Antibiotic Resistance in Oral Microbiota

A study on prevalence, molecular analysis, and possible contributing factors in Yemen and Norway

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I dedicate my dissertation to my wife, children and for the memory of my supervisor professor Nils Skaug

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Acknowledgments

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List of publications

The present thesis and the following papers are submitted as partial fulfilment of the requirements for the Ph.D. degree at the Faculty of Dentistry, University of Bergen, Bergen, Norway. In the present thesis, the following papers will be referred to in the text by Arabic numerals.

1. Al-Haroni MH, Skaug N, Al-Hebshi NN. (2006). Prevalence of subgingival bacteria resistant to aminopenicillins and metronidazole in dental patients from Yemen and Norway. *Int J Antimicrob Agents* ; **27**: 217–223.

2. Al-Haroni M, Skaug N, Bakken V, Cash P. (2008). Proteomic analysis of Ampicillin-resistant oral *Fusobacterium nucleatum*. *Oral Microbiol Immunol*; **23**: 1–7

3. Al-Haroni M, Skaug N. (2006). Knowledge of prescribing antimicrobials among Yemeni general dentists. *Acta Odontol Scand* ; **64**: 274–280.

4. Al-Haroni M, Skaug N. (2007). Incidence of antibiotic prescribing in dental practice in Norway and its contribution to national consumption. J Antimicrob Chemother; **59**:1161–1166.

Abstract

Worldwide antibiotic resistance among oral microbiota is an increasing problem and information regarding such resistance is completely lacking for Yemen and very limited data is available for Norway. The **aims** of the current thesis were to (1) disclose the prevalence of ampicillin and metronidazole resistance among selected subgingival microbial species obtained from individuals in Yemen and Norway **(paper 1)**, (2) determine the susceptibility pattern of *Fusobacterium nucleatum* isolated from Yemen and characterize the aminopenicillins-resistance determinant of *F. nucleatum* **(paper 2)**, and (3) assess if antimicrobial prescription practices by dentists in Yemen and Norway could possibly contribute to the current prevalence and the emergence of bacterial resistance in these geographically separate locations **(papers 3 and 4)**. **Materials**: Thirty-four and 21 subgingival plaque samples from Yemen and Norway, respectively, were cultivated on fastidious anaerobic blood agar containing 2 μg/mL of either ampicillin or metronidazole. The bacterial growth from each plate was then screened using DNA-DNA checkerboard hybridization technique for the presence of ampicillin and metronidazole resistance among 18 selected subgingival species **(paper 1)**. Ampicillin-resistant *F. nucleatum* strains were isolated from Yemen by cultivating subgingival plaque samples on crystal violet erythromycin (CVE) plates supplemented with or without 2 μg/mL ampicillin. The molecular basis of ampicillin resistance among *F. nucleatum* strains was studied using two-dimensional gel electrophoresis and mass spectrometry **(paper 2)**.The antimicrobials prescription knowledge of Yemeni dentists was investigated by distributing a structured questionnaire to all working dentists in the three major governorates in Yemen. The questionnaire aimed at investigating the therapeutic and prophylactic use of antimicrobials with relevant clinical and non-clinical parameters **(paper 3)**. The Norwegian dentists' antimicrobial prescription practices were revealed by analyzing aggregated data obtained from the **Nor**wegian **P**rescription **D**atabase (NorPD) on the basis of their total prescriptions of 11 antibiotics issued in 2004 and 2005. Consumptions of these antibiotics in dental practice were measured using the WHO measurement unit, the Defined Daily Dose

(DDD) **(paper 4)**. **Results**: A statistically significant higher resistance to metronidazole and ampicillin among nine and seven species (P <0.05), respectively, was found in Yemeni isolates compared with Norwegian ones. The molecular characterization of ampicillin-resistant *F. nucleatum* isolates showed that ampicillin-resistant *F. nucleatum* isolates harboured a class D β-lactamase enzyme. In addition, increased synthesis of two proteins, enolase and ABC transporter ATP-binding proteins, was associated with β-lactamase enzyme production. A sound knowledge of antimicrobials prescription among Yemeni dentists was lacking with a trend of overuse. NorPD data showed antimicrobial prescribing trend in favour of narrow-spectrum penicillins among Norwegian dentists. **Conclusions**: The findings indicate that high prevalence of resistance among oral bacteria in Yemen maybe a potential threat in the management of dental infections in the region. Antimicrobial overuse by dentists in Yemen could exacerbate the existing dilemma of antimicrobial resistance of oral bacteria. In contrast, the low prevalence of resistance among subgingival species in Norway is most probably a result of the judicious use of antimicrobials, that is, more restrictive prescribing practices in the country. The presence of class D β-lactamase among ampicillin-resistant *F. nucleatum* strains increases their virulence and cost of treatments as these enzymes might present resistance to several classes of β-lactam antibiotics. **Recommendations**: Halting resistance development and saving effectiveness of antimicrobials need strict, practical, and feasible approaches. There is an urgent need to formulate an action plan to counter the revealed situation of antimicrobial resistance in Yemen. A proposed strategy to be adopted in the country for the control of antimicrobial resistance should be based on the prevention of communicable diseases and infection control to reduce the need for antimicrobial agents. An antimicrobial resistance surveillance system and a multidisciplinary committee that monitors antimicrobial use in the country should form integral parts of a structured approach to reduce antimicrobial resistance by improving antimicrobial prescribing. Finally, such an effective strategy requires close cooperation and consultation between Yemen and other involved parties, both at national and international levels.

1. Introduction

1.1 Oral microbiota

The oral cavity is the first part of the gastrointestinal tract and it has a number of features that makes it a distinct microbial habitat. The various surfaces in the oral cavity are continuously bathed with saliva and they represent different ecological niches in which distinct inhabitants exist within this complex environment. The ecological characteristics of the different surfaces found in the oral cavity, each with different key ecological factors such as adhesion ligands, pH, nutrients, redox potential, oxygen tension, and temperature, make it a unique microbial habitat in the human body [1]. The composition of microbiota in the oral cavity is complex and such complicity was noticed in as early as 1683 by Antonie van Leeuwenhoek [2]. The oral microbiota is composed predominantly of bacteria, but fungi, viruses, mycoplasmas, and even protozoa and archaea can be found. It is estimated that more than 700 cultivable and noncultivable species are present in the oral cavity [3]. Over 400 of the 700 oral species have been identified from the periodontal pocket and 300 species from other locations in the oral cavity. Any particular individual is thought to have approximately 100–200 of these 700 species and is thought to harbour around 50 species in the periodontal pocket [4].

Several Gram-positive and Gram-negative bacterial genera are found in the oral cavity. Among the Gram-positive ones are *Enterococcus, Peptostreptococcus, Streptococcus, Staphylococcus, Actinomyces, Corynebacterium, Eubacterium*, and *Lactobacillus* species, whereas *Aggregatibacter* (formerly *Actinobacillus*), *Haemophilus, Bacteroides, Campylobacter, Leptotrichia, Prophyromonas, Capnocytophaga, Prevotella, Tannerella, Eikenella, Treponema, Fusobacterium*, and *Wolinella* species are among the Gram-negative ones [1].

Adhesion of bacteria species to oral surfaces is the initial event in their establishment as a distinct microbial community in different niches within the oral cavity.

The initial adhesion is characterized by the presence of the same bacterial species that later on may modify the surrounding environment, making it suitable for other species to colonize [1].

Despite the diverse community of microorganisms found within the oral cavity, it is characterized by a high degree of stability. Such a stable community is referred to as climax community [1]. It is maintained in spite of host defence and modest environmental stress, such as, changes in saliva flow, diets, regular exposure to mouth rinses and tooth pastes, challenge by exogenous species, and exposure to antimicrobials. This stability, referred to as microbial homeostasis, is of great importance to oral health as it insures that potentially harmful species remain in low numbers [1]. Major environmental perturbations, such as pH or redox potential changes, are necessary to break the microbial homeostasis, resulting in deteriorated oral health and development of diseases, such as periodontitis and dental caries [5].

Dental plaque and oral diseases

Coaggregation is the physical interaction between bacteria of different species. It is not random among oral bacteria; each species binds specifically to other bacteria. The diverse community of microorganisms found on a tooth surface is known as dental plaque. It is defined clinically as the soft, tenacious deposit that forms on tooth surfaces that is not readily removed by rinsing with water [6]. Microbiologically, it can be defined as the diverse community of microorganisms found on a tooth surface as a biofilm, embedded in an extracelluar matrix of polymers of host, and is of microbial origin [7]. Recently, the classical name of bacterial deposits on tooth surfaces known as “dental plaque” is increasingly substituted by the more appropriate name “dental biofilm”. According to its location, dental biofilm can be found supragingivally or subgingivally. The general properties of a biofilm make the involved microorganisms dramatically different from their planktonic counterparts, that is, bacteria that are suspended or growing in a fluid. Such properties include open architecture, protection from host defences, enhanced resistance to antimicrobial agents, neutralization of in-

hibitors, novel gene expression, coordinated gene responses, spatial and environmental heterogeneity, broader habitat range, and more efficient metabolism [7].

It is well known that periodontal diseases [8] and dental caries, the most prevalent microbial diseases in humans, are dental biofilm-mediated diseases [2, 9]. There has been an ongoing controversy as to which bacteria or bacterial species within the dental biofilm are involved in the causation of these diseases. The issue is even more complicated in the case of periodontal diseases, principally because these diseases occurs at sites with a preexisting complex normal flora, making discrimination of opportunistic pathogens from host-compatible species a real challenge, especially the fact that the pathogens may be carried in low numbers in a healthy oral cavity [10, 11]. In addition, periodontal infections seem to be mixed in nature, involving more than one bacterial species, rendering evaluation of the aetiology of periodontitis a difficult task. For this and others reasons, Koch's postulates have been replaced by a set of criteria to define periodontal pathogens. These criteria include (1) association (the species is found more frequently and at higher levels in disease compared to health), (2) elimination (elimination of the species is paralleled by remission of disease), (3) host response (presence of immune response against that species), (4) possession of virulence factors, and (5) induction of disease in animals [10]. These criteria assisted researches in pointing out some candidates as etiological agents of periodontal diseases. In light of these criteria, there was a strong evidence to support a consensus implicating *Porphyromonas gingivalis* and *Tannerella forsythia* as etiological agents of chronic periodontitis, and *Aggregatibacter actinomycetemcomitans* as that of aggressive periodontitis [12].

It is well known that in dental biofilm, certain bacteria often cluster together and if one member of a particular cluster is detected in a sample, other members of that cluster are also most likely to be detected [13], indicating that these bacteria prefer similar living environment. There are five microbial complexes described, namely, red, orange, yellow, purple, and green complexes, in subgingival plaque [13]. The red complex is composed of *P. gingivalis*, *T. forsythia* and *Treponema denticola*, and it is strongly associated with the clinical signs of chronic periodontitis,

whereas bacteria of the genera *Fusobacterium, Prevotella, Peptostreptococcus, Eubacterium*, and *Campylobacter*, which are members of the orange complex, are moderately associated with the disease.

Fusobacterium nucleatum

The *Fusobacterium* species is an old genus and currently includes 13 species from both human and animals [14-16]. *Fusobacterium nucleatum* species are most frequently isolated from the oral cavity. The bacterium is an anaerobic, nonsporing, nonmotile and Gram-negative rod bacterium with fused ends [17]. The heterogeneity of *F. nucleatum* is well known and five subspecies of *F. nucleatum* have been described [14, 15, 18]. The taxonomy of *F. nucleatum* subspecies is on a shaky ground, as there seems to be much heterogeneity within this species [19, 20]. The five described *F. nucleatum* subspecies are: *F. nucleatum* subspecies *nucleatum*, *F. nucleatum* subspecies *vincentii*, *F. nucleatum* subspecies *polymorphum*, *F. nucleatum* subspecies *fusiforme* and *F. nucleatum* subspecies *animalis*. All *F. nucleatum* subspecies are human isolates except *F. nucleatum* subspecies *animalis* which is of animal origin [14, 15].

F. nucleatum can be encountered in their niches within the oral cavity in the early months of life [21]. It acts as a bridge between early and late colonizers in dental plaque and coaggregates with many species found in the oral cavity including periodontal pathogens [22-25]. The proportion and number of *F. nucleatum* isolates are higher in individuals with compromised periodontal tissues. During periodontal infections, the cell mass of *F.nucleatum* increases as much as 10,000-fold, making it one of the most abundant anaerobic species in the disease sites [26]. However, the actual role of *F. nucleatum* in periodontal disease pathogenesis is probably masked by the bacterium being a common isolate in healthy individuals also [11]. In addition, virulence factors of *F. nucleatum* are less studied than those in other bacteria known as etiological factors of periodontal diseases. The pathogenic role of *F. nucleatum* in otitis media, orofacial and skin infections, tonsillar abscesses, septic arthritis, and bacterial endocarditis has been documented [17, 27].

The genome of both *F. nucleatum* subspecies *nucleatum* type strain ATCC 25586 and *F. nucleatum* subspecies *vincentii* type strain ATCC 49256 has been published [28] [29]. The genome of *F. nucleatum* subspecies *nucleatum* (ATCC 25586) contains 2.17 Mbp encoding 2067 open reading frames (ORFs) and its comparison with *F. nucleatum* subspecies *vincentii* (ATCC 49256) underscores the heterogeneity of *F. nucleatum* subspecies [29]. Despite the fact that *F. nucleatum* is a Gram-negative bacterium, interesting phylogenetic inferences, based on conserved indels (i.e. protein domain(s) insertion and deletion), place it at an intermediate position between Gram-positive and Gram-negative taxa [30]. In line with this, based on 16S rRNA sequence analysis, *Fusobacterium* species appear as a separate cluster only distantly associated to the low G+C Gram-positive bacteria [18, 31].

Strains of *F. nucleatum* are intrinsically resistant to erythromycin. Thus, erythromycin is used in the selective medium for isolation of *F. nucleatum* in plaque samples [32]. Most strains of *F. nucleatum* are susceptible to penicillin, but an ongoing increase in the proportions of *F. nucleatum* isolates resistant to penicillins have been reported [33, 34]. β-Lactamase enzyme production was detected in penicillin resistant *F. nucleatum* species [34-36].

1.2 Antibiotics and antimicrobials

The word antibiotic originally described a substance, such as penicillin or cephalosporin, produced by or derived mostly from certain fungi, bacteria, and other organisms, that can directly kill or inhibit the growth of other microorganisms. Later, these substances were replaced by synthetic or semisynthetic derivatives that were designated antimicrobials or microbial agents to distinguish them from the former. However, nowadays, the term antibiotic is often used informally for a drug that according to this definition is an antimicrobial. In the present thesis, both terms will be used synonymously for antibacterial agents.

The first antibiotic, penicillin, was discovered in 1928 by Sir Alexander Fleming. Ten years after the Fleming's discovery of penicillin, sulfonamide was discovered, and as time passed, new drug discoveries led to an explosive development of numerous antibiotics from the 1950s till the early 1990s. It was not surprising that shortly after numerous antibiotic discoveries that were active against both Gram-positive and Gram-negative bacteria, surgeons believed at that time that the ongoing ancient fight between human and infectious diseases was becoming to an end.

The antimicrobial agents can be divided into two major groups: bactericidal agents, which kill bacteria; and bacteriostatic agents, which inhibit bacterial multiplication without actually killing them. It is found that these agents inhibit the growth of or kill microorganisms by a variety of mechanisms. In general, their action on bacteria involves cell wall, ribosomes, cytoplasmic membrane and nucleic acid replication sites.

Antibiotics in dental practice

It is well known that periodontitis and dental caries are dental biofilm-mediated diseases [2, 9]. Therefore, reduction of dental biofilm accumulation is regarded a premium goal in controlling these diseases. This is achieved mainly by patient's oral hygiene efforts with regular professional help by dental hygienists. Systemic antibiotic therapy has no effect on reducing supragingival plaque accumulation and solely dedicating them to control the dental plaque-mediated periodontal diseases is not an appropriate practice [37]. Mechanical debridement of dental biofilm alone is usually, but not always, sufficient for the control of these diseases. Therefore, chemotherapy is sometimes needed to aid the mechanical debridement.

Dental practitioners, by law, have the right to prescribe a battery of antibiotics in dental practice. In general, antimicrobial prescribing in dental clinics are justified as: (1) therapeutic aid to surgical treatment of an acute or chronic infection, (2) therapeutic to treat active infectious disease, for example, acute ulcerative gingivitis, and (3) prophylactic to prevent metastatic infection, such as bacterial endocarditis [38-42]. It

is worth mentioning in this respect that prophylaxis in medically compromised patients (MCPs) who receive dental treatment is not always a clear-cut matter, because different guidelines may have different recommendations and various regimens exist. Furthermore, these guidelines are always under revision and, therefore, dentists are required to update themselves regularly. Just recently, the **A**merican **H**eart **A**ssociation (AHA) recommends that some patients who have taken prophylactic antibiotics routinely in the past are no longer in need of prophylactic antibiotics as a preventive measure before their dental treatment. This includes patients with mitral valve prolapse, rheumatic heart disease, bicuspid valve disease, calcified aortic stenosis, and congenital heart conditions such as ventricular septal defect, atrial septal defect, and hypertrophic cardiomyopathy [43]. Clinical signs and symptoms of active infections include tachycardia, facial swelling, limited mouth opening, raised temperature, difficulty in swallowing, and regional lymphadenitis. Single or combined drug therapies have gained increasing importance in dental practice, but, whenever possible, single drug therapy should be prescribed to reduce incidence of side effects, emergence of resistance, and the cost of therapy.

Antibiotic prescription should be based on microbiological testing for the following clinical diagnosis: aggressive periodontitis, generalized severe chronic periodontitis, periodontitis exhibiting progressive attachment loss despite thorough adequate treatment, and severe periodontitis associated with systemic diseases, for example, human immunodeficiency virus [44]. Reports show that many antimicrobial classes are utilized by dentists [37, 45-50]. For empiric therapy, that is, the proper selection of which antibiotic to prescribe for patients, the dentists should consider the pharmacological characteristic of the antibiotic, the patient's safety, the probable infectious agent, and the cost of the drug.

Dentists' antimicrobials prescription attitude seems to be biased toward certain classes of antimicrobials, mainly penicillins and metronidazole [45, 47, 51]. Penicillins and metronidazole prescriptions accounted for about 68 and 26% of the total antibiotic prescriptions issued by 10% of the dentists working in England [47]. Metroni-

dazole prescriptions issued by dentists accounted for 45% of all metronidazole prescriptions in the United Kingdom [52]. It is estimated that the total dentists' prescriptions contributed to 7–9% of the total prescriptions issued for the community [52, 53]. On an average, 159 antibiotic courses per year are prescribed by each dentist in the United Kingdom [54]. The average number of prescriptions per dentist per week ranged from three in the United Kingdom to 4.45 in Canada [45, 54]. The actual contribution of dentists' prescriptions to the national antimicrobials consumption is not clear.

Antibiotics commonly used in dental practice

Most oral infections are polymicrobial because of involvement of Gram-positives and Gram-negatives of both anaerobes and aerobic bacteria. In the following section, a description of the most-prescribed antibiotics in dental practice is given. This is far from being a comprehensive reference of these antibiotics, but may serve as a general overview of these agents.

β-Lactam antibiotics

Although, Sir Alexander Fleming discovered the penicillin in 1928, the mass production of this antibiotic actually began from 1939 when a joint effort was made by Great Britain, Canada, and the United States to mass produce penicillin for the alliance troops. A wide array of penicillins and other β-lactams antibiotics have been synthesized by incorporating various side chains into the β-lactam ring. Of all β-lactams antibiotics, penicillins are the most widely used antimicrobial agents in dentistry. The narrow-spectrum penicillinase-sensitive agents, such as penicillin G and penicillin V, and the broad-spectrum aminopenicillins, for example, ampicillin and amoxicillin, are of primary interest to dental practitioners. Penicillin V, phenoxymethylpenicillin, is orally administered and it is active against streptococci and most oral anaerobes [33]. Phenoxymethylpenicillin is effective against a majority of α-haemolytic streptococci and penicillinase-negative staphylococci. Aerobic Gram-positive organisms, including *Actinomyces, Eubacterium,* and

Peptostreptococcus species, are sensitive together with anaerobic Gram-negative organisms, such as, *Bacteroides, Prevotella, Porphyromonas, Fusobacterium,* and *Veillonella* species. The majority of *Staphylococcus aureus* strains have developed resistance to the drug. Phenoxymethylpenicillin is commonly used by dental practitioners in the treatment of acute purulent infections, post-extraction infections, and salivary gland infections [33, 40].

The mode of activity of aminopenicillins is similar to that of phenoxymethylpenicillin, that is, inhibiting cell wall synthesis, but the former is effective against a broader spectrum of organisms, including Gram-negative organisms such as *Haemophilus* and *Proteus* species. The aminopenicillins owe their extended spectrum to an increased ability to penetrate the outer membrane of Gram-negative bacteria. Ampicillin is sometimes used in the empirical treatment of dento-alveolar infections when the antibiotic sensitivity patterns of the causative organisms are unknown [40]. Amoxicillin is the drug of choice for prophylaxis of infective endocarditis [42, 55], because of its predictable and reliable absorption after oral administration rather than its increased spectrum, in patients undergoing dental treatment procedures requiring prophylaxis [33, 42]. It is also common to combine some penicillins with β-lactam inhibitory substances such as clavulanic acid, sulbactam, or tazobactam. These inhibitors block the β-lactamase enzyme produced by the bacteria from functioning and increase the ability of the β-lactam antibiotic to work.

Metronidazole

Metronidazole was introduced in the mid-1950s by Rhone-Poulenc under the brand name Flagyl. It was the first drug of the group that is now called nitroimidazoles. Flagyl was first introduced as a drug in the treatment of trichomonas vaginalis, a sexually transmitted disease, and it revolutionized the therapy for that condition. In 1964, a dentist noted that patients with gingivitis treated with Flagyl for trichomonas vaginalis were cured and the second major indication was then established. Flagyl was also found useful in the treatment of protozoan parasite *Giardia lamblia* and in the treatment of *Entamoeba histolytica* during the late 1960s and 1970s. In the early

1970s, it was found that Flagyl was very active against the obligate anaerobes of which the two best-known families are *Bacteroides* and *Clostridia.* Flagyl is regarded as the gold standard for treating these infections.

The exquisite anaerobic activity of this drug makes it exceedingly effective against anaerobic bacteria. Metronidazole exerts its effect on bacteria by inhibiting microbial RNA synthesis. The drug is active against almost all strict anaerobes including *Bacteroides, Eubacterium, Fusobacterium*, and *Peptostreptococcus* species. The drug is indicated in the treatment of acute necrotizing ulcerative gingivitis and for moderate to severe odontogenic infections, frequently in combination with penicillins [33, 40].

Tetracyclines

Tetracyclines are broad-spectrum bacteriostatic drugs that bind to the 30S ribosomal subunit of bacteria, and specifically inhibit the binding of aminoacyl-t-RNA synthetases to the ribosomal acceptor site, thus inhibiting protein synthesis [40]. Tetracycline, doxycycline, and minocycline are the best-known members of this family of antibiotics. In dentistry, tetracyclines are used with some success as adjunctive treatment in localized aggressive periodontitis [40]. Tetracyclines have few side effects but are not recommended for children or pregnant women because they can discolor developing teeth and alter bone growth [56]. Tetracyclines also have nonantibacterial properties that include antiinflammatory, immunosuppressive properties, suppression of antibody production in lymphocytes, reduction in phagocytic function of polymorphonuclear leukocytes, and reduction of leukocyte and neutrophil chemotaxis. It also acts as an inhibitor of lipase and collagenase activity, as an enhancer of gingival fibroblast cell attachment, and even has antitumor activity [56-58].

Macrolides and lincosamides

The macrolide-lincosamide-streptogramin B class (MLS) antibiotics contain structurally different but functionally similar drugs. Macrolides are bacteriostatic drugs that exert their action by interfering with bacterial protein synthesis by binding to the 50S

ribosomal subunit; it is thought to bind to the donor site during the translocation step [56]. Erythromycin, clarithromycin, and azithromycin are members in this family.

Macrolides have activity against streptococci, staphylococci, and some oral anaerobes [33]. Erythromycin is used instead of penicillins in penicillin-allergic patients with an added advantage of being active against β–lactamase producing strains [40]. Clindamycin is a lincosamide and is effective against both aerobic and anaerobic species of bacteria and has a wider host range than erythromycin. It is a potent bactericidal antibiotic that exerts its action by interfering with protein synthesis. In dentistry, clindamycin has its main indication in penicillin-allergic patients who require antibiotic prophylaxis prior to dental treatments [59].

1.3 Antibiotic resistance

Bacterial resistance to antimicrobials can be defined either genotypically, where the bacteria carries certain resistance elements, phenotypically, where the bacteria can survive and grow above a certain level of antibiotics in the laboratory; or clinically, where the bacteria are able to multiply in humans in the presence of drug concentrations during therapy [60]. Bacterial resistance to antimicrobial agents can be either natural (inherent, intrinsic) or acquired.

Natural (inherent, intrinsic) resistance

In this type of resistance all isolates of a certain bacterial species are not sensitive to the antimicrobial in question. This could be because of a lack of certain structures in bacteria that serve as the target molecules for the antimicrobial or the lack of metabolic processes essential for the activation of the antimicrobial. In agreement with this, bacteria without a cell wall (e.g., the *Mycoplasma* species) are naturally resistant to antimicrobial agents such as β–lactam antibiotics, having activity against the cell wall. Another example of natural resistance is in the case of enterococci and cephalosporins. There are no penicillin-binding proteins in enterococci that bind these drugs with high affinity, and thus enterococci are intrinsically resistant to these

agents. Intrinsic resistance attributable to lack of metabolic processes is also noticed among oral bacteria. For example, *Actinomyces* species, *Streptococcus* species, and *A. actinomycetemcomitans* lack the enzyme nitroreductase necessary to convert metronidazole to its active metabolites, and are not affected by the drug at normal therapeutic concentrations. Intrinsic resistance because of a missing metabolic process is found in aminoglycosides resistance in enterococci in which the facultative anaerobic metabolism limits the uptake of aminoglycosides because of the absence of an electron-transport chain.

Acquired resistance

In contrast to natural resistance, acquired resistance is found only in some isolates of a certain bacterial species. However, sometimes the percentage of resistant isolates could reach high figures and susceptible isolates are hardly found. Acquired resistance in bacteria is evolved because of genetic alteration that can be achieved by two mechanisms: chromosomal mutation in the preexisting bacterial genome or, most frequently, by horizontal gene transfer between bacteria both within and outside species. Horizontal gene transfer allows bacterial population to develop antibiotic resistance at a rate significantly greater than would be afforded by mutation of chromosomal DNA. Indeed, horizontal gene transfer is the most frequent pathway for the dissemination of antibiotic resistance genes.

For the dissemination of antibiotic resistance gene or genes by horizontal gene transfer, a resistance gene may be inserted into transferable genetic elements (plasmids, transposons, and integrons) and may be linked within them to other resistance genes. The movement and introduction of transposons, integrons, or plasmids (each may carry antibiotic resistance gene or genes), into a bacterium occur via three mechanisms, namely, transformation, transduction, and conjugation. In **transformation**, free exogenous segments of DNA carrying resistance genes are acquired by the bacteria from their environment, and the bacteria is required to undergo a physiological state termed competence – altered bacterial phenotype by which bacteria are able to take up and integrate exogenous free DNA from their environment.

Natural transformation was first demonstrated in *streptococcus pneumonia* in 1928 by Griffith [61]. Transformation occurs in bacterial species that are naturally competent, such as pneumococci, haemophilus, and some oral streptococci. In fact, transformation is thought to be responsible for the devolvement and appearance of mosaic genes and the mosaic structure of Penicillin Binding Proteins (PBP) responsible for penicillin resistance in streptococci. **Transduction,** firstly described in 1953, is similar to transformation except that the exogenous bacterial DNA carrying resistance determinants is transferred from one bacterium to another by insertion in a phage particle. The last mechanism in horizontal gene transfer is **Conjugation**. Edward Tatum and Joshua Lederberg discovered the principle of conjugation in 1947 [62]. They mixed two different strains of *Escherichia coli* and discovered the appearance of recombinant types that were different from the two strains they had mixed. It was later shown that this phenomenon was a result of direct physical contact between the two different strains and this facilitates the transfer of plasmid DNA from a donor to a recipient bacterium (Figure 1A). Many conjugative plasmids have been shown to present resistance to a variety of antibiotics. Perhaps even more insidious is their demonstrated capacity to transfer to a wide range of bacteria. Some plasmids are not conjugative, but rather are termed mobilizable [63]. Such plasmids can be transferred to a recipient if the conjugative functions are provided by a separate self-transmissible plasmid that is also harbored within the bacteria (Figure 1B). Mobilizable plasmids have not been as thoroughly studied as conjugative plasmids but may be equally responsible for the spread of antibiotic resistance genes and development of multidrug-resistant bacteria [63].

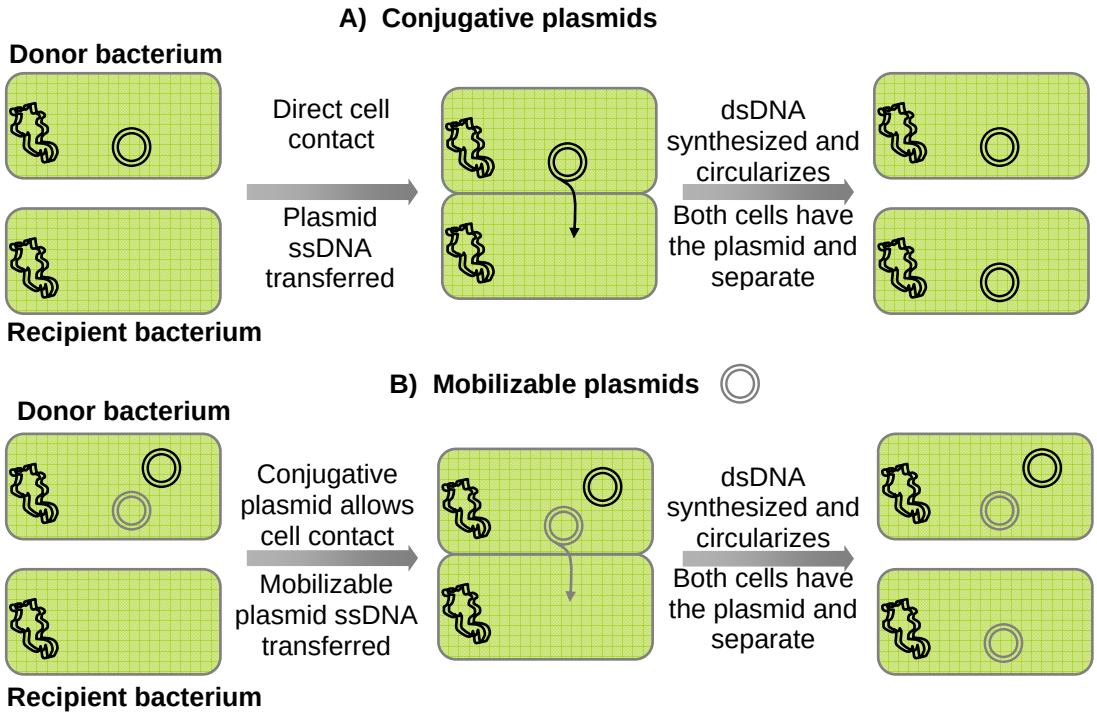

Figure 1. (A) Conjugative transfer of plasmid between cells. (B) Transfer of a mobilizable plasmid between cells assessed by a conjugative plasmid (adopted from Moritz and Hergenrother, 2007).

Transposons and integrons are mobile DNA elements that can insert and be integrated into regions of DNA on the chromosome or plasmids of Gram-positive and Gram-negative bacteria. Transposons associated with antibiotic resistance fall into three major classes based on their general structure and method of insertion. The first two classes consist of composite and noncomposite transposons that integrate into target DNA by generating direct repeats in the target sequence (Figure 2A) [63]. Composite and noncomposite transposons typically contain genes that are not essential for their transposition (such as antibiotic resistance determinants) in between flanking terminal insertion sequences (composite) or inverted repeats (noncomposite). Because transposition can involve excision and transfer of the entire element, such transposons are important in the spread of antibiotic resistance genes [63]. The third class, defined as conjugative transposons, are capable of excision from the chromosome or a plasmid of the donor cell to transfer DNA via conjugation into a recipient bacterium (Figure 2B). Conjugative transposons have a broad host range and their transfer is not constrained to closely related bacteria; this has been demonstrated by the Tn916 and Tn1545 family of transposons, which have been found or introduced into 50 different species and 24 genera of bacteria encompassing both Gram-negatives and Gram-positives [63]. Conjugation of a conjugative transposon begins with the excision of the transposon from either the bacterial chromosome or plasmid DNA. The transposon DNA becomes circular and conjugative transfer of a single-stranded DNA copy to the recipient cell proceeds in a manner identical to plasmid conjugation. A wide variety of antibiotic resistance genes have been discovered on large conjugative transposons, and they are thought to be a significant contributor to the spread and increase of antibiotic resistance in Gram-positive bacteria. The last type of mobile genetic elements is the integrons. These mobile genetic elements consist of an integrase gene, two promoters transcribing in opposite direction, and an array of other genes, which often contain antibiotic resistance genes (Figure 2C) [63]. Integrons differ from transposons in that the former possess a site-specific recombination system and do not randomly excise or insert into DNA regions. Importantly, many antibiotic resistance genes have been found as part of integrons and such gene cassettes are capable of insertion and

excision from other mobile genetic elements or the bacterial chromosome. Thus, resistance determinants present on some transposons and plasmids may be the result of integron insertion [63].

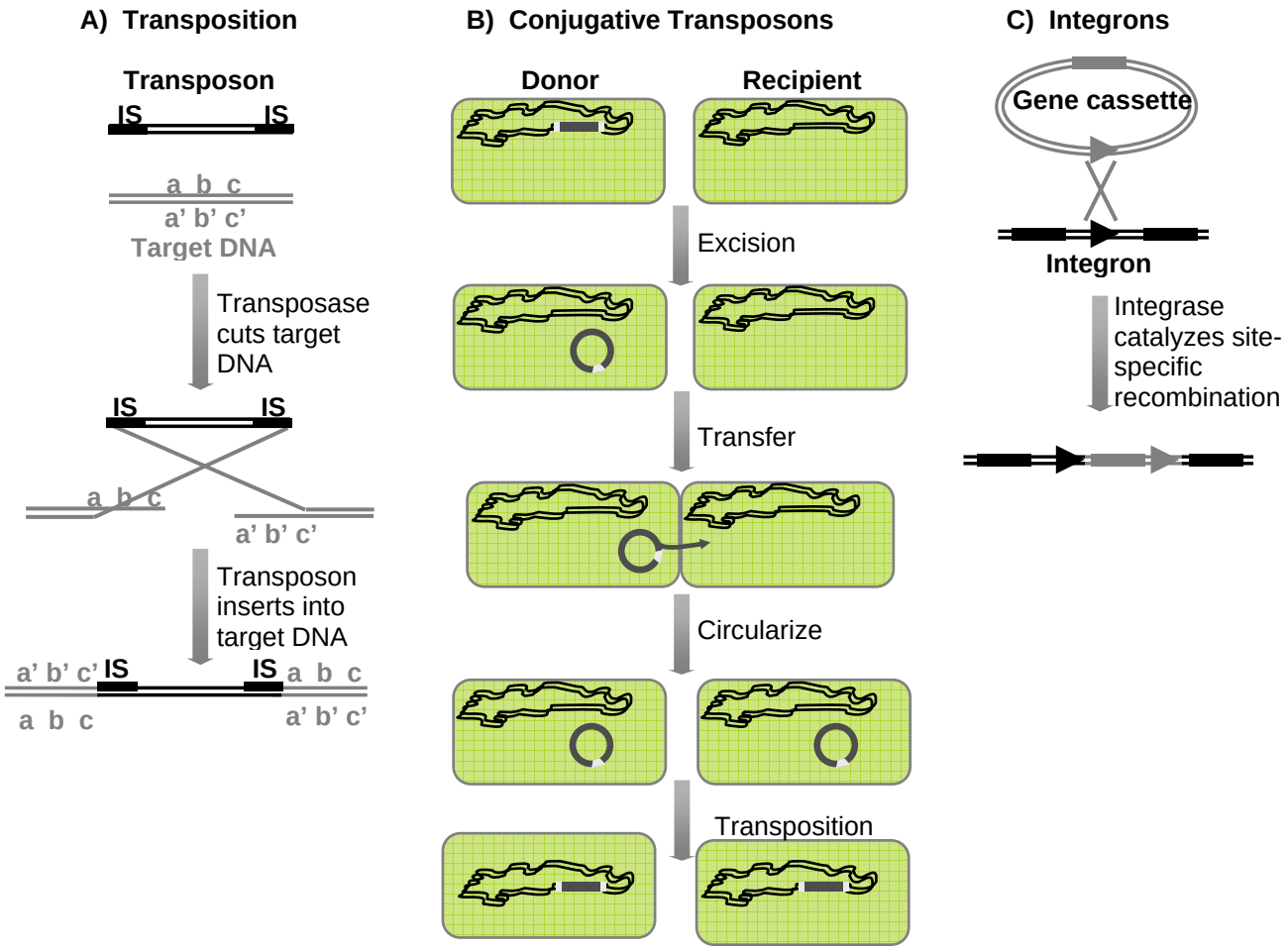

Figure 2.(A) Integration of a transposon into a target DNA (transposition); IS, insertion sequance. (B) Excision, circulation and subsequent conjugative transfer by a conjugative transposon into a recipient cell. (C) Acquisition of an array of genes by an integron via sitespecific recombination (adopted from Moritz and Hergenrother, 2007).

Mechanisms of antibiotic resistance

Specialized defence mechanisms, encoded by the acquired resistant genes, are utilized by bacteria for their survival in an environment in which antimicrobials are designed to kill them. Generally, one or more of four principal ways are utilized by bacteria to render antimicrobials ineffective (Figure 3) [60]. These are the following:

(1) The target molecules are structurally altered to prevent antibiotic binding. An example of this includes the alteration of ribosomal target sites in the DNA gyrase/topisomerase genes that are the targets of fluoroquinolones. Modification of the PBPs may occur through mutation in the chromosomal genes encoding the enzymes or through the acquisition of foreign homologous genes or fragments of genes from related species encoding new PBPs, a mechanism which is prevalent in Gram-positive cocci but seen less frequently in Gram-negative bacteria. Methicillin-resistant *S. aureus* (MRSA) is known to produce an alternative PBP (2a) that bypasses the effect of the antibiotic. Resistance to β-lactam antibiotics might be caused by the production of low-affinity PBPs. This resistance mechanism is widespread among the oral viridans streptococci, such as, *Streptococcus oralis*, *Streptococcus sanguis*, and *Streptococcus mitis*.

(2) Antibiotics are excluded from cell entry. Several antibiotics utilize porin channels when entering Gram-negative bacteria. So, the decreased expression of porins results in impermeability or decreased uptake that often leads to antibiotic resistance.

(3) Antibiotics are pumped out of the cell through a mechanism known as efflux pump. The bacteria can actively efflux the antimicrobial agent. Five major families of the efflux system are present. These are MFS: Major Facilitator Superfamily; RND: Resistance Nodulation-Division; SMR: Small Multidrug Resistance; ABC: ATP-Binding Cassette; and MATE: Multidrug and Toxic Extrusion.

(4) Antibiotics are inactivated, for example, through enzymatic degradation. The most common example of this mechanism is resistance against β-lactam antibiotics because of β-lactamases. These enzymes present resistance to the most widely used

antimicrobials in medical and dental practice, that is, β–lactams. Therefore, a special account, with some detailed information, for these enzymes is given.

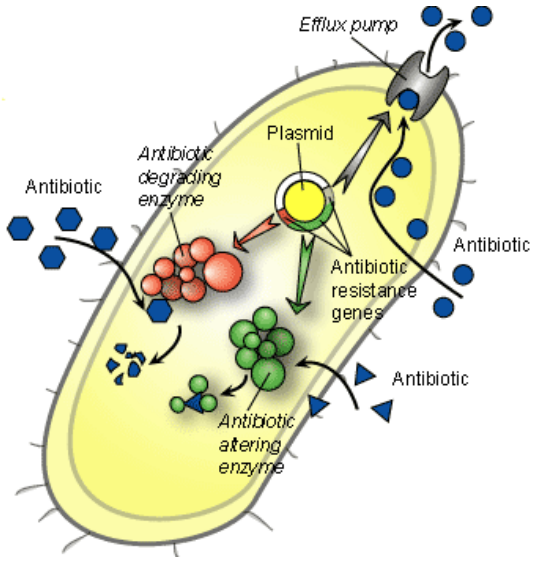

Figure 3. Resistance mechanisms of bacteria (reproduced with permission from Dr. Grace Yim).

Table 1. summarizes the mechanisms of action, antimicrobial spectra and main antimicrobial resistance mechanisms of the main antimicrobials used in dentistry.

Table 1. Main antibiotics used in dentistry, mechanism of action, their spectrum, and main bacterial resistance mechanisms involved.

Drug	Mechanism of action	Spectrum	Main resistance mechanism(s)
Phenoxymethyl-penicillin	inhibition of cell wall synthesis	aerobic G+, anaerobic G+, anaerobic G – (narrow-spectrum)	enzymatic (β-lactamases), alteration of the target site (mosaic PBP) [64, 65]
Amoxicillin, Ampicillin	inhibition of cell wall synthesis	as above plus *haemoghilus* spp. (broad-spectrum)	as above
Metronidazole	inhibition of RNA synthesis	strict anaerobic bacteria, some facultative anaerobes	enzymatic (5- nitroimidazole reductase) [66]
Erythromycin	inhibition of protein synthesis	mainly G +	target site modification, enzymatic inactivation, and active efflux [67, 68]
Clindamycin	inhibition of protein synthesis	as above plus additional activity on anaerobes	as above
Tetracycline	inhibition of protein synthesis	many G + and G –	active efflux, enzymatic inactivation, ribosomal protection proteins [69, 70]

β-Lactamases

More than 300 β-lactamases from various bacteria differing in substrate profiles, potential for inhibition, and physiological characteristics, have been described. These enzymes catalyze the hydrolysis of the β-lactam ring of the β-lactam antibiotics, resulting in microbiologically inactive compounds. Several classification schemes for β-lactamases have been proposed [71-74]. The molecular classification scheme is the simplest and it is comprised of four distinct molecular classes (A,B,C, and D) based on amino acid sequences [74]. Classes A, C, and D comprise evolutionarily distinct groups of serine enzymes, and class B contains the zinc types. The most threatening of these enzymes are the metallo-β-lactamases; molecular class B β-lactamases, that inactivate almost all β-lactam drugs, even the carbapenem antibiotics [64, 72]. Additionally, extended spectrum β-lactamases (ESPLs) have evolved from mutations around the active site of the parental β-lactamases (class A and D) and extending their substrate spectrum to hydrolyze a panel of β-lactam antibiotics [64, 75]. The genes encoding for the production of β-lactamase could be chromosomal or inserted on a mobile genetic element, for example, plasmid, that travel from one bacterium to another [64].

The β-lactamases production by bacteria could be copious, whether constitutively or inducibly [64, 73]. The β-lactamases of Gram-positive bacteria are generally excreted in large amounts and may therefore, in mixed infection, also protect other organisms present at the infection site. This could be of particular clinical importance in biofilm-associated diseases. The permeability characteristics may act in concert with β-lactamase production to protect the microorganism from the effects of β-lactam antibiotics. Therefore, decreased permeability of the antibacterial agent allows small amounts of strategically located β-lactamase to present high resistance [76].

Previous studies shown a high penicillins susceptibility (90–99%) of oral bacteria recovered from subgingival plaque samples [77, 78]. It was in the 1980s when reports began to emerge and information about clinical failure of penicillin therapy in the treatment of oro-facial infections from which penicillin-resistant and β-lactamase

producing oral *Bacteroides*, *Capnocytophaga*, *Veillonella*, and *Streptococcus* strains were isolated [23, 79, 80]. In addition, β-lactamase producing *Bacillus* and *Pseudomonas* species were isolated from subgingival plaque in patients with chronic periodontitis [81]. The *Prevotella* species have been reported to be the most frequent β-lactamase producing species found in the periodontal pockets and saliva [35, 81, 82]. The β-lactamase producing *Prevotella* species were frequently found to colonize infants and healthy young children [35, 83]. Isolates of *F. nucleatum* have earlier been shown to produce significant amounts of β-lactamase in patients suffering from tonsillitis [36]. In addition, Könönen *et al.* demonstrated that penicillin resistance caused by β-lactamase production by oral strains of *F. nucleatum* frequently occurs in childhood [84]. The susceptibility of *P. gingivalis* isolates to penicillins have been reported to be 100% and it was thought that the bacterium has no β-lactamase enzymes [82, 85-87]. However, Nagy *et al*. investigated 183 clinical isolates of *Bacteroides, Porphyromonas*, and *Prevotella* species, from severe infections after abdominal, gynaecological, and oral surgery, to detect β-lactamase production and β-lactamase producing *Porphyromonas*; *P. gingivalis* were detected mainly in 47% of the isolated strains [88]. In addition, Prieto-Prieto *et al.* referred to a study conducted in Spain in which 59% of *Porphyromonas* species were resistant to penicillin G [89].

Molecular characterization of β-lactamases produced by oral bacteria was started in 1991 by Lacroix *et al*. in which a β-lactamase gene (TEM-1) in an *E. corrodens* strain, isolated from the periodontal pocket, was sequenced [90, 91]. More studies were then published on the characterization of β-lactamases produced by oral bacteria, mainly in Bacteroidaceae, as members of this family had been implicated in the etiology of acute oral infections and periodontal disease [92, 93]. More recently, detection and characterization of a panel of β-lactamase genes produced by different subgingival oral bacteria isolated from patients with refractory periodontitis, currently known as recurrent periodontitis, were reported and in this study the CfxA β-lactamase gene was found to be prevalent in *Prevotella* and *Capnocytophaga* species [94, 95].

Use of antibiotics and development of resistance

"Antibiotic therapy, if indiscriminately used, may turn out to be a medicinal flood that temporarily cleans and heals, but ultimately destroys life itself" Felix Marti-Ibanez, 1955.

Resistance development is a natural biological outcome of antibiotic use [96]. It represents a particular aspect of the general evolution of bacteria that is genetically determined and presents a survival advantage. The selection pressure applied on bacterial population when antimicrobials are used is the driving force for the resistant bacteria to emerge [96-98]. Therefore, resistant bacterial clones have been continuously selected as an evolutionary response to the use of antibiotics. The magnitude of this selection is determined by the total consumption of antibiotics within the particular setting in which these antibiotics are used. Indeed, the correlation between the antibiotic use and emergence of bacterial resistance is well established and is clearly seen by the frequency of resistant bacteria that is considerably higher in countries with high antibiotic consumption [99]. However, a quantitative relation between the two factors, that is, antibiotic use and bacterial resistance, was not clear.

The development of resistance appears to follow a sigmoid distribution, with a lag phase before resistance appears, then a relatively rapid increase in the proportion of resistant bacteria, followed by a third phase in which the proportion between susceptible and resistant bacteria reaches an equilibrium [100]. This equilibrium level is determined by the relative fitness of resistant and sensitive strains including transmission ability, the genetic basis and stability of resistance, and the magnitude of the antibiotic selection pressure. When this level of resistance has been reached, measures to contain or potentially lower the resistance level seem very difficult [101]. One strategy that has been widely adopted to curtail the rapid emergence and subsequent dissemination of resistance genes is to restrain the use of antibacterial drugs [102-104]. Despite the fact that many countries adopt antibiotic treatment guidelines, restriction of antibiotic use outside human medicine, and improved diagnostic tools for bacterial infections, the global trend of antibiotic resistance is still on the increase. Globally there is an extensive overuse of antibiotics, for example, use based on incorrect medical indications as well as misuse by using the wrong agent, administration

route, dose and treatment duration [105, 106]. In industrialized countries, around 80–90% of antibiotic consumption in humans takes place in the community, and at least half of this is considered to be based on incorrect indications, mostly viral infections [106-108]. Easy access to antimicrobial agents in several countries is a big problem as well. There are also nonclinical factors that influence the use of antibiotics. Those include cultural conceptions, patient demands, economic incentives, and advertising to prescribers, consumers, and providers from the pharmaceutical industry [109, 110]. Consequently, the patterns of antibiotic use differ substantially between and within countries [99]. In Europe, for example, antibiotic consumption is four times higher in France than in the Netherlands, although there is no reason to believe that the burden of disease differs between the two countries [99].

In developing countries, a high infectious disease burden commonly coexists with high antibiotic consumption and rapid emergence and spread of microbial resistance [111, 112]. Several risk factors for resistance emergence particularly pertinent to, but not limited to, developing countries exist [111, 112]. Among these are misuse and easy access of antibiotics, poor quality antimicrobials, and lack of patient compliance to the prescribed drug and regimen. In addition, dissemination of resistant bacteria in developing countries is facilitated by inadequate infection control measures in health facilities and shortfalls in hygiene, sanitation, and public health [111, 113].

The potential reversibility of resistance is a debatable issue, and the chances of success differ greatly between the hospital setting and the community. The rationale for reversibility is that resistant bacteria will have a disadvantage over susceptible strains in environments without antibiotics, as most resistance mechanisms will present a reduction in bacterial fitness, for example, a slower growth rate, reduced virulence or transmission rate [114-116]. Thus, a decreased volume of antibiotic use should lead to lower selection pressure and a reduction in the proportion of bacteria resistant to a certain antibiotic. In line with this, Feres *et al*. found that the prevalence of amoxicillin-resistant subgingival bacteria that emerged with a 14-day amoxicillin therapy decreased from 37% to the baseline value (0.5%) in a 90-day period [117].

Thereby, resistant organisms are replaced by susceptible ones as a consequence of removal of the selective-pressure-driving force, that is, antibiotics. However, the issue of reversibility is complicated by the fact that resistant bacteria may reduce the biological costs associated with resistance through compensatory evolution [118, 119], therefore, reversibility is difficult to achieve in highly adapted resistant strains. It is expected that reversibility would be higher in communities than in hospital settings, especially as the continuous antibiotics use in hospitals would allow better adaptability to reduce the biological costs associated with resistance. However, it was noticed that in hospital settings the rate and extent of reversibility of antibiotic resistance among isolates are much higher than in communities. The reason for this difference is that the main driving force for reversibility in hospitals, in contrast to communities, is not the biological cost of resistance. Instead, in hospitals a "dilution effect" is observed as patients admitted from the community in most cases bring susceptible bacteria into clinical wards and therefore affect the levels of resistant bacteria [120].

It is generally agreed that more the antimicrobials used, the more is the selection pressure applied on bacterial population, and resistant bacteria began to emerge. Therefore, detailed and extensive information on antibiotic utilization has gained interest in many communities, and the antibiotic consumption measurement is increasingly being recognized as an important factor of monitoring emerging resistance.

Measuring antibiotic consumption

The pioneering work of Arthur Engel in Sweden and Pieter Siderius in Holland alerted many investigators to the importance of comparing drug use between different countries and regions [121]. However, in the early work, drug utilization data obtained from different countries did not permit detailed comparisons because the source and form of the information varied between them. To overcome this difficulty, researchers in Northern Ireland, Norway, and Sweden developed a new unit of measurement, initially called the agreed daily dose [122] and later the Defined Daily Dose (DDD) [123]. The DDD unit is defined as the average maintenance dose of the drug

when used for its major indication in adults. Another important methodological advance was the development of the uniform Anatomical Therapeutic Chemical (ATC) classification of drugs by Norwegian researchers. In the ATC classification, drugs are classified in groups at five different levels. The drugs are divided into 14 main groups (first level); the second level in the ATC classification system is the therapeutic group. The third and fourth levels are the chemical/pharmacological/therapeutic subgroups. The fifth level is the chemical substance. The complete classification of amoxicillin illustrates the ATC classification system (Table 2).

The "DDD/ATC system" is adopted by WHO and researchers are advised to use this standardized methodology that allows meaningful comparisons of drug use in a country or between different countries to be made. The WHO Collaborating Centre for Drug Statistics Methodology, located at the Norwegian Institute of Public Health, Department of Pharmacoepidemiology, Oslo, is responsible for the development and maintenance of the "ATC/DDD system" [124].

Antibiotic consumption measurements can describe the extent of use at a certain moment and/or in a certain area (e.g. in a country, region, community or hospital). It could be presented as numbers of DDDs per 1000 inhabitants per day or, when antibiotics use by inpatients is considered, as DDDs per 1000 bed-days [125]. The number of DDDs/1000 inhabitants/day gives an estimate of the proportion of the population exposed daily to a particular drug. This figure is a rough estimate and should be read with caution.

Table 2. Description of ATC classification system (amoxicillin is used as an example)

Description of ATC classification	ATC classification	System corresponding to ATC classification
1st level, anatomical main group	J	Anti-infective for systemic use
2nd level, therapeutic group	J01	Antibacterial for systemic use
3rd level, chemical subgroup	J01C	Beta-lactam antibacterial, penicillins
4th level, therapeutic subgroup	J01CA	Penicillins with extended spectrum
5th level, chemical substance	J01CA04	Amoxicillin

2. Rationale and aims of the study

The work in this thesis was prompted by the very liberal use of antibiotics in many developing countries compared to some developed countries and the international concern regarding increasing antibiotic resistance of oral bacteria. The author hypothesized that Yemen is one of these antibiotic-misusing developing countries and, therefore, decided to investigate the situation in his homeland, Yemen, and compare it with that in a developed country, Norway. The general aim of the current study was to investigate antibiotic resistance in oral bacteria in both Yemen and Norway. The specific objectives were:

(1) To assess and compare the prevalence of selected subgingival bacteria resistant to aminopenicillins and metronidazole, in Yemen and Norway (**paper 1**).

(2) To assess the susceptibility pattern of *F. nucleatum* species isolated from Yemen, and to characterize its aminopenicillins-resistant determinants **(paper 2)**.

(3) To elucidate antimicrobials prescription practices among dentists in Yemen **(paper 3)** and Norway **(paper 4)**, and the possible contribution of these practices to the emergence of bacterial resistance.

3. Material and Methods

3.1 Study subjects and study data (papers 1, 2, 3, and 4)

A total of 55 patients were enrolled as plaque donors of whom 34 and 21 were from Yemen and Norway, respectively **(paper 1)**. Another 23 plaque donors from Yemen were enrolled in **paper 2**. All plaque donors were dental-clinic attendees for various treatment demands, and were asked for verbal consent prior to sampling; Plaque collection was approved by the Regional Ethical Committee for Medical Research, West-Norway. None of them had any antimicrobial therapy in the previous three months. In **paper 3**, a questionnaire comprised of 65 close-ended questions that sought answers to 11 parameters were distributed by hand (the author) to 280 dentists working in all the governmental as well as private dental clinics in the three major governorates (Sana'a, Aden, and Taiz) in Yemen. The questionnaire investigated demographic data of the dentists and their knowledge on therapeutic and prophylactic antimicrobial usage in clinical dentistry. Aggregated data on antibiotic prescriptions by dentists in Norway were analyzed **(paper 4)**. The data were obtained from the Norwegian Prescription Database (NorPD) and it contained the numbers of prescriptions issued by dentists, the number of dentists having prescribed the antibiotics, and the total DDDs in 2004 and 2005 of 11 antibiotics (phenoxymethylpenicillin, amoxicillin, erythromycin, doxycycline, metronidazole, clindamycin, tetracycline, spiramycin, oxytetracyclin, clarithromycin, and azithromycin) used in dentistry.

3.2 Plaque sampling and primary cultures (papers 1 and 2)

After clinical examination and removal of supragingival plaque, three randomly selected posterior teeth in each patient were assigned for isolation and subgingival sampling. The subgingival samples were collected using sterile paper points size 50

(Dentsply, USA) that were inserted as far as possible subgingivally parallel to the long axes of the sampled teeth and removed after 20 s. The three samples thus obtained from each subject were placed in a sterile vial containing 1.5 mL VMGA III transport medium [126]. All samples were transported to the Laboratory of Oral Microbiology, University of Bergen, within 48 h after sampling at ambient temperature in plastic bags under anaerobic condition using the Anaerocult® system (Merck). Upon arrival at the laboratory, 3–5 sterile glass beads were added aseptically to each vial. The samples were then vortexed (WhirliMixer, Fisons Scientific Equipment, England) for 2 min. Thereafter, 10 μL of each sample suspension were plated on fastidious anaerobic blood agar (Lab M, UK) or crystal violet erythromycin (CVE) plates [32] with or without either 2 μg/mL ampicillin or 2 μg/mL metronidazole. The inoculated plates were incubated anaerobically (5% CO_2, 10% H_2 and 85% N_2) for 10 days at 37°C using the Anoxomat System™ (MART Microbiology BV, The Netherlands).

3.3 Bacterial strains (Papers 1 and 2)

Paper 1 investigated the presence of 18 selected subgingival bacteria resistant to ampicillin and metronidazole in dental plaque samples from Yemen and Norway. The list of the bacterial strains used for the preparation of whole genomic DNA probes, used for detection of studied strains, is listed in Table 3. Paper 2 screened *F. nucleatum* isolates from Yemen (n=23) for ampicillin minimum inhibitory concentrations (MICs) and then the assessment for β-lactamase production of resistant strains was done. Five highly ampicillin-resistant (MIC > 8 µg/mL) and four susceptible *F. nucleatum* isolates were used for proteomic analysis. In the E-test, *Eggerthella lenta* ATCC 43055 and *Bacteroides fragilis* ATCC 25285 reference strains were used for quality control strains. The *E. coli* ATCC 25922 strain was used as a negative control in the β-lactamase production test. In addition, a β-lactamase positive clinical strain of *F. nucleatum* [35] was used as a positive control in the chromogenic cephalosporin disk (Fluka, Germany) test for β-lactamase production.

Table 3. Bacterial strains used in papers 1 and 2.

Species	Strain	Species	Strain
Porphyromonas gingivalis *	ATCC 33277	*Streptococcus sanguinis* *	ATCC 10556
Prevotella intermedia *	VPI 4197	*Streptococcus constellatus* *	ATCC 27823
Aggregatibacter actinomycetemcomitans *	ATCC 33384	*Streptococcus gordonii* *	CCUG 33482
		Streptococcus mitis *	ATCC 9811
Eikenella corrodens *	ATCC 23834	*Streptococcus intermedius* *	ATCC 27335
Camphylobacter rectus *	ATCC 33238	*Tanerella forsythensis* *	FDC 2008
Capnocytophaga gingivalis *	ATCC 33624	*Veillonella parvula* *	ATCC 10790
Fusobacterium nucleatum *	ATCC 23736	*Eubacterium nodatum* *	CCUG 15996
Peptostreptococcus micros *	CCUG 17638	*Eggerthella lenta* #	ATCC 43055
Streptococcus mutans *	ATCC 25175	*Bacteroides fragilis* #	ATCC 25285
Streptococcus oralis *	ATCC 10557	*Escherichia coli* #	ATCC 25922

* Bacterial strains used for preparation of whole genomic DNA probe [127]
# Bacterial strains used for the E-test and β-lactamase production test
ATCC: American Type Culture Collection, USA; VPI: Virginia Polytechnic Institute and State University, USA; CCUG: Culture Collection, University of Gothenburg, Sweden; FDC: Forsyth Dental Center, Boston, USA.

3.4 Identification of studied species

DNA-DNA hybridization (paper 1)

Standardized bacterial sample suspensions were prepared for DNA-DNA checker-board hybridization technique [10] for detection of the presence and identification of the studied species in the cultivated plaque samples. Hybrids were detected by chemiluminescence, and then they were exposed to X-ray films (Roche Diagnostic, Basel, Switzerland) to detect bound probes. Inspection of hybirds was done visually on digitized images of the X-ray films at least for three times on different occasions. Hybrids were interpreted according to standard signals (controls 10^5 and 10^6 cells) of the 18 studied species.

Phenotypic tests and biochemical assays (paper 2)

Fusobacterium nucleatum isolates were recovered from plaque samples on the CVE plates [32]. Subsequent identification was based on stereomicroscopic colony morphology, Gram staining, anaerobosis, and a biochemical profile of 29 miniaturized enzymatic tests using the commercial assay Rapid ID 32 A system (Biomerieux® Sa, France).

3.5 Antimicrobial susceptibility testing

Agar dilution method (paper 1)

Direct plating of 10 μL of bacterial sample suspensions on fastidious anaerobic blood agar (Lab M, UK) plates supplemented with either 2 μg/mL ampicillin or 2 μg/mL metronidazole were used for testing the presence of resistant species [117].

The Epsilometer test (E-test) (paper 2)

Determination of the MICs of the isolated *F. nucleatum* strains were performed using the E-test (AB Biodisk, Sweden) on brucella agar plates (SBA; BBL), 5% sheep blood, supplemented with 1 μg/mL vitamin K, and 5 μg/mL hemin. Resistant isolates were then screened for β-lactamase production using the chromogenic cephalosporin disk test (Fluka, Germany) for β-lactamase production.

3.6 Proteomics (paper 2)

Sample preparation

Ampicillin-resistant and susceptible *F. nucleatum* isolates were cultured on fastidious anaerobic blood agar plates (Lab M, UK) and incubated anaerobically (5% CO_2, 10% H_2 and 85% N_2) using the Anoxomat System™ for 48 h at 37°C. The colonies of each *F. nucleatum* isolate were removed from the agar surface using a disposable sterile plastic loop and suspended in PBS-A buffer for washing. Extraction of soluble proteins of *F. nucleatum* isolates were performed using a hand-held homogenizer in a

buffer containing 7 M deionized urea, 2 M thiourea, 4% (w/v) 3-[3-(cholamidopropyl)-dimethylammonio]-1-propane sulphonate (CHAPS), 0.3% (w/v) dithiothreitol (DTT), and 0.2% carrier ampholyte (Bio-Lyte® 3/10 and/or 5/8 Ampholytes). Determination of protein concentration of the samples was based on the Lowry methods using (Bio-Rad RC-DC™ protein assay).

Two dimensional gel electrophoresis and protein identification by peptide mass mapping and MALDI-TOF/TOF MS

The isoelectric focusing (IEF) of the samples (first dimension separation) was performed on immobilized pH gradient (IPG) gel strips (7 cm length and ranges pH 3–10 and 5–8 linear gradient; Bio-Rad Ltd) using the PROTEAN IEF Cell (Bio-Rad). The second dimension electrophoresis was performed on sodium dodecyl sulfate (SDS) polyacrylamide gels (12% precast SDS-PAGE gels; Bio-Rad) using the Mini Protean II gel system (Bio-Rad). Localization of protein on gels was done using Biosafe coomassie blue stain (Bio-Rad). LabScan (GE Healthcare) and ImageMaster 2D Platinum software version 5 (GE Healthcare) were used for image acquisition and subsequent analysis of digitized images.

Acquisition of the peptide mass spectra for protein identification was done on an Ultraflex **M**atrix **A**ssessed **L**aser **D**esorption **I**onization- **T**ime **o**f **F**light/**T**ime **o**f **F**light **M**ass **S**pectrometer (MALDI-ToF/ToF MS; BRUKER Daltonic GmbH, Germany). The acquired mass spectra of peptides were used to search in the NCBInr, MSDB and Swissprot protein databases using the Mascot search engine (http://www.matrixscience.com/).

4. Results

Paper 1

In both Yemeni and Norwegian subgingival plaque samples, all the 18 studied species (Table 2) were detected but at different frequencies. Significant differences were found in species prevalence in the two study population. *P. gingivalis*, *Eubacterium nodatum*, *Streptococcus sanguinis*, and *Veillonella parvula* were significantly more prevalent in the Yemeni subjects ($P < 0.05$). On the other hand, *A. actinomycetemcomitans*, *Capnocytophaga gingivalis*, and *S. mitis* showed a significantly higher prevalence in the Norwegian samples.

In the samples from the Yemeni subjects, 28.9 and 60.3% of all detected species were resistant to ampicillin and metronidazole, respectively. The corresponding figures for the Norwegian samples were 7.9 and 11.3%. Yemeni samples exhibited a significantly higher prevalence of metronidazole resistance among *Eikenella corrodens*, *Streptococcus mutans*, *S. oralis*, *S. sanguinis*, *Streptococcus constellatus*, *Streptococcus gordonii*, *S. mitis*, *Streptococcus intermedius*, and *V. parvula* as well as a significantly increased prevalence

of ampicillin-resistant *P. gingivalis*, *Prevotella intermedia*, *S. constellatus*, *S. intermedius*, *T. forsythensis*, *F. nucleatum*, and *V. parvula*.

Paper 2

The ampicillin MICs of *F. nucleatum* isolates ranged between 0.125 and 256 μg/mL. All the ampicillin-resistant *F. nucleatum* isolates turned the nitrocefin-impregnated discs into red indicating hydrolysis because of β-lactamase production. The analysis of the ampicillin-resistant *F. nucleatum* global gene expression at the level of the proteome revealed the presence of a 29 KDa protein. This protein was identified using Mascot search of the obtained peptide mass fingerprint spectra as class D β-lactamase. There was increased synthesis of two proteins at 37 and 46 KDa that were

significantly associated with ampicillin-resistant *F. nucleatum* isolates. These proteins were identified using the Mascot search of the obtained peptide mass fingerprint spectra as ABC transporter ATP-binding protein and enolase, respectively.

Paper 3

A total of 150 properly completed questionnaires by Yemeni dentists were analyzed and knowledge scores were calculated. Yemeni dentists' knowledge of conditions for prescribing antimicrobials in the various parts of the questionnaire was generally poor (mean score 28.5, s.d. 5.6). The patients' clinical conditions, dental treatment procedures, and the medical background of the patients were the parameters that scored poorest, with a high tendency of overprescribing antibiotics, as compared to current international recommendations. The data showed that penicillins, mostly broad-spectrum, were the first choice of antibiotics (72%) followed by spiramycin (10%) when treating dental infections. No statistically significant difference was found among age band groups. Significant difference in scores was found ($p<0.001$) between genders with lower female mean score than male mean score (27.6, s.d. 4.6 and 29.57, s.d. 5.9, respectively).

Paper 4

A total of 268,834 prescriptions issued by 4765 different dentists working in all types of dental settings in Norway in both 2004 and 2005 were analyzed. The narrow-spectrum-penicillin phenoxymethylpenicillin was the most frequently prescribed antibiotic by dentists in Norway followed by metronidazole. Phenoxymethylpenicillin and metronidazole prescriptions accounted for 75 and 6.3% in 2004, respectively, versus 73 and 6.9% in 2005 of all the dentists' antibiotic prescriptions. The prescription frequencies of the 11 antibiotics prescribed by dentists in Norway are presented in Table 4. The average number of prescriptions per dentist per week during the study period was 0.59. The broad-spectrum amoxicillin accounted for approximately 4.6% of all the prescriptions issued in 2004 and 2005.

Dentists' antibiotic prescriptions in Norway contributed to about 8% to the total consumption of the 11 drugs.

Table 4. The prescription frequencies of the 11 antibiotics prescribed by dentist in Norway in 2004 and 2005

Prescribed antibiotics	Dentists' prescriptions		Prescribed antibiotics	Dentists' prescriptions	
	2004	2005		2004	2005
Phenoxymethyl-penicillin	75%	73%	Azithromycin	1.2%	1.1%
Metronidazole	6.3%	6.9%	Spiramycin	0.7%	0.6%
Erythromycin	4.9%	5%	Tetracycline	0.7%	0.5%
Amoxicillin	4.6%	4.7%	Oxytetracyclin	0.2%	0.2%
Clindamycin	3.7%	4.4%	Clarithromycin	0.08%	0.00%
Doxycycline	2.3%	2.2%			

5. Discussion

The present study investigated, for the first time, antibiotic resistance among selected oral bacteria in Yemen and compared the findings from Yemen and Norway. The main findings were the high prevalence of antibiotic resistance among isolates from Yemen, thus confirming my hypothesis of antibiotic misuse, and the association with poor knowledge of antibiotic prescription among Yemeni dentists. It was shown that aminopenicillin resistance among *F. nucleatum* isolates from Yemen was mainly because of class D β-lactamase production. In addition, dentists' contribution to the national consumption of antibiotics in Norway was measured, also for the first time, using the WHO DDD/ATC system. The results indicate that the prescription practices among dentists in Norway are conservative.

5.1 General discussion

Antibiotics and other antimicrobial drugs have revolutionized the treatment of infectious diseases since their introduction in the beginning of the last century. In 1945, Sir Alexander Fleming, during his Nobel Prize acceptance speech for his penicillin discovery in 1928, warned the scientific community about the danger of antibiotic resistance that microbes can develop, and he informed that it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to nonlethal quantities of penicillin. The use of different antibiotics for many years led to the emergence of infectious bacteria that are resistant not only to one but to several antibiotics. As a result, there are strains of bacteria today for which only one effective drug treatment is available or, in some cases, none at all [128]. Antimicrobial resistance by microbes is now considered as a major threat to public health and its control is now an international priority for action.

Nowadays, the world is considered smaller than before as a result of better communications. The public health issues are increasingly regarded and seen as one

global issue that affect all countries. Unfortunately, in many parts of the globe there are still some countries that are bounded by so many restrains, making them less developed. Many major global health problems continue to present a dilemma in these countries.

Antibiotic resistance in oral microbes is an increasing problem when treating dental infection. In the recent years, a shift from narrow-spectrum antibiotic prescriptions to broad-spectrum ones by dentist was reported, and the increase of bacterial isolates resistant to the former antibiotics is blamed for such a shift in prescription practices. On the other hand, the issue of antibiotic resistance is neglected in Yemen, and scientific studies that deal with this topic are lacking. Therefore, there was a strong motive for the author to conduct this work. The author tried to measure antibiotic resistance among some selected oral microbes in Yemen to compare it to that in Norway in a reliable way and from different aspects. Norway is a country that possesses several characteristics to be an ideal model for comparison. It is aimed to be the initial step to appreciate how big the problem is in Yemen and to improve the nation's ability to anticipate, avert, and contain resistance. The study also aims to initiate appropriate actions to be adopted as soon as possible.

The author wanted to document the prevalence of aminopenicillin and metronidazole resistance among 18 selected oral bacteria. The presence of aminopenicillin- and metronidazole-resistant oral bacteria in dental practice is worrisome, principally because these antimicrobials are used most frequently by dentists, as revealed in the current study, for empiric therapy in many encountered oral infections [47, 51, 129].

At the beginning of this study, ampicillin was chosen for the performance of aminopenicillins susceptibility testing for investigating the prevalence of resistance to these agents among the studied subgingival species. On proceeding with the dentists' antimicrobial prescription practices, the author, unexpectedly, discovered that ampicillin is not one of the antimicrobials utilized by dentists in Norway. This unexpected discovery was further investigated to disclose reasons behind dentists' zero-

ampicillin-prescriptions. It was found that only one formulation exists in Norway for ampicillin, that is, injection. The existence of only this form of ampicillin explains the zero-ampicillin-prescriptions by Norwegian dentists, principally because dentists in Norway do not prescribe injections. Nevertheless, the issue was not of big concern, because the ampicillin resistance prevalence obtained earlier in paper 1 is also valid for amoxicillin [130], which is prescribed frequently by dentists.

5.2 Specific discussion

Prevalence of resistance and resistance determinants

The occurrence of the 18 studied oral bacteria in the two study groups varies with some significant differences. These differences are in line with studies that have shown differences in the mean proportions of subgingival species in samples from periodontitis or healthy subjects in different countries, which may be explained by variations in oral hygiene, race, age, diet, genetics, disease susceptibility, and disease manifestations [131-134]. There were significant differences in the prevalence of ampicillin and metronidazole resistance among the studied oral bacteria between Yemen and Norway with higher resistance found in Yemen. In fact, this result was somewhat expected owing to the presence of many risk factors that fuel resistance emergence in Yemen. The huge misuse and easy access of antimicrobials were observed by many health workers before conducting this study. However, such observations were not scientifically documented.

The present situation of high resistance among oral bacteria in Yemen moves dentists into a vicious circle. Increasing levels of resistance necessitate the use of broader and more potent antimicrobials to secure patients, but using these broader and more potent antimicrobials exacerbates the problem of bacterial resistance, especially if misuse is common, and even more resistance develops and creates a situation where effective antimicrobials become more difficult to obtain, is expensive, or even lacking.

The bacterium *F. nucleatum* was chosen for a further investigation (paper 2) to elucidate its resistance determinants to ampicillin. Several reasons existed behind this selection. Firstly, *F. nucleatum* is an important oral microorganism and its importance is mentioned in the introduction section of this thesis. Secondly, β-lactam resistance in *F. nucleatum* has been on the rise [33, 34], and although β-lactamase production was blamed for such resistance, interestingly, the molecular characterization of this enzymatic resistance was not documented or fully understood [35, 95]. The results of this study informed about the presence of β-lactamase enzyme in ampicillin resistant *F. nucleatum* strains isolated from Yemen. The peptide mass fingerprint spectra significantly identify a class D β-lactamase in ampicillin-resistant *F. nucleatum* strains isolated from Yemen. The matched class D β-lactamase enzyme is recorded in the NCBInr protein database in 2003, and even more recently it was found in one strain of *F. nucleatum* isolated in France [135]. In addition, class D β-lactamase enzyme production in ampicillin-resistant *F. nucleatum* strains was significantly associated with increased synthesis of two proteins, namely enolase and ABC transporter ATP-binding protein. However, there is no evidence that any of the involved changes seen in enolase and ABC transporter ATP-binding protein synthesis are actually involved in ampicillin resistance.

Antibiotic prescription practices

The results obtained from **paper 3** indicated that a proper antimicrobial prescription practice is lacking, and antimicrobial overuse by dentists in Yemen is common. A relationship between high rates of antibiotic resistance and high antibiotic consumption was shown, which is probably related to higher consumption driving more selective pressure, and it has been noticed that after introduction of an antibiotic, clinical resistance has emerged [99]. The obtained results of high resistance prevalence found among the studied oral bacteria in Yemen with the poor knowledge of proper prescribing and misuse of antimicrobial by dentists in the country are in line with the previously reported association between these two situations, that is, high antimicrobial consumption and high bacterial resistance [96-98]. However, the antimicrobials

use in dental practice is much less than that in medical practice. Thus, it is most probable that the selective pressure applied on oral bacterial population by the use of antimicrobials in dental practice is less than that applied by medical practitioners. Therefore, the high prevalence of resistance found among oral bacteria in Yemen compared to Norway could not be attributed only to dentists' misuse of antimicrobials in the country. Instead, dentists' misuse of antimicrobials in Yemen could explain, only in part, the high prevalence of resistance found among oral bacteria. Most importantly, it is the probable antimicrobial misuse by other health workers in the country. Unfortunately, only a few reports highlight antimicrobial misuse by health workers in Yemen [136, 137].

On the other hand, a conservative antimicrobial prescribing practice prevails in Norway. This is observed by the strong trend of prescribing the narrow-spectrum penicillin phenoxymethylpenicillin by little more than 70% of all the prescriptions analyzed. The reliance of dentists in Norway on phenoxymethylpenicillin as their first choice confirms the result of low prevalence of antibiotic resistance among oral bacteria in Norway. The high frequency of prescribing narrow-spectrum penicillins in dental practice in Norway is in line with the general trend in the country [138].

The low prevalence of ampicillin resistance among the studied subgingival species in Norway could be explained by the antimicrobial prescription practices revealed in the current study. In Norway, the aminopenicillins prescriptions issued by dentists, and even by medical practitioners, are much less than that of the narrow-spectrum penicillins prescriptions, which may suggest that the selection pressure, as a fundamental force in the emergence of resistant bacteria, applied on bacterial populations because of aminopenicillins use, is considerably low in the country. In addition, it was found that the average number of prescriptions per dentist per week, during the study period, is 0.59. The reported figures from United Kingdom and Canada are 3 and 4.45, respectively [45, 54]. This finding is regarded as an additional reflection of the Norwegian dentists' conservative and restricted practice in using antimicrobials.

Conclusions

1. High prevalence of oral bacteria in Yemen being resistant to ampicillin and metronidazole compared to Norway.
2. The high prevalence of resistant oral bacteria in Yemen is explained, at least in part, by the common misuse of antimicrobials by Yemeni dentists.
3. A newly recorded class D β-lactamase is produced by ampicillin-resistant *F. nucleatum* isolates from Yemen. This enzyme could complicate antimicrobial treatment because these enzymes might present resistance to several classes of β-lactam antibiotics.
4. The presence of conservative antibiotic prescription practices by dentists in Norway
5. The continued reliance on narrow-spectrum penicillins prescriptions suggests that oral bacterial resistance is rare in the country.

Reflections on the current situation of antibiotic resistance and recommendatory suggestions to the health policy makers in Yemen

Antimicrobials resistance by microbes is an issue of great concern. It is not a disease by itself but could be a part in any infectious disease treatment course. It can exacerbate the underlining patient conditions, from simple wound infection to car accidents. The emergence of multidrug resistance microbes make it even worse and more challenging for the treatment of these super-bugs. Besides the medical consequences, antibiotic resistance is associated with large costs to society. The most concrete example and the easiest to measure is the cost of drugs, as new empirical treatments are needed to combat resistant pathogens. Among other factors that influence the cost are increased period of hospitalization, increased risks of complications and mortality, costs associated with isolation of patients, and the need to temporarily dismiss carriers of resistant bacteria within the medical staff.

In the recent years, understanding of how microbes resist these miracle drugs has made health professionals more cautious in handling and using these drugs, and national polices were established to guide prescribers on when and how these agents should be used. This is mainly true for the developed countries, where their use and access is very controlled. However, the use of antimicrobials and their easy access is still a problem in most developing countries. On the other hand, it is true to say that polices aimed to regulate antimicrobials (prescription, sale, handling, etc.) exists in many developing countries, but their implementation is not in place. As a result, misuse of these agents in developing countries is huge, and such presence of antimicrobial polices proves to be of no actual benefit in these societies.

Halting resistance development and saving antimicrobials effectiveness need strict, practical, and feasible approaches. The author hereby would like to give a brief message to health policy makers in Yemen and argue for the urgent need to have an action plan for the halt of antimicrobial resistance in the country. The author proposes the following strategy to be adopted in Yemen for the control of antimicrobial resistance:

- **Prevention:** prevention of communicable diseases and infection control to reduce the needs for antimicrobial agents.
- **Surveillance:** the establishment of a proper antimicrobial resistance surveillance system in several major hospitals in the country is a crucial step that is lacking in the country.
- **Antimicrobials committee and subcommittees:** The establishment of a multidisciplinary committee that monitors antimicrobial use in the country and implementation of policies that ban the access of antimicrobials without prescriptions to improve the present resistance situation in Yemen. In addition, organizing antimicrobial teams in health academic institutes would ensure that medical education is following antimicrobial guidelines. Furthermore, these teams could closely monitor authorized prescribers' practices and knowledge on proper prescribing.

- **International cooperation:** an effective strategy requires close cooperation and consultation between Yemen and other involved parties at both national and international levels.

6. Methodological considerations

Sampling and samples transportation (papers 1 and 2)

Traditionally, subgingival samples have been taken using either a curette or paper points and some variations have been documented between and even within these sampling methods [139, 140]. In line with this, a recent review points out that a curette collects plaque from the entire pocket, whereas plaque that is adsorbed onto a paper point is derived mostly from the outer layers of the biofilm, which may contain higher proportions of putative pathogenic bacteria relative to curette samples [141]. In addition, the paper points sampling is a less invasive procedure than sampling by curettes, and is the preferred method, especially when sampling from healthy periodontal pockets [139]. Therefore, paper points sampling was preferred in this study for the analysis of samples by the checkerboard DNA-DNA hybridization method using whole genomic probes.

For transportation of microbial samples, an ideal transport medium keeps the microbes alive and preserves their proportions in the sample. Anaerobiosis and low redox potential of the transport media is essential for survival of anaerobes [126]. In this study, the transport media VMGA III was chosen because of its superior properties, especially for preservation of anaerobes.

DNA-DNA checkerboard hybridization technique (paper 1)

Species identification was carried out using a whole genomic DNA-DNA hybridization approach in checkerboard format [10], which is an acknowledged method for species identification for complex microbial populations such as oral flora. The checkerboard design enables the analysis of a wide range of bacteria (up to 40) in a large number of samples (up to 40) in one run. The sensitivity of the assay is in the range of 10^4 bacterial cells [142]. However, this technique has its disadvantages. Firstly, the detection is semiquantitative and is limited to species for which probes are

available. Secondly, the technique employs whole genomic DNA probes, which may increase the probability of cross-reactions between closely related species. In this study, hybridization signals were interpreted as true positive reactions when they were stronger than those of the standard 10^5 cells thus minimizing false positive readings caused by cross-reactions between DNA related species.

Antimicrobial susceptibility testing (papers 1 and 2)

The purpose of undertaking susceptibility testing, by whatever method, is to attempt to establish and integrate the pattern of *in vitro* potency of an antimicrobial agent against a population of potential pathogens into a relationship in the light of clinical experience. Thus breakpoints, discriminatory antimicrobial concentrations were used in the interpretation of results of susceptibility testing to define isolates as susceptible, intermediate, or resistant. In the United States, the **C**linical and **L**aboratory **S**tandard **I**nstitute (CLSI) - previously called the **N**ational **C**ommittee for **C**linical **L**aboratory **S**tandards (NCCLS) - publishes consensus standards and guidelines for susceptibility testing that are adopted in many parts of the world. These standards and susceptibility breakpoints have undergone considerable changes from the late 1970s and up to the present. Within Europe, the **Eu**ropean **C**ommittee on **A**ntimicrobial **S**usceptibility **T**esting (EUCAST), which is a standing committee of the **E**uropean **S**ociety for **C**linical **M**icrobiology and **I**nfectious **D**iseases (ECCMID), adopted two separate breakpoints [143]. The first one depends on the normal distribution of bacteria's MICs and is named epidemiological cut-off. It aims at detecting bacteria with resistance mechanisms and at monitoring development of resistance. The second breakpoint is known as clinical breakpoints and is intended for the guidance of therapy. The epidemiological cut-off breakpoint defines an organism as resistant (non-wild type) if the observed MIC or inhibition zone falls outside the normal distribution of MICs or zones for isolates without specific resistance mechanisms (wild type). Therefore, it automatically gives importance to small reductions in bacterial susceptibility and allows emerging low-level resistance to be detected and monitored.

In this thesis, one of the main objectives was to document the prevalence of aminopenicillins and metronidazole resistant-subgingival species, in both Yemen and Norway, for comparison rather than for clinical oriented aims. Therefore, the epidemiological cut-off breakpoint was thought best suitable for use in the current study to compare resistance prevalence in Yemen and Norway. However, the epidemiological cut-off breakpoints of the 18 investigated subgingival spices were not reported by the EUCAST. Nevertheless, the author adopted a breakpoint of 2 μg/mL for both ampicillin and metronidazole in the study based on literature review [24, 144-148]. This concentration is higher than the in vitro MIC90s (i.e. the MIC of an antimicrobial agent that inhibits the growth of 90% of susceptible isolates) values shown in the in vitro susceptibility studies of oral bacteria referred to in the study. Ampicillin and amoxicillin are aminopenicillins with the same broad antimicrobial spectrum; however, for the susceptibility testing ampicillin was chosen. This decision was based only on practical reasons, provided that results from antimicrobial resistance testing with one of them is valid for the other one as well [130].

The E-test (AB Biodisk, Sweden) has been shown to be a reliable method for antimicrobials MICs determinations of isolated bacterial strains, with results comparable to those obtained by the NCCLS methods [16]. Therefore, this method was used to determine the ampicillin MIC values of the 23 *F. nucleatum* strains isolated from Yemen.

Proteome analysis (paper 2)

The word proteomics was first introduced in 1995 and was defined as the large-scale characterization of the entire protein complement of a cell line, tissue or organism. Proteome analysis is a direct measurement of proteins in terms of their presence and relative abundance. The introduction of the two-dimensional gel electrophoresis (2-DE) method in 1975 by O'Farrell and its development was a major step forward in protein separation. Today, the 2-DE with immobilized pH gradients for proteins separation and the subsequent identification of these proteins by MALDI-TOF mass spectrometry is one of the techniques preferred for proteomic studies in Europe. However,

the technique has its disadvantages and limitations. Generally, it is a time consuming technique; the general workflow of the experiment could last several days. Moreover, sample preparation for 2-DE could be problematic, especially to achieve a desirable degree of solublization for hydrophobic proteins such as membranous proteins. In addition, the detection threshold of the resolved proteins in the gel is limited by the sensitivity of the used stain, so low abundance-proteins could be difficult to detect and analyze.

Optimization of the 2D-E technique to better resolve and analyze the proteins of *F. nucleatum* took a considerable time, especially to deal with and reduce the streaking business, until satisfactory 2D-E images were obtained. In addition, the subsequent analysis of *F. nucleatum* proteome maps was confronted by, but not limited to, the heterogeneity of the *F. nucleatum* strains. Thus, it was concluded that a significant proportion of the variability in the observed proteome between the ampicillin-susceptible and ampicillin-resistant *F. nucleatum* strains was unrelated to differences in ampicillin resistant phenotype and was because of background strain variation.

Antibiotic prescription practices (papers 3 and 4)

Two different approaches were used to investigate dentists' antimicrobial prescription practices in Yemen and Norway for any possible contribution on the emergence of bacterial resistance. In Yemen, obtaining real prescription data is rather difficult, if not impossible. This is principally because of a lack of prescriptions registry database. Therefore, the approach to retrieve information about Yemeni dentists' antimicrobial prescription practices was based on investigating their knowledge of conditions for the proper prescribing of antimicrobial agents to their patients. On the other hand, prescriptions registration database, namely NorPD, exists in Norway [149]. The dentists' prescriptions data is stored and systematized in NorPD to enable researchers to retrieve information on frequencies of antibiotic prescriptions by dentists, but most importantly, the data is aggregated using the WHO antibiotic consumption unit known as DDD [124]. The data stored in NorPD lacks information

on doses used, frequency of administration, duration of treatment, and reasons for individual prescriptions. Such missing information would have also reflected on the knowledge for proper prescribing. However, knowledge for proper prescribing among dentists in Norway was investigated [150, 151], and these studies revealed a general good prescription knowledge. Therefore, the use of the available data stored in NorPD was considered sufficient to satisfy one of this study's main aims, that is, assessing dentists' antimicrobial prescription practices.

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Paper 1

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Prevalence of subgingival bacteria resistant to aminopenicillins and metronidazole in dental patients from Yemen and Norway

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Abstract

The purpose of this study was to assess the prevalence of resistance to aminopenicillins and metronidazole among selected subgingival species in dental patients from Yemen and Norway. Three subgingival samples were collected by paper points from each of 34 Yemeni and 21 Norwegian adult volunteers and then pooled. Each of the 55 pooled samples was plated on fastidious anaerobic blood agar containing 2 μg/mL ampicillin or metronidazole, or no antimicrobial. Species identification of growth was done using DNA–DNA checkerboard hybridisation. The overall proportion of ampicillin resistance among the 18 identified species was 28.9% and 7.9% in the Yemeni and Norwegian samples, respectively, whereas for metronidazole it was 60.3% and 11.3%. The number of species resistant to ampicillin and metronidazole was significantly higher ($P < 0.016$ and $P = 0.0000$, respectively) in the Yemeni than in the Norwegian samples.

Keywords: Antimicrobial resistance; Developing countries; Oral bacteria

1. Introduction

The oral flora is comprised of more than 600 cultivable and non-cultivable species [1]. In certain conditions and when the ecology of the oral microbiota is disturbed, a subset of these species become virulent and cause infections. Utilising antimicrobial agents is one way to fight such infections. However, like most medically significant bacteria, antimicrobial resistance among oral bacteria is an evolving problem. Several studies on susceptibility testing of oral bacteria reported the presence of isolates that were resistant to penicillin, metronidazole, tetracycline and macrolides [2]. Antimicrobial resistance is a global problem and one of the biggest challenges facing public health today [3]. The problem is complex and multifactorial [4–6] and also of increasing concern to dentistry [2,7]. Development of antimicrobial resistance mechanisms by pathogenic microorganisms is their way to evade antimicrobials and thus survive [8].

Most studies that deal with the emergence of antimicrobial resistance of oral bacteria have been done in the western world. Little information is therefore available from developing countries. Yemen, like most developing countries, possesses socioeconomic and behavioural factors that promote bacterial resistance [9]. In Yemen, self-prescription by individuals and misuse due to healthcare provider-related factors are common practices [10]. Data from Yemen are sparse and the few published reports regarding bacterial resistance call for urgent need of national surveillance [11]. On the other hand, to the best of our knowledge there is only one report from Norway regarding antibiotic resistance of oral bacteria [12]. This study found that 68% of 25 patients with refractory marginal periodontitis harboured β-lactamase-producing bacteria.

Our aim was to assess the prevalence in a developing country (Yemen) and a developed country (Norway) of selected subgingival bacteria resistant to aminopenicillins and metronidazole, two antimicrobials commonly prescribed by dentists [13–16], to determine whether the information in the literature regarding antimicrobial-resistant human medical bacteria in the two countries is also valid for oral bacteria.

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2. Materials and methods

2.1. Study subjects

Thirty-four Yemeni dental patients (mean age 30.1 ± 1.9 years) who visited three dental clinics in Sana'a, Yemen, during the summer of 2003, and 21 Norwegian dental patients (mean age 40.4 ± 2.3 years) who visited two clinics in Bergen, Norway, during the spring of 2004 were recruited for the study. All patients were in need of dental treatment and were selected consecutively among volunteers. Subjects were excluded from the study if they: (1) had general medical problems known to influence their subgingival microflora; (2) suffered from aggressive periodontitis or acute necrotizing gingivitis or periodontitis; and/or (3) had taken any antimicrobial(s) during the last 3 months prior to sampling. Sampling and examination of the samples were approved by the Regional Committee for Medical Research Ethics, West Norway.

2.2. Subgingival sampling

After clinical examination and removal of supragingival plaque, three randomly selected posterior teeth in each patient were assigned for isolation and subgingival sampling. The subgingival samples were collected using sterile paper points size 50 (Dentsply, York, PA, USA) that were inserted as far as possible subgingivally and removed after 20 s. The three samples obtained from each subject were pooled by placing them in a sterile vial containing 1.5 mL of locally produced VMGA III transport medium [17]. All the pooled samples were transported at ambient temperature in plastic bags under anaerobic condition using the Anaerocult® system (Merck, Darmstadt, Germany).

2.3. Cultivation of samples

Samples arrived at the Laboratory of Oral Microbiology, University of Bergen within 48 h after sampling. On arrival, three to five sterile glass beads were added aseptically to each vial. The samples were then vortexed (WhirliMixer; Fisons Scientific Equipment, Loughborough, UK) for 2 min. Thereafter, 10 μL of each sample suspension was plated on fastidious anaerobic blood agar (Lab M, Bury, UK) with either 2 μg/mL ampicillin or 2 μg/mL metronidazole, or with no antimicrobial [18]. The inoculated plates were incubated anaerobically (5% CO_2, 10% H_2 and 85% N_2) for 10 days at 37 °C using the Anoxomat System™ (MART Microbiology BV, Lichtenvoorde, The Netherlands).

After incubation, the growth on each plate was suspended in 1 mL TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.6) using sterile L-shaped plastic rods before being transferred into individual sterile 10 mL glass tubes and sonicated for 10 s to disintegrate clumps. For standardisation, optical density at 600 nm (OD_{600}) of the suspensions was adjusted to a final OD_{600nm} of 1.0 (≈10^9 cells/mL). To prepare samples for analysis, 10 μL of each adjusted suspension (≈10^7 cells) was transferred to individual Eppendorf tubes, each containing 140 μL TE buffer. All the Eppendorf tubes were stored at 5 °C.

2.4. Preparation of DNA probes

The 18 probe species (Table 1) were cultivated, harvested and prepared for DNA extraction as explained elsewhere [19]. DNA extraction was performed according to the method of Smith et al. [20]. The quantity of extracted DNA was determined spectrophotometrically as the absorbance at 260 nm and its purity as the ratio of absorbance at 260 nm and 280 nm. A ratio of 1.8 corresponds to DNA of 100% purity. DNA with at least 90% purity was accepted for probe preparation. Whole genomic DNA probes were prepared from each strain by labelling 1–3 μg of DNA with digoxigenin (DIG-high prime; Roche Diagnostics, Basel, Switzerland) using the random primer technique [21].

2.5. Identification of probe species in the samples

The samples were analysed using whole genomic DNA probes and DNA–DNA checkerboard hybridisation [22]. In brief, the prepared samples were lysed and vacuum-filtered onto a 15 cm × 15 cm positively charged nylon membrane using the 'Minislot-30' device (Immunetics, Cambridge, MA). Two pooled standards containing 1 ng and 10 ng of

Table 1
Bacterial strains used for preparation of whole genomic DNA probes

Species	Strains	Species	Strains
Porphyromonas gingivalis	ATCC 33277	*Streptococcus oralis*	ATCC 10557
Prevotella intermedia	VPI 4197	*Streptococcus sanguinis*	ATCC 10556
Actinobacillus actinomycetemcomitans	ATCC 33384	*Streptococcus constellatus*	ATCC 27823
Eikenella corrodens	ATCC 23834	*Streptococcus gordonii*	CCUG 33482
Campylobacter rectus	ATCC 33238	*Streptococcus mitis*	ATCC 9811
Capnocytophaga gingivalis	ATCC 33624	*Streptococcus intermedius*	ATCC 27335
Fusobacterium nucleatum	ATCC 23736	*Tannerella forsythensis*	FDC 2008
Peptostreptococcus micros	CCUG 17638	*Veillonella parvula*	ATCC 10790
Streptococcus mutans	ATCC 25175	*Eubacterium nodatum*	CCUG 15996

ATCC, American Type Culture Collection, USA; VPI, Virginia Polytechnic Institute and State University, USA; CCUG, Culture Collection, University of Gothenburg, Sweden; FDC, Forsyth Dental Center, Boston, USA.

DNA corresponding to 10^5 and 10^6 bacteria [23], respectively, of each probe species were included in each run. The membranes were allowed to dry at room temperature and the samples were fixed by exposure to 70 mJ/cm^2 of ultraviolet light. The membranes were pre-hybridised and then hybridised with the digoxigenin-labelled whole genomic DNA probes using the 'Miniblot 45' device (Immunetics). Hybrids were detected by chemiluminescence as described by Wall-Manning et al. [23] except that skim milk was used instead of casein in the blocking solution and the stringency washes were performed in sodium dodecyl sulfate (SDS) buffer (0.1% sodium chloride sodium citrate, 0.1% SDS). Hybridisation signals were interpreted as true-positive reactions when they were stronger than those of the standard 10^5 cells, thus minimising false-positive readings caused by cross-reactions between DNA-related species.

2.6. Statistical analysis

Data available for each individual were: (1) absence/presence of each probe species, recorded as 0/1; (2) absence/presence of each resistant species, recorded as 0/1; and (3) number of all resistant species detected, recorded from 0 to 18 as appropriate. The overall resistance among the probe species was calculated by dividing the total number of resistant species detected for each antimicrobial with the total number of species shown. The two study groups were compared for prevalence of probe species as well as resistant ones using the χ^2 test. Differences in resistance to both drugs between the two groups were sought using the Mann–Whitney *U*-test. Differences with $P < 0.05$ were considered statistically significant. SPSS 12.0 for Windows was used for computerisation and statistical analysis.

3. Results

3.1. Prevalence of the probe species

All the probe species were detected in subgingival samples from both patient groups, but at different frequencies (Fig. 1). *Porphyromonas gingivalis*, *Eubacterium nodatum*, *Streptococcus sanguinis* and *Veillonella parvula* were significantly ($P < 0.05$) more prevalent in the Yemeni subjects, whilst *Actinobacillus actinomycetemcomitans*, *Capnocytophaga gingivalis* and *Streptococcus mitis* showed a significantly higher prevalence in the Norwegian samples.

3.2. Prevalence of resistant species

In the samples from the Yemeni subjects, 28.9% and 60.3% of all detected species were resistant to ampicillin and metronidazole, respectively. The corresponding figures for the Norwegian samples were 7.9% and 11.3%. Fig. 2 shows the prevalence of individual species resistant to ampicillin or metronidazole in the two study groups. Yemeni samples

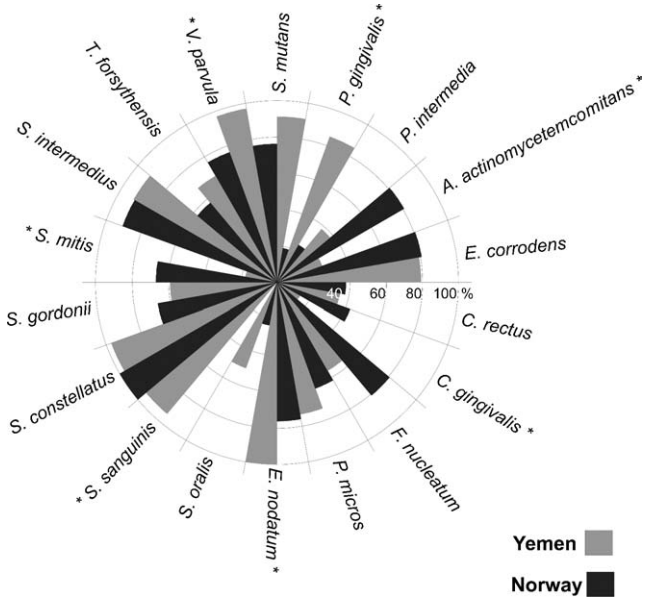

Fig. 1. Percentages of the probe species testing positive in the two study populations. * $P < 0.05$.

exhibited a significantly higher prevalence of metronidazole-resistant *Eikenella corrodens*, *Streptococcus mutans*, *Streptococcus oralis*, *S. sanguinis*, *Streptococcus constellatus*, *Streptococcus gordonii*, *S. mitis*, *Streptococcus intermedius* and *V. parvula* (Fig. 2A) as well as a significantly increased prevalence of ampicillin-resistant *P. gingivalis*, *Prevotella intermedia*, *S. constellatus*, *S. intermedius*, *Tannerella forsythensis*, *Fusobacterium nucleatum* and *V. parvula* (Fig. 2B). The most frequently detected species in the Yemeni samples resistant to both antibiotics was *V. parvula*.

4. Discussion

Our aim was to assess the resistance to two commonly used antimicrobials (aminopenicillin and metronidazole) among 18 subgingival species in adults who visited a dental clinic in Yemen or Norway for various reasons. Most previous studies on antimicrobial resistance of oral bacteria referred to periodontitis patients [12,24]. Few of the sample donors in our study suffered from periodontitis. By using paper points, subgingival planktonic bacteria and loosely attached dental biofilm, which contains the more pathogenic microflora [25], were sampled. Our data provide information regarding the prevalence of the 18 probe species in the subgingival microbiota of these subjects. The significant differences in this regard between the Yemeni and Norwegian dental patients are in line with studies that have shown differences in the mean proportions of subgingival species in samples from periodontitis or healthy subjects in different countries, which may be explained by differences in oral hygiene, race, age, diet, genetics as well as disease susceptibility and manifestations [26–28].

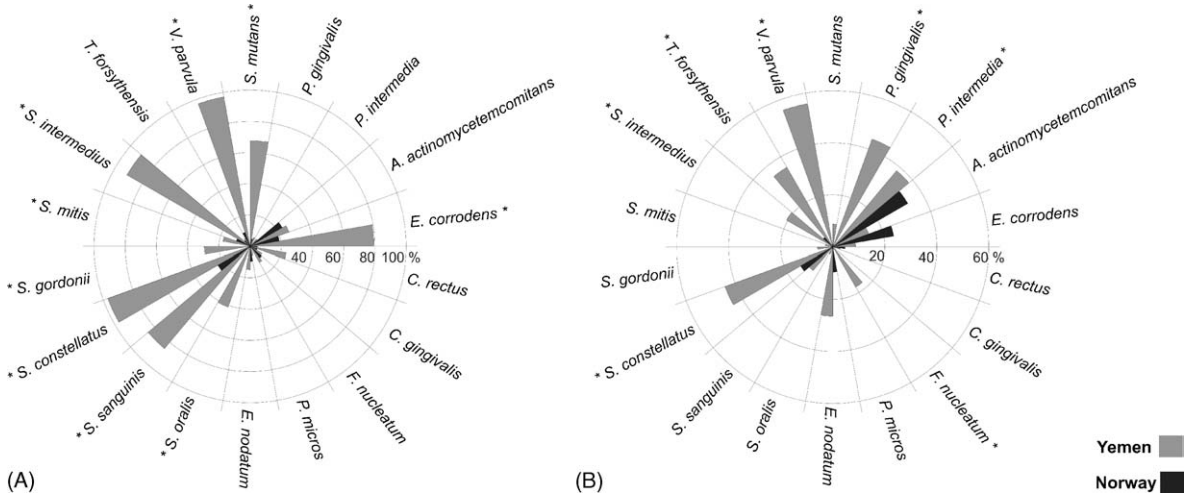

Fig. 2. Prevalence of the probe species resistant to (A) 2 μg/mL metronidazole and (B) 2 μg/mL ampicillin in the two study populations. $^{*}P < 0.05$.

Ampicillin and amoxicillin are aminopenicillins with the same broad antimicrobial spectrum [29] and results from antimicrobial resistance testing with one of them is also valid for the other [30]. Aminopenicillins are one of the three antimicrobials most commonly prescribed in dentistry [31]. The proportion of ampicillin-resistant species was significantly higher in Yemeni than in Norwegian subgingival samples (28.9% versus 7.9%). Previously, a very small proportion of the subgingival microbiota has been found to be resistant to penicillins in vitro [29,32,33]. Recently, Feres et al. [18] reported the percentage of bacterial isolates resistant to amoxicillin in subgingival plaque samples at baseline to be 0.5%. During 14 days of amoxicillin treatment, the prevalence of resistant isolates increased to 37% and dropped to baseline levels 76 days post treatment. Since the subjects in our study confirmed not having taken any antimicrobial during the 90 days prior to sampling, they harboured permanently resistant species.

Our findings of 19.4% and 3.3% ampicillin-resistant streptococcal isolates in the Yemeni and Norwegian samples, respectively, are in line with the high levels of penicillin resistance that are now being demonstrated in the α-haemolytic streptococci [2]. Diaz-Mejia et al. [34] tested the ampicillin susceptibility of oral streptococci from healthy Cuban and Mexican volunteers and found the proportion of resistant strains to be less than 5%. More recently, it was reported that the susceptibility rate of viridans group streptococci to ampicillin was 95% [35], giving a proportion of resistant isolates similar to that reported in our study from Norway as well as reported from Mexico and Cuba [34].

The species that accounted for the significantly increased proportion of ampicillin-resistant species in the Yemeni samples compared with the Norwegian samples were *P. intermedia*, *V. parvula*, *A. actinomycetemcomitans*, *T. forsythensis* and *P. gingivalis*. β-Lactamase production by *Prevotella* species is well known and such species have been reported to be the most frequent β-lactamase-producing species in the periodontal pockets [36,37]. In the Yemeni samples, all the *Prevotella* isolates were resistant to ampicillin. Furthermore, ampicillin-resistant *P. gingivalis* was detected in 15 Yemeni samples but in none of the Norwegian ones. The susceptibility of *P. gingivalis* to penicillins has been reported to be 100% [24,38] and the bacterium has so far not been reported to produce β-lactamase [12,38–40] or to have β-lactamase genes [41]. However, Prieto-Prieto and Calvo [42] referred to a study conducted in Spain in which 59% of *Porphyromonas* species were resistant to penicillin G; Spain is known to have high antibiotic consumption [24].

Metronidazole is mainly active against strict anaerobes [43,44] and dentists are frequent prescribers of this antimicrobial [45]. Susceptible organisms rarely develop resistance to metronidazole, but some species may require a high concentration for susceptibility [46]. The proportions of metronidazole-resistant species in the Yemeni and Norwegian samples were 60% and 11%, respectively. In comparison, Feres et al. [18] reported that over 50% of the isolates in their study were resistant to 2 μg/mL metronidazole in vitro prior to administration of that agent. As expected, the streptococci species comprised a high proportion (84% and 8% from Yemen and Norway, respectively) of the metronidazole-resistant species in the current study; aerobic bacteria can utilise the aerobic metabolism pathway and consequently bypass the crucial reduction step intracellularly that is required for this drug to be active [47]. However, *V. parvula*, which is an obligate anaerobe that has been reported to be highly sensitive to metronidazole [18,24], was frequently detected as metronidazole resistant in the Yemeni subjects. Recently, and in line with our findings, one study has shown that treatment of *Helicobacter pylori* with metronidazole-based triple therapy in Yemen was unsatisfactory [48]. This finding was attributed to the misuse of metronidazole in the country and subsequent bacterial resistance that affected complete eradication of *H. pylori*.

The proportion of resistant isolates demonstrated in vitro in microbiological samples will depend on the concentration of the antimicrobial agent used for growth inhibition and the interspecies variability in susceptibility to that agent. It was recently concluded that the interindividual susceptibility of principal periodontal pathogens is not homogeneous and appears to vary according to bacterial species and antimicrobial molecules [49]. This variability seemed to be greater with older agents than with newer ones (e.g. ampicillin and amoxicillin). In our study, we adopted a breakpoint of 2 μg/mL for both ampicillin and metronidazole. This concentration is higher than the in vitro MIC_{90} values (i.e. the minimum inhibitory concentration of an antimicrobial agent that inhibits the growth of 90% of susceptible isolates) shown in the in vitro susceptibility studies of oral bacteria referred to in our study [50–55]. Thus, 2 μg/mL theoretically falls outside the normal distribution of MICs for each of the species under the current study.

The definition of microbial resistance is a tenuous issue and a topic of debate. Therefore, the term breakpoint was established to define the borderline between susceptible and resistant isolates. However, from the late 1970s up to now, such breakpoints have undergone considerable changes. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) adopted recently two separate breakpoints [56]. The first one, depending on the normal distribution of a bacteria's MICs and named the epidemiological cut-off, aims at detecting bacteria with resistance mechanisms and at monitoring development of resistance. The second breakpoint is known as the clinical breakpoint and is intended for guidance of therapy.

Lack of harmonisation of breakpoints and methods creates difficulties in the comparison of our results of proportions of resistant molecular isolates with those of the other studies. Feres et al. [18] and Rodrigues et al. [57] identified and determined the percentages of resistant species in the same way as was done in our study. However, Feres et al. [18] related the proportions of resistant isolates to individual sample sites whilst in our study the results are related to one pooled sample per individual. In the Rodrigues et al. study [57], four periodontal pockets were sampled per individual and the mean percentage of tetracycline-resistant isolates was computed by averaging these values within a subject and then in each treatment group at each visit.

Limitations of the current study are that: no MICs could be established for the isolates; isolates that might have been resistant to both 2 μg/mL ampicillin and 2 μg/mL metronidazole could not be revealed; and resistant isolates could not be harvested and stored. However, our findings did disclose significant differences in the in vitro prevalence of ampicillin- and metronidazole-resistant subgingival species between Yemen and Norway. These differences can be attributed to the different levels of factors promoting microbial resistance in the two countries. In Norway, usage of antimicrobials is well controlled and probably low compared with Yemen where misuse by health professionals and self-prescription of antibiotics are common practices [10]. A horrifying example of the latter practice was a patient excluded from our study who reported daily use of an antibiotic just to increase his appetite. In fact, it was a difficult task to find Yemeni subjects who fulfilled the inclusion criteria of our study. However, like in most developing countries, socioeconomic and behavioural factors also lead to and promote bacterial resistance [9].

In conclusion, this is the first report on ampicillin and metronidazole resistance among the subgingival microbiota in Yemen. The results indicate a high prevalence of oral bacteria in Yemen with resistance to ampicillin and metronidazole. This difference is probably attributed to easy access, misuse and availability of poor quality drugs in the country. In contrast, this is a limited problem in Norway. Our findings should be taken into consideration when managing oral infections as well as extraoral infections of oral origin in Yemeni patients.

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Paper 2

Proteomic analysis of ampicillin-resistant oral *Fusobacterium nucleatum*. Oral Microbiology Immunology 2008: 23: 1–7

By

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Proteomic analysis of ampicillinresistant oral Fusobacterium nucleatum

Abstract

Introduction: Fusobacterium nucleatum represents one of the predominant anaerobic species in the oral microbiota. Penicillin-resistant F. nucleatum have been isolated from intra- and extraoral infections. This study aimed to assess ampicillin resistance in F. nucleatum by investigating the synthesis of resistance-associated proteins.
Methods: Ampicillin-resistant and ampicillin-susceptible F. nucleatum isolates were obtained from 22 dental plaque samples. Two-dimensional gel electrophoresis and mass spectrometry were used to investigate bacterial protein synthesis. Proteins exhibiting statistically significant quantitative changes between sensitive and resistant isolates were identified using peptide mass mapping and matrix-assisted laser desorption/ionization – time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry.
Results: Twenty-three F. nucleatum isolates were recovered from plaque samples and their ampicillin minimum inhibitory concentrations ranged between 0.125 lg/ml and 256 lg/ml. Analysis of the bacterial cellular proteins by two-dimensional gel electrophoresis resolved 154–246 distinct protein spots (mean 212, n = 9). Between 32% and 83% of the protein spots were common for the F. nucleatum isolates. Comparisons of the protein profiles of sensitive and resistant isolates revealed the presence of a 29 kDa protein and significant increases in the synthesis of two proteins at 37 and 46 kDa in the ampicillin-resistant F. nucleatum isolates. These proteins were identified as a class D b-lactamase, ATP-binding cassette (ABC) transporter ATP-binding protein and enolase, respectively.
Conclusion: Synthesis of a class D b-lactamase by ampicillin-resistant F. nucleatum isolates could complicate antimicrobial treatment because these enzymes might confer resistance to many classes of b-lactam antibiotics. The differences observed in protein synthesis between ampicillin-resistant and ampicillin-susceptible F. nucleatum may contribute to the antibiotic resistance and virulence of these bacteria.

Key words: ampicillin; Fusobacterium; proteomics; resistance; Yemen

Paper 3

Knowledge of prescribing antimicrobials among Yemeni general dentists. Acta Odontologica Scandinavica, 2006; 64: 274–280

By

MOHAMMED AL-HARONI & NILS SKAUG

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ORIGINAL ARTICLE

Knowledge of prescribing antimicrobials among Yemeni general dentists

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Abstract

Objective. Overuse of antimicrobial agents is closely related to an increase in bacterial resistance. A sound knowledge of appropriate prescribing of antimicrobials among health professionals is thus critical in combating the resistance. The objectives of this study were to assess the rationale for and patterns of antimicrobial prescriptions by general dental practitioners in Yemen. ***Material and Methods.*** A questionnaire containing 65 closed questions was used for this cross-sectional study and distributed to 280 dentists in the three major governorates in Yemen. The anonymously completed questionnaires sought answers to demographic questions and to questions on the therapeutic and prophylactic use of antimicrobial agents in dentistry. Correct and incorrect answers were defined according to information available in the current authoritative literature. Each correct answer was given a score of 1 while an incorrect answer scored 0. Thus, the total score had an attainable range from 0 to 65. Frequencies, means, and associations were assessed statistically. ***Results.*** Out of 181 collected forms (response rate 64.6%), 150 were appropriately completed and used for data analyses. Penicillins were the most frequently prescribed drugs (72%), followed by spiramycin (10%). It was found that up to 84% of practitioners were likely to prescribe an antimicrobial agent when there was no clinical indication for such a medication. Many respondents (70%) would consider antibiotics for at least one of the given non-clinical factors. ***Conclusions.*** The results suggest that dental practitioners in Yemen lack uniformity in the rationale for appropriate prescribing of antimicrobials to their patients. Consequently, to reduce overuse, there is an urgent need for the dental community in the country to be informed about evidence-based guidelines and the appropriate use of antimicrobial agents in clinical dental practice.

Key Words: *Antibiotics, general dental, prescription, Yemen*

Introduction

Resistance to antimicrobial agents is the ability of microbes to remain impervious to the inhibitory or lethal effects of these drugs and this has increased in conjunction with the ever widening use of antimicrobials in recent years. Thus, resistance to all antimicrobial agents was already noticed within the first couple of years after they were introduced in clinical medicine. The prevalence of resistant isolates and their level of resistance have reached a critical point, and alarming cases are increasingly being reported which are also a cause of concern to dentistry [1,2]. In addition, the continuous emergence of multiresistant species is reported from different parts of the globe and shows their ability to tolerate a panel of antibiotics and to cause serious mortality. This global problem is one of the biggest challenges facing public health today [3].

Bacterial resistance to antimicrobials is a result of a complex interplay of several factors [3,4]. The selective pressure exerted by widespread use of antimicrobial drugs is regarded as the driving force behind the evolution of microbial defense mechanisms. It has been shown, however, that a significant reduction in the use of antibiotics can be followed by a significant reduction in antimicrobial resistance and it is only through prudent and appropriate use of these drugs that their efficacy can be prolonged [5].

Misuse of antibiotics can be traced to the prescribers, patient compliance to prescriptions, and the drug sellers. Physicians' perceptions of patients' expectations might influence their prescription of antibiotics [6] and this is probably also the case with

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dentists. The few published studies on dentists' knowledge of antibiotic prescription have revealed that factors other than a sound knowledge might influence their prescription practices [7–10]. The misuse is more widespread when antibiotics are obtainable without prescriptions and where general knowledge and beliefs among the public are based on poor understanding [11]. Furthermore, many dental infections are mixed infections that provide an optimum environment for exchange of resistance determinants between bacterial species, thus resulting in even more resistance if combined with improper use of antimicrobials [12].

The prescribing of antimicrobials in dental practice is generally considerably less than that in medical practice [13,14]. However, in England and Scotland, dentists accounted for about 10% of all community prescriptions and in the USA, in the period 1995–97, for almost 9% of the most commonly used antimicrobial agents in Western countries [2,7]. Furthermore, antimicrobials are the most common medication prescribed by dental practitioners in developed [15] and developing countries [16]. Therefore, the contribution by dentistry to microbial resistance should not be neglected. In line with this, in 1999 the Féderation Dentaire International (World Dental Federation) Commission issued guidelines for appropriate use of antimicrobial agents to minimize development of resistance in dentistry [17]. Following other current guidelines and recommendations for therapeutic and prophylactic use of antimicrobial agents in immunocompetent [18,19] and immunocompromised [20,21] patients, will also contribute towards reaching this goal and subsequently will have an influence on combating resistance emergence.

Recently, we found a significantly higher prevalence of resistance to ampicillin and metronidazole among 18 selected oral microbes from Yemeni dental patients compared with those in Norway [22]. This finding prompted the current study, the aim of which was to assess the knowledge of general dental practitioners in Yemen in understanding the conditions for appropriate prescribing of antimicrobial agents to their patients.

Material and methods

Questionnaire

This cross-sectional questionnaire study was performed during the summer of 2004. The questionnaire comprised 65 close-ended questions. The questionnaire was identical to the one designed and first used by Palmer et al. [7] with the exception that the first three questions were omitted: 1) "Have you attended any postgraduate courses on antibiotic prescribing within the past two years?", 2) "Year of first dental degree", and 3) "Place of qualification".

The questionnaire sought answers to the following 11 parameters : (i) gender, (ii) age bands (21–30, 31–40, 41–50, 51–60, and above 61 years), (iii) clinical signs, (iv) antimicrobial treatment of dental infection (choice of antibiotic, dose and frequency used, and number of days treated), (v) non-clinical factors, (vi) choice of antimicrobial agent to dental infection in the case of patients allergic to penicillin, (vii) clinical conditions, (viii) treatment procedure with no relevant medical history, (ix) a relevant medical history, (x) antibiotic regimen used for prophylaxis with adult medically compromised patients (MCPs) not allergic to penicillin, and (xi) antibiotic regimen used for medically compromised patients allergic to penicillin. The categories of questions designated (iii), (v), (vii), and (viii) had various alternative answers (see Table II) and the respondents were asked to indicate "Yes" or "No" according to their opinion on each of these answers. Category (ix), "A relevant medical history", comprised 19 medical conditions (see Table III) that the respondents had to mark according to whether they thought prophylactic antibiotics were required or not.

A list of dentists was obtained from the databases of three pharmaceutical companies that operate in Yemen. Because there is no postal system in the country, the questionnaires were distributed by hand by one of the investigators (M.A-H.) to 280 dentists working in all the governmental as well as private dental clinics in the three major governorates (Sana'a, Aden, and Taiz). The purpose and importance of the study were explained to all recipients of the questionnaire. The questionnaires were collected later during a maximum of three different visits before a recipient was reported as a non-respondent.

Data analysis

Response rate and gender distribution were computed. Scores of knowledge were calculated by giving each correct answer a score of 1 while an incorrect answer scored 0. Thus, the total knowledge score of a questionnaire had an obtainable range from 0 to 65 based on information in guidelines, recommendations, and expert literature [18,19,23]. This authoritative information defined correct answers as follows: In the "Clinical signs" category, "Yes" would be a correct answer for elevated temperature and evidence of systemic spread, gross or diffuse swelling, difficulty in swallowing, and closure of the eye because of swelling. Similarly, "No" would be the correct answer for all answer alternatives in "Non-clinical factors" and "No relevant medical history". Of the 13 alternatives in "Clinical conditions", "Yes" is a correct answer only for cellulitis and acute ulcerative gingivitis. Regarding the need for prophylaxis in MCPs, the medical conditions considered to be the indications

Table I. Maximum possible scores, ranges, mean and standard deviation (SD) of scores, and respondents' level of knowledge of the five categories of questions in the questionnaire

Categories of questions	Maximum score	Range	Mean ± SD	Level of knowledge (%)
Clinical signs*	6	0–6	3.72 ± 1.03	Intermediate (62)
Clinical conditions*	14	0–14	3.25 ± 1.26	Poor (40)
Non-clinical factors*	5	0–5	5.99 ± 2.08	Intermediate (65)
Treatment procedure with no relevant medical history*	7	0–7	3.48 ± 1.40	Poor (49)
Relevant medical history**	19	0–19	6.50 ± 3.65	Poor (34)

*For details, see Table II; **For details, see Table III.

for antimicrobial prophylaxis are: previous bacterial endocarditis, prosthetic heart valves, ventricular septal defect, rheumatic heart disease, aortic stenosis, and radiotherapy to head and neck. Dental extractions, scaling, and polishing are the dental procedures requiring prophylaxis in MCPs.

The respondents were grouped according to the scores gained as having: 1) good knowledge (above 80% correct answers), 2) intermediate knowledge (between 50% and 80% correct answers), and poor knowledge (fewer than 50% correct answers). Average knowledge scores for the question categories (iii), (v), (vii), (viii), and (ix), respectively, were expressed as percentages and calculated by dividing the sum of the scores obtained by the sum of the maximum possible scores for the given category. Normal distribution of the data was checked, and the type of statistical test was chosen accordingly. For each of the categories of questions, the mean and the standard deviation were calculated. Significant differences with gender as a grouping variable were tested with the Mann-Whitney U-test. When age bands were used as grouping variable, the Kruskal-Wallis test was used for statistical computation. Differences with a p-value ≤ 0.05 were considered as statistically significant. All analyses were conducted using SPSS 12.00 for Windows.

Results

A total of 181 questionnaires were collected, giving a response rate of 64.6%. Of these, 150 had been properly completed and were analyzed. Sixty-six percent of the usable forms were submitted by males. The mean score was 28.9 with a standard deviation of 5.6. Test of significance (Mann-Whitney test, $p < 0.001$) showed a lower female mean score than male mean score (27.6, SD 4.6 and 29.57, SD 5.9, respectively). Group statistics using gender as a grouping test variable against the different

Table II. Distribution of respondents' "Yes" and "No" answers regarding clinical signs and conditions, non-clinical factors and no relevant medical history that require/do not require prophylactic antibiotics

Clinical signs	Yes/No	Non-clinical factors	Yes/No
1. Elevated temperature and evidence of systemic spread	115/35	1. Patient expectation of a prescription	22/128
2. Localized fluctuant swelling	102/48	2. Pressure of time and workload	36/114
3. Gross or diffuse swelling	135/15	3. Patient's social history	37/113
4. Unrestricted mouth opening	43/107	4. Uncertainty of diagnosis	42/108
5. Difficulty in swallowing	69/81	5. Where treatment has to be delayed	78/72
6. Closure of the eye owing to swelling	117/33		

Clinical conditions	Yes/No	No relevant medical history	Yes/No
1. Acute pulpitis	48/102	1. Extraction	
2. Acute periapical infection		a) routine	27/123
a) before drainage	105/45	b) surgical	135/15
b) after drainage	94/56	2. Apicectomy	139/11
3. Chronic apical infection	108/42	3. Root canal therapy	
4. Pericoronitis	126/24	a) preoperative	42/108
5. Cellulitis	117/33	b) postoperative	52/98
6. Periodontal abscess	124/26	4. Scaling and polishing	52/98
7. Acute ulcerative gingivitis	120/30	5. Restorative treatment	4/146
8. Chronic marginal gingivitis	81/69		
9. Sinusitis	123/27		
10. Chronic periodontitis	100/50		
11. Dry socket	102/48		
12. Trismus	49/101		
13. Reimplantation of teeth	114/36		

Table III. Relative distribution (%) of respondents according to their opinion on dental treatment and patients with medical conditions requiring antibiotic prophylaxis

Relevant medical conditions	Scaling and polishing (%)	Subgingival class II fillings (%)	Subgingival class V fillings (%)	Root canal therapy (%)	Extractions (%)	Impressions (%)	Seek specialist advice (%)
1. Diabetes mellitus	50.6	26	22.6	34.6	74.6	17.3	24
2. Hemodialysis patients	26.6	16.6	18	22.6	40.6	12	44
3. Hodgkin's disease	18.6	12.6	10	12.6	22.6	6	44.6
4. Aids	36	28.6	28	34	38.6	16	50.6
5. Patients on immunosuppressives	40	26	26	32.6	40.6	14	46.6
6. Autoimmune disease patients	16	8.6	8	14.6	26.6	4.6	46
7. Renal transplant patients	38	28.6	30	32	52	16.6	46.6
8. Head and neck irradiated patients	18	10	10	16	30.6	10.6	42.6
9. Patients with prosthetic joints	26.6	16.6	18	24	42.6	10	34
10. History of infective endocarditis	74.6	58.6	58.6	60	82.6	28.6	40
11. Cardiac valve prosthesis	64	52	50.6	54	78	22	42
12. Rheumatic heart disease	60	46.6	44.6	52	76.6	22.6	40.6
13. Aortic stenosis	32	26.6	26.6	31	44	14	52
14. Ventricular septal defect	34	28	26	36	48	14	52.6
15. Coronary bypass surgery	26.6	22.6	22.6	30.6	40	12	52.6
16. Rheumatic fever – no valvular dysfunction	30	22.6	22.6	26.6	40.6	12	44
17. Coronary heart disease	40.6	30.6	32.6	36	48	18	48.6
18. Pacemaker	26.6	22.6	22	26	34.6	12.6	48.6
19. Physiological/functional/innocent murmurs	14.6	14	12.6	18	26.6	8	48

categories revealed a statistically significant difference (Mann-Whitney test, $p \leq 0.00$) in the prescription of antibiotics in relation to clinical diagnosis. No significant difference was found among age-band groups (Kruskal-Wallis test). The ranges of scores in question categories with mean and standard deviation are presented in Table I. The table also shows the general knowledge of when it is appropriate or inappropriate to use antibiotics in the different parts of the questionnaire.

Penicillins were prescribed by 72% as the first drug of choice for treating dental infections, followed by spiramycin (10%). Three percent of the respondents prescribed erythromycin and lincomycin, 2% clindamycin and metronidazole, while other antimicrobial agents were prescribed by 5% of the respondents. Many respondents (70%) would consider antimicrobials for at least one of the given non-clinical reasons. Table II shows that elevated temperature and evidence of systemic spread, gross or diffuse swelling, closure of the eye because of swelling, and localized fluctuant swelling represent the main clinical signs taken by dentists to indicate the need to prescribe antibiotics to their patients. Approximately one-third of the respondents indicated that they would prescribe antimicrobials for patients with acute pulpitis, and around two-thirds would consider antimicrobials appropriate for chronic periodontitis and chronic apical infections.

Table III presents the respondents' opinions on dental treatment and patients with medical conditions requiring antibiotic prophylaxis. It reveals that a history of previous infective endocarditis, followed by cardiac valve prosthesis are the conditions that most respondents indicated as requiring antibiotic prophylaxis in all investigated treatment procedures. On the other hand, patients with autoimmune disease and Hodgkin's disease received little such attention.

Discussion

The questionnaire investigated the dentists' knowledge of therapeutic and prophylactic antimicrobial usage in clinical dentistry. This is the first study of its kind in Yemen. The knowledge of the respondents in some aspects was better than in others, but a general lack of uniformity and compliance with the expert literature [19,23,24] predominated. Palmer et al. [7] suggested that 29% of antimicrobial usage has no rational basis. Our findings revealed that the respondents would prescribe antimicrobials on the demand of patients or their social history, by 15% and 25%, respectively. Twenty-eight percent of the respondents would prescribe antimicrobials based on no diagnosis. This figure was found to be only 9% in England and Scotland [7] but reached 20% in a study conducted in Kuwait [9]. Lack of time and pressure of workload, with no clinical background had an influence on 24% of our respondents when prescribing antimicrobial agents. It is noticeable that a large number claimed they would not prescribe antibiotics after conservative treatment. However, 32% of them believed in the use of antibiotics in patients presenting with acute pulpitis, which is proven to be of no benefit at all in such cases [25]. The controversy between the two answers highlights the misconception of relating clinical observations and underlying pathological conditions.

The majority of chronic or even acute dental infections can be successfully treated by eliminating the source of infection, pulp extirpation, drainage of abscess, or tooth extraction without the need for antibiotics. Exceptions are when there is evidence of systemic involvement and gross, rapid, and diffuse spread of infection [19]. However, a large proportion of the surveyed Yemeni dentists indicated they would prescribe antimicrobial agents for treatment of several dental clinical conditions for which such drugs have no justification at all. For example, 72% and 54% would prescribe antibiotics for chronic apical infections and chronic marginal gingivitis, respectively. Routine use of antimicrobials by many respondents was illustrated by their treatment of dry sockets and pericoronitis, where evidence-based practice indicates local treatment alone as being sufficient [19]. However, in some clinical situations, e.g. pericoronitis with widespread infection or systemic involvement [26], prescribing antibiotics is justified. Such exceptional situations were not intercepted by our questionnaire. The general tendency of respondents to over-prescribe antimicrobials may be a consequence of lack of aseptic techniques, thus a "just in case" principle is practiced. This opinion would in itself be a justification for overuse of antibiotics, and such a practice is totally unacceptable because there is increasing evidence that it leads to a serious rise in bacterial resistance [27].

Our results indicate that penicillins are the family of antimicrobials that most dentists in Yemen would prescribe for treatment of dental infections. However, 10% would use a macrolide antimicrobial (spiramycin) as their first choice in such cases. This choice is difficult to explain and is not in line with the practice of dentists in other countries [10,28].

Participants scored better on clinical signs and symptoms and on non-clinical factors than they scored on prophylaxis in MCPs, suggesting that dentists need to extend their knowledge from just treating patients' teeth to treating patients with teeth. Prophylaxis in MCPs who receive dental treatment is not always a clear-cut matter, because different guidelines may have different recommendations and different regimens exist [23,29]. These differences may lead to controversy in a subgroup of medically compromised dental patients, e.g. MCPs

requiring placement of a rubber dam, in which antibiotic prophylaxis is mandatory according to the British Cardiac Society (BCS) guidelines, while they are neglected in the American Heart Association (AHA) guidelines [23]. Clinical judgment might also influence a dentist's decision on antimicrobial coverage in patients with a compromised immune system, such as AIDS and diabetic patients, who generally do not require such prophylaxis [23]. Furthermore, the same guidelines could be refused by one dental school and adopted by another in the same country, owing to the lack of convincing scientific background information [30]. These examples highlight the need for international guidelines that are generally agreed on and followed, as several different opinions might be a gateway to misuse.

Despite the domination of poor and low intermediate knowledge among respondents, a significant difference was found in the general knowledge between genders with regard to antimicrobial prescription in acute pulpitis, acute and chronic periapical infection, cellulitis, pericoronitis, periodontal abscess, acute ulcerative gingivitis, chronic marginal gingivitis, chronic periodontitis, sinusitis, and dry socket, in which females scored less than males. This favorable and advantageous male prescription pattern might be due to males being more confident than females. Or females, besides being introduced relatively recently to the labor force, might be more afraid of being accused of infection sequelae of dental treatment.

Our findings indicate that the scientific basis for prescribing antimicrobial agents was neglected by the majority of the respondents. This situation is not surprising as similar findings were reported among other health professionals in Yemen [31] and by general dental practitioners in other countries [7,9,10]. An exception is a study conducted in Norway some years ago [32]. Inferences from our study and similar studies raise questions about whether it is lack of, or ignorance of guidelines that lies behind antibiotics overuse. Such irrational use of antibiotics can be corrected by arranging an audit of clinical antibiotic prescription in dentistry, which is reported to improve general dental practitioners' attitudes to prescribing antimicrobials by, in some circumstances, 50% [33,34]. It is also worth mentioning here that general medical practitioners were found to prescribe more antibiotics and more broad-spectrum ones than did dentists when dealing with acute dental emergencies [35]. The use of antimicrobial agents will select for resistant isolates, and if this use is unnecessary, the situation will be worsened [5]. Nowadays it is not unusual to see an isolate with multidrug resistance or a "superbug" that does not respond to any antibiotic.

No official records indicate how many dentists there are in Yemen. However, we gained access to a list of all established governmental hospitals as well as private dental clinics. Dentistry is considered a relatively new professional discipline in this country, the first dental college having been established in 1994. Before that, dentists gained their degree abroad, mainly from eastern European countries and neighboring Arab universities. Yemeni dentists are not willing to reveal their professional identity by giving information about the place and year of their graduation; furthermore, no postgraduate courses on the prescribing of antimicrobials are offered to them in Yemen. On these bases, the first three questions in the original questionnaire of Palmer et al. [7] were omitted in our study.

One hundred and fifty usable forms out of 280 distributed (53.5% usable) and 181 collected (82.8% usable) forms were analyzed. This may raise questions about the representativeness of the data for Yemeni dentists in general and whether the non-respondents might have affected the study outcomes if they had responded. However, the demographic features of the non-respondents or of the 31 dentists that returned partially completed questionnaires yielded no new information, compared to the usable ones. In fact, investigating health workers' knowledge is considered a sensitive issue. This might partly explain the non-respondents' refusal to give information about their reasons for not complying, when further approached. It is probable that non-respondents might have an even poorer knowledge than those who responded. The low response rates among health practitioners are not uncommon [36]. We addressed the importance of the study and the study subjects were reminded at two subsequent visits, but not more than about two-thirds of them responded positively. This lack of responsiveness might be due to high workload, loss of the questionnaire, or even lack of interest [37].

In conclusion, our study is the first survey to date among Yemeni dentists. The findings, being representative of about 53.6% of all general dental practitioners in Yemen and studied in the light of the authoritative international literature in the field, indicate that too few Yemeni dentists have a good knowledge of antimicrobial indications and contra-indications. A consequence will be overuse of antimicrobial agents, which is most probably one explanation for the greater prevalence of resistant subgingival species among dental patients in Yemen, when compared with Norway [22]. Consequently, it is a matter of urgency that the dental community in Yemen is informed about the accepted current antibiotic prescription guidelines and the related evidence-based clinical practice. This is significant, since the implication of these recommendations will be one important step towards restricting the inappropriate use of antimicrobial agents in this country.

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Paper 4

Incidence of antibiotic prescribing in dental practice in Norway and its contribution to national consumption. Journal of Antimicrobial Chemotherapy (2007) 59, 1161–1166

By

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Incidence of antibiotic prescribing in dental practice in Norway and its contribution to national consumption

Abstract

Objectives: To assess dentistry-based utilization of the 11 antibiotics prescribed by dentists in Norway and its relative contribution to national outpatient consumption and to determine the relationship between numbers of prescriptions and the consumption of these antibiotics.
Methods: Data on national antibiotic prescriptions by dentists in 2004 and 2005 were used. Consumption of the antibiotics was expressed using WHO defined daily doses (DDDs), DDDs per 1000 inhabitants per day (DIDs) and numbers of prescriptions per 1000 inhabitants (PIDs).
Results: Analysis of 268 834 prescriptions issued by 4765 dentists showed that the dentists' prescriptions contributed 8% of the total national consumption of the 11 antibiotics and 13.5%, 2.8% and 1.2% of the national b-lactam penicillins, macrolides and lincosamides and tetracyclines utilization, respectively. The dentists' contributions to the national phenoxymethylpenicillin, spiramycin and metronidazole consumptions were considerably higher (_13.2%) than for the other prescribed antibiotics (_8.6%). There was a strong positive correlation between numbers of DDDs and numbers of prescriptions and between DIDs and numbers of PIDs.
Conclusions: Reliance of Norwegian dentists on phenoxymethylpenicillin as their first choice suggests a low prevalence of antibiotic resistance among oral bacteria in Norway. Norwegian dentists prefer to prescribe narrow-spectrum antibiotics; their prescribing is conservative and relatively low compared with that of physicians.

Keywords: antibiotics, dentists, prescriptions, utilization