Charles University in Prague

Faculty of Medicine in Hradec Králové

Department of Dentistry

Effect of Antimicrobial Agents on Oral Microorganisms

Dissertation

Kudiyirickal Marina George

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Antibiotics **abbreviations:**

AMI -Amikacin	ERY- Erythromycin
AMOK- Amoxicillin/	FUR -Furantoin
Clavulanic acid	GEN -Gentamicin
AMP- Ampicillin	IMP -Imipenem
AMPI- Ampicillin/ inhibitor	KYS -Oxolinic acid
AMPS- Ampicillin/ Sulbactam	LIN -Lincomycin
API- Aminopen/ inhibitor	LVF -Levofloxacin
AZL- Azlocillin	MEP -Meropenem
AZR -Aztreonam	MTZ -Metronidazole
AZT- Azithromycin	MUP -Mupirocin
CEF1-Cephalothin	NET -Netilmicin
CETX -Cefotaxime	NOR -Norfloxacin
CFA -Ceftazidime	OFL -Ofloxacin
CFI- Cefpirome	OXA -Oxacillin
CFM- Cefepime	PEN -Penicillin
CFN -Cefazolin	PIP -Piperacillin
CFP -Cefoperazone	PIPT -Piperacillin/ tazobactam
CFPS -Cefoperazone/ sulbactam	ROX- Roxithromycin
CFR -Ceftriaxone	SPI -Spiramycin
CFT- Cefoxitin	TEI -Teicoplanin
CFTX -Ceftizoxime	TET -Tetracycline
CFX -Cefuroxime	TIC -Ticarcillin
CIP -Ciprofloxacin	TICI -Ticarcillin/ inhibitor
CLI -Clindamycin	TMP -Trimethoprim
CMP- Chloramphenicol	TOB -Tobramycin
COL -Colistin	VAN -Vancomycin
COT -Cotrimoxazole	

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1. INTRODUCTION

Microorganisms in the oral cavity have been implicated as the causative agents in caries, pulpitis, abscess, periodontal disease and halitosis, bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birthweight, coronary heart disease and cerebral infarction (91). Orofacial infections may be odontogenic or non-odontogenic in nature. Diseases of the pulp and periodontium such as dental caries, endodontic infections, dental abscesses, periodontitis and pericoronitis, which constitute the vast proportion of odontogenic infections, are mainly caused by the endogenous bacterial microbiota in the oral cavity, (36) while non-odontogenic infections vary depending on the nature and site of infection (164). Unlike odontogenic infections, the non - odontogenic infections do not affect the teeth. Mucosal infections of viral and fungal origin account for the majority of the oral non-odontogenic infections, but bacteria also play an important role (33).

There are more than 500 distinct bacterial species which have been known to constitute the normal oral microbiota (60). The major etiologic factor for odontogenic infections is the normal bacterial flora in the plaque. Under favourable circumstances i.e. when there is abundance of microbes, or at an unusual site these microbiota have the potential to cause infection and lead to progression of the disease. Gram-positive facultative acid-forming bacteriae have been implicated in dental caries while diseases of the periodontium are mainly due to anaerobic proteolytic Gram-negative bacteria (37).

The specificity of non-odontogenic infections can be observed in several strains. *Staphylococcus aureus* is ubiquitous in nature and causes skin infections, abscesses, oral lesions like angular stomatitis and cheilitis. However they are rarely isolated from patients with upper respiratory infections (tonsillitis and otitis media). *Streptococcus pyogenes* is known to cause skin infections and tonsillitis and is hardly ever known to cause orofacial or other respiratory tract infections.

Streptococcus pneumoniae and *Haemophilus influenzae* are major aetiologic agents in respiratory diseases and are not usually seen in oral infections. *Helicobacter pylori* is implicated in diseases of stomach and may occasionally be found in oral cavity but they do not cause respiratory infections. Enterococci and aerobic Gram-negative bacilli like *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are opportunistic pathogens that cause infections in respiratory tract especially in patients who are immunocompromised and are rarely found on skin. *Legionella pneumophila* is known to cause only pneumonia (37).

With the advent of sophisticated culture techniques, isolation and identification, it is now known that anaerobic infections are more common. In dentistry the most common approach to the treatment of such infections is drainage and use of systemic antibiotics where indicated (20). Among the anaerobes the most predominant species include *Bacteroides*, fusobacteria, peptococci, peptostreptococci, and viridans streptococci (61). The lack of specificity of pathogenic microbiota and polymicrobial nature of odontogenic infections in contrast to the greater specificity of pathogenic microbiota in non-odontogenic infections (37) highlights the importance of adequate culture and identification of potential pathogens in the prompt and appropriate treatment of these infections.

The first line of treatment for majority of orofacial infections is debridement and/ or incision and drainage and in some cases extraction of the offending tooth. Odontogenic infections like periodontal abscess, pericoronitis, acute periapical abscess and deep fascial space infections require antimicrobial therapy. Such therapy becomes more effective when it is commenced immediately after diagnosis and prior to surgery as this is known to reduce the duration of infection and risks associated with such dental pathology (164). Most of the microorganisms causing orofacial infections are treated using drugs belonging to the β-lactam antibiotics, cephalosporins, fluoroquinolones, aminoglycosides, macrolides and other broad spectrum antibiotics.

The role of antimicrobial agents for treatment of oral infections is in preventing the spread of infection and in reducing extent of damage (98). Culture and antimicrobial susceptibility testing are pivotal to the effective treatment of bacterial infections with the appropriate antibiotics. As

there is an inevitable delay of 3 to 5 days in obtaining these culture results from clinical diagnostic microbiology laboratories, often the physician has to start on an empirical therapy in case of life threatening infections. Among the wide range of therapeutic agents available to the dentist, the drug of choice in most instances belongs to the penicillin group and in particular amoxicillin. The wide antimicrobial spectrum, less side effects and low cost make this the preferred group of drugs (73). In cases where patient is allergic to a particular antibiotic, suitable alternatives have to be used. The same holds true for antibiotic resistant cases. There is a vast array of antimicrobial agents for the treatment of orofacial and odontogenic infections but growing prevalence of antimicrobial resistance is an area of great concern.

Interestingly, antibiotic resistance was first brought to the notice of the scientific community by Sir Alexander Fleming himself, whose discovery of penicillin has been the cornerstone in antimicrobial research (52). In an extensive study by Hughes and Datta (77) on the duration of existence of antibiotic resistance among *E. coli,* it was found from the *E. coli* specimens obtained in the early 20th century (nearly 50 years before antibiotics were introduced) revealed plasmids that did not show antibiotic resistance. They may have become resistant following largescale use of antimicrobials. Various reports suggest that excessive and inappropriate use of antibiotics for human diseases and in agriculture have resulted in development of bacterial resistance to antimicrobials (77,109).

The rational use of antibiotics is important to prevent development of resistant strains and unwanted side effects of drugs. The choice of antibiotic is case-specific and it is important to take into consideration the age and health of the patient, history of allergy, drug absorption and distribution, plasma concentration and laboratory data (61,164). In addition, the type and site of infection, antibiotic usage prior to an infection, cost effectiveness of the drug, drug metabolism and penetration (61,164) along with the recent domestic antimicrobial susceptibility patterns are also factors which determine the drug of choice and finally outcome of infection (94).

2. LITERATURE REVIEW

2.1 ORAL MICROBIOTA AND NOMENCLATURE

The literature on the role of microorganisms in dental infections has an interesting historical background in view of the pathbreaking molecular techniques which have shed a new light on our present knowledge in oral microbiology. The Chinese believed that a white worm with a black head lived in the tooth and it caused abscesses (116). The worm theory which was followed until the middle of 18th century and thereafter the hollow tube theory (142) prevented the pursuit for a bacterial cause for pulpal disease. Van Leeuwenhoek in the 17th century described the presence of microorganisms which he termed “animalcules”. He was the first to make such an observation using the material scraped from his own teeth and in his words “a little white matter, which is as thick as if ’twere batter... I then most always saw… that in the said matter there were many very little living animalcules.” He also wrote about a sample which he had obtained from an old man who had not cleaned his teeth “an unbelievably great company of living animalcules, a swimming more nimbly than any I had ever seen up to this time. Moreover the other animalcules were in such enormous numbers, that all the water… seemed to be alive.” It has been estimated that nearly a million microorganisms are present per milliliter of saliva. Bacteria and a small proportion of fungi constitute the organisms found in the saliva and they are shed from the hard and soft tissues of the oral cavity and nasopharynx into the saliva. These then multiply in the retained pools of saliva. The normal commensals which comprise the oral microbiota play a pivotal role in maintaining oral health and a disturbance in this dynamic equilibrium leads to the oral disease (21). The recent methods of microbial identification like PCR for sequencing have opened the floodgates to reveal novel taxa and difficult to culture species, which means that the oral microbiota is constantly being added upon leading to a better understanding of their role in health and disease process.

The oral cavity has a wide range of microbial species (111,186,199) and they constitute ~300–500 species of bacteria, fungi and protozoa. Interestingly, only ~10% have been isolated by conventional culture methods. Previous investigation has revealed that nearly 40% of isolates were novel bacterial phenotypes determined using 16S rRNA amplification methods and among these many could not be isolated conventionally (142). In the oral cavity α-haemolytic streptococci are the most commonly isolated microorganism followed by oral commensals like coagulase-negative staphylococci, Gram-negative cocci belonging to the families Neisseriaceae and Veillonellaceae, lactobacilli, spirochaetes, corynebacteria and mycoplasmas. Certain other microorganisms are also found which can cause orofacial infection includes *Staph. aureus, Enterococcus faecalis, Str. pneumoniae, Str. pyogenes, Neisseria meningitidis,* members of the family Enterobacteriaceae, *H. influenzae* and actinomycetes. The oral cavity is similar to a dynamic ecosystem where any disturbance in this equilibrium results in disease by the endogenous microbiota. Microorganisms which are isolated from orofacial infections include Gram-positive aerobic cocci, α-haemolytic streptococci, peptostreptococci and Gram-negative anaerobes (50). The ability of a microorganism to colonize and cause disease depends on their cell surface components which helps them in attaching to the tissue surface, further on metabolic activity and utilization of nutrients (181).

Classification for Bacteria

The most widely used system for classification of all forms of life on Earth has been that developed by Carl Linnaeus in the 18th century originally for classification of plant and animals. In general all forms of life can be grouped under three domains:

i) Eukarya- eukaryotic organisms.
ii) Bacteria- prokaryotic organisms.
iii) Archaea- these are also prokaryotes but are genetically and metabolically different from true bacteria and are thought to be the evolutionary link between prokaryotes and eukaryotes (Table 1)

The oral cavity has been known to have microorganisms belonging to these three domains. However, a large proportion of the microbiota is constituted by bacteria and only a small proportion by archaea and fungi (21).

Table 1. Characteristics of Eukarya, Bacteria and Archaea

	Domain		
Property	**Eukarya**	**Bacteria**	**Archaea**
Nuclear membrane	+	-	-
Chromosomes	>1	1	1
Chromosome organization	Linear	Circular	Circular
Murein in cell wall	-	+	-
Cell membrane lipids	Ester-linked glycerides; unbranched, polyunsaturated	Ester-linked glycerides; unbranched; saturated or monounsaturated	Ether-linked; branched; saturated
Cell membrane sterols	Present	Absent*	Absent
Organelles**	Present	Absent	Absent
Ribosome size	80S	70S	70S
Transcription/translation coupling	No	Yes	Yes

* except mycoplasmas
** except ribosomes

Classification of Bacteria

Bacteria can be classified on the basis of their cell shape, characteristics, genus and family (76).

(i) Gram-negative bacteria

Cell Shape	**Characteristics**	**Genus**	**Family**
Cocci	Aerobic	*Neisseria*	Neisseriaceae
		Veillonella	
Coccobacilli		*Brucella, Bordetella Pasteurella, Haemophilus*	Brucellaceae
Bacilli	Facultative anaerobic, motile with peritrichous flagella or immotile	*Escherichia, Shigella, Salmonella, Proteus, Erwinia, Yersinia, Enterobacter, Serratia*	Enterobacteriaceae
	Aerobic, motile with peritrichous flagella or immobile	*Azotobacter Rhizobium*	Azotobacteraceae Rhizobiaceae
	Aerobic, motile with polar flagella	*Nitrosomonas, Nitrobacter, Thiobacillus*	Nitrobacteraceae
		Pseudomonas, Acetobacter, Legionella	Pseudomona-daceae
	Facultative anaerobic with polar flagella	*Campylobacter, Zymomonas, Aeromonas*	
	Curved rods with polar flagella	*Vibrio, Spirillum, Desulfovibrio*	Spirillaceae

(ii) Gram-Positive Bacteria

Cell Shape	**Characteristics**	**Genus**	**Family**
Cocci	Cells in irregular clusters	*Staphylococcus Micrococcus, Sarcina*	Micrococcaceae
	Cells in chains	*Streptococcus Leuconostoc*	Streptococcaceae
Bacilli	Aerobic sporing	*Bacillus*	Bacillaceae
	Anaerobic sporing	*Clostridium*	
	Lactic fermentation	*Lactobacillus*	Lactobacillaceae
	Propionic fermentation	*Propionibacterium*	Propioni-bacteriaceae
	Oxidative, weakly fermentative	*Corynebacterium Listeria, Erysipelothrix*	

(iii) Other Major Groups

Cell Shape	**Characteristics**	**Genus**	**Family**
Acid-fast rods Ray-forming rods		*Mycobacterium Actinomyces, Nocardia Streptomyces*	Actinomycetales
Spiral organisms	Motile	*Treponema, Borrelia, Leptospira, Spirocheta*	Spirochetales
Small pleomorphic	Lack rigid wall	*Mycoplasma*	Mollicutes
Small intracellular parasites		*Rickettsia, Coxiella, Chlamydia*	Rickettsiaceae Chlamydiaceae
Intracellular parasites	Bordeline with protozoa	*Bartonella*	Bartonellae

2.2 CULTURE AND IDENTIFICATION OF MICROBIOTA

2.2.1 Culture of Microbiota

In the 18th century Spallanzani developed the first culture media for bacterial growth which was later modified in the 19th century by Pasteur. The originally developed culture media consisted of broth obtained from infusion or by enzymatic digestion of meat (55, 31). It was Robert Koch who saw the need to have solid culture media for physical separation of bacterial colonies as a broth medium would have a mixture of microbes. He used specimen from the infected lesions and placed this on aseptically divided potatoes which was thereafter incubated at body temperature to obtain bacterial colonies. Pure cultures were subsequently obtained by subculture on potatoes. This was the forerunner to the development of solid media from broths by the addition of solidifying agents such as gelatin and agar (198).

The most commonly used culture medium for the isolation of bacteria which are capable of growing aerobically is the Mueller-Hinton medium sheep blood agar, liver broth, Brain-heart infusion (BHI) agar and BHI broth to detect anaerobic bacteria. However, growth of certain microorganisms require specialized media like *Haemophilus* testing media, chocolate agar for *Haemophilus* species, cation-adjusted Mueller-Hinton medium with lysed sheep blood for *Str. pneumoniae*, GC agar base defined growth supplement for *N. gonorrhoeae*, Middlebrook 7H10 medium for mycobacteria and RPMI medium for yeasts (29).

After 18-24 hours of incubation at body temperature 36±1°C of inoculated agars, most bacteria grows in small colonies with characteristic morphology. In case of slow bacterial growth incubation time is prolonged to four days to rule out false-negative results. If *Actinomyces* etiology is suspected, anaerobic culture is prolonged to 10 days. Culture and following identification of bacteria (with susceptibility testing) is relatively time-consuming.

2.2.2 Identification of grown bacteria

Microscopy

Microscopy may divide bacteria in several groups in according with characteristic morphologic picture in optical microscope. Suspension of growing bacteria is put onto the microscopic glass

and then it is coloured by Gram stain. In optical microscope with immerse objective it may be distinguished as Gram-positive (blue) cocci or rods and Gram-negative (pink) cocci or rods.

Biochemical identification

This identification is based on characteristic patterns of biochemical activity of bacteria. Grown colonies are inoculated into the test tube with specifical biochemical tests (i.e. coagulase or catalase production, utility of sugar etc.). There exists a lot of commercial identification set based on biochemical micromethod (i.e. VITEK – Bio Mérieux, API system, BBL Crystal etc.). These methods can determine species and subspecies of the majority of bacteria.

2.2.3 Molecular identification of bacteria

Molecular techniques for identification of bacteria have resulted in greater ease in the study of mixed bacterial communities without the need to use cumbersome conventional culture techniques directly from the patient's sample (198). Zuckerandl and Pauling were the first to propose the use of biological macromolecules to determine the evolution of organisms (203). This analyses the DNA sequences of genes of common ancestry, or proteins in a large number of microbes and the similarity of these sequences are determined using mathematical techniques to develop phylogenetic trees showing the evolution of the organisms. The subunit (16S) ribosomal RNA gene is widely used as these are commonly found in most microorganisms and allows greater ease in the alignment of the sequences, as their important function has conserved them during evolution (201). For this method the sequences of the same gene from different organisms are aligned and matrix of similarities is elucidated from the genetic distance between pairs of organisms in the dataset and again analysed to finally construct the phylogenetic tree or dendrogram (137). The identity of the microbe can then be found either by adding the sequence of its gene to the tree or by performing a similarity search in the database against other sequences of the same gene (131,115). Using polymerase chain reaction (PCR) and cloning steps to this technique can help in the detection of microorganisms in mixed bacterial communities (198).

2.3 OROFACIAL INFECTIONS

Orofacial infections may be caused by bacteria, fungi, viruses and parasites. Clinical diagnosis is aided by microbiological sampling and analysis of the causative microorganism. Bacterial infections of oral mucosa constitute especially non-odontogenic infections (33). Yeasts are implicated in the aetiology of white mucosal lesions while viruses are known to cause majority of the oral ulcers. On the contrary, knowledge on the etiology of majority of the oral infections is by far lacking, and coupled with this, is the underutilization of microbiological sampling for oral diagnosis by the majority of dental practitioners (35).

In a paper by Dahlén, the significance of microorganisms in oral mucosal specimen as an etiologic agent or carrier with no role in the pathogenesis has been explained (33). Orofacial infections are as a result of an imbalance in the microbial homeostasis leading to proliferation of opportunistic microorganisms like *S. aureus*, enterococci, beta-hemolytic streptococci and aerobic gram-negative bacilli (*E. coli, Enterobacter* spp., *Klebsiella* spp., *Pseudomonas* spp.). Some of these species can be resistant to antibiotics and pose difficulty in adequate antimicrobial treatment (37).

Oral mycoses are frequently encountered among individuals who are on broad spectrum antibiotic therapy, immunosuppressants, use of steroids in inhalers, diseases like AIDS, cancer, denture wearers with poor oral hygiene, xerostomia, and smoking. Extensive studies by Samaranayake et al. (161) have shown that mycotic infections are an increasing source of concern in individuals who are human immunodeficiency virus (HIV)-positive. Oral candidiasis is the most common fungal infection seen in the oral cavity and *C. albicans* is the major causative agent. Other *Candida* spp., like *C. tropicalis, C. krusei* and *C. dublinensis* have also been isolated from oral candidiasis. The increasing number of *Candida* spp. isolated from oral mycotic infections may be attributed to the rise in antibiotic resistance coupled with the overuse and or misuse of systemic antifungal agents, especially ketoconazole and fluconazole (37). Patients with diseases like AIDS, cancer and transplant cases are increasingly being treated with immunosuppressants which increase the risk of oral candidal infection.

Viral infections like HIV and hepatitis B and C has received a lot of attention in the recent years due to the risk of disease transmission. Nevertheless, the importance of viruses in the

pathogenesis of periodontitis (170) has also received great interest. Research by Slots (170) showed that there was greater tissue destruction in periodontal sites with Epstein–Barr virus or cytomegalovirus than in areas where they were absent. This type of herpes virus-related periodontitis may also be partly explained by the synergism between viruses and bacteria wherein herpetic infection leads to localised immunosupression and resultant proliferation of bacteria (37).

Parasitic infections are less commonly encountered in the oral cavity. However Bergquist (13) states that these are more prevalent and parasites like *Trichomonas tenax* have been implicated in periodontal disease. Other parasitic infections like leishmaniasis are also being observed more frequently than before (37).

Table 2. Major infectious agents of non-odontogenic infections in dentistry*

Infectious agent	**Etiology**	**Disease** (reference)
BACTERIA	*Staphylococcus aureus*	Oral mucosal infections (33), abscesses (18), skin infections (49)
	Streptococcus pyogenes (beta-hemolytic group A streptococci)	Pharyngotonsillitis (177), skin infections (49)
	Streptococcus pneumoniae (pneumococci)	Middle ear infections (63), sinusitis (19)
	Enterococcus faecalis (enterococci)	Oral mucosal infections (33)
	Propionibacterium acnes	Skin infections (49)
	Moraxella catarrhalis	Middle ear infections (63)
	Haemophilus influenzae	Middle ear infections (63), sinusitis (19)
	Legionella pneumophilia	Respiratory infections (118)
	Helicobacter pylori	Stomach and esophagus infections (148)
	Mycobacterium tuberculosis	Tuberculosis, respiratory infections (118)
	Esherichia coli, Klebsiella pneumoniae, Enterobacter spp., *Pseudomonas* spp. (aerobic gram-negative bacilli)	Oral mucosal infections (33) Respiratory infections (118)

	Anaerobic species	Abscesses (18), sinusitis (19)
FUNGI	*Candida* spp.	Oral infections (161)
	Aspergillus spp.	Respiratory infections (118)
	Trichophyton spp., *Malassezia* spp.	Skin infections (49)
VIRUSES	Herpes simplex virus-1, mumps virus, varicella–zoster virus, morbilli virus (measles), influenza virus, Coxackievirus A and B, respiratory syncytical viruses, human cytomegalovirus, Epstein–Barr virus, human papillomaviruses, human immunodeficiency virus (HIV)	Oral viral infections in children (160) Oral viral infections in adults (171) Respiratory infections (118)
PROTOZOANS	*Leishmania* spp.	Leishmaniasis (13)

* adapted from Gunnar Dahlén (37)

Pulpal infection

Pulpal necrosis and alveolar abscess are primarily due to entry of bacteria either from carious lesion or trauma. These diseases are endogenous in nature resulting in a parasitic symbiosis and can potentially be harmful to the host. The etiologic agents are mainly obligate anaerobes. The low redox potential (Eh) during endodontic infection results in the selection of anaerobes like *Bacteroides* species, *Porphyromonas endodontalis, Eubacterium* species, *Fusobacterium nucleatum* and *Peptostreptococcus micros* (9, 179).

Diseases of the Periodontium

The etiology of periodontal diseases is also attributed to the endogenous microflora found in the gingival sulcus or the periodontal pocket with greater than 500 species when the disease is in the active phase. Gnotobiotic animals do not develop periodontal infections and the efficacy of antibiotics in the treatment of periodontal diseases implicate bacteria as the causative organism (175). Majority of the Gram-positive, facultative anaerobes like *Streptococcus anginosus* and *Actinomyces naeslundii* (117) constitute the microorganisms commonly isolated from the healthy gingival sulcus. However if oral hygiene is absent, then the dental plaque that forms has a large

number of Gram-negative species and motile forms which is proportional to the degree of gingivitis. (112).

In the early stage of gingival inflammation a large no. of species can be detected and these include obligate anaerobes like spirochetes, *Fusobacterium nucleatum* and *Bacteroides* species (121, 122). In advanced periodontitis, microorganisms like *Porphyphomonas gingivalis, Bacteroides forsythus* and *Treponema denticola* (the so-called 'red complex') (174) are implicated. Thus, the causative factor of periodontitis is polymicrobial (39) and it is an endogenous infection with host response also playing a vital role in the disease process.

Oral tissues and prostheses - Candidiasis

Epithelial cells, inert polymers of dentures, orthodontic appliances, teeth and oral bacteria can provide surface for the adherence of Candida which are normal commensals in the oral cavity. *C. albicans* can be found in 20% of the healthy persons and in 40% of those who are hospitalized. Thus it can also be a nosocomial exogenous infection. Candida species can be detected in large numbers in patients undergoing broad spectrum antibiotic therapy, xerostomia, immunosuppressive therapy, use of steroids in inhalers, diseases like AIDS and in patients wearing dentures or certain orthodontic appliances.

Denture stomatitis usually occurs in the maxilla among 30% to 75% denture wearers and in individuals with palatal expansion appliances, orthodontic appliances, and partial or complete dentures. Based on the severity they are classified as Newton Type I- minor inflammation, Newton Type II – severe , and Newton Type III - irreversible hyperplasia. Selective pressure of dentures in mouth is responsible for infection by *C. albicans* in 85% of dentures wearers, and only in 20% of individuals not wearing dentures (23). There is a predominance in the colonization of *C. albicans* to dentures in comparison to the mucosa underlying the denture.

2.4 ANTIBACTERIAL AGENTS

The indication for use of antibiotics in dentistry is for treatment of systemic effects of orofacial infection and prophylaxis. Their routine use for treatment of infection is not warranted as simple measures including operative measures such as providing drainage, root canal treatment or

extraction of the offending tooth and oral hygiene can resolve most infections. Antibiotic usage for the treatment of orofacial infections should adhere to current clinical and best practice guidelines.

7% – 11% of most commonly used antibiotics by dentists include betalactams, macrolides, tetracyclines, clindamycin, and metronidazole (27). The various side–effects of antibiotics include GI upset, anaphylaxis and antibiotic resistance which is mainly due to inappropriate use of broad spectrum antimicrobials like cephalosporins and fluoro-quinolones (200). Methicillin-resistant *Staphylococcus aureus* is known to have resistance to most antibiotics (106). Widespread emergence of antibiotic resistance has been a great concern in recent years and strict adherence to antibiotic prescribing protocols within the primary dental care has great importance in curtailing this problem. The most prevalent orofacial infections include pulpitis and periapical periodontitis. These require simple intervention like restoration, endodontic treatment or extraction. However several studies reported that antibiotics are used as first line of treatment (140, 41,159).

There are only limited clinical situations in dental practice such as infections with rise in body temperature and systemic effects like lymphadenopathy, trismus, and facial cellulitis, wherein antibiotic therapy on empirical basis is justified. Pericoronitis and periodontal conditions like periodontal abscess, and acute necrotizing ulcerative gingivitis are few other indications for antibiotic usage in dentistry (159).

Penicillins

Penicillins which belong to the β-lactam antibiotics are the most widely used antibiotic by dentists (2). Antimicrobials like amoxicillin (2), penicillin V (41) , metronidazole (140) , followed by amoxicillin and clavulanate (147) were found to be used in the order of decreasing frequency. In a study published by Kuriyama et al., the clinical efficacy of penicillin V, amoxicillin, or amoxicillin and clavulanate was found to be similar (92).

In a research paper by Lewis et al. it was reported that resistance to amoxicillin/clavulanic acid was detected in 5% of the main isolates from dental abscesses (108). However, another study showed total susceptibility to amoxicillin/ clavulanic acid in endodontic infections (12).

Cephalosporins

Cephalosporins are bactericidal drugs. First generation of cephalosporins inhibit mainly Gram-positive bacteria, second and third generations have greater bactericidal activity against Gram-negative bacteria while fourth generation cephalosporins are broad spectrum antibiotics with bactericidal activity against both Gram-negative and Gram-positive bacteria (188).

Carbapenems

Active against both Gram-positive and Gram-negative bacteria, but not to intracellular bacteria. In a study to evaluate the susceptibility of microorganisms isolated from endodontic infections to β-lactams, a total susceptibilty to imipenem and 99.3% to amoxicillin/clavulanate was observed, while 16.1% showed resistance to amoxicillin and penicillin G, and 4.89% to cefoxitin. (57)

Fluoroquinolones and Clindamycin

Non-odontogenic bone infections affecting orofacial structures are commonly treated with clindamycin, as it reaches high concentrations in the bone (22), and fluorquinolones (ciprofloxacin, norfloxacin, moxifloxacin) which are highly effective against Gram-negative bacilli, Gram-positive aerobic cocci and, third generation fluorquinolones (moxifloxacin), which are active against anaerobes (141).

Tetracyclines

Tetracyclines are active against a wide range of Gram-positive and Gram-negative organisms and are useful in the treatment of periodontal diseases and also in local delivery devices (196). Though Walker reported high prevalence of antibiotic resistance to tetracyclines in the periodontal flora, a recent study by Brescó-Salinas M et al. showed better susceptibility in odontogenic infections. (197,15).

Macrolides

Commonly used macrolide antibiotics include erythromycin, clarithromycin, roxithromycin and azithromycin. They are mainly bacteriostatic but in high concentrations they can also be bactericidal. Macrolides are active against many Gram-positive bacteria but not *Enterococcus* spp. However, resistance to erythromycin and azithromycin has been reported to be high in odontogenic infections (Brescó-Salinas M, et al). Routine use of these agents for common infections may explain the high rates of resistance. (11, 189, 14)

Frequency and duration of antimicrobial use can be found in various resources (16) and therapeutic guidelines, which are mainly based on advice by experts (156). Average duration of antibiotic use for dental infection was found to be 6.92 days in a Canadian study whereas a US study published on antimicrobial prescribing practice among endodontist showed that an average of 7.58 days (202). Few studies found that Eastern Mediterranean dentists prescribed smaller doses for longer duration (159,38). Effectiveness of a two dose, 3 gm amoxicillin has been reported in some clinical cases (182). However, 2 or 3 days of antibiotic use in acute dentoalveolar infections have been recommended in appropriate dosage by the British National Formulary (113). Co-amoxiclav is usually prescribed in doses ranging from 375 mg to 625 mg every 8 hours (17). In patients allergic to penicillin, clindamycin in doses ranging from 50 mg to 450 mg every 6 hours or metronidazole in a dose of 200 mg every 8 hours for 3–7 days can be used as effective alternative (17).

2.5 ANTIMICROBIAL SUSCEPTIBILITY TESTS

Antibiotic susceptibility tests are used to determine the inhibitory effect of an antibacterial agent against microbes, and they help in selecting appropriate therapy and determining the sensitivity pattern of an organism for epidemiological reasons. The guidelines for culture medium preparation, incubation criteria, and interpretation of the test results for antimicrobial susceptibility testing methods like disk diffusion, broth dilution, and agar dilution (126-130) are laid down by the CSLI (formerly NCCLS) for selected aerobic and anaerobic bacteria, mycobacteria (125) and fungi. These guidelines are constantly updated. The two principal types of susceptibility testing methods include the conventional (phenotypic) culture-based and the genetic susceptibility testing methods.

2.5.1 Conventional culture-based antimicrobial susceptibility testing

Conventional culture-based antimicrobial susceptibility testing methods evaluate the *in vitro* effects of the antimicrobial agent on the growth of the test organism or assess directly the antimicrobial modifying enzymes. These tests are used to determine the antimicrobial resistance phenotypes, which may be intrinsic or acquired (29). They are divided into two types depending on the principle used. They include (101):

Diffusion method	Dilution method
Stokes method	Minimum Inhibitory Concentration
Kirby-Bauer method	i) Broth dilution
E-test method	ii)Agar Dilution

(i) Diffusion tests are more commonly used as they are simple, flexible and cost effective. In this method, the antimicrobial agent diffuses from a cellulose filter paper disk into the solid medium inoculated with a test strain. Following 18 to 24 hours of incubation, “zones of inhibition” of bacterial growth may be present around the antibiotic disk. This is a qualitative test as the results based on the “zones of inhibition” of bacterial growth are denoted as resistant, susceptible, or intermediate susceptible to the antimicrobial agent (29).

(ii) Dilution tests are quantitative assays and are used to estimate the minimal concentration (mg/l) of the tested antibiotic that inhibits the growth of the microorganism. This is known as the minimum inhibitory concentration (MIC). Subculture of the dilutions with no visible growth onto antibiotic-free agar media gives the value of the concentration of antibiotic that kills rather than inhibits the microorganism and this is known as the minimum bactericidal concentration (MBC) (29). Dilution tests can be grouped into three types namely, macrobroth, microbroth, and agar dilution method.

(a) **Macrobroth Dilution.** It is done manually using standard-size test tubes containing liquid medium with a standard inoculum of the test organism and different concentrations of antimicrobial agents are added. Incubation is done for 18 to 24 hours for aerobic bacteria

whereas for anaerobic bacteria, mycobacteria, and yeasts the incubation can be done for a much longer duration (29).

(b) **Microbroth Dilution**. This may be an automated technique or done manually using microtiter plates and is similar to that for macrobroth dilution except for the detection of growth which is done by optical density analyses and direct visualization of microtiter wells.

(c) **Agar Dilution.** In this technique instead of a liquid medium the antibiotic is present in a solid medium containing agar in Petri dishes. "Spot" inoculation of a standard concentration of microorganism on this solid medium is done and thereafter incubated upto 18 to 24 hours in case of aerobic bacteria or longer in case of anaerobic bacteria and mycobacteria. Absence of growth of microorganism denotes that it is susceptible to the given concentration of the antibiotic in the medium. This method provides very specific MIC and also allows many different isolates to be tested at the same time by spot inoculation of the same plate (29).

(iii) Gradient Diffusion (Etest, Epsilometer testing)

Gradient diffusion test involves the use of a single plastic-coated strip in which an increasing concentration gradient of the test-antibiotic is present. This strip is placed on solid agar containing the streaked microorganism and the results are read after 18 to 24 hours. MIC is determined by the intersection of the lowest point of the elliptical zone of growth inhibition and the gradient plastic strip. The advantage of this method is the ability to test a wider range of antibiotic concentration in comparison to the other methods. The commercial tests include Etest (AB Biodisk NA, Piscataway, New Jersey) (29).

2.5.2 Genetic Susceptibility Testing Methods

The genetic susceptibility testing method is done more quickly and has greater reliability than the phenotypic methods as they are done using the clinical sample thereby eliminating the need to do culture and also the "genotype" of the organism is evaluated. With this method there is a low risk for the patient and hence useful in the case of life-threatening diseases like meningitis, endocarditis, or osteomyelitis wherein longer duration of antibiotic therapy is essential. Other benefits of this technique are that the genotype may be known much rapidly in case of slow growing microbes or where the microorganism is difficult or impossible to culture. Also, the biohazard associated with genotypic testing method is far less than the conventional phenotypic

testing methods. For example, these methods are found to provide better results than the phenotypic testing methods used for detecting methicillin-resistance in coagulase-negative *Staphylococcus* species (88), and *Enterococcus* species with low-level vancomycin-resistance (87).

The disadvantages (29) of this method are as follows:

1. The most relevant objection to using genetic method is the problem of expression of a given gene i.e. the presence of a resistance gene does not necessarily mean the phenotypic expression of such gene. In addition, resistance can arise from different mechanisms, which does not have to be covered by a given genetic test.
2. Poor sensitivity when the test specimen has small number of microorganisms
3. Individual antimicrobial agents require varying types of assays
4. Certain antimicrobial agents may have yet-unknown genetic method of resistance.
5. Specimens contaminated with extraneous nucleic acid may give false-positive results especially in tests using polymerase chain reaction (PCR).

In this technique the PCR is used to amplify the "target" nucleic acid and the resultant product known as amplicon is then assessed as to whether it is the desired target DNA containing part or the entire resistance-associated gene. Following the detection of antimicrobial-resistance genes by PCR amplification of the target DNA, the amplicon confirmation is performed by electrophoretic mobility (Gel electrophoresis), probe hybridization assays (Southern blotting, slot, dot-blot, enzyme-linked immunosorbent assay, or liquid hybridization formats), restriction fragment length polymorphism (RLFP) analysis, or DNA sequencing formats (29).

2.5.1 Principle of Antimicrobial Susceptibility Testing

The antibiotic susceptibility test (AST) is a valuable tool for the clinician in choosing an initial empiric regimen and, drugs on a case-specific basis. On the basis of the most prevalent susceptibility profile, an antibiotic panel is chosen for test and is regularly reviewed and changes made when deemed necessary (101).

The early works by Rideal,Walker and other investigators paved the way for evaluating the effectivity of noxious agents to bacteria. However, these tests and their subsequent modifications

became very tedious following the introduction of antibiotics and the need for numerous routine tests for analyzing their effectiveness. Alexander Fleming was the first to introduce the ditch plate method of agar diffusion. This was followed by numerous other agar diffusion methods which were put to use by the Oxford Group to assay the blood antibiotic level. This was done by the placing reservoirs in containers on the surface of the medium thereby facilitating the diffusion of the antibiotic into the surrounding medium. This method is still in use although it is now more common for most laboratories to follow the disc diffusion method of AST which uses antimicrobial impregnated absorbent paper disc. The basic principle of newer methods comprise of diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth (101).

2.5.2 Factors Influencing Antimicrobial Susceptibility Testing

There are several factors which affect the AST. The size of the zone of inhibition is dependant on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism, the inoculum size, and the growth rate of the bacterium (29). Other factors include (101):

1. pH of the agar medium: Ideally a pH between 7.2 and 7.4 at room temperature after gelling is appropriate. Drugs like aminoglycosides, quinolones, and macrolides become inactive when the pH is low, while others like tetracycline have greater effect. These effects are reversed when the pH is very high.
2. Moisture: The presence of excess moisture on the agar plate also affects the antimicrobial susceptibility test results.
3. Thymidine or thymine: When the agar medium contains excessive thymidine or thymine they can cause false–resistance values. In case of sulfonamides and trimethoprim, due to reversal of the inhibitory effect of these drugs by excessive thymidine or thymine, the zone of inhibition is small, less distinct or absent thus leading to false results.
4. Divalent cations: Divalent cations like magnesium and calcium in excess decrease the zone diameter and vice versa in cases of drugs like aminoglycosides and tetracyclines. Zinc ions in excess can also result in smaller zone of inhibition for carbapenems.
5. Type of agar medium: Aerobic or facultative bacteria grow well on unsupplemented Müeller-Hinton agar. Fastidious bacteria such as *Haemophilus* spp., *N. gonorrhoeae*, *S. pneumoniae*, and viridans and ß-haemolytic streptococci grow only on supplemented

Müeller-Hinton agar or other media. Thus appropriate culture medium should be used to obtain correct results.

2.6 Resistance to antimicrobial agents

Antimicrobial drug resistance is mediated by various mechanisms including mutation and uptake of genes by vertical or horizontal transmission (101). There are two types of resistance to antimicrobial drugs i.e. acquired and intrinsic. Acquired resistance develops due to recombination and mutation in the genes of the microorganism. This in turn is due to various complex mechanisms. These mainly include:

(i) Inactivation of the antimicrobial agent e.g. by β-lactamases.

(ii) Accessibility of the antibiotic into the microorganism may be impeded especially during downregulation of porins

(iii) Excretion of the antibiotic which may occur when there is upregulation of the efflux pumps

(iv) Mutation of the target site on the microorganism on which the antibiotic exerts its effect can result in antibiotic failure as the site of action is no longer present. The microbe can also produce alternative target sites and this confers a protective action against the antibiotic. There may be other modes of protection of the target which also helps resist the antimicrobial agents (72).

2.6.1 Studies on antimicrobial drug-resistance in the oral microbiota

The emergence of antimicrobial drug resistance is of growing global concern due to the increased morbidity and mortality from failure of treatment and the associated increase in cost of management of diseases and health care. The inappropriate prescription of antimicrobials by physicians and dentists, lack of adherence to established protocols for management of infections by chemoprophylaxis and in some countries the availability of over-the-counter antibiotics have led to increasing incidence of antibiotic resistance (101).

The transfer of resistance factors especially those on mobile elements can lead to development of resistance in human and animals at a greater pace in susceptible hosts. The prevalence and variation in the antibiotic susceptibility profiles of multidrug-resistant strains locally and globally have given

rise to the need to have sentinel sources for surveillance database and analyse these reports. As the susceptibility profile varies over a period of time it needs to be constantly updated for future public health and clinical healthcare policies, improving patient outcomes and preventing drug-resistance and reducing the cost of healthcare (101).

Resistance to Aminopenicillins

Veillonella spp. and *Prevotella denticola* isolated from root canals have been known to be resistant to amoxicillin. A recent study showed amoxicillin susceptibility (breakpoint for amoxicillin was 8 mg/L) by 34 strains of facultative anaerobic bacterial isolates belonging to the same root canal and 52 of 54 (96%) isolates of obligate anaerobes (102). This study used the NCCLS agar dilution method and confirms that amoxicillin resistance is not common among oral anaerobes in deep-seated orofacial infections. Another study by Fosse et al. revealed high susceptibility of Gram-negative bacilli such as *Prevotella* to amoxicillin and clavulanic acid combination, but in 53.2% of patients and 39.4% of the periodontal pockets one ß-lactamase producing isolate of *Prevotella* sp. was detected (54). Further studies are needed to determine the amoxicillin resistance among oral microbiota. Reproducibility of results is a cause for major concern owing to the hardships with regards to antimicrobial susceptibility testing for anaerobes and the lack of universally accepted standardized methodology for antimicrobial susceptibility testing for anaerobes (181).

Resistance to Penicillins

ß-lactamase production is rarely observed among streptococci. The resistance to penicillin among streptococci is due to alterations of the penicillin-binding proteins (24, 32, 69). The earliest reported case of ß-lactamase producing streptococci in subgingival plaque of adults with periodontitis was in 1986 (86). Another investigation on 207 isolates of nine species of alpha-haemolytic streptococci which comprised of species like *Streptococcus mutans, S. salivarius, S. oralis* and *S. mitis* revealed total susceptibility to penicillin by only *S. mutans* (185). Resistance of four blood culture isolates of *S. mitis* to penicillin (MICs 16–32 mg/L) has also been reported in another study. These also showed resistance to the aminoglycosides, gentamicin, kanamycin and tobramycin (185). A high susceptibility to penicillin and other antimicrobials (5,104,83,84) have been seen among *S. mutans* strains.

All the 424 isolates of *S. mutans* obtained from a study in 116 children and students were found to be susceptible to penicillin, amoxicillin, trimethoprim, tetracycline and erythromycin (84). Reports from other studies have revealed uniform susceptibility to penicillin by 839 isolates of *S. mutans* in 209 patients who were exposed and not exposed to dental amalgam fillings (104). Selection of antibiotic resistance has not been reported to mercury in dental amalgam (48,194). Alpha-haemolytic streptococci like *S. oralis* and *S. mitis* show the greatest penicillin resistance. The degree of resistance may vary, however oral bacteria do always show resistance. It has been reported in the literature that interspecies transfer of resistance determinants occurs between *S. pneumoniae* and other α-haemolytic streptococci (151,89). These are mosaic genes that have areas with nucleotide sequences identical to those from strains known to be susceptible to penicillin and are interspersed with regions of nucleotide sequence divergence. These are responsible for the resistance (45) and are also found in *S. sanguis, S. oralis* and *S. mitis* (45,30, 146,69).

Porphyromonas gingivalis, Prevotella intermedia and *P. nigrescens,* which are also detected in oral infections, have also been known to exhibit antibiotic resistance especially *P. gingivalis,* which was found in one Spanish study to produce ß-lactamase (93,80). However in this study *P. gingivalis* isolates were less commonly isolated from the periodontal pockets. Penicillin resistance is also found more commonly in *Prevotella* spp. (90,3), although the resistance has been found to be similar for both pigmented and non-pigmented *Prevotella* species (93). *Fusobacterium* and *Veillonella* species also demonstrate resistance to penicillin (89,192,191). Beta-lactamase producing fusobacteria (31%) in odontogenic abscesses have also been reported in a study (47).

Resistance to Metronidazole

Mobile genetic elements are thought to be responsible for the resistance to metronidazole (187). Other reasons for resistance may be due to mutations in the enzymes causing reduction of the drug to its active form, mutations leading to decreased entry of the antibiotic into the cell and mutations to transporters causing efflux of the drug (153). Studies on antibiotic susceptibility by Roche & Yoshimori on isolates obtained from odontogenic abscesses showed that eight out of 97 isolates which included five isolates of *Lactobacillus* spp., two isolates of *Gemella morbillorum*

and an isolate of *Actinomyces israelii* were all resistant to metronidazole. However, other isolates in this study including *Prevotella* spp., *Peptostreptococcus* spp., *Bacteroides* spp. and *Porphyromonas* spp. were susceptible (154).

Investigations by Eick et al. described resistance to metronidazole by capnophiles like *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* isolated from periodontal and odontogenic abscesses. This resistance may be explained by a mechanism of intrinsic resistance (47). Similar results have been reported by Madinier et al., in their study where among the 50 test strains 72% were resistant to metronidazole (114). Metronidazole resistance has also been found in *Helicobacter pylori* (139) and the anaerobic protozoa (190).

Resistance to Cephalosporins

High-level resistance to cephalosporins has been detected among alpha-haemolytic streptococci and their MIC for cefotaxime has been reported to be very high (128 mg/L). First and second generation cephalosporins also have high MIC values. In a laboratory study the resistance determinant in cefotaxime was transferred to *S. pneumoniae* having a low level of resistance with great ease (151). Root canal exudates containing *Enterococcus* spp. in periodontal patients are known to exhibit greater resistance to cephalosporins than the Gram-negative bacteria (134). Although the transfer of resistance determinant is low, owing to the greater frequency of antibiotic exposure there is a possibility of higher rate of transfer of the resistance determinant with an increase in the selection pressure (181).

Oral staphylococci were susceptible to cephalosporins (81) in one study but another study found methicillin-resistant *S. aureus* (155), which is hard to eliminate from the oropharynx once they colonize (173). *Peptostreptococcus, Porphyromonas* and *Fusobacterium* are also susceptible to cephalosporins (93). However the MIC50s and MIC90s were greater for the fourth generation cephalosporins in comparison to the cephalosporins of the older generation, which may be due to the misuse of these cephalosporins. *Prevotella* species has been reported to demonstrate resistance to a wide variety of cephalosporins (93). In contrast to the above findings, in a study by Eick et al. resistance to cefoxitin was found in one-third of *Fusobacterium* spp. and one-third of *Veillonella* spp. (47).

More studies are required to have a clear picture about the prevalence and degree of resistance to penicillins and cephalosporins by members of the oral cavity, as it is now known that transfer of high-level resistance to *S. pneumoniae* can occur (181).

Resistance to Tetracyclines

The mechanism of tetracycline resistance is by synthesis of efflux proteins, production of ribosome protection proteins and enzymatic modification of the antibiotic. 27 types of *tet* genes have been identified in oral microbiota which encode for tetracycline resistance (153). In a study conducted on healthy Greek children, 23% of alpha-haemolytic streptococci mainly *Str. mitis* isolated from the oropharynx showed resistance to tetracycline (79). Okamoto et al. in their study comparing the prevalence of black-pigmented anaerobes of the genus *Porphyromonas* and of *Prevotella* spp., and the distribution of the *tet* (Q) gene found that 27.5% of *P. nigrescens* and 6.4% of *P. intermedia* isolates carried *tet* (Q) gene (136). In another study 21% of *P. intermedia* isolates and 15.2% of *P. nigrescens* exhibited the *tet* (Q) gene while *P. gingivalis* isolates also carried *tet* (Q) in combination with the erythromycin resistance determinant *erm* (F). More frequently, *tet* (Q) and *erm* (F) are found to be carried in combination (132,138,184).

Resistance to tetracycline is also a co-marker in penicillin-resistant isolates of oral cavity (66,100). This association has been reported by Fosse et al. (54). They observed in their study that 50% of Gram-negative oral anaerobes were resistant to tetracycline and penicillins thus associating tetracycline resistance with ß-lactamase production. The likelihood of spread of resistance factors is of significance as there have also been similar studies on association between tetracyclines, penicillin and erythromycin resistance (162,195,149,150). Previous investigators have detailed the resistance to tetracycline lasting for a longer duration after discontinuing treatment (149,168).

Macrolides

The mechanism of macrolide resistance include acquisition of one of the 21 *erm* genes which code for rRNA methylases which cause methylation of adenine residues in 23S rRNA, and thus inhibits the binding of macrolides to the 50S ribosomal subunit. Another mechanism is the

inactivation of macrolide by an enzyme encoded by *mph*, and efflux of macrolides by an ATP-binding transporter encoded by *msrA* which has been found in *S. aureus* (153). In addition expression of genes in the *mef* family, which encodes another efflux pump, may be responsible for the low-level of macrolide resistance seen in the members of the oral cavity (4,168).

In a study by Ioannidou et al. among the 200 isolates of α-haemolytic streptococci from the oral cavity of healthy Greek children, 38.5% showed erythromycin resistance, and 33.5% showed clarithromycin resistance, however for erythromycin the MIC90 was twice than that when compared with clarithromycin. The prevalence of resistance to erythromycin was greatest for *S. oralis* species at 53%, while it was 48% for *S. salivarius* and 44% for *S. sanguis* (79).

2.7 Guidelines For Antimicrobial Susceptibility Testing

Table 3. Suggested battery of antibiotics for susceptibility testing (101)

Staphylococcus	**Gram negative bacilli**	***Streptococcus Enterococcus***	***Haemophilus***	***N. gonorrhoeae***
Penicillin	Ampicillin	Penicillin	Ampicillin	Penicillin
Oxacillin	Piperacillin	Oxacillin	Amoxycillin/ Clavulanic acid	Cefazolin
Cephalothin	Cephalothin	Ampicillin	Cefuroxime	Ceftriaxone
Gentamicin	Cefotaxime	Cefotaxime	Cefotaxime	Chloramphenicol
Netilmicin	Ceftazidime	Erythromycin	Tetracycline	Ciprofloxacin
Amikacin	Gentamicin	Chloramphenicol	Erythromycin	
Chloramphenicol	Netilmicin	Tetracycline	Chloramphenicol	
Tetracycline	Amikacin	Vancomycin		
Erythromycin	Chloramphenicol			
Co-trimoxazole	Tetracycline			
Clindamycin	Co-trimoxazole			
Ofloxacin	Nalidixic Acid			
Rifampicin	Ciprofloxacin			
Vancomycin	Ofloxacin			
Teicoplanin	Nitrofurantoin			
	Imipenem			
	Meropenem			

Note: The choice of antibiotic depends on the susceptibility pattern exhibited locally. The selection of antibiotics varies based on specimen and the isolates under consideration (101).

3. AIMS OF THE STUDY

General Aim

The general aim of the present study was to report the long-term surveillance of antibiotic susceptibility of the subjects reporting with bacterial infection of odontogenic and non-odontogenic origin at the University Hospital in Hradec Králové from 1996 through 2007.

Specific Aims

The specific aims were:

(i) to isolate and determine the prevalence of bacterial species in oral samples of patients with bacterial infection reporting at the Dept. of Dentistry (1996-2007),

(ii) to assess the age, site of infection and sex distribution and,

(iii) species-specific relationships,

(iv) to determine the most effective antimicrobial therapy for orofacial infections of odontogenic and non-odontogenic origin based on the *in vitro* antibiotic susceptibility test.

4. MATERIALS AND METHODS

4. Materials and methods

4.1 Patient selection and bacterial sampling procedure

The study involved the patients attending the Department of Dentistry, University Hospital in Hradec Králové with suspected or proven orofacial bacterial infections during the period from 1996 through 2007. These patients were subjected to a comprehensive oral examination after obtaining a detailed dental and medical history. Sampling was performed routinely on patients with orofacial odontogenic and non odontogenic infections by swabbing or obtaining a liquid material or pus from oral cavity or neighbouring structures and transported in anaerobic transport devices (sterile test tube for anaerobic transport with stopper or swab containing Amies transport medium (Dispolab) to the laboratories at the Dept. of Clinical Microbiology.

4.2 Culture

After admission all samples were cultivated on blood agar with 5% sheep blood (BA), chocolate agar (CA) with ATB (bacitracin, vancomycin and clindamycin), McConkey agar (MC), and Brain Heart Infusion agar (BHI). At the same time, these samples were inserted to liver broth, which was incubated for 18-24 hours at 36±1°C and then inoculated onto BA and MC again. Aerobic cultivation was done on BA and MC at 36±1°C for 18-24 and 48 hours, and Sabouraud agar (for yeasts) for 48 hours. CA plates were incubated in a special atmosphere of 5% carbon dioxide (CO_2) for 48 hours, BHI agars were put in BUG BOX to ensure anaerobic condition (5% CO_2, 10% H_2, and 85% N_2) at 36±1°C for 48 to 72 hours. In case of slow bacterial growth incubation time was prolonged to four days to rule out false-negative results. If *Actinomyces* etiology was suspected then, anaerobic culture was prolonged to 10 days. Mycobacteriological examination was carried out using rapid culture technique in MGIT (MB BacT, Becton Dickinson) and convential cultivation on Löwenstein-Jensen agar (Trios, Prague) for 3 to 9 weeks in accordance with standard methods in microbiology of mycobacterial infection (144).

The culture plates were then examined for bacterial growth each 18-24 hours and quantity or semiquantity was evaluated for each sample. Pure bacterial isolates for identification and antibacterial susceptibility testing were obtained by subculture.

4.3. Identification

Presumptive identification of the pure bacterial colonies of strict/ facultative anaerobes/ aerobes, gram-positive/ negative rods and cocci was based on colony morphology and pigmentation on culture media, and on microscopy picture according to Gram-staining and oxidase test (Lachema). Bacterial isolates were identified by standard methods by means of plasmacoagulase test (ITEST plus) for staphylococci, porfyrin production test for hemofils (Trios), hydrolysis of tributyrin (ITEST plus), test of the susceptibility to optochin (Oxoid), the specific battery of biochemical tests (Hajn agar, indol production, Simmons citrate assimilation, urease production, Trios) for gram-negative rods. All gram-negative nonfermentative rods and other unidentified isolates were, if needed, further identified using commercial systems BBL Crystal GP, E/NF, AN, NH and VITEK 2 (Bio Mériéux). Serological identification of ß-hemolytic streptococci were performed by latex aglutination (ITEST plus) into a Lancefield´s group.

4.4. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility tests for the obligate and facultative anaerobes and aerobes were done (in accordance with 2) using disc diffusion test or microdilution broth method. Production of beta-lactamase was identified by nitrocephin test (Lachema), confirmation of MRSA was done by latex aglutination (MRSA-Screen Denka Seiken).

Quality controls (QC) of antibiotic susceptibility tests were used in the validation of antibiotic susceptibility tests and performed in accordance with laboratory standards (2) using reference strains: *Escherichia coli* CCM 3954, *Escherichia coli* CCM 4225, *Pseudomonas aeruginosa* CCM 3955, *Staphylococcus aureus* CCM 3953, *Enterococcus faecalis* 4224 and *Haemophilus influenzae* CCM 4456. QC results were obtained for antibiotics, respectively, of which more than 99% were within the acceptable limits.

The bacterial strains were manually divided into appropriate susceptibility categories (resistant, intermediate susceptibility, or susceptibility) based on the guidelines for interpretation of diameter of inhibition zone for individual antibiotics (164). Species, drug, zone diameter, susceptibility category, and quality control results were read manually and the results were recorded into the central laboratory information system.

The demographic, bacteriologic and antibiotic susceptibility data were collected retrospectively using the hospital records at the Department of Clinical Microbiology.

Exclusion criteria were negative laboratory results and test results of the same patient but not related to the oral cavity. In addition, bacteria regarded as normal commensals and duplicate isolates from a given patient with identical species within different samples, and mycological results of the patients were not considered. Samples with mixed isolates without potencial pathogenicity were grouped together as microflora and no attempt was made to find the antibiotic susceptibility profile of their individual species separately in this study.

4.5 Method of Statistical Analysis

Data were analysed to evaluate the relationships between specific microbes and gender. Chi-square test and simple linear regression analysis were performed to determine temporal trends in occurence of microbial species. Unpaired *t*-test was done to determine if there was any gender prevalence. Significance was determined at $p < 0.05$ level. Relationship between specific microbes and their antibiotic drug-sensitivity profile was also analysed.

5. RESULTS

5. Results

During the 11year study period (1996 to 2007), a total of 678 patients were studied. 350 (51.6%) were males and 328 (48.4%) females. Overall, 1609 strains were isolated. Some of the patients made multiple visits on different occasions for repeated infections.

5.1 Age

The age of the study cohort ranged from 2 to 94 years. The mean age was 41.2 (± 18.03 SD) years for males and 43.7 (+/- 19.5 SD) years for males.

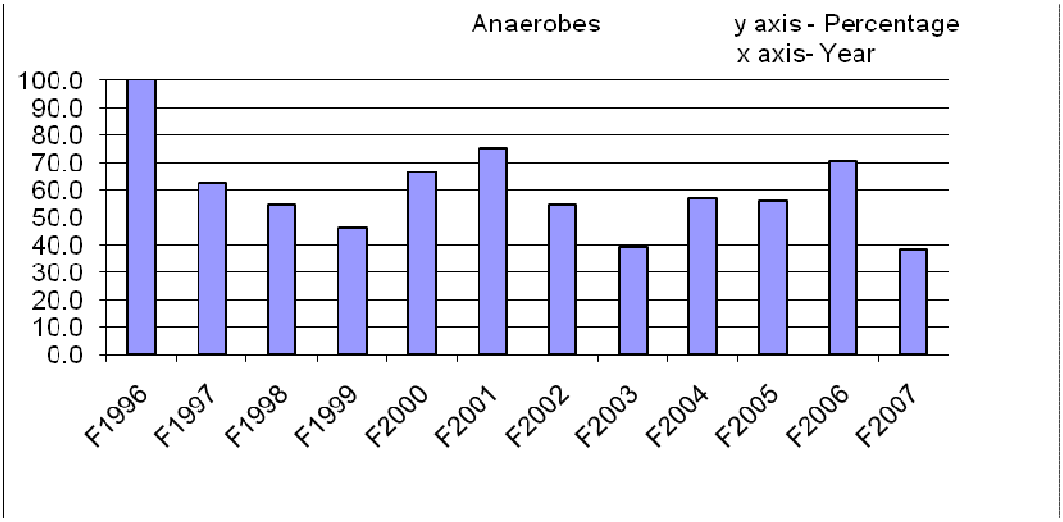
5.2 Gender

Gender distribution of cases during the study period (1996-2007) is in the Graph 8. The proportions of various bacterial species isolated from males and females during the study were comparable (p = 0.082) (See Table 4). Enterobacteria were more prevalent among males whereas *Moraxella catarrhalis,* obligate anaerobes, *H. influenzae,* oral streptococci, *S. aureus,* coagulase-negative staphylococci and beta-haemolytic streptococci were slightly higher among females. However these findings were not statistically significant (See Table 4 and Graphs 1 to 8).

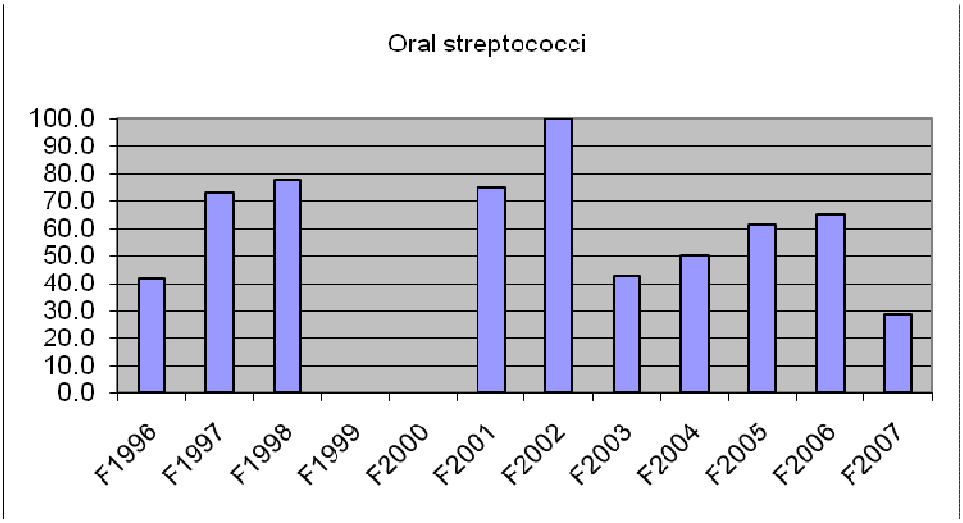
Table 4. Species distribution of patients by gender

Microbe – group	**Female** %	**Male** %
Moraxella catarrhalis	60.0	40.0
Anaerobes	56.4	43.6
Haemophilus influenza	56.3	43.8
Oral streptococci	51.8	46.4
Staphylococcus aureus	52.8	47.2
Coagulase-negative staphylococci	51.6	48.4
Streptococcus beta haemolytic	51.3	48.7
Corynebacterium spp.	50.0	50.0
G- non fermentative rods	50.0	50.0

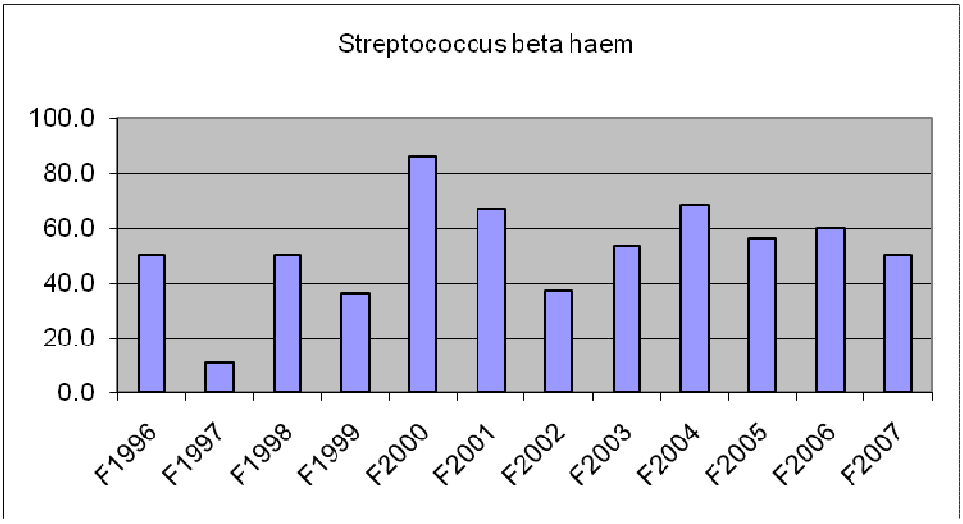
Enterobacteria	47.7	52.3
Indigenous microbiota	41.2	58.8



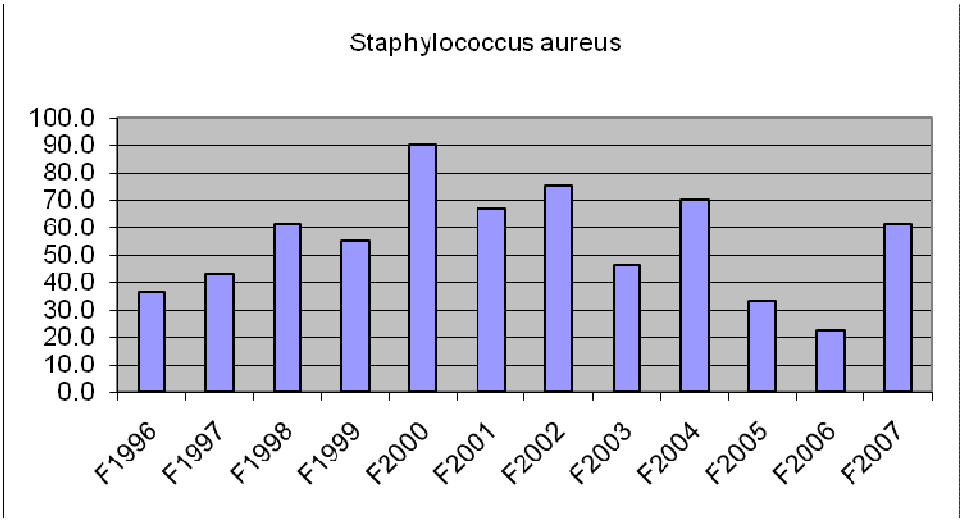
Graph 1. Distribution of obligate anaerobes isolated from females during the study period (1996-2007)



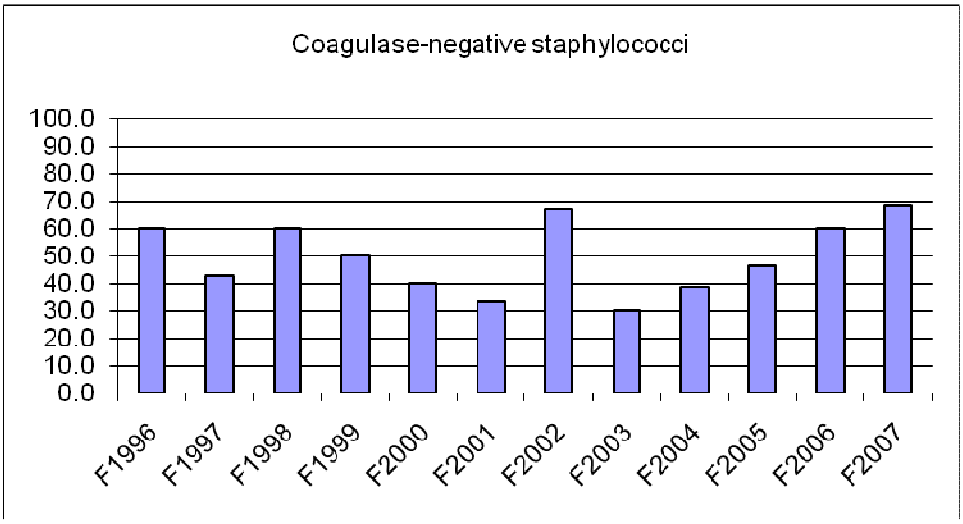
Graph 2. Distribution of oral streptococci isolated from females during the study period (1996-2007)



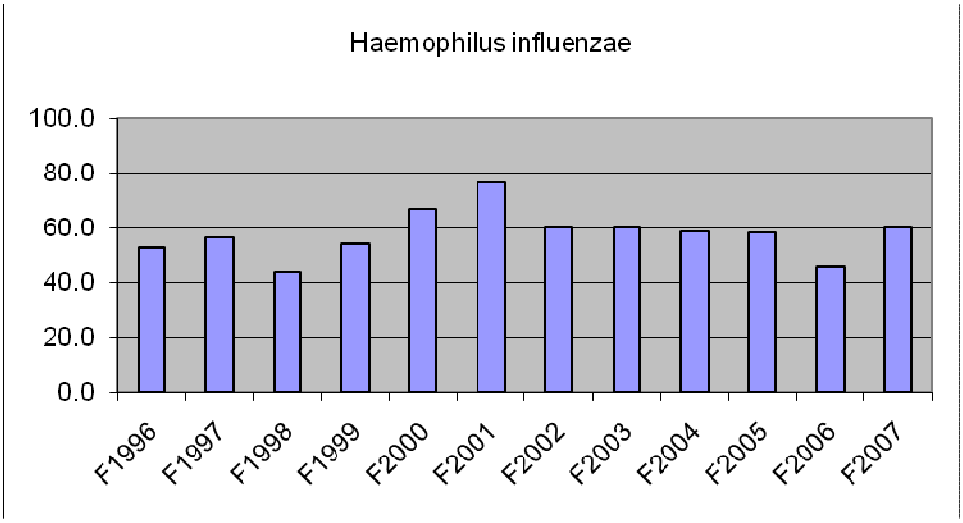
Graph 3. Distribution of beta haemolytic streptococci isolated from females during the study period (1996-2007)



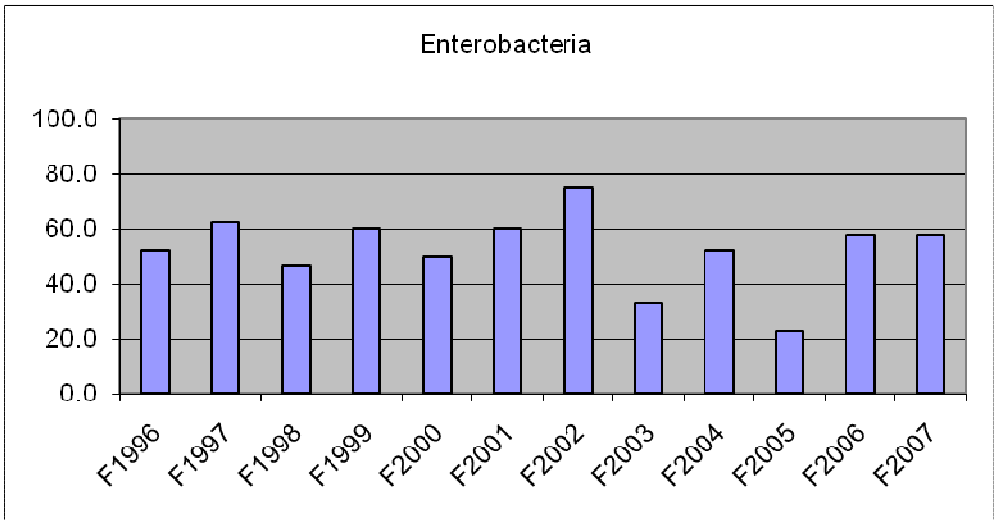
Graph 4. Distribution of *Staphylococcus aureus* isolated from females during the study period (1996-2007)



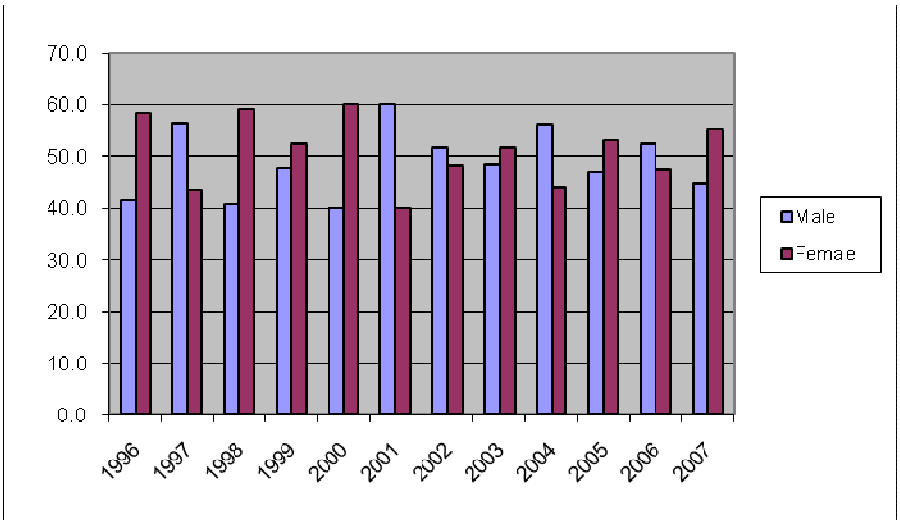
Graph 5. Distribution of coagulase-negative staphylococci isolated from females during the study period (1996-2007)



Graph 6. Distribution of *Haemophilus influenzae* isolated from females during the study period (1996-2007)



Graph 7. Distribution of enterobacteria isolated from females during the study period (1996-2007)



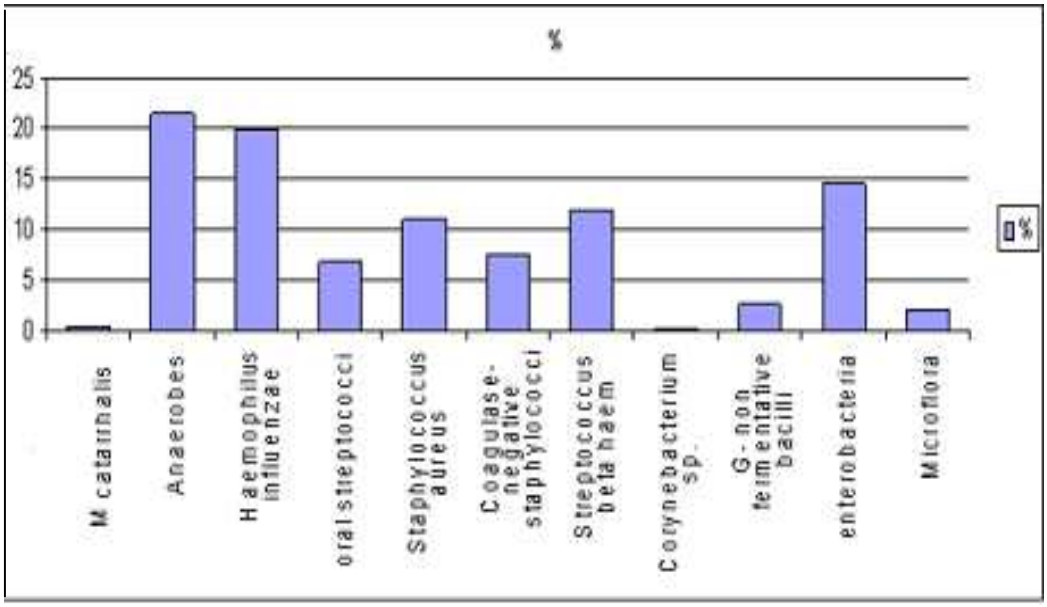
Graph 8. Gender distribution of cases during the study period (1996-2007)

5.3 Site of specimen

Nearly 52 different types of isolates were identified from the specimens. The most frequent sites were throat (18.1%), salivary gland (16.2%) and abscess (14.1%).

5.4 Spectrum of microorganisms

A total of 48 species were identified of 1609 isolates from 678 patients. The spectrum of microorganisms during the study period comprised of predominantly facultative anaerobes 78.5% (n=1263) and obligate anaerobes 21.5% (n=346). Among the facultative anaerobes the most common species was *H. influenzae* (n=320; 19.9%) followed by enterobacteria (n=235; 14.6%), and beta-haemolytic streptococci (n=193; 12%), *S. aureus* (n=176; 10.9%), coagulase-negative staphylococci (n=122; 7.6%), oral streptococci (n=134; 8.3 %) and Gram-negative non-fermentative rods (n=40; 2.5%), *M. catarrhalis* (n=5; 0.3%), and *Corynebacterium* spp. (n=4; 0.3%). The microflora isolated in this study is profiled in the table no. 5 and 6 and graph 9.



Graph 9. Proportion of main bacterial microbiota during the study period (1996-2007)

Table 5. Spectrum of bacteria isolated from orofacial infections with their numbers during the study years.

	Year												
Microbe/s	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**Total**
Anaerobes	3	16	31	13	3	12	11	51	58	50	72	26	**346**
M. catarrhalis	2	0	1	0	1	1	0	0	0	0	0	0	**5**
Coag-neg staph.	5	7	10	8	5	6	3	10	13	13	20	22	**122**
Corynebacterium	0	1	1	0	0	1	0	0	1	0	0	0	**4**
G- non-fermentive rods	2	1	0	0	0	1	0	18	4	6	2	6	**40**
H. influenzae	19	23	32	37	15	17	15	30	34	31	37	30	**320**
Oral? Microbiota	8	3	0	3	0	4	0	0	4	4	4	4	**34**
Staph. aureus	11	14	26	20	10	9	4	13	20	18	18	13	**176**
Streptococcus beta haemolytic	10	18	12	22	7	12	8	13	22	23	30	16	**193**
Enterobacteria	21	8	13	15	6	20	4	24	21	44	33	26	**235**
Oral streptococci	14	16	11	7	1	4	5	7	14	19	21	15	**134**
Total	**95**	**107**	**137**	**125**	**48**	**87**	**50**	**166**	**191**	**208**	**237**	**158**	**1609**

Note – Details of bacteria names see Table 6

5.5 Trends in the species isolated

There was a change in the total number of species during the period and it was found that the total species of microbes increased with respect to the study period ($p = 0.0284$) except for a substantial decrease during the years 2000 to 2002.

There was a steady increase in the number of cases of anaerobic infections during the study period. *Moraxella catarrhalis* and *Corynebacterium* sp. were isolated more frequently during the initial study period. Greater number of cases of coagulase-negative staphylococci and Gram-negative non-fermentative rods were isolated during the last five years of the study. There was not much variation in the number of isolates of *H. influenzae,* oral streptococci, *S. aureus,* and beta–haemolytic streptococci.

Table 6. Spectrum of bacterial species isolated from orofacial infections

Anaerobes	**Gram negative non fermentative bacilli**
Actinomyces israelii	*Acinetobacter* sp.
Bacteroides fragilis	*Pseudomonas aeruginosa*
Bacteroides melaninogenicus	*Stenotrophomonas maltophilia*
Bacteroides sp.	**Coagulase-negative staphylococci**
Bifidobacterium sp.	*Staphylococcus epidermidis*
Fusobacterium sp.	*Staphylococcus* plasmacoagulase negative
Mobiluncus mulieris	**Oral streptococci**
Peptococcus sp.	Alpha haemolytic *Streptococcus*
Peptostreptococcus micros	*Streptococcus intermedius*
Peptostreptococcus sp.	*Streptococcus milleri*
Porphyromonas endodontalis	*Streptococcus pneumoniae*
Prevotella buccalis - non pigmented	**Beta haemolytic *Streptococcus***
Prevotella melaninogenica - pigmented	Group A beta - haemolytic *Streptococcus*
Propionibacterium propionicum	Group B beta - haemolytic *Streptococcus*
Propionibacterium sp.	Group C beta - haemolytic *Streptococcus*
Veilonella sp.	Group F beta - haemolytic *Streptococcus*
Enterobacteria	Group G beta - haemolytic *Streptococcus*
Citrobacter sp.	Non AB beta - haemolytic *Streptococcus*
Enterobacter sp.	***Corynebacterium*** sp.
Enterococcus sp.	*Corynebacterium pseudodiphteriae*
Escherichia coli	**Others**
Escherichia coli haemolytica	*Staphylococcus aureus*
Klebsiella oxytoca	*Haemophilus influenzae*
Klebsiella pneumoniae	*Moraxella catarrhalis*
Morganella morganii	
Proteus mirabilis	
Proteus vulgaris	
Serratia sp.	

5.6 Antimicrobial Susceptibility Results

In general, β-lactam antibiotics like meropenem and ampicillin in combination with β-lactamase inhibitor, macrolide antibiotics like azithromycin and roxithromycin, third generation cephalosporins like ceftizoxime and cefoperazone with β-lactamase inhibitor (sulbactam), fluoroquinolones like ofloxacin and other drugs like nitrofurantoin, mupirocin and teicoplanin demonstrated high levels of antimicrobial activity. Among the different antibiotics used in the study, the maximum resistance was shown by first generation cephalosporins like cefazolin followed by β-lactam antibiotics like ticarcillin, azlocillin, ampicillin and other drugs like metronidazole, cotrimoxazole, tetracycline and erythromycin. These results are presented in table 7. Antibiotic susceptibility values for each species in this study are indicated in tables 8 to 17.

Table 7. Percentage of susceptible bacterial strains to antibiotics

Cefazolin	51.5	Amikacin	97.1
Ticarcillin	63.0	Clindamycin	97.2
Azlocillin	63.6	Chloramphenicol	97.2
Ampicillin	78.0	Cefepime	97.4
Metronidazole	82.1	Vancomycin	97.8
Cotrimoxazole	81.6	Ceftazidime	98.0
Tetracycline	84.7	Levofloxacin	98.0
Erythromycin	88.7	Ciprofloxacin	98.4
Ampicillin/ sulbactam	90.5	Amoxicillin/ clavulanic acid	98.5
Aminopen/ inhibitor	90.5	Piperacillin/ tazobactam	98.8
Cefoperazone	90.9	Cefoxitin	98.9
Ticarcillin/ inhibitor	91.7	Imipenem	99.8
Colistin	91.8	Ampicillin/ inhibitor	100
Oxacillin	92.2	Azithromycin	100
Lincomycin	92.6	Cefoperazone/ sulbactam	100
Cefuroxime	92.8	Ceftizoxime	100
Cefotaxime	93.2	Furantoin	100
Gentamicin	94.0	Meropenem	100
Netilmicin	94.7	Mupirocin	100
Piperacillin	94.7	Ofloxacin	100
Cephalothin	95.0	Teicoplanin	100
Penicillin	95.9	Roxithromycin	100
Spiramycin	96.2		

1. Obligate Anaerobes

Among the 1609 strains of microbes studied, 346 were obligate anaerobes and were highly susceptible to amoxicillin/clavulanic acid combination. 94.1% were susceptible to penicillin. Bacterial isolates (n=4) tested were susceptible to erythromycin also. Available data for 336 isolates of obligate anaerobes demonstrated that they were highly susceptible to imipenem while 2 strains exhibited decreased susceptibility (50%) to tetracycline. All the 9 strains tested of obligate anaerobes were resistant to gentamicin. Less than 1% resistance was observed with chloramphenicol, cefoxitin, and clindamycin. These values are presented in table 8.

Table 8. Antibiotic susceptibility pattern of obligate anaerobes

Obligate anaerobes		
Atb	N	S (%)
GEN	9	0.0
TET	4	50.0
MTZ	322	83.5
PEN	339	94.1
CLI	341	99.4
CFT	344	99.4
CMP	344	99.7
ERY	4	100.0
AMOK	346	100.0
LIN	4	100.0
IMP	336	100.0
CFTX	6	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

2. Oral streptococci

Oral streptococci remained highly susceptible to chloramphenicol, vancomycin, amoxicillin/ clavulanic acid and teicoplanin but less susceptible to cotrimoxazole (66.3%) and tetracycline (71.6%). Isolates also exhibited good susceptibility to penicillin (95.9%), clindamycin (96.7%) and ampicillin (98.7%) (See table 9).

Table 9. Antibiotic susceptibility pattern of oral streptococci

Oral streptococci		
Atb	n	S (%)
MTZ	2	0.0
COT	102	70.6
TET	122	73.0
ERY	109	93.6
PEN	120	96.7
CLI	40	97.5
AMP	93	98.9
CMP	132	99.2
GEN	1	100.0
AMOK	30	100.0
FUR	2	100.0
CIP	1	100.0
API	6	100.0
CFT	3	100.0
IMP	3	100.0
VAN	95	100.0
OXA	1	100.0
TEI	20	100.0
PIPT	3	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

3. *Staphylococcus aureus* and **beta-haemolytic *Streptococcus***

The antibiotic susceptibility of *Staph. aureus* strains was as follows: 92% to tetracycline, 93.2% to erythromycin, 97.2% to chloramphenicol, 98.4% to lincomycin, 99.3% to gentamicin, and 99.4% to cotrimoxazole. Isolates of *Staph. aureus* were highly susceptible to all the other tested antibiotics. All the tested antibiotics worked well in the case of beta-haemolytic streptococci (See table 10 & 11).

Table 10. Antibiotic susceptibility pattern of *Staphylococcus aureus*

Atb	**N**	**S** (%)
TET	174	92.0
ERY	176	93.2
CMP	141	97.2
LIN	124	98.4
GEN	137	99.3
COT	176	99.4
AMOK	76	100.0
FUR	1	100.0
CLI	47	100.0
CIP	82	100.0
API	95	100.0
CFT	57	100.0
VAN	138	100.0
OXA	176	100.0
TEI	65	100.0
CEF1	1	100.0
OFL	1	100.0
MUP	1	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

Table 11. Antibiotic susceptibility pattern of beta-haemolytic *Streptococcus*

Atb	N	S (%)
SPI	78	96.2
CLI	108	96.3
ERY	190	98.4
TET	24	100.0
CMP	23	100.0
COT	21	100.0
AMOK	62	100.0
AMP	70	100.0
PEN	193	100.0
VAN	23	100.0
TEI	2	100.0
CEF1	90	100.0
AMPI	1	100.0
AMPS	18	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

4. Coagulase-negative staphylococci

Coagulase-negative staphylococci were highly susceptible (100%) to amoxicillin/ clavulanic acid (n=38) and teicoplanin (n=64). They were less susceptible to erythromycin (68.9%) while the rest of the antibiotics tested were 75% or more susceptible (See table 12).

Table 12. Antibiotic susceptibility pattern of coagulase-negative staphylococci

Atb	N	S (%)
ERY	119	68.9
TET	120	75.0
COT	118	80.5
OXA	112	83.9
CLI	54	85.2
LIN	66	86.4
GEN	112	93.8
CIP	79	96.2
CMP	114	96.5
CFT	61	98.4
API	86	98.8
VAN	114	99.1
AMOK	38	100.0
AMP	1	100.0
TEI	64	100.0
SPI	1	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

5. Gram-negative non-fermentative bacilli

Gram-negative non-fermentative bacilli were highly susceptible to cefoperazone/ sulbactam and colistin. The susceptibility rates to ampicillin, cefotaxime and tetracycline were 7.7 %, 30%, and 50% respectively. In a few cases, piperacillin, ticarcillin, ofloxacin, netilmicin and azlocillin were very effective. However, some strains of Gram-negative non-fermentative rods were unsusceptible to certain drugs like cefuroxime and cefazolin. (See table 13).

Table 13. Antibiotic susceptibility pattern of Gram-negative non-fermentative rods

Atb	N	S (%)
CMP	3	0.0
AMOK	1	0.0
CFX	9	0.0
CEF1	1	0.0
CFN	12	0.0
AMP	13	7.7
CETX	10	30.0
TET	10	50.0
API	8	62.5
COT	12	75.0
GEN	23	87.0
CFM	16	87.5
TICI	9	88.9
CFP	10	90.0
CFA	16	93.8
PIPT	16	93.8
IMP	17	94.1
LVF	18	94.4
AMI	20	95.0
CIP	21	95.2
COL	22	100.0
PIP	1	100.0
TIC	2	100.0
CFPS	17	100.0
OFL	1	100.0
NET	5	100.0
AZL	1	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

6. *Haemophilus influenzae*

Azithromycin, cefuroxime, aminopen/ inhibitor, amoxicillin/ clavulanic acid, chloramphenicol and in a small number of cases ampicillin/ inhibitor showed strong antimicrobial activity against *H. influenzae*. However, some strains of *H. influenzae* showed greater resistance to cotrimoxazole, tetracycline, and ampicillin. (See table 14)

Table 14. Antibiotic susceptibility pattern of *Haemophilus influenzae*

Haemophilus influenzae		
Atb	n	S (%)
COT	263	79.8
TET	316	98.1
AMP	319	98.1
CMP	80	100.0
AMOK	123	100.0
API	194	100.0
CFX	234	100.0
AZT	293	100.0
AMPI	3	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

7. *Enterobacteria*

Enterobacteria were highly susceptible (100%) to drugs like piperacillin/ tazobactam and third and fourth generation cephalosporins like cefoperazone/ sulbactam and cefepime, respectively. Imipenem, piperacillin, vancomycin, and teicoplanin also exhibited high antimicrobial activity against Enterobacteriaceae. More than 75% of isolates were susceptible to several other drugs including amoxicillin/ clavulanic acid. High order of resistance (69.7%) to ampicillin was observed. The bacteria was less susceptible (< 75%) to lincomycin, azlocillin, erythromycin, aminopen/ inhibitor, first generation cephalosporins (cephalothin and cefazolin), tetracycline and ticarcillin. (See table 15)

Table 15. Antibiotic susceptibility pattern of enterobacteria

Atb	n	S (%)
CFT	0	0.0
OXA	2	0.0
AMPS	2	0.0
ROX	1	0.0
AMP	165	30.3
LIN	2	50.0
CEF1	6	50.0
AZL	7	57.1
ERY	19	57.9
API	94	59.6
CFN	134	60.4
TET	162	70.4
TIC	18	72.2
AMOK	33	78.8
CFX	107	86.0
CMP	79	86.1
COL	132	90.2
COT	158	90.5
NET	57	94.7
AMI	100	97.0
GEN	142	97.2
CETX	133	97.7
CFA	71	98.6
LVF	77	98.7
CIP	112	99.1
PEN	1	100.0
FUR	1	100.0
IMP	55	100.0
VAN	29	100.0
TEI	12	100.0
PIP	30	100.0
CFPS	58	100.0
OFL	4	100.0
PIPT	59	100.0
MEP	4	100.0
CFP	1	100.0
CFM	56	100.0
TICI	3	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

8. *Moraxella catarrhalis* and *Corynebacterium* species

All isolates belonging to *Moraxella (Branhamella) catarrhalis* were susceptible to tetracycline, chloramphenicol, amoxicillin/ clavulanic acid, ampicillin and aminopenicillin/ inhibitor. Most of the strains of *Corynebacterium* were susceptible to the antibiotics tested except for one resistant strain each for oxacillin, erythromycin, lincomycin, and cotrimoxazole (See table 16 and 17).

Table 16. Antibiotic susceptibility profile of *Moraxella catarrhalis*

Atb	n	S (%)
VAN	5	0.0
ERY	5	60.0
PEN	4	75.0
TET	5	100.0
CMP	5	100.0
AMOK	3	100.0
AMP	5	100.0
API	1	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

Table 17. Antibiotic susceptibility profile of *Corynebacterium* sp.

Atb	N	S (%)
CFT	0	-
OXA	2	50.0
ERY	3	66.7
LIN	3	66.7
COT	4	75.0
TET	4	100.0
CMP	3	100.0
GEN	4	100.0
AMOK	2	100.0
AMP	2	100.0
CIP	2	100.0
API	2	100.0
CFX	1	100.0
VAN	3	100.0
TEI	1	100.0
CETX	1	100.0
CFN	1	100.0
PIP	1	100.0
NET	1	100.0

Atb- abbreviation of antibiotic; n- number of tested strains; S- % of susceptible strains

6. DISCUSSION

6.1 Bacteriological profiles

The majority of suppurative odontogenic infections is polymicrobial in nature and consists of both mixed aerobic and anaerobic bacteria (64, 85, 193) with anaerobes two to four times greater in proportion than aerobes (157,158,25,10,1,99,120,6,165,166). Only very few long-term studies have examined the species distribution profiles and gender dominance in oral infections. The aim of this retrospective study was to investigate the prevalence of bacterial species in oral samples of patients with suspected orofacial infection reporting at the Dept of Dentistry (1996-2007), the species distribution of bacteria, to assess the sex and species specific relation in odontogenic and non-odontogenic infections. A total of 678 culture-positive patients were included in this study with 1609 strains comprising of 48 different species isolated (see table 6). The total number of species of microbes isolated in this study was high. However, a substantial decrease in the number occurred during the years 2000 to 2002 which may be attributed to change in methodology. Nearly 52 different types of isolates were identified from the specimens. 18.1% of microorganisms were isolated from the throat, 16.2% from salivary glands and 14.1% from abscesses.

Age and gender

This study showed an age distribution between 2 and 94 years, with a mean age of 41.2 (± 18.03) years among males and 43.7 (± 19.5) years among females. This is in partial agreement with earlier studies comprising of 25-35, 20-29, and 23-70 years age groups (85,10,71). The proportion of males and females in the study were comparable (p 0.082). These findings are in agreement with earlier studies (85,78,43,123). Infections caused by *M. catarrhalis*, anaerobes, *H. influenzae*, oral streptococci, *Staph. aureus*, coagulase-negative staphylococci and beta haemolytic *Streptococcus* were slightly higher among females and enterobacteria in males, however this differences in percentage distribution of isolates among either genders did not show statistical significance.

Spectrum of microorganisms

Isolates comprised of predominantly facultative anaerobes. Facultative anaerobes and obligate anaerobes accounted for 78.5% (n= 1263) and 21.5% (n=346) respectively. The most frequently isolated facultative anaerobe were identified as *H. influenzae* (n=320, 19.9%) followed by, enterobacteria (n=235, 14.6%), beta-haemolytic *Streptococcus* spp. (n=193, 12%), *Staph. aureus* (n=176, 10.9%), coagulase-negative staphylococci (n=122, 7.6%), oral streptococci (n=110, 6.8%), and Gram-negative non-fermentative rods (n=40, 2.5%). However, *M. catarrhalis* and *Corynebacterium* sp. were the least common. This is in contrast to a study by Heimdahl et al that demonstrated predominance of obligate anaerobes like *Bacteroides, Prevotella* and *Fusobacterium* (73). Earlier studies by other investigators have reported *Porphyromonas, Prevotella, Fusobacterium, Peptostreptococcus,* and streptococci, to be the major pathogenic bacteria isolated from dental infections (61,164,6,73,143,95,107,20).

Study of oral infections by Hunt and co-workers demonstrated the prevalence of 57% streptococci, 34% staphylococci and 15% anaerobic bacteria (78). Dahlen et al. reported *Staph. aureus,* coliform bacteria and *Klebsiella* in majority of their cases while *Streptococcus pyogenes, H. influenzae, Pseudomonas* or other gram-negative aerobic bacteria were observed in some cases (34). The results of this study are in agreement with the findings of obligate and facultative anaerobes by Kuriyama et al. (94). In their study involving 664 strains isolated from dentoalveolar infections, periodontitis and pericoronitis, the majority of the isolates belonged to viridans streptococci, *Peptostreptococcus, Gemella,* pigmented and nonpigmented *Prevotella, Porphyromonas,* and *Fusobacterium.*

Enteric gram-negative rods, have been isolated from normal oral flora in 27.9% cases with enterobacteria accounting for 57% of isolates in a study by Sedgley et al (167). These strains have also been found in immunocompromised persons undergoing chemotherapy (58). The proportion of enterobacteria varies depending on the consumption of contaminated food and water and personal hygiene (172,7). In this study enterobacteria like *Citrobacter, Enterobacter, Enterococcus, Escherichia coli, E. coli haemolytica, Klebsiella oxytoca, Kl. pneumoniae, Morganella morganii, Proteus mirabilis,* and *P. vulgaris* were more commonly isolated. In a study by Gonçalves et al enteric rods like *Enterobacter cloacae* (7 strains), *E. aerogenes* (1 strain), *Pantoea* (*Enterobacter*) *agglomerans* (1 strain), *Serratia marcescens* (5 strains), *Klebsiella pneumoniae* (1 strain) and

Citrobacter freundii (1 strain) were isolated from periodontal pockets of patients with chronic periodontitis. The isolation of pathogens like *E. coli, Kl. pneumoniae* and *Ps. aeruginosa* from mouth that may cause opportunistic infections in respiratory tract especially in patients who are immunocompromised highlights the importance of early identification of these potentially harmful microorganisms (62).

6.2 Antimicrobial susceptibility profiles

Most odontogenic orofacial infections are caused predominantly by anaerobes but there have been only a few long-term studies that have examined the bacteriologic and antimicrobial susceptibility profiles in oral infection. Currently there are several surveillance programs involving a large number of countries across the globe to monitor the antimicrobial susceptibility profiles of microorganisms isolated from blood, urine and other specimens. However similar profiling for oral specimens is yet to take shape and a serious move in this direction is long due. Administration of antibiotics through oral or other routes affect the microbiota throughout the body and hence it will be useful to compare the susceptibility profiles of oral bacteria.

Obligate anaerobes

In this study, obligate anaerobes did not show resistance to amoxicillin/ clavulanic acid which is similar to the observation reported by Lana et al (102) wherein all the facultative anaerobic bacterial isolates (34 strains) and majority of obligate anaerobes (52 of 54 strains) showed high susceptibility. In another study by Fosse et al, amoxicillin with clavulanic acid worked well against Gram-negative bacilli like *Prevotella* except for the presence of one ß-lactamase producing strain (54). *Prevotella* spp. is known to demonstrate resistance to penicillin commonly (90,3) and this resistance has been found to be similar for both pigmented and non-pigmented species (93).

In this investigation obligate anaerobes showed higher susceptibility to penicillin (94.1%) and the majority of obligate anaerobic strains were susceptible to all the drugs tested, with the exception of tetracycline and gentamicin. Similar observations were made by Kuriyama et al. wherein the susceptibility rates to penicillin G for *Peptostreptococcus*, *Porphyromonas*, and *Fusobacterium* were 86%, 100%, and 89% respectively while 72% of pigmented and 82% of nonpigmented strains of *Prevotella* showed high susceptibility (94). Certain studies show that in *Prevotella* the resistance

mechanisms against tetracycline are genetically-determined like the β-lactam-resistance (54,100).

In this study it was observed that third generation cephalosporin like ceftizoxime worked very well against all the strains whereas 2 of 342 strains of obligate anaerobes were resistant to second generation cephalosporin like cefoxitin. Greater antimicrobial activity of the newer generation cephalosporins than the older ones may be attributed to the higher stability of the former in the presence of β-lactamases (116).

Other drugs which proved equally effective were erythromycin and lincomycin. Most of the strains were also susceptible to chloramphenicol. Metronidazole and clindamycin were also effective and this is in agreement with the reports by other investigators (98). Thus the high susceptibility to β-lactam antibiotics favours their continued use in the management of infections by obligate anaerobic strains.

Oral streptococci

In this study, oral streptococci exhibited 95.9% susceptibility rate to penicillin. This is in contrast to certain other studies where only 77% of viridans streptococci were susceptible to penicillin G (94). All the tested strains in this study were also susceptible to other drugs except for some resistance to cotrimoxazole, tetracycline, erythromycin, penicillin, clindamycin and ampicillin.

There have been conflicting reports in the literature regarding the efficacy of penicillins against viridans streptococci and β-lactamase–producing anaerobes (94,61,164,6,73,143,95,52). In a study by Kuriyama et al most anaerobes and facultative anaerobes (especially viridans streptococci) were susceptible to penicillin except β-lactamase–positive *Prevotella*. They reported that viridans streptococci and majority of the strains of *Fusobacterium* were resistant to erythromycin while anaerobes were susceptible to clindamycin (94). In patients who have penicillin allergy, alternative drugs like erythromycin and clindamycin are administered. (61,164,6,73,143,95). Their effectiveness make them suitable for orofacial infections in such patients. In contrast to the bacteriologic data from other studies (94), this study showed that 92% of oral streptococci were susceptible to erythromycin. Previous studies have shown that the serum concentration of the ß-lactam antibiotics and erythromycin is greater than that achieved in the saliva (178,133,74,46). However oral streptococcal

species have been found to be susceptible to low ß-lactam antibiotic concentrations in the saliva (178,133).

Clindamycin was effective against both oral streptococci and obligate anaerobes which is in agreement with the study by Kuriyama et al (94). The high bactericidal activity of clindamycin against β-lactamase–producing bacteria coupled with their ability to achieve high alveolar concentrations (78) and clinical efficacy at the recommended dosage (164) make them more suitable for treating infections by β-lactamase–producing obligate anaerobes (61,164,6,73,143,51). There is an inhibitory action on the formation of β-lactamase (163) and greater host defence achieved on administration of clindamycin (59,110,70). Antibiotic-associated colitis, the major side effect, restricts the use of clindamycin to treat severe oral infections or where treatment with penicillin has been ineffective (94,61,6).

Previous studies showed that viridans streptococci, *Peptostreptococcus, Porphyromonas,* and *Fusobacterium* were highly susceptible to cefazolin (1^{st} generation cephalosporin) and cefmetazole (2^{nd} generation cephalosporin). However some strains of *Prevotella* showed lower susceptibility only to cefazolin (94). Similar to the above observations, a high susceptibility of obligate anaerobes and a few strains of oral streptococci to cefoxitin (2^{nd} generation cephalosporin) were observed in this study. Cephalosporins show cross-reactivity with β-lactam antibiotics and hence should not be administered to patients with immediate hypersensitivity reactions to penicillin (116). However the broad spectrum and strong bactericidal action against oral pathogens make them drugs of choice in the treatment of dental infections (116).

This study also showed an increased resistance to tetracycline similar to other studies (61,6), but oral streptococci showed 71.6% susceptibility to tetracycline. However in another study, minocycline worked well against viridans streptococci and strict anaerobic bacteria which is attributed to its powerful bacteriostatic effect than tetracycline (94,6,180).

The results also demonstrated that alpha haemolytic streptococci were highly susceptible to erythromycin, penicillin, ampicillin, vancomycin but resistance was noted against tetracycline, cotrimoxazole and chloramphenicol. *S. pneumoniae* and other alpha-haemolytic streptococci (151,89)

are known to transfer resistance traits to each other. This inter-species transfer of resistance genes poses great concern in the treatment of resistant strains.

Although some strains among oral streptococci were resistant to penicillin (4.1%) and ampicillin (1.3%), all the strains of alpha-haemolytic streptococci and, beta-haemolytic streptococci tested against penicillin and ampicillin were highly susceptible while cephalosporins were equally effective for oral streptococci and beta-haemolytic streptococci. In another study by Teng et al, among the 207 isolates of alpha-haemolytic streptococci, including *Streptococcus mutans, S. salivarius, S. oralis* and *S. mitis*, only *S. mutans* showed no resistance to penicillin (185). Potgieter et al. reported that a few strains of *S. mitis* were not susceptible to penicillin, aminoglycosides like gentamicin, kanamycin and tobramycin (145).

Several other studies have also demonstrated susceptibility of *S. mutans* to penicillin, amoxicillin, trimethoprim, tetracycline and erythromycin (84). 8% of *S. salivarius* strains, 20% of *S. mitis* strains and 35% of *S. oralis* strains demonstrated resistance to penicillin in another study (185). It has been reported that greatest resistance to penicillin is demonstrated by *S. oralis* and *S. mitis* in comparison to other members of alpha-haemolytic streptococci (181).

Hunt et al. reported susceptibility of streptococci to ampicillin, cephalothin, and penicillin (78). The results in this investigation are in agreement with the above study as ampicillin, cephalosporins and penicillin worked well against oral streptococci, and beta-haemolytic streptococci. However, in contrast to their study, the present study results found a greater antimicrobial activity of erythromycin against all the tested streptococcal species.

Staphylococci

In this study, coagulase-negative staphylococci showed a 98.4% susceptibility to cephalosporin agents like cefoxitin (second generation cephalosporin) while all isolates of *Staph. aureus* (n=57) tested with cefoxitin (second generation cephalosporin) and only 1 of the isolate tested with cephalothin (first generation cephalosporin) were susceptible. This is in agreement with a previous study by Jacobson et al (81).

Overall 31.1% of coagulase-negative staphylococci and 6.8% of *Staph. aureus* were resistant to erythromycin. *Staphylococcus aureus* demonstrated greater antimicrobial activity to the tested antibiotics than the coagulase–negative staphylococci. Higher resistance in the range of 50% for streptococcal and staphylococcoccal species has also been reported in a study by Hunt and coworkers (78).

In the case of tetracycline, lower susceptibility was demonstrated by all the tested staphylococcal and streptococcal strains except beta-haemolytic streptococci. On the contrary, all the above strains showed higher susceptibility for chloramphenicol. Certain studies have shown a decrease in susceptibility of oral microbiota to minocycline following administration of minimal dose of minocycline. This reveals that antibiotic concentration is closely related with the development of resistant strains within the members of the oral microbiota (181,135). All the streptococcal and *Staph. aureus* strains were highly susceptible to amoxicillin/ clavulanic acid and vancomycin. Only one vancomycin resistant strain of coagulase negative staphylococci was detected.

Gram-negative non-fermentative bacilli

Antimicrobial agents like ciprofloxacin, colistin, cefoperazone/ sulbactam tested in this study were highly effective against Gram-negative non-fermentative bacilli. In another study by Sots et al., ciprofloxacin worked well against *Enterobacter cloacae, Kl. pneumoniae, Ps. aeruginosa, Kl. oxytoca* and *Enterobacter agglomerans* (169). In this study all the 3 cases of Gram-negative non-fermentative bacilli were found to be resistant to chloramphenicol. Although chloramphenicol is cheap and has broad spectrum of activity, high rates of side effects and adverse reactions prompt the clinicians to use this agent as a reserve-drug only for severe and CNS infections (152). Very few strains of *Staph. aureus,* enterobacteria, Gram-negative non-fermentative rods tested against ofloxacin were all found to be susceptible.

Haemophilus influenzae

In this study, *H. influenzae* showed high susceptibility to cefuroxime which may be attributed to the inhibition of their adherence to the buccal epithelial cells by cefuroxime (82). The resistance mechanism in *H. influenzae* has been attributed to chromosomal mutation and changes in the penicillin binding proteins (PBPs) by interspecies recombination resulting in decreased susceptibility

to β-lactam antibiotic (26,44). In this study, 98.1% of *H. influenzae* were susceptible to ampicillin. Isolates of *H. influenzae* did not show resistance to azithromycin, cefuroxime, aminopen/ inhibitor, amoxicillin/ clavulanic acid, chloramphenicol and ampicillin / inhibitor. In a study by Guggenbichler & Kastner it was noted that there was longer carriage of resistant strains following therapy with azithromycin for upper respiratory tract infection in children in comparison to clarithromycin (65,40). The microorganisms in their investigation comprised of *S. pneumoniae, S. pyogenes, H.influenzae, M. catarrhalis, S.viridans, S.salivarius,* etc. This selection and/or persistance of resistant strains of commensal and pathogenic microbiota following prolonged exposure to antibiotics is an area of increasing concern (40).

Enterobacteria

In this study, Enterobacteriaceae were susceptible to cephalosporins, imipenem and fluoroquinolones like ciprofloxacin, levofloxacin and ofloxacin, while a high level of resistance was seen against ampicillin (69.7%), amoxicillin/ clavulanic acid (21.2%), tetracycline (29.6), and chloramphenicol (13.9%). This is similar to the findings by Gonçalves et al. in which 93·75% of the enteric rods were susceptible to cephalosporins and aztreonam but showed resistance to tetracycline (25%), and chloramphenicol (18·8%). Susceptibility of Enterobacteriaceae to ciprofloxacin and norfloxacin has also been reported by Barbosa et al (7).

However, there was high susceptibility to gentamicin (97.2%), amikacin (97%) in contrast to the findings by Gonçalves et al (62). In this study, susceptibility was also seen to penicillin while a few cases were erythromycin-resistant which is similar to a study by Stillerman et al (176). The susceptibility to cotrimoxazole was 90.5%. High resistance to ampicillin (69.7%) and amoxicillin/ clavulanic acid (21.2%) seen in this study may be attributed to β-lactamase activity whereas the resistance to ampicillin, azlocillin, ticarcillin and susceptibility to β-lactam antibiotics like piperacillin / tazobactam, may be explained by the production of a nonextended spectrum beta-lactamase by the enterobacteria. Results for enterobacteria demonstrated that there was greater susceptibility to the 3rd and 4th generation cephalosporins.

Overall 99.1% susceptibility rate was seen against ciprofloxacin. In a study by Slots et al, 50% of strains comprising of *Enterobacter cloacae, Kl. pneumoniae, Ps. aeruginosa, Kl. oxytoca* and

Enterobacter agglomerans were highly susceptible to ciprofloxacin (169).

Moraxella catarrhalis and Corynebacterium species

Susceptibility rate of 100% was evident in all of the isolates belonging to species like *M. catarrhalis* for tetracycline, chloramphenicol, amoxicillin/ clavulanic acid, ampicillin and aminopen/ inhibitor. A few isolates were resistant to erythromycin, penicillin and cotrimoxazole. This is in partial agreement with another study (119).

Most of the strains of *Corynebacterium* were susceptible to the antibiotics tested including ampicillin and amoxicillin/ clavulanic acid, except for one resistant strain each for oxacillin, erythromycin, lincomycin and cotrimoxazole. In another study, all the strains of *Corynebacterium* were susceptible to penicillin G, ampicillin, amoxicillin, erythromycin, lincomycin, clindamycin, kanamycin and vancomycin (183).

This study cohort comprised of a mixed collection of patients and the microorganisms were subjected to a standard set of antibiotics with additional sets of antibiotics used depending on the susceptibility profiles of the data. These have lead to difficulty in direct comparison of susceptibility profiles within each individual species as different sets of antibiotics were used to determine the most appropriate drug of choice for treatment of orofacial infections on a case by case basis. Besides, there can be a difference in the *in vivo* and *in vitro* activity of antibiotics (103). However, the presence of numerous causative organisms for orofacial infections highlight the need for appropriate antimicrobial agent to treat these infections (20,96,97).

7. CONCLUSIONS

The microorganisms most commonly implicated in orofacial infections in this study were facultative anaerobes like *Haemophilus influenzae* and enterobacteria followed by obligate anaerobes. The predominance of facultative anaerobic bacteria and the presence of obligate anaerobes reveal the complex polymicrobial nature of odontogenic and non odontogenic lesions. Both sexes had equal predilection for the disease and there had been no significant change in the male/ female ratio over the 11 year study period. However, there had been an increase in the total number of bacterial species. In future, large-scale oral bacteriological surveillance programmes are required to corroborate the results of the present study.

Obligate anaerobes were highly susceptible to most antibiotics including penicillins while resistance to gentamicin and tetracyline was noted among these species. Greater than 95% susceptibility was demonstrated by oral streptococci to β-lactam antibiotics in comparison to erythromycin and broad-spectrum drugs like tetracycline and cotrimoxazole. However, most isolates of alpha and beta-haemolytic streptococci showed greater susceptibility to antimicrobials than the oral streptococcal species. The susceptibility rate of coagulase-negative staphylococci was significantly lower than that of the *Staphylococcus aureus* strains although both groups exhibited greater susceptibility to β-lactam antibiotics than broad spectrum drugs. Enterobacteria showed the highest susceptibility to piperacillin/ tazobactam, third and fourth generation cephalosporins, whereas there was unusually high resistance to ampicillin. Gram-negative non-fermentative bacilli were more susceptible to third-generation cephalosporins and polypeptide antibiotics. Isolates of *H. influenzae* were susceptible to a wide range of β-lactams, broad-spectrum antibiotics like chloramphenicol and second-generation cephalosporins. *Moraxella catarrhalis* and *Corynebacterium* species were also found to be susceptible to β-lactam antibiotics.

The findings in this study suggest that β-lactam antibiotics are still the mainstay in the antimicrobial management of orofacial infections as they are effective in eradicating strict and facultative anaerobes but appropriate and adequate antibiotic regimen on a case-specific basis is essential to prevent the emergence of resistance to antimicrobials in future. Towards this goal,

large scale surveillance programs will help in improving patient outcome and formulating public health policies.

8. CLINICAL IMPLICATIONS OF THE STUDY

Significance of the Study

The bacteriological and antibiotic susceptibility profiles in this study can help the clinical microbiologist and the dental practitioner to make a rational choice of appropriate antibiotic drug therapy and the study highlights the importance of prompt and accurate microbiological investigation in the management of oral infection.

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