 1 ng of DNA obtained from saliva samples, r = 0.216 (p-value <0.05).

In contrast, none of the binary variables that describe consumption of antibiotics throughout life were correlated with the log10 transformed counts of tet(M) gene per 1 million copies of 16S rRNA gene (p-value >0.05).

The "antibiotic consumption" variable was included to the linear regression model with "counts of tet(M) gene per 1 ng of DNA" as the response variable. The consumption of 3-10 courses of
antibiotics and more than 10 courses of antibiotics were significantly associated with, respectively, 0.439 (CI95=0.155–0.723) and 0.480 (CI95=0.117-0.842) increase in log10 \( \text{tet}(M) \) gene copy numbers compared to those who had never taken antibiotics (see table 2).

When adjusted for age and gender, consumption of 1-2 courses of antibiotics throughout life became significantly associated with 0.337 (CI95=0.029–0.646) increase in amount of log10 \( \text{tet}(M) \) gene copies in saliva compared to those who had never taken antibiotics. Adjusted for age and gender, consumption of 3-10 antibiotic courses was significantly associated with 0.501 (CI95=0.193-0.808) increases in log10 \( \text{tet}(M) \) genes when compared to never-takers. Adjusted for age and gender, consumption of more than 10 courses of antibiotics was significantly associated with 0.561 (CI95=0.169-0.954) increases in log10 \( \text{tet}(M) \) gene copy number when compared to never-takers. The age and gender were not significantly associated with log10 \( \text{tet}(M) \) gene copy number (see table 2).

The unadjusted model could explain 12.6% of variance of \( \text{tet}(M) \) gene in the study sample. If model is generalized above the sample studied, it could explain 9.3% of variance in \( \text{tet}(M) \) gene. The adjustment of the model for age and gender did not significantly improved the unadjusted model in terms of predictive accuracy, p-value of \( F \) change 0.543.
**Table 2.** The linear regression models. The outcome variable is log10 tet(M) gene copy number. The predictors are: antibiotic consumption, age, and gender. Model 1 includes dummy variables of antibiotic consumption with "zero courses" as the reference. Model 2 is the Model 1 adjusted for age and gender.

<table>
<thead>
<tr>
<th></th>
<th>AR²</th>
<th>R²</th>
<th>Adj.R²</th>
<th>Unstandardized coef.</th>
<th>95.0% CI for B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>Std. Error</td>
</tr>
<tr>
<td>(Constant)</td>
<td>0.126</td>
<td>0.126</td>
<td>0.093</td>
<td>3.283</td>
<td>0.1221</td>
</tr>
<tr>
<td>Antibiotic consumption: 1-2 courses</td>
<td>0.2677</td>
<td>0.1410</td>
<td>0.061</td>
<td>-0.013</td>
<td>0.549</td>
</tr>
<tr>
<td>Antibiotic consumption: 3-10 courses</td>
<td>0.4389</td>
<td>0.1428</td>
<td>0.003</td>
<td>0.155</td>
<td>0.723</td>
</tr>
<tr>
<td>Antibiotic consumption: 10+ courses</td>
<td>0.4797</td>
<td>0.1821</td>
<td>0.010</td>
<td>0.117</td>
<td>0.842</td>
</tr>
<tr>
<td>(Constant)</td>
<td>0.013³</td>
<td>0.139</td>
<td>0.084</td>
<td>3.569</td>
<td>0.296</td>
</tr>
<tr>
<td>Antibiotic consumption: 1-2 courses</td>
<td>0.337</td>
<td>0.155</td>
<td>0.033</td>
<td>0.029</td>
<td>0.646</td>
</tr>
<tr>
<td>Antibiotic consumption: 3-10 courses</td>
<td>0.501</td>
<td>0.155</td>
<td>0.002</td>
<td>0.193</td>
<td>0.808</td>
</tr>
<tr>
<td>Antibiotic consumption: 10+ courses</td>
<td>0.561</td>
<td>0.197</td>
<td>0.006</td>
<td>0.169</td>
<td>0.954</td>
</tr>
<tr>
<td>age</td>
<td>-0.015</td>
<td>0.013</td>
<td>0.276</td>
<td>-0.041</td>
<td>0.012</td>
</tr>
<tr>
<td>gender (0=females)</td>
<td>0.016</td>
<td>0.106</td>
<td>0.882</td>
<td>-0.195</td>
<td>0.227</td>
</tr>
</tbody>
</table>

³ p-value of F change statistics more than 0.05
3.4.1 Diagnostics of the adjusted model

Outliers / influential cases: the adjusted model had two potential outliers with standardized residuals greater than 2. Their hat values, 0.09 and 0.11, were below the average hat value, 0.15. The covariance ratios (CVR) for both of the potential outliers were slightly below the lower boundary of 0.78. The values of CVR were 0.78 and 0.72. Taking the Cook’s distances of these two potential outliers into consideration, there should not be concern neither for outliers nor for influential cases. Assumption of independence: the assessment of the assumption of independent errors produced value 2.042 of Durbin-Watson statistic that indicates the assumption has been met. Assumption of no multicollinearity: the tolerance values were above 0.2. The values of variance inflation factor were sufficiently less than 10 and their average was 1.95. This points the assumption of no multicollinearity has been met. Residual's assumptions: the assumption of linearity and homoscedasticity has been met. The scatterplot of standardized residuals against standardized predicted values is shown in Appendix 4. The predictive model adjusted for age and gender can predict 13.9% of variance in the outcome variable. The shrinkage of the model was estimated as 8.4%.
4 Discussion

4.1 Summary of the findings and interpretation

In the current study, the presence and levels of \textit{tet}(M) gene in saliva of dental students was evaluated by droplet digital PCR. All samples were positive for the presence of \textit{tet}(M) resistance gene. This results support the hypothesis of Chopra and Roberts that the majority of population is most likely to have some oral bacteria carrying \textit{tet}(M) gene (62). Our findings were in line with the results of the study by Seville \textit{et al.}, who identified \textit{tet}(M) in all 20 saliva samples collected from healthy individuals (35). In the report presented by Lancaster \textit{et al.}, 15 out of 18 bacterial isolates obtained from the oral cavity of 4-6 years children were resistant to tetracycline (69). However, there was a principal difference between the abovementioned studies and our work in the method used for \textit{tet}(M) identification. Lancaster \textit{et al.} examined only those bacteria that could be cultivated. In contrast, in the current study, bacterial DNA was examined for the presence of \textit{tet}(M) irrespective of the ability of bacteria to grow in lab or not.

While mere detection of \textit{tet}(M) gene in saliva of every tested student is an interesting finding, it is not easy to interpret data on \textit{tet}(M) gene copy numbers in regards to their contribution to clinical resistance; the resistance gene could be present but inactive. In our study, we use \textit{16S rRNA} gene copy number as a representative of total bacterial counts in saliva samples. However, the \textit{16S rRNA} copy number per genome varies from one bacterial species to another. Therefore, the absolute counts of resistances genes were reported per 1 million copies of \textit{16Sr RNA} gene rather than bacterial cell numbers (42).

The level of \textit{tet}(M) gene copy number in saliva was not different in the dental students at the start of education and those dental students who had been already exposed to dental clinical practice settings. The difference in \textit{tet}(M) levels in the saliva samples form two study groups was not statistically
significant. Even if there was a statistical difference between the two groups, maybe it would have been difficult to interpret the results beyond just being statistically significant. To illustrate this further, for instance, a count of 10 copies of \textit{tet(M)} gene in one sample and 100 copies in another sample does not imply that the latter sample is 10 times "more resistant" to tetracycline. Sandegren and Andersson suggested that the increased number of resistance gene copies, in general, could lead to increased levels of clinical antibiotic resistance (70). However, the authors point out that it is still difficult to correspond a resistance gene copy number to clinical antibiotic resistance. The study by Grohs et al., presented experimental evidences that clinical antibiotic resistance to tetracycline in \textit{Streptococcus pneumoniae} isolates was dependent on structural changes within identified \textit{tet(M)} genes (71). In the current study, it was possible to quantify the copy number of \textit{tet(M)} in each sample without their qualitative, or functional, assessment. Therefore, even if there had been no difference in average of \textit{tet(M)} gene copies between the two groups, we would not be able to conclude that the levels of clinical antibiotic resistance were equal in the observational groups.

It is still an open question how many copies of a given resistance gene are associated with clinical antibiotic resistance (66). It would therefore be tempting to design a study which could investigate the link between a resistance gene copy number detected in saliva and any observed clinical resistance related to that gene.

A significant positive association between \textit{tet(M)} gene copy number and number of antibiotic courses taken throughout life has been found when data expressed per 1 ng of DNA, but not when expressed per 1 million copies of \textit{16S rRNA} gene. The most commonly used method for quantitation of genes in samples with bacterial DNA is its normalization to bacterial \textit{16S rRNA} gene (41, 42, 49). Normalization to a certain amount of DNA is less commonly used but employed in the current study for a purely methodological interest.
The fact that correlation only exists when a rather uncommon normalization method is used, raises some questions that deserve further investigation.

4.2 Limitations

The sample size in the study was based on the previous works rather than on the computation using the effect size, the alpha- and beta-levels. However, similar studies used fewer study participants than this study. For example, Ready et al., found statistically significant difference in prevalence of amoxicillin-resistant oral bacteria when studying two observational groups of children. The two groups consisted of 25 and 15 children who had not taken antibiotics within 3 months prior to the beginning of the study (45). In the study by Feres et al., 20 participants with chronic periodontitis were examined for the change in the prevalence of antibiotic resistance bacterial strains due to administration of either amoxicillin or metronidazole (72). Al-Haroni et al., collected plaque samples from two groups composed of 21 and 34 participants to explore the prevalence of oral bacterial resistant to ampicillin and metronidazole (23).

The sampling frame for the current study was the Department of Clinical Dentistry, UiT, Tromsø. As we aimed to examine whether dental practice per se is associated with higher levels of resistance genes, the 5th-year dental students were chosen as the proxy for the graduated dentists and match this group with other group of students that has no dental clinical attachment at all, i.e. 1st-year student. The reason for choosing the abovementioned study subjects is better access to the participants and, most likely, a better response rate. In fact, the latter was at its maximum, 97 from 97 invited students provided their consent to enter the study. However, the enrolled dental students might lack representativeness to generalize the results beyond the chosen sample frame. A better sample frame could be, for example, all dentists and GPs in Tromsø who spent several years in practice.
The two observational groups were different in age at statistically significant level of 0.05 (data not shown). The age variable was included to the regression model to ensure the results controlled for any possible confounder. Since there is evidence that the antibiotic selective pressure implied by their use is associated with increase in clinical antibiotic resistance (14, 15, 62), and Norwegian males in the age group 15-34 tend to receive more tetracycline than females (28), the gender variable was included to the regression analysis as a possible confounder. Intragroup variations in gender distribution was not likely to affect the comparison of the average level of \( \text{tet}(M) \) copy numbers between the two groups because there was no difference in gender distribution between the two groups (Table 1). The results of the linear regression showed age and gender were not confounders in our study. We accounted for possible interactions among the predictor variables but no significant interactions were identified (data not shown).

The copy number of \( \text{tet}(M) \) in saliva was determined for both the 1\textsuperscript{st}- and the 5\textsuperscript{th}- year dental students. Since all participants in principle shared the same campus, there was a theoretical possibility of transferring bacterial DNA between those groups. From this perspective the two groups may not be considered entirely unrelated. A better group for comparison would be 5\textsuperscript{th}-year students of a non-dental faculty, preferably from another university.

It is well-known that AR is associated with antibiotic consumption (19). In the questionnaire, the question number 10 "How many times in life have you been subjected to an antibiotic treatment course?" was used as a proxy explanatory variable for prediction of variation in the level of antibiotic resistance genes in saliva of the dental students. It is unlikely that most of the participants could be aware of all the occasions of antibacterial treatment back to their early years of life. Although the study findings detected association between antibiotic consumption and the level of the \( \text{tet}(M) \) gene in saliva, the magnitude of the effect might be measured with imprecision. Moreover, recall bias was
possible to be introduced to the study: the participants with lower overall health state or presence of a chronic disease may remember better the number of antibiotic course administered to them.

According to Roberts and Kreth, the transient bacteria can transfer accessory genes to oral bacteria via HGT (12). Taking this into consideration, it would be worthwhile to identify what transient bacteria can be found in the study participants. In a previous study to examine bacteria isolated from poultry and salmon, the presence of clinically relevant tetracycline resistance bacteria was reported which raises questions about their carry over into the food chain (73, 74). The mode of delivery is shown also to be a factor that influence the profile of antibiotic resistance detected in new-borns’ oral microflora. While those babies delivered vaginally are positive for $tet(M)$ and $tet(O)$ genes in their oral bacteria, those delivered by Caesarean-section are positive for presence of $tet(O)$ and $tet(W)$ genes in their oral bacteria (44). Hence, the information as diverse as the dietary pattern and mode of delivery may be relevant for future studies.

In the study by Ready et al., it has been suggested that amalgam restorations could be associated with the emergence of mercury-resistant bacterial found in the oral cavity. Furthermore, the authors stressed that the possibility of co-selection of tetracycline resistance genes along with mercury-resistance genes was biologically plausible (50). Although the amalgam in dental fillings was banned in 2008 in Norway, it would have been helpful to obtain information about the presence of amalgam tooth restorations in the oral cavity of participants.

The questionnaire could invoke underreporting bias by asking questions regarding health-related behaviour such as smoking or snuff use. The possible underreporting could stem from the acknowledgement that smoking and snuff use are considered socially undesirable behaviours. The incentive of underreporting may be well enhanced by awareness that medical students should promote healthy lifestyle as a part of medical ethics.
4.3 Generalizability

It is well known that antibiotics exert selective pressure on bacterial population (14, 15, 62). Furthermore, a number of experimental studies provided evidence that the volume of antibiotics was one of the most important factors for the development of resistance and resistance levels maintenance (75, 76). In the present work we studied participants that were, in general, of good health. They might be different from other “ordinary” students or not-students in terms of socioeconomic status, health, intelligence, or health-related behaviour. The majority of the participants reported consumption of antibiotics of less than 10 antibiotic courses throughout life (Table 1). We thus believe that our results of tet(M) quantitation may not be applicable to those individuals with systemic diseases treated regularly with antibiotics or to those individuals whose antibiotic consumption is substantially higher than in our study population.

Overall consumption of antibiotics in Norway is lower than in other European countries (26) However, antibiotic consumption in Norway is similar to other Scandinavian countries (27). This suggests that our findings may be applicable to Scandinavian countries but not to other European countries.

The study reported by Blix and co-workers demonstrated that consumption of antibiotics was associated with age of antibiotic receivers (28). Since the mean age of the participants of the present study was 24 years it may not be possible to generalize our findings to both more and less aged populations.

Our initial aim was to examine exposure to dental office environment as an occupational risk in view of antibiotic resistance. The "exposure" of the students to dental practice was limited to 1,5 years. It may be the case that this period of time was insufficient to observe the association with the change in resistance genes if the effect exists. Neither this period of time dedicated to dental practice is comparable to those of more experienced graduated dentists. In addition, the results may not be
applicable to other professionals in the field. For instance, dental hygienists rarely works with assistants (51) which, in turn, makes it difficult to use HVEs as a protective mean.
5 Conclusion

This work was an attempt to gain more knowledge of the problem of antibiotic resistance through examining the prevalence and levels of \textit{tet(M)} resistance gene in two observational groups. This is the first study in Norway that investigated the presence and levels of \textit{tet(M)} in saliva samples. In the current study, the levels of \textit{tet(M)} gene were not significantly different between the 1\textsuperscript{st}-year students and the 5\textsuperscript{th}-year students. Therefore, we could not conclude that working in a dental clinic can be considered a risk factor for getting more antibiotic resistance genes. However, it seems that the use of saliva as a biological sample accompanied by the highly sensitive method of ddPCR could be used as a diagnostic tool to reveal the presence and levels of resistance genes in a given individual. It seems also that the high prevalence (100\%) of \textit{tet(M)} reflects the use of tetracycline in the population where this drug is one of the mostly prescribed antibiotics nationally. It is still an open question what is the threshold level of a particular resistance gene in the saliva that would predict a failure in antibiotic treatment. As we know the presence of resistant genes could threaten the effectiveness of antibiotic therapy. Therefore, it would be interesting to design a future study to link failure of empirical antibiotic therapy to the levels of antibiotic resistance genes that could be detected in the saliva.

In conclusion, this study suggests that there is no evidence that support any claim that acquisition of antibiotic resistance can be an occupational risk for dental professionals.
References


73. Cantas L, Fraser TWK, Fjelldal PG, Mayer I, Sørum H. The culturable intestinal microbiota of triploid and diploid juvenile Atlantic salmon (Salmo salar) - a comparison of composition and drug resistance. BMC Veterinary Research. 2011;7:71-.


Appendix 1. **Questionnaire**

**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. Sjovløst godt
   2. God
   3. Verken godt eller dårlig
   4. Ikke helt godt
   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. Sjovløst godt
   2. God
   3. Verken godt eller dårlig
   4. Ikke helt godt
   5. Dårlig

7. **Er du fornøyd med utseende til tennene dine?**
   1. Sjovløst fornøyd
   2. Noen lunde fornøyd
   3. Temmelig misfornøyd
   4. Sjovløst misfornøyd

8. **Bruker du medikamenter/medisiner daglig?**
   1. 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
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)

<table>
<thead>
<tr>
<th></th>
<th>Hele tiden</th>
<th>Ofte</th>
<th>Mer enn halve tiden</th>
<th>Mindre enn halve tiden</th>
<th>Sjelden</th>
<th>Aldri</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Jeg føler meg trist og nedfor</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>B. Jeg føler meg rolig og avslappet</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>C. Jeg føler meg energisk, sprek og aktiv</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>D. Jeg våkner opp og føler jeg meg frisk og uttvilt</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>E. Hverdagen min er full av ting som interesserer meg</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

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

- Ja
- Nei
- Usikker

14. Røyker du? Om ja, hvor ofte?

- Røyker hver dag
- Røyker av og til
- Røyker aldri

15. Hvor lenge har du røykt?

- Jeg røyker ikke
- Mindre enn i 3 år
- 1-3 eller flere år

16. Snuser du? Om ja, hvor mye?

- Snuser hver dag
- Snuser av og til
- Snuser aldri

17. Hvor lenge har du snust?

- Jeg snuser ikke
- Mindre enn i 3 år
- 1-3 eller flere år

18. Hvor mange sigaretter/snus porsjoner per dag?

- Antall sigaretter:
- Antall snusporsjoner:

19. Hvor ofte pusser du tennene dine?

- Morgen og kveld
- En gang per dag
- En gang i blant

20. Hvor ofte bruker du tanntråd/tannstikker?

- Etter hver tannpuss
- En gang per dag
- En gang per uke
- Sjeldnere
- Aldri

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

- Etter telefonbruk
- Etter å ha skrevet journal
- Etter å ha tatt røntgenbilder
- Etter hver pasient
- Prøver å begrense antall håndvask
HOLDNINGER

22. Anser du at tannleg 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

23. Hvem er mest utsatt for smitte på et tannlegekontor?
1 Tannlegen
2 Pasienten
3 Tannhelsesekretæren
4 Vanskelig å besvare

24. Kjenner du til/har hørt om tilfeller der en pasient har blitt smittet etter et tannlegebesøk?
1 Ja
2 Nei

25. Kjenner du til/har hørt om tilfeller der tannlegen har blitt smittet på tannlegekontoret?
1 Ja
2 Nei

26. 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

27. Hva er detiktigste enkelt-tiltaket mot smitte?
1 Unngå nærkontakt med pasient (dråpesmitte)
2 God håndhygiene
3 Engangsartiklene
4 God rengjøring av utstyr
5 Sikre rutiner for å deponere klinisk avfall
6 Vanskelig å besvare

28. På hvilken måte kan tannlegen beskytte seg når han/hun har en «vanlig» pasient i stolen?
(Merk: her kan du gi flere svar)
1 Sprute alle arbeidsflater mellom hver pasient
2 Godt såpe-håndvask mellom hver pasient
3 Godt håndvask med sprøt 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 arbeidsstøy
7 Ved å bruke øyebeskyttelse/ visir
8 Ved å bruke papir- 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

29. Kan tannlegen eliminere smitterisiko ved å følge «hygieneveilederen»?
1 Ja
2 Nei
3 Vanskelig å besvare

30. Hva er viktige barrierer for god hygiene på tannkliniken?
1 Glemsomhet
2 Behandlingen tar lengre tid
3 Ubekvemme arbeidsforhold
4 Manglende kunnskap om smittevern
5 Pasienten forteller ikke om mulige smitte

3
31. Hvilken metode ville du benyttet for å reingjøre de forskjellige redskapene?
(Se *en* ring rundt det tallet som passer best for deg)

<table>
<thead>
<tr>
<th></th>
<th>Varme-desinfisering</th>
<th>Kjemisk-desinfisering</th>
<th>Damp-auto-klavering</th>
<th>Vakum-auto-klavering</th>
<th>Tørr-sterilisering</th>
<th>Hydrogen-peroksid</th>
<th>Sprit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ekstraksjons-tenger</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>Rotkanalinstrumenter</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>Håndstykker (bor)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>Avtrykk</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>Undersøkelses brett</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>F</td>
<td>Skarpe instrumenter</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>G</td>
<td>Kirurgisk utstyr</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

32. I hvilke situasjoner ville du brukt de nedenfor nevnte hjelpemidlene?
(Se *en* ring rundt det tallet som passer best for deg)

<table>
<thead>
<tr>
<th></th>
<th>For alle prosedyrer</th>
<th>For noen prosedyrer</th>
<th>For infektiøse pasienter</th>
<th>For noen prosedyrer og infektiøse pasienter</th>
<th>Aldri</th>
<th>Vanskelig å besvare</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Hansker</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>To par hansker</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>Munnbind</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>Øyebeskyttelse</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>Visir</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>Engangsartikler</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>Plast over hele stolen</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>Plast også på tastatur, lampe, blyant, etc</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

TAKK FOR DINE SVAR!

En problemstilling i studien er: Er utbredelsen av resistens gener avhengig av hvor lenge tannlegen/tannlegestudenten har vært i klinisk praksis?

Personene som blir aksepterte 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). Studien vil avsluttes juni 2016.

**UNNØDSELJEN**


Referanseverdier for stimulert helsaliva

<table>
<thead>
<tr>
<th>0,70 – 1,00 ml/minutt</th>
<th>Lav sekresjon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,00 – 3,00 ml/minutt</td>
<td>Normal sekresjon</td>
</tr>
</tbody>
</table>

Informasjon om holdninger og kunnskap vil innhentes fra spørreskjema. Du kan når som helst trekke deg fra studien, uten noen spesielt «god» grunn.

**INNSAMLET DATA**


Fra din spyttprøve vil vi analysere antimikrobielle resistensgener fra ditt DNA. Vi vil KUN analysere DNA mht antimikrobiell resistens. Vi garanterer at verken informasjon om dette eller noen annen informasjon fra ditt DNA vil bli spredt utenfor vår forskergruppe. Din spyttprøve og informasjonen som registreres om deg fra spørreskjemaet skal kun brukes slik som beskrevet i hensikten med studien.
Etter at studien er avsluttet vil alle innsamlede data bli ødelagt og spyttprøvene destruerte. Resultatet fra DNA-analysen kan du få ved å henvende deg til prosjektlederne med ditt identifikasjonsnummer. Prosjektet er godkjent av Regional komité for medisinsk forskningsetikk (Helseregion nord; REK Nord).

**RISIKO**
Det er så og si ingen risiko med undersøkelsen. Det er ingen kjente bivirkninger ved å avgi en spyttprøve.

**KOSTNAD**
Konsultasjoner og undersøkelsen er uten kostnad for deltakeren. Som for all behandling av helse- og tannhelsepersonell i Norge er deltakerne i studien dekket av Norsk Pasientskadeerstatning (NPE).

**OPPFOLGINGSBEHANDLING**
Ingen oppfølgingsbehandling er nødvendig. Dersom du likevel ikke ønsker å delta, eller bestemmer deg for å gå ut av studien før den er avsluttet (om ca. 1 år) er det fullt ut akseptabelt. Behandling for eventuell annen sykdom eller skade i munnhulen som kan bli avdekket under undersøkelsen, vil måtte utføres hos egen tannlege og betales av deltakeren. Resultatene fra studien vil bli publiserte i nasjonale og internasjonale tidsskrifter. Deltakerne kan henvende seg til en av prosjektlederne for informasjon.

**INNSAMLET SPYTT**
Under analyseperioden (ca. 2 mnd.) vil spyttprøven som ble tatt bli lagret på forskningslaboratoriet ved Institutt for klinisk odontologi, UiT. Det biologiske materialet kan bare brukes etter godkjenning fra REK. Alle innsamlede prøver vil bli destruerte etter at studien er avsluttet.

**KONFIDENSIALITET**
Alle opplysninger behandles konfidentielt og ingen vil kunne gjenkjennes i publikasjoner. Ved prosjektets slutt vil alle forskningsdata bli destruerte.

**SPØRSMÅL**
Det er frivillig å delta! Ta gjerne kontakt med oss dersom du lurer på noe.

**PROSJEKTLEDERE**
- Førsteamanuensis Mohammed Al-Haroni, IKO – UiT
  - tlf. 776 49151
  - mohammed.al-haroni@uit.no
- Professor Tordis A Trovik, ISM - UiT
  - tlf. 776 44297
  - tordis.a.trovik@uit.no
- Master of Public Health (MPH) Student Stanislav Iakhno, ISM - UiT
  - stanislav.iakhno@gmail.com

**SAMTYKKEERKLÆRING**
Jeg har mottatt skriftlig og muntlig informasjon om studien, og sier meg villig til å delta.

**DELTAKER:**

<table>
<thead>
<tr>
<th>sted</th>
<th>dato</th>
<th>navn</th>
</tr>
</thead>
</table>

**TANNLEGE:** Jeg bekrefter å ha gitt informasjon om studien

<table>
<thead>
<tr>
<th>sted</th>
<th>dato</th>
<th>navn</th>
</tr>
</thead>
</table>


## Appendix 3. PCR conditions.

<table>
<thead>
<tr>
<th>Phase</th>
<th>temp</th>
<th>time</th>
<th>cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preheating and enzyme activation</td>
<td>95 °C</td>
<td>10min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
<td>-</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>58 °C</td>
<td>60 sec</td>
<td>40</td>
</tr>
<tr>
<td>Enzyme deactivation</td>
<td>98 °C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Store</td>
<td>4 °C</td>
<td>infinite</td>
<td>-</td>
</tr>
</tbody>
</table>

The ramp rate – 2.5 °C/sec. The temperature of the lid – 105 °C.
Appendix 4. Assessment of residuals of the adjusted regression model. Top: plot of standardized residuals against predicted values. Middle: histogram of residuals distribution. Bottom: P-P plot of expected cumulative probability of normal distribution against observed cumulative probability of distribution.