The prevalence of β-lactamase-producing anaerobic oral bacteria and the genes responsible for this enzyme production in patients with chronic periodontitis

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A dissertation submitted to the Faculty of Health Sciences, University of The Witwatersrand, in fulfilment of the requirements for the degree of

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Declaration

I, Buhle Ntandokazi Binta declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of The Witwatersrand, Johannesburg. It has not been submitted before for any degree or examinations at this or any other University.

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…….day of…….., 2014

Dedication

This thesis is dedicated to my mother Celiwe Binta and my sister Zipho Binta, who have helped me reach this point in my academic career through their endless support and encouragement.

Publications and presentations arising from this thesis

1. 5th Cross faculty graduate symposium, University of the Witwatersrand, Johannesburg, 2013: Poster presentation
2. University of the Witwatersrand School of Oral Health Sciences Research Day, Johannesburg, 2013: Poster presentation
3. FIDSSA 5 - Changing attitudes congress, 2013, Champagne Sports Resort, Drakensburg, South Africa: Poster presentation
4. A paper has been Submitted for a possible publication to Journal of Periodontal Research, 2014

Abstract

Introduction: Chronic peridontitis is an inflammatory disease that is caused by the accumulation of bacteria in the form of a biofilm in the periodontal pocket. It can be treated with oral hygiene in conjunction with β-lactam antibiotics. Many oral anaerobic bacteria associated with chronic periodontal diseases have developed resistance to β-lactam antibiotics by virtue of their production of β-lactamase enzymes. This study investigated the prevalence of β-lactamase-producing anaerobic bacteria in the oral cavities of South African patients with periodontitis and the genes responsible for these enzymes production.

Methods: Periodontal pocket debri was collected from 48 patients with chronic periodontitis and cultured anaerobically on blood agar plates with and without β-lactam antibiotics. Presumptive β-lactamase-producing isolates were evaluated for definite β-lactamase production using the nitrocefin slide method and identified using the API Rapid 32A system. Antimicrobial sensitivity was performed using a disc diffusion test. Isolates were screened for the presence of the Bla_{TEM} and Bla_{cfxA} genes using Polymerase Chain Reaction (PCR). Amplified PCR products were sequenced and the Bla_{cfxA} gene was further characterized using Genbank databases. Seventeen isolates containing Bla_{cfxA} gene were subjected to broth microdilution technique to determine minimum inhibitory concentrations of Amoxycillin, Augmentin, and Penicillin.

Results: Seventy five percent (36 of 48) of patients carried, on average 2 strains of β-lactamase-producing oral anaerobic bacteria, which constituted 10% of the total cultivable oral flora. A total of 85 oral anaerobes were isolated from patients. The

predominant isolates were gram negative species such as *Prevotella* spp (58%), *Bacteroides* spp (18%) and *Porphyromonas* spp (7%). The disc diffusion antimicrobial sensitivity test showed that 40% of the strains were resistant to β-lactam antibiotics. PCR results revealed that none of the anaerobes carried Bla_{TEM}. The Bla_{cfxA} gene was identified in 51% of the β-lactamase-producing bacteria. Variants of the Bla_{cfxA} gene included *cfxA2* (77%), *cfxA3* (14%) and *cfxA6* (9%). Minimum inhibitory concenration antimicrobial susceptibility test results showed that more than 53% of the strains were resistant to β-lactam antibiotics when the Bla_{cfxA} gene was present.

Conclusions: A high prevalence of β-lactamase-producing oral anaerobic bacteria was found in South African patients with chronic periodontitis. Although, it comprised 10% of their oral flora these anaerobes can protect non-β-lactamase-producers by releasing these enzymes into the environment. The most prevalent β-lactamase gene in this population was Bla_{cfxA} subcategory *cfxA2* which has epidemiological implications and genetic transfer can occur among these bacteria. On average fifty percent of the isolates that carried this gene were resistant to β-lactam antibiotics therefore alternative antimicrobial agents should be considered in patients that are non-responsive to β-lactam antibiotics. This study indicates that there is a need for education in the dental community regarding antibiotic resistance and regular surveillance with diagnostic testing is needed.

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Chapter 1 Introduction

Chronic periodontal disease is an inflammatory disease of gingiva which affects 70% - 80% of adults worldwide (Marsh and Martin, 1999). This disease is more prevalent in developing countries. It is caused by accumulation of subgingival plaque which is a bacterial biofilm containing predominantly gram negative anaerobic oral bacteria, such as *Prevotella spp, Porphyromonas spp,* and *Fusobacterium spp*. Bacterial by products and host response causes tissue damage which results in loosening of the tooth, occasional pain, discomfort and eventually tooth loss. Treatment of periodontitis is by oral hygiene techniques used in conjunction with β-lactam antibiotics. However studies have demonstrated that a wide variety of periodontal pathogens have developed resistance to β-lactam antibiotics by virtue of their production of enzymes known as β-lactamases.

β -lactamase-producing bacteria release the β-lactamase enzyme into their environment resulting in resistance to antimicrobial therapy and they may also convey protection from antimicrobials to other susceptible oral bacteria. Mechanisms of bacterial resistance to antimicrobials have been attributed to resistance genes which are transferred between related species, and commensal and pathogenic bacteria in the oral biofilm.

A preliminary study conducted in South Africa showed 69% of patients with chronic periodontitis harbouring β-lactamase-producing anaerobes with a mean of one to two strains per patient. However this study did not determine the prevalence of the β-lactamase genes that encode for the β-lactamse enzymes, and did not test the

antimicrobial susceptibility of the periodontal pathogens. Therefore, this study was conducted to isolate and identify β-lactamase-producing oral anaerobes from periodontal pocket debris of patients with chronic periodontitis, determine their prevalence, analyse their antimicrobial sensitivity profile and identify the genes responsible for β-lactamase production in oral anaerobes in this population.

1 Literature review

1.1 Periodontitis and Gingivitis

Gingivitis is the mildest form of periodontal disease that affects 30-50% of adults worldwide (Pihlstrom et al., 2005). It is caused by the dental plaque which is a bacterial biofilm on the teeth adjacent to the gingiva. Although gingivitis is a mild form of periodontitis, it does not affect the underlying structures of the teeth and is reversible, but progresses to periodontitis if left untreated (Pihlstrom et al., 2005). However, in some cases gingivitis may exist for prolonged periods before developing into periodontitis. The transition into chronic periodontitis may be due to selective overgrowth of plaque species due to impairment of the host defences, infection and proliferation of a newly arrived pathogen in the gingival area or activation of immune responses that damage host tissue (Samaranayake, 2002).

Periodontitis is an extension of the inflammatory process that extends into the periodontal ligament, cementum and the alveolar bone surrounding the teeth (Nisengard and Newman, 1994). A localized inflammatory response occurs due to the formation of a periodontal pocket forming between the gingiva and tooth root from the accumulation of subgingival plaque (Samaranayake, 2002).

The periodontal pocket gets deeper as the disease progresses with further destruction of the tooth's supporting structures (Figure 1.1) such as the alveolar bone (Pihlstrom et al., 2005).

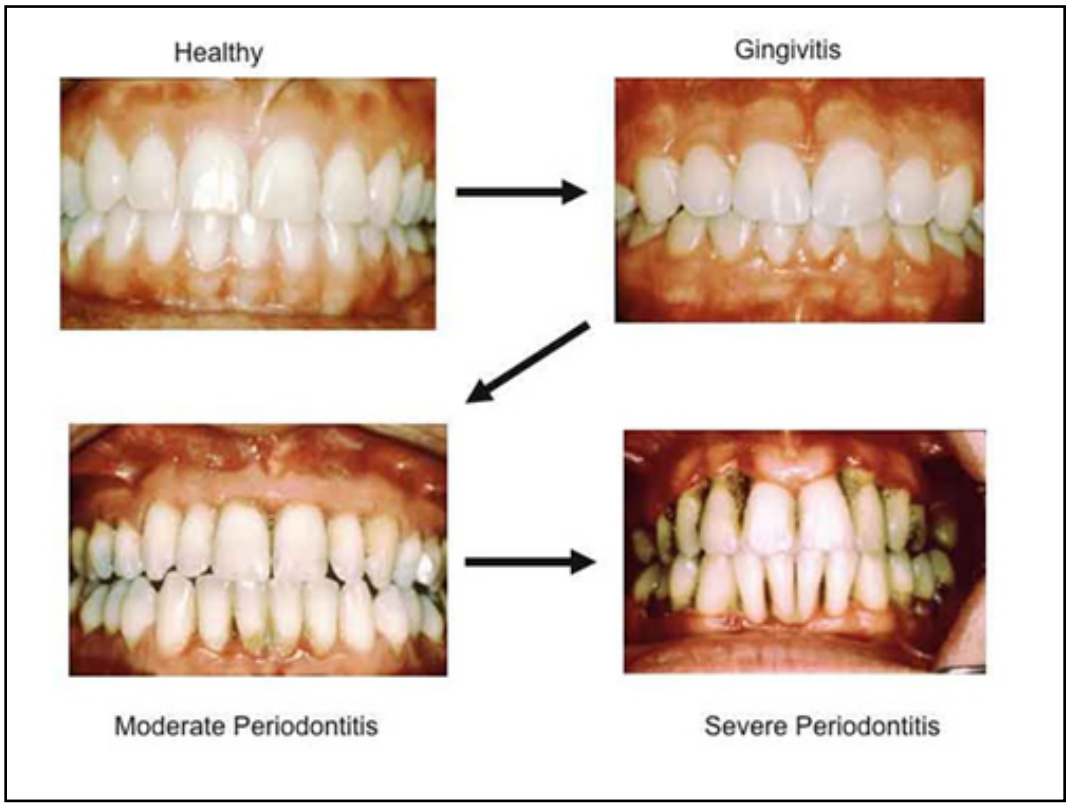


Figure 1.1 Progression of periodontal diseases (Bingham, 2010)

The depth of the pocket indicates an inflammatory response that results in the swelling of gingival tissues at the top of the pocket and the loss of collagen attachment of the tooth to the alveolar bone at the base of the pocket. Pockets can extend from 4 to 12 mm in depth and can harbour from 10^7 to 10^9 bacterial cells (Loesche and Grossman, 2001). Destruction of the tooth's supporting structures results in loosening of the tooth, occasional pain and discomfort and eventual tooth loss (Samaranayake, 2002, Southard and Godowski, 1998, Fosse et al., 2002, Pihlstrom et al., 2005).

Chronic periodontitis occurs mostly in adults as a slowly progressive chronic disease which is very common amongst the general population affecting about 70% - 80% of all adults (Marsh and Martin, 1999, Nisengard and Newman, 1994). Periodontitis with slight to moderate destruction is characterized by loss of up to one third of the teeth's supporting tissues and probing depths of up to 6mm with clinical attachment loss of up to 4 mm. The disease may be localized, involving one area of a tooth's attachment or it may be generalized, involving several teeth or the entire dentition. Advanced destruction of the teeth's periodontal tissues, periodontal probing depths greater than 6 mm with attachment loss greater than 4 mm, and radiographic evidence of bone loss and tooth mobility are signs of an advanced level of chronic periodontitis (Armitage, 1999, Loesche and Grossman, 2001). This disease is more prevalent in developing countries, and it has been found that prevalence and severity increase with age (Pihlstrom et al., 2005).

Chronic periodontitis results in the inflammation of the peridontium which then releases inflammatory cytokines, lipopolysaccharides, bacterial products and bacteria

into the systemic circulation. The presence of these products, bacteria and immune cells promotes atherosclerosis and affects blood coagulation and the function of platelets, which all in all contributes to the onset of a stroke (Li et al, 2000). A number of proposed mechanisms exist in which oral anaerobic bacteria may trigger pathways leading to cardiovascular disease. Oral anaerobes can be distributed to distant sites of the body especially in immuno-compromised patients such as those that are suffering from diabetes, malignancies or rheumatoid arthritis (Li et al, 2000). Diabetes mellitus is due to an absolute or relative deficiency of insulin. This syndrome is a risk factor for severe periodontal disease, and severe periodontitis often coexists with diabetes (Li et al, 2000).

1.2 Causative organisms

The microflora of the mouth consists of more than seven hundred different aerobic and anaerobic bacteria which exist in the form of dental plaque (Legg and Wilson, 1990). The oral cavity represents a perfect example of microbial ecology. Below the gum line, the number of bacteria ranges from 1×10^3 in a healthy shallow crevice to more than 1×10^8 in a periodontal pocket (Nisengard and Newman, 1994).

Normally bacteria in the oral cavity coexist mutually, but under certain conditions which favour some putative pathogens over other species, periodontal diseases are initiated (Mayrand and Grenier, 1998). As dental plaque matures to a state that is associated with periodontal disease, and increasing severity of the disease the prevalence and concentration of gram negative and anaerobic bacteria increases (Pihlstrom et al., 2005). Anaerobic bacteria have long been recognised as the

microorganisms that cause gingivitis and periodontitis and many of these bacteria are responsible for the initiation and progression of periodontal disease as are gram negative species (Legg and Wilson, 1990, Kim et al., 2011).

Oral bacterial species exist in microbial complexes in supragingival and subgingival plaque, by growing in these complexes oral bacteria are able to express resistance to the host's immune system and antimicrobial agents, therefore the purpose of these microbial complexes is to promote growth and survival of oral bacteria (Socransky et al., 1998, Haffajee et al., 2008). The different microbial complexes have been associated with the sequence of colonization of the oral bacteria as well as periodontal disease severity (Holt and Ebersole, 2005).

The microbial complex that is affiliated with periodontal diseases is known as the "red complex", this complex includes putative periodontal pathogens such as *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* (Socransky et al., 1998*).* Oral anaerobic bacteria species of the red complex appear later in biofilm development in the periodontal pocket. The red complex oral anaerobes are associated with clinical periodontal symptoms such as; bleeding upon probing of diseased sites, deep periodontal pockets and advanced lesions (Holt and Ebersole, 2005).

The second microbial complex that has been observed in subgingival and supragingival plaque of patients diagnosed with chronic periodontitis is known as the "orange complex ". Members of the orange complex are *Fusobacterium nucleatum, Prevotella intermedia, Campylobacter rectus, Prevotella nigrescens,*

Peptostreptococcus micros and *Eubacterium nodatum* (Socransky et al., 1998). Oral anaerobes of the orange complex are associated with the gingival redness, bleeding upon probing of the periodontal pocket and deeper peridontal pockets. These bacterial species are also associated with the red microbial complex (Haffajee et al., 2008).
Deeper periodontal pockets harbour more plaque containing red and orange complex bacterial species. These pockets produce more gingival crevicular fluid thus providing essential nutrients for the orange and red complex bacterial species (Haffajee et al., 2008).

Other oral anaerobes that have been associated with periodontal disease include *Capnocytophaga gingivalis, Bacteroides capillosus, Prevotella spp, Bacteroides ureolyticus, Eikenella corrodens* and *Veillonella spp* (Savitt and Socransky, 1984). *Veillonella* species which are normally found in the human intestinal and respiratory tract have been isolated from human dental plaque in patients with periodontal disease (Nisengard and Newman, 1994). Many of these bacteria derive some of their nutrients from the gingival crevicular fluid, which is a tissue transudate that seeps into the periodontal area (Loesche and Grossman, 2001).

Infection of tissue with these and other organisms is usually accompanied by the release of bacterial leucotoxins, fibrolysins, endotoxins and proteases which damage the gingival tissues and trigger host cell populations to express hydrolytic enzymes, and evoke both antibody mediated and cell-mediated immune responses.
These immune responses are usually protective, but a sustained microbial challenge and immune response results in the breakdown of tissues (Nisengard and Newman, 1994, Mayrand and Grenier, 1998).

1.3 Pathogenesis

Both the host and oral bacteria in the periodontal biofilm play a role in damage of the tissue by release of proteolytic enzymes that recruit polymorphonuclear leucocytes into the tissues (Nisengard and Newman, 1994, Pihlstrom et al., 2005). The neutrophils, lymphocytes, plasma cells and macrophages vary in number depending on the disease status of the tissue (Nisengard and Newman, 1994). Several components of the host's immune system are active in the pathogenesis of periodontal diseases and these immune responses may be beneficial or destructive.

1.3.1 Host immune response

The immune response removes bacterial products such as antigens and enzymes that have penetrated the tissue, it also prevents bacterial growth (Loesche and Grossman, 2001). These responses are usually protective, but a sustained microbial challenge and presence of effector molecules released by resident and migrating cells together with inflammatory mediators results in the breakdown of both soft and hard tissue, mediated by cytokine and prostanoid cascades (Pihlstrom et al., 2005, Bartold et al., 2010). Both hypo-responsiveness and hyper-responsiveness of certain pathways that form part of the host inflammatory response result in tissue destruction (Bartold et al., 2010).

Bacterial antigens can penetrate the crevicular epithelium and evoke both humoral antibody-mediated and cell-mediated immune responses (Pihlstrom et al., 2005).

Prostaglandins and cytokines generated during the inflammatory response can stimulate bone resorption (Marsh and Martin, 1999). In chronic periodontitis osteoclast activity is enhanced without a corresponding increase in bone formation,

which results in inflammatory-mediated bone loss. Osteoclasts are multinucleated cells that are responsible for bone resorption, these cells have been shown to resorb alveolar bone in periodontal disease studies (Bartold et al., 2010).

1.3.2 Bacterial pathogenesis

Periodontal pathogens possess numerous mechanisms that permit them to directly damage the periodontium or indirectly compromise the host response (Nisengard and Newman, 1994). These include factors influencing bacterial colonization, bacterial adhesion, coaggregation, proliferation, interbacteria relationships and host factors and tissue destruction (Marsh and Martin, 1999). *P. gingivalis* produces a number of factors that can be associated with virulence including fimbriae, collagenase, lipopolysaccharide, endotoxins, toxic proteases and a capsular polysaccharide which provides resistance to host defenses such as antibodies and inhibition of phagocytosis by the hosts immune cells (Nisengard and Newman, 1994). The collegenase produced by *P. gingivalis* degrades fibrogen, and another protease called thiol-proteinase contributes to the degradation of the collagenous periodontal ligament that connects teeth to alveolar bone (Marsh and Martin, 1999). *P. endodontalis* produces type IV collagen which may contribute to the pathogenesis of endodontic infections. *P. intermedia* and *P. gingivalis* possess the ability to destroy immunoglobulins and complement components (Nisengard and Newman, 1994).

F. nucleatum along with members of the red complex secrete serine proteases. These proteases degrade elements of the periodontal connective tissue and host defense systems. The 65 kDa *F. nucleatum* protease was found to degrade extracellular matrix

proteins and is thought to play a role in both the nutrition and pathogenicity of periodontal pathogens. The breakdown of the extracellular matrix proteins may contribute to the damage of periodontal tissues (Signat et al., 2011). *T. denticola* is an oral spirochete that is resistant to human β-defensins. Defensins interact strongly with lipopolysaccharides (LPS) due to the negative charge of LPS.

These bacteria lack a traditional LPS which numerous gram negative bacteria posses, therefore β-defensins cannot interact with the LPS of this oral spirochete. This resistance confers a survival advantage allowing it to survive in the periodontal pocket (Brissette and Lukehart, 2002). Another bacterial enzyme known as phospholipase A may initiate alveolar bone resorption as a precursor for prostaglandin. The combination of the direct effects of the bacteria on the periodontal tissues and indirect effects achieved by influencing host responses both influence the responses of the periodontium to the periodontal pathogens (Nisengard and Newman, 1994). Once a periodontal pocket has formed and the pocket is full of periodontal pathogens and there is no adequate treatment active periodontitis commences. This leads to loss of the tooth's supporting structures and will eventually lead to tooth loss (Pihlstrom et al., 2005).

1.4 Treatment of periodontal disease

The main aim of periodontal therapy is to control the infection by reducing the number of bacteria which are in the form of dental plaque in the periodontal pocket. The rationale of treatment depends upon the identification of as many environmental and host factors as possible (Nisengard and Newman, 1994).

Treatment includes implementing oral hygiene measures and antibiotic therapy (Nisengard and Newman, 1994).

1.4.1 Oral hygiene measures

Oral hygiene involves mechanical procedures such as scaling and root planning that remove subgingival calculus, reducing the infection in shallow to medium depth pockets. Patient home care which involves brushing, use of antimicrobial mouth rinses and flossing regularly can maintain the health of the pocket. Chlorhexidine di-gluconate mouth rinse is considered the most effective antimicrobial compound for oral use. Chlorhexidine has the advantage of inhibiting the development of plaque and gingivitis (Loe, 2000). Cetylpyridinium chloride is a quaternary ammonium compound that is used in some mouthwashes, this compound has demonstrated a moderate degree of efficacy as an antiplaque agent and in the reduction of gingivitis (Santos et al., 2004).

A clinical-trial done by Santos et al (2004) evaluated the short-term clinical and microbiological efficacy of 0.05% chlorhexidine and cetylpyridinium chloride used as an adjunctive oral-hygiene method for patients with periodontitis. They found that the plaque levels and the total subgingival anaerobic microflora had been reduced significantly in patients who used the mouth rinse. Although chlorhexidine and cetylpyridinium chloride are effective in decreasing the number of periodontal pathogens they have undesirable side effects such as staining of the teeth, and irritation of soft tissue (Loe, 2000, Santos et al., 2004)

Stannous fluoride is used in toothpastes and oral mouth rinses. In a study in 1985 it was noted that a single subgingival application of stannous fluoride reduced the amount of black pigmented gram negative anaerobic bacteria but had little effect in reducing the total bacterial count (Schmid et al., 1985). There are few investigations on the effect of fluoride in periodontics although it is effective in controlling gingivitis by reducing plaque accumulation (Brecx et al., 1990, Paine et al., 1998).

Oral hygiene has the advantage of being a localized method of removal of the pathogenic bacteria, but does not always eliminate all the bacteria due to their presence within the periodontal tissues, or in the presence of deeper pockets their inaccessibility to the instrumentation, therefore the numbers of bacteria remain relatively constant in these deep pockets (Southard and Godowski, 1998, Loesche and Grossman, 2001). Antibiotics are frequently prescribed for patients with periodontitis usually as adjuncts to conventional mechanical treatment. Serrano et al. (2011) demonstrated that systemic antibiotics significantly improved the clinical outcome of periodontal therapy.

1.4.2 Antibiotics

β-lactam antibiotics (Figure 1.2) are the most widely used group of antibiotics for treating periodontal conditions because of their suitable antimicrobial spectrum, bactericidal activity, low incidence of adverse effects and cost effectiveness (Wilke et al., 2005, Ioannidis et al., 2009, Iwahara et al., 2006). They are classified together as a result of their common core structure which is the β-lactam ring and are separated on the basis of another ring structure bound to the β-lactam ring (Wilke et al., 2005,

Williams, 1999). These antibiotics also have structural similarities with the binding sites of the bacterial substrates which enable them to attach to and inactivate the transpeptidases involved in the synthesis of the bacterial cell wall (Williams, 1999).

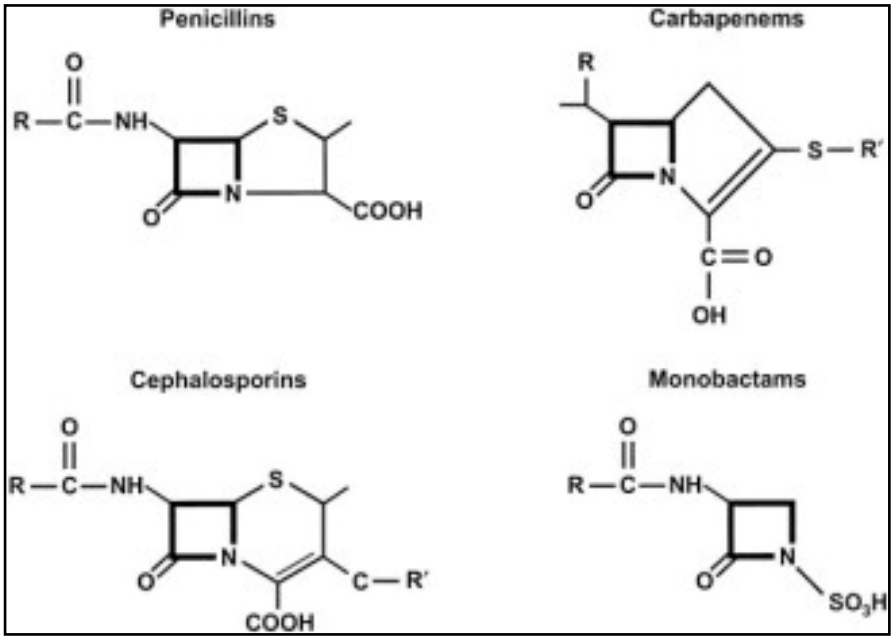


Figure 1.2 Chemical structure of β-lactam antibiotics (Lilly et al,. 2002)

Tetracyclines are also used in the treatment of periodontal diseases. These antibiotics inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit of bacteria and preventing access of aminoacyl tRNA to the acceptor site on the mRNA-ribosome complex. This results in the disruption of the formation of the initiation complex required for amino acid protein synthesis (Soares et al., 2012). Tetracyclines have the advantage of being able to inhibit collagenase therefore inhibiting tissue breakdown in periodontal disease. However bacteria have developed resistance to tetracycline over the years and use various mechanisms to resist the antimicrobial agents.

These strategies include (i) limitation of access of tetracycline to the target site, (ii) alteration of the ribosome to prevent binding of the antibiotic, and (iii) producing tetracycline inactivating enzymes (Soares et al., 2012, Ramos M M et al., 2009).

The most common tetracycline resistance genes that have been found to confer resistance to gram negative periodontal pathogens are tet(M) and tet(Q) (Lacroix and Walker, 1996, Ioannidis et al., 2009). Other antibiotics used in the treatment of chronic periodontitis include metronidazole, clindamycin, and doxycycline (Kapoor et al., 2012).

Studies have shown that periodontal microorganisms in patients with chronic periodontitis can be resistant to the antibiotics that are commonly used including β-lactam antibiotics (Ardila et al., 2010, Handal and Olsen, 2002, Iwahara et al., 2006, Ramos M M et al., 2009, Wilke et al., 2005). Various studies have also shown an increase in the levels of resistance to tetracycline antibiotics over the years in patients with periodontal diseases (Fiehn and Westergaard, 1990, Kornman and Karl, 1982, Abu Fanas et al., 1991).

1.5 Drug resistance

Antimicrobial resistance has become a widespread phenomenon compromising the efficacy of antibiotics. The main reason for the rapid growth in resistance can be attributed to the misuse of antibiotics in a region (Ardila et al., 2010, Ioannidis et al., 2009). Studies have indicated that antibiotic misuse and overuse affect the commensals and pathogenic bacteria, which could result in the commensals serving as reservoirs of antibiotic resistance determinants for the pathogens (Wilke et al., 2005,

Kim et al., 2011). Bacterial resistance to these antibiotics has been extensively described and attributed to resistance genes (Ioannidis et al., 2009). Antimicrobial resistance can be classified into three groups: intrinsic, mutational and acquired resistance.

Intrinsic resistance is innate resistance to antibiotics that occurs naturally in microorganisms. Mutational resistance is due to mutations in the chromosome of bacterial species. Upon reproduction of the microorganisms the progeny produced will be genetically altered and result in bacterial populations that are resistant to antimicrobial agents. Acquired resistance occurs when a microorganism acquires genes that code for antibiotic resistance from another microorganism (Soares et al., 2012).

Resistance to β-lactam antibiotics arises through several mechanisms such as: (i) modification of the penicillin-binding protein which may occur through the mutations in the chromosomal genes encoding the enzymes which is known as intrinsic resistance. Another strategy is through the acquisition of foreign homologous genes or fragments of genes from related species encoding new penicillin-binding-proteins (Gjermo et al., 2002, Wilke et al., 2005). (ii) Decreased access of the antibiotic to the targets in the bacterial cell by reduced permeability of the outer-membrane of the pathogenic bacteria, this mechanism is observed in gram negative bacteria due to the composition and structure of the cell wall of these microorganisms. The outer membrane of gram negative bacteria functions as an impenetrable barrier to some antibiotics, however some β-lactam antibiotics such as ampicillin and amoxicillin are small enough to penetrate through porin pores of the microbes (Soares et al., 2012).

(iii) The final resistance mechanism is inactivation of the antibiotic by bacterial production of inactivating destructive enzymes (Figure 1.3) known as β-lactamases (Handal and Olsen, 2000, Williams, 1999).

Kim et al. (2011) suggested that horizontal gene transfer of resistance determinants can occur in the oral biofilm, therefore exchange of mobile genetic elements between commensals pathogenic bacteria can contribute to the emergence of drug resistance in the oral cavity. A study by Tribble et al. (2007) demonstrated that *Porphyromonas gingivalis* is capable of conjugal transfer of chromosomal and plasmid DNA which provide a useful way to transfer resistance genes. The most frequent and most efficient mechanism of resistance to β-lactam antibiotics is the production of β-lactamase enzymes which have been found in a variety of putative periodontal anaerobic bacteria such as *Prevotella spp* and *Fusobacterium spp* (Wilke et al., 2005, Iwahara et al., 2006, Williams, 1999).

1.6 β-lactamase enzymes

β-lactamase enzymes are the major cause of bacterial resistance to β-lactam antibiotics (Bush et al., 1995). These enzymes are commonly detected in diseased periodontal sites and have been proven to be positively correlated with increased periodontal pocket depth (Soares et al., 2012). Number of different types of β-lactamase enzymes have been isolated and characterized. They have been organized into four classes (A to D) on the basis of their sequence similarities and biochemical characteristics (Williams, 1999, Wilke et al., 2005).

These destructive enzymes are widespread amongst gram-negative and gram-positive bacteria (Handal and Olsen, 2000, Wilke et al., 2005, Brook, 2009). In the oral cavity containing a mixed population of both gram-negative and gram-positive bacteria, β-lactamase enzymes are generally excreted into the environment and confer protection to the microorganisms producing the enzyme and non- β-lactamase producers present at the site of infection (Herrera et al., 2000, Brook, 2009).These enzymes are important in gram negative bacteria as they are the major defense mechanism of these pathogens against β-lactam antibiotics (Wilke et al., 2005). The outer-membrane of the gram negative pathogens forms a permeable barrier that limits the entry of the β-lactam compounds into the cell. Decreased permeability in concert with production of β-lactamases confers maximal protection of the microbes from β-lactam antibiotics (Handal and Olsen, 2000).

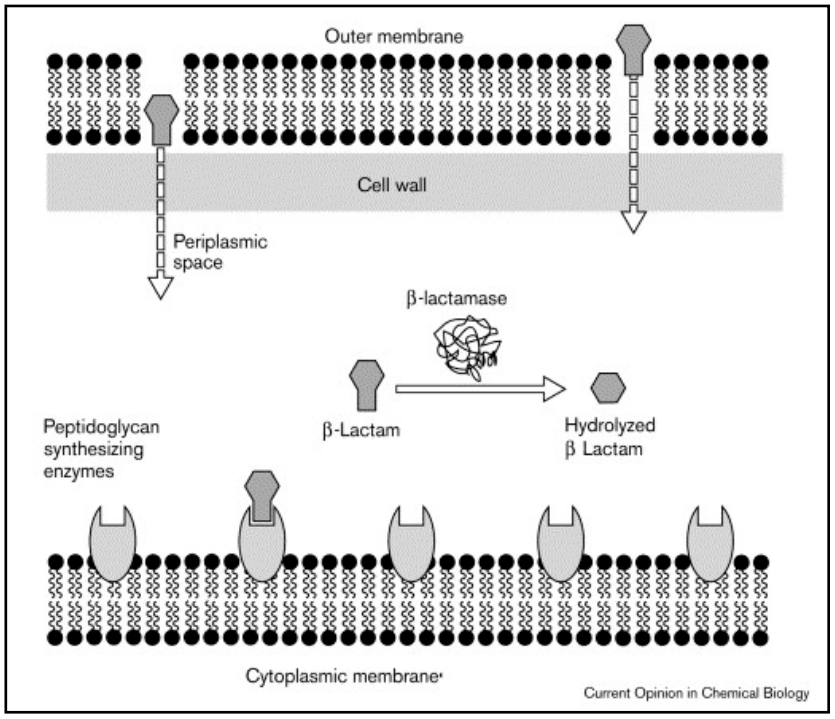


Figure 1.3 Activity of β-lactam antibiotics and β-lactamases in Gram negative bacteria (Wang et al,. 1999)

β-lactamases are almost ubiquitous in bacteria, when produced in small quantities, many contribute little to antibiotic resistance and may play a physiological role in peptidoglycan metabolism (Livermore, 1993, Medeiros, 1997). They have been detected in dark pigmented *Prevotella* species and *Capnocytophaga* species in patients diagnosed with chronic periodontitis (van Winkelhoff et al., 1997). The β-lactamase enzymes produced by *Prevotella* strains have the properties of the class A group of β-lactamases, which hydrolyze most penicillins (Iwahara et al., 2006). These enzymes catalyze the hydrolysis of the β-lactam ring of the antibiotics which results in the splitting of the amide bond. This then results in the production of inactive products and the antibiotic can no longer inhibit bacterial cell wall synthesis (van Winkelhoff et al., 1997, Williams, 1999, Handal and Olsen, 2000).

Studies have demonstrated that the most common β-lactamase producing oral anaerobic bacteria belong to the genus *Prevotella*, *Fusobacterium*, *Capnocytophaga* and *Veillonella* (van Winkelhoff et al., 1997, Handal et al., 2005, Patel, 2011). A high prevalence of β-lactamase producing oral anaerobic bacteria has been reported in different countries. In the Spanish population the prevalence was 87%, in Dutch population 73%, in France 53% whereas in South African patients 69% (van Winkelhoff et al., 1997, Fosse et al., 1999, Herrera et al., 2000, Patel, 2011).

1.7 β-lactamase inhibitors

Strategies have been implemented to inhibit β-lactamase resistance to β-lactam antibiotics, these strategies include modification of the antibiotic structure so that it is no longer a substrate for the enzyme and inhibition of the β-lactamase enzyme using a

compound known as a β-lactamase inhibitor (Williams, 1999, Handal and Olsen, 2000). β-lactamase inhibitors are structurally related to penicillin as they consist of the amide bond of the β-lactam group of antibiotics, but they have a modified side chain. These structural features enable the inhibitors to bind irreversibly to β-lactamases and inactivate the enzymes (Handal and Olsen, 2000). β-lactamases often exhibit a high affinity for those compounds, and the success or failure of the compounds depends on their ability to inactivate clinically important β-lactamases (Livermore, 1993, Handal and Olsen, 2000).

The combination of a β-lactamase inhibitor with a substrate β-lactam antibiotic can prove a useful treatment option, as the β-lactamase inhibitor restores the activity of the antibiotic (Livermore, 1993, Williams, 1999). Clavulanic acid is an example of these inhibitors and it is usually administered in combination with amoxicillin forming a compound known as Augmentin ® (Handal and Olsen, 2000). Susceptibility tests including clavulanic acid are considered reliable, since all of the TEM- and SHV- derived β-lactamases are inhibited by clavulanic acid (Handal and Olsen, 2000). Amoxicillin-clavulanic acid is amongst the most widely used agent for treating periodontal diseases (Syed and Loesche, 1972).

1.8 β-lactamase genes

β-lactamase enzymes are encoded by chromosomal DNA or plasmid DNA. Chromosomal DNA is relatively stable and chromosomal β-lactamases are universal in a specific bacterial species. The spread of β-lactamase genes has been attributed to

their integration within mobile genetic elements such as plasmids or transposons which carry the genes and facilitate the transfer of genetic material between microbes. This mobility is important as it allows for the spread of resistance genes through several bacterial communities (Williams, 1999, Wilke et al., 2005). Studies of these transposons suggest that they play a significant role in the spread of drug resistance (Arzese et al., 2000).

Plasmid β-lactamases are present in many species of gram negative bacteria, and the most common of these β-lactamases is the TEM-type enzyme. A study by Lacroix and Walker (1992) found a strain of *Eikenella corrodens* isolated from a periodontal pocket, and containing the *TEM-1* β-lactamase gene in association with a streptomycin resistance gene. They found that the sequence of this β-lactamase gene had one nucleotide difference with the β-lactamase gene carried on transposon Tn3 (Handal et al., 2005, Lacroix and Walker, 1992).

Another mechanism used in the circulation of resistance genes involves integrons. These genetic elements consist of an integrase gene with adjacent gene cassettes that commonly contain antibiotic resistance genes. Integrons have been identified carrying genes for β-lactamases of Ambler classes A, B, and D (Handal et al., 2005).

Gram negative bacteria such as most periodontal pathogens can synthesize all four classes of β-lactamases, and expression of the genes is either constitutive or inducible (Handal and Olsen, 2000). Constitutive production of genes is when they are continuously expressed as a resistance mechanism, whereas when genes are only induced to produce their products by exposure to a challenging substance they are termed as inducible genes (Handal and Olsen, 2000). Once expressed β-lactamase

enzymes are secreted into the periplasmic space in gram negative bacteria (Wilke et al., 2005).

β-lactam resistance is also associated with resistance to tetracycline by production of *tet* and *erm* genes which results in resistance to erythromycin (Handal et al., 2005). A high prevalence of *tetM, tetQ* and bla_{TEM} genes in the subgingival plaque and tongue of patients with periodontitis has been noted (Ioannidis et al., 2009) but resistance due to the enzymes in these patients was not established. Although the genetic basis of β-lactamase production by oral anaerobic bacteria has not been clarified, bla_{CfxA} genes are known to be present in these organisms.

1.8.1 *Bla*$_{\text{CfxA}}$ genes

Bla$_{\text{CfxA}}$ *(CfxA)* genes are highly prevalent in *Prevotella* species and *Capnocytophaga* species isolated from periodontal pockets (Fosse et al., 2002, Handal et al., 2005). Horizontal gene transfer might explain the spread of closely related gene sequences among these periodontal species (García et al., 2008). *CfxA* has also been shown to transfer among *Bacteroides* strains, transference amongst this species has been found to be associated with the conjugative transposon Tn 4555 (García et al., 2008).

A study by Fosse et al (2002) identified the *CepA/cblA* β-lactamase gene in a *Prevotella bivia* strain isolated from a periodontal pocket. This gene belongs to the main β-lactamase resistance gene families (bla_{TEM}, bla_{OXA}, bla_{AmpC}, bla_{CfxA}, and $bla_{\text{CepA/}cblA}$), and is commonly associated with *Bacteroides fragilis* (Fosse et al., 2002). They proposed further studies on the eventuality of a simultaneous carriage of *CepA/CblA* and *CfxA* on the same chromosomal transposon.

CfxA and CfxA2 genes have been isolated from oral infection sites as well as from the causative organisms isolated from these infection sites which suggests that these genes are responsible for the production of β-lactamases (Fosse et al., 2002, Iwahara et al., 2006). Giraud-Morin et al. (2003) suggested that the *CfxA/CfxA2* partition could be partly related to the genus and partly to the geographical origin of the enzyme-producing strains because *CfxA* gene predominated in North America whereas *CfxA2* predominated in France (Parker and Smith, 1993, Madinier et al., 2001).Whereas a study in the United Kingdom showed the presence of both the genes present in *Prevotella* species (Iwahara et al., 2006). However not much is known about β-lactamase-producing bacteria in South Africa.

1.9 Aim

The purpose of this study was to investigate the prevalence of β-lactamase-producing anaerobic oral bacteria in the oral cavities of South African patients suffering with periodontitis and identify the genes responsible for this enzyme production.

1.10 Objectives

1. To isolate and identify β-lactamase producing oral anaerobic bacteria from the periodontal pocket debris
2. To determine the prevalence of β-lactamase producing oral anaerobic bacteria
3. To analyse the antimicrobial sensitivity profile of β-lactamase-producing oral anaerobic bacteria
4. To determine the most prevalent gene/s responsible for β-lactamase production in oral anaerobic bacteria in this population.

Chapter 2 Materials and Methods

2.1 Study population

This study was conducted at the Oral and Dental teaching Hospital of the University of the Witwatersrand, Johannesburg.

Sample size estimation for the confidence interval around a proportion was done using the formula:

$$N = \frac{4 z_{crit}^2 \, p(1-p)}{D^2}$$ (Eng, 2003)

The parameters used for the sample size estimation are as follows:
Confidence level 95% and Confidence width 0.30
Proportion estimate from a previous study by Patel (2011) is 0.31.

Based on this sample size calculation, at least 37 patients were supposed to be included which was increased to 48 in case of laboratory accidents. Bacterial samples were obtained from a total of forty eight patients diagnosed with chronic periodontitis. Patients diagnosed with severe to moderate forms of chronic periodontitis (Figure 2.1) and with pocket depths of more than five millimeters (≥ 5 mm) were asked to participate in the study. Ethics clearance was obtained from the Human Research Ethics Committee (certificate number: M 110112) and written consent was obtained from all the participants (Appendix 1.1, 1.2). Patients with a history of previous periodontal treatment, necrotizing ulcerative gingivitis, diabetes or those that had consumed systemic antimicrobials or anti-inflammatory drugs four weeks prior to the study, were excluded from participating.

2.2 Sample collection

Pocket depths were measured using periodontal probes (Figure 2.1) and the two deepest periodontal pockets in the oral cavity were selected for microbiological sampling. Samples were collected over a time period of 7 months by clinicians in the presence of the investigator. After careful removal of supragingival plaque and isolation of samples with cotton rolls, a fine sterile paper point (DiaDent, Diamond Dental Industries) was inserted into the pocket (subgingival area) and left in place for ten seconds. Paper points from the two selected sites were pooled in one milliliter of reduced transport fluid (Syed and Loesche, 1972) and processed within an hour of sampling to ensure the viability of anaerobic bacteria. The laboratory procedure is depicted in a flow diagram in Chapter 7, Appendix 1.3.

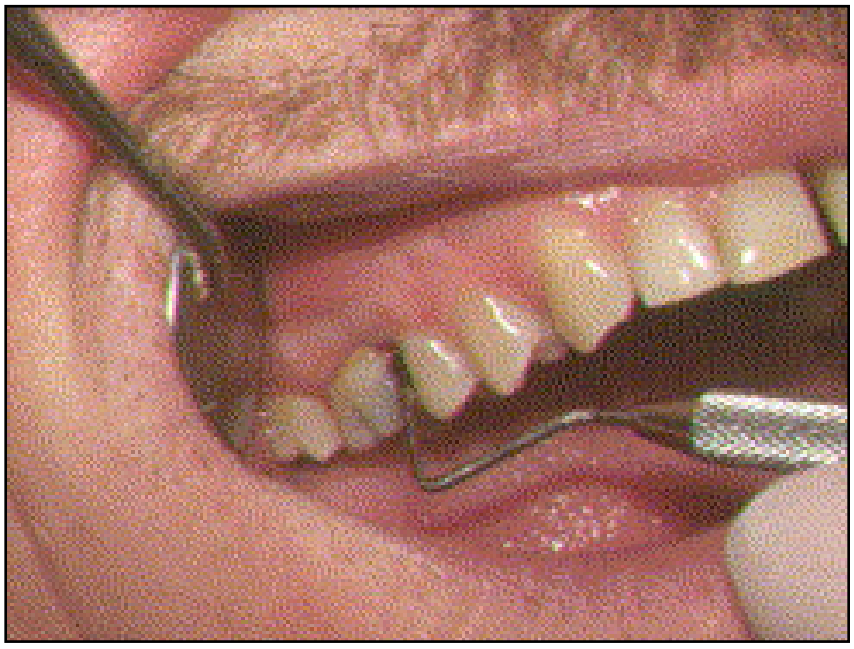


Figure 2.1 Probe in periodontal pocket (Raffetto, 2004)

2.3 Isolation of bacteria

Samples were vortexed for thirty seconds using the Vortex Genie 2 (Lasec | SA, South Africa). Three serial ten fold dilutions were prepared using 900 µl of phosphate buffered saline. A 100 µl of 10^{-2}, 10^{-3}, 10^{-4} dilutions containing the sample was spread on non-selective blood agar plates supplemented with 5mg/l of haemin (Sigma-Aldrich, South Africa) and 1 mg/l of menadione (Sigma-Aldrich, South Africa) for the enumeration of total anaerobic bacteria.

To determine the proportions of subgingival microflora resistant to amoxicillin based on β-lactamase production of the anaerobic bacteria, a 100 µl of the appropriate dilution (10^{-1} and 10^{-2}) containing the sample was spread onto blood agar plates enriched with haemin and menadione and supplemented with 3µg/ml of amoxicillin only (Smithkline Beecham). A 100 µl of the dilutions was also spread onto blood agar plates supplemented with 3µg/ml of amoxicillin and 0.75 µg/ml of clavulanic acid (Smithkline Beecham).

All the inoculated blood agar plates were incubated for one week at 37º C in a jar sealed with silicone, containing an anaerobic gaspak (Davies diagnostic, South Africa) and an anaerobic indicator strip (Becton, Dickson and Company, USA).
The number of colony forming units (cfu) was determined in each plate. Colonies that grew on amoxicillin supplemented plates but did not grow on amoxicillin-clavulanic acid supplemented plates were considered as presumptive producers of β-lactamase and were sub-cultured (further 7 days) onto non-selective blood agar plates under anaerobic and aerobic conditions to eliminate any facultative bacteria.

The blood agar plates sub-cultured to test for aerobic colonies were placed in an anaerobic jar and a candle was placed in the jar to create a carbon dioxide (CO_2) environment (Figure 2.2). The blood agar sub-cultured for anaerobic conditions were placed in a jar containing an anaerobic gaspak (Davies diagnostic, South Africa) and an anaerobic indicator strip (Becton, Dickson and Company, USA). Both jars were incubated at 37ºC for one week.

Figure 2.2 Anaerobic jar with anaerobic gaspak and an anaerobic indicator strip for anaerobic conditions. Candle jar for creating CO_2 conditions.

2.4 Identification of β-lactamase producing bacteria

Colonies that grew under anaerobic conditions only were considered as strict anaerobes and they were then further evaluated for β-lactamase production using the nitrocefin paper disc spot test (Figure 2.3), in which a filter paper disc (diameter 7cm) was placed in a petri dish and impregnated with nitrocefin solution (1 ml). An isolated colony was then applied to the impregnated paper with a loop; and if a pink to red reaction developed within 5 - 15 minutes, it indicated β-lactamase presence and was considered positive (Montgomery et al., 1979).

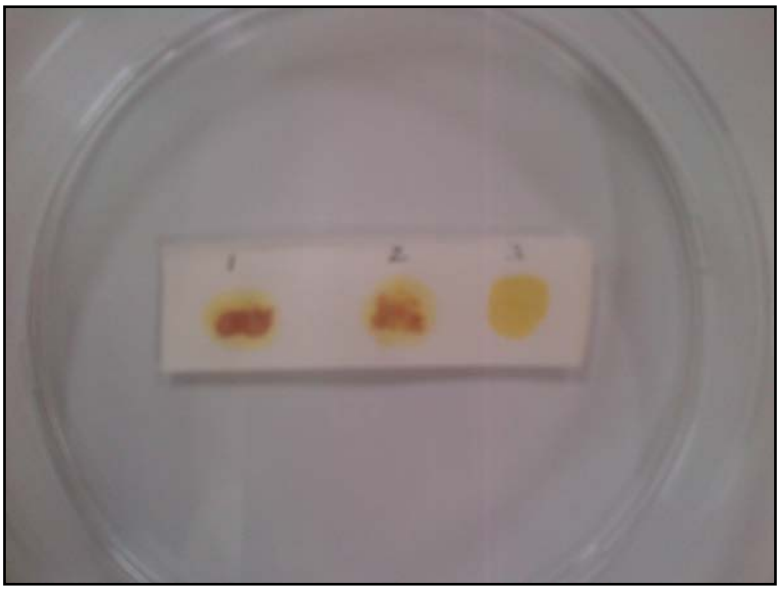


Figure 2.3 Nitrocefin Test in which bacterial cultures that are positive for β-lactamase production change the colour of the reagent from yellow to pinkish-red as seen in cultures 1 and 2. Bacteria that don't produce β-lactamase enzymes do not produce a colour change as seen with culture 3

Microbial colonies that had a positive result for the Nitrocefin Paper Disc Spot test were identified using the gram stain technique and API Rapid 32-A system (Biomérieux, La Balmes Les Grottes, France), which is a standardized system for identification of anaerobes. This system uses 29 miniaturized enzymatic tests and a database to identify anaerobic microorganisms. Test procedure was followed as recommended by the manufacturer. Microbial colonies harvested from blood agar were suspended in 2ml of sterile distilled water using a swab. Fifty five microlitres of the inoculum was dispensed into each cupule of the API strip (Figure 2.4). The URE Cupule (1.0) was covered with 2 drops of mineral oil, then the lid was placed on the strip, followed by incubation of the strip at 37 ºC for 4 - 4 ½ hours in aerobic conditions.

Figure 2.4 API Rapid ID 32 A strip

The following reagents were added to the applicable test's to reveal reactions:

- NIT test (cupule 0.0) : 1 drop of NIT 1 and NIT 2 reagents
- IND test (cupule 0.1) : 1 drop of James reagent
- PAL to SerA test's (cupules 0.2 to 0.E) : FB reagent (1 drop)

The reactions were read after 5 minutes according to the reading table (Table 2.1), and results recorded on the result sheet. Results were interpreted by coding them into a numerical profile (Figure 2.5 to 2.8) and identification of the microbial colony was obtained using the APIweb ™ database. Isolates were stored in Microbank™ vials (Davies Diagnostics, South Africa) and 2% skim milk and stored at – 70 º C for further research.

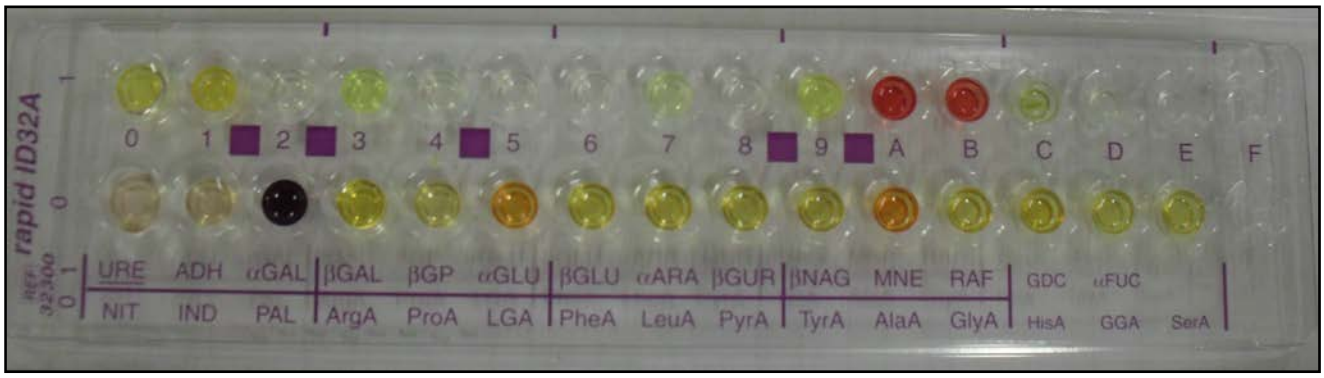


Figure 2.5 API Rapid 32A color changes of *Porphyromonas gingivalis*

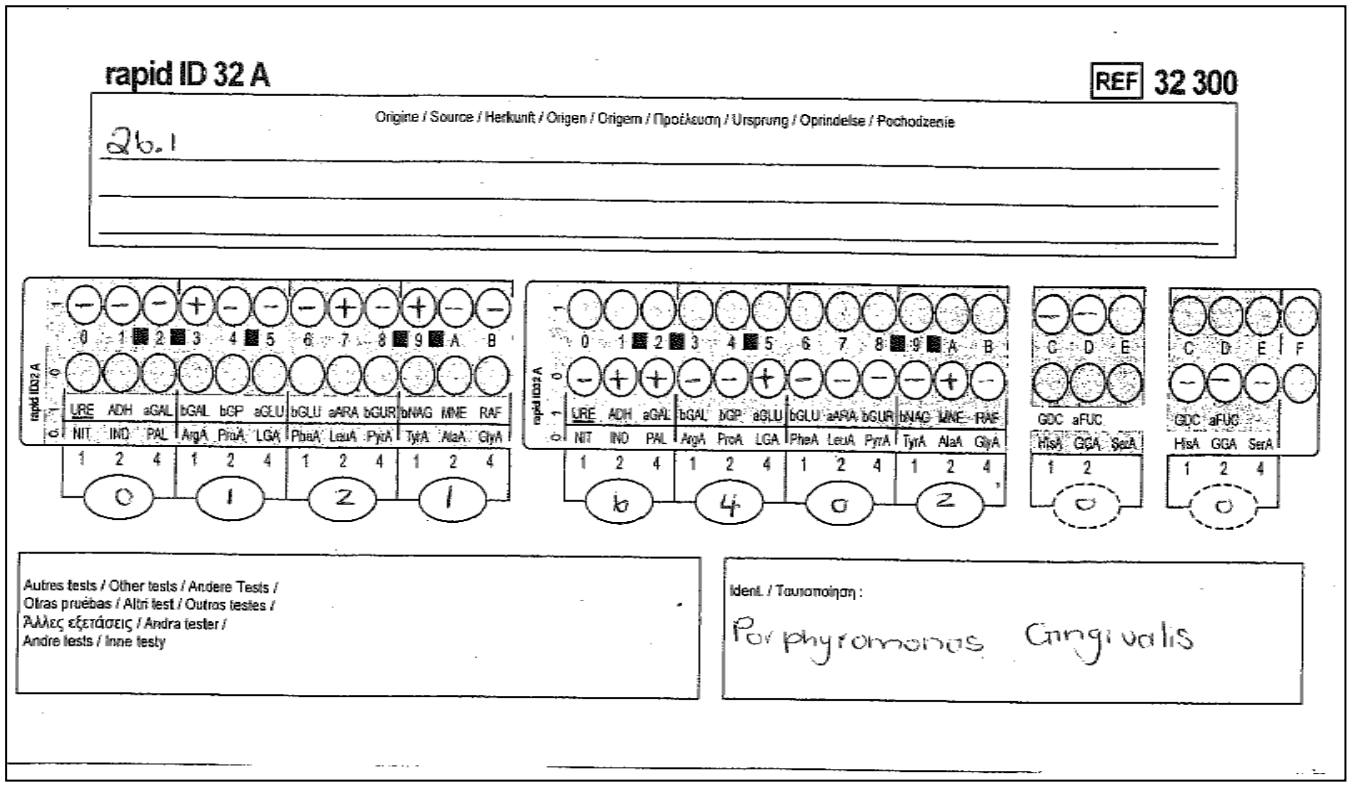


Figure 2.6 API Rapid 32 A numerical profile of *Porphyromonas gingivalis*

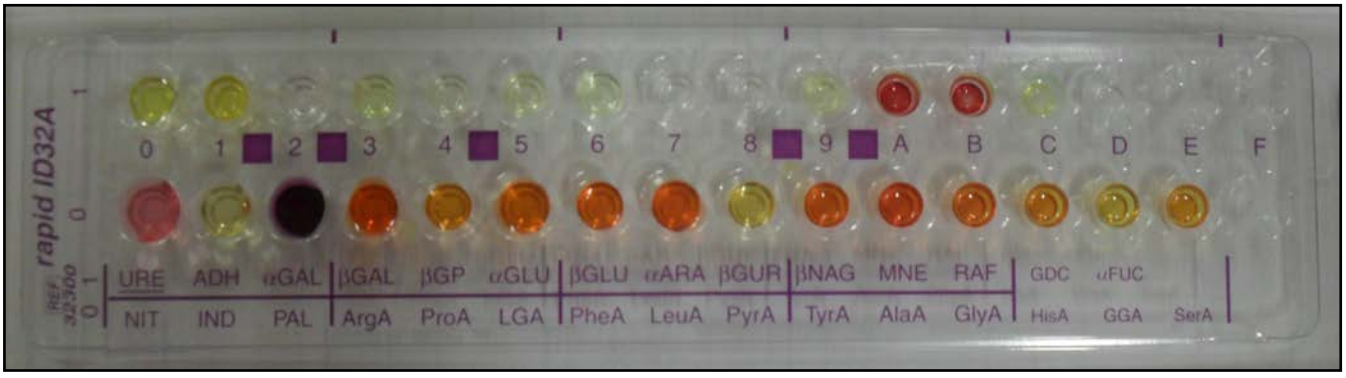


Figure 2.7 API Rapid 32 A colour change reaction of *Actinomyces meyeri*

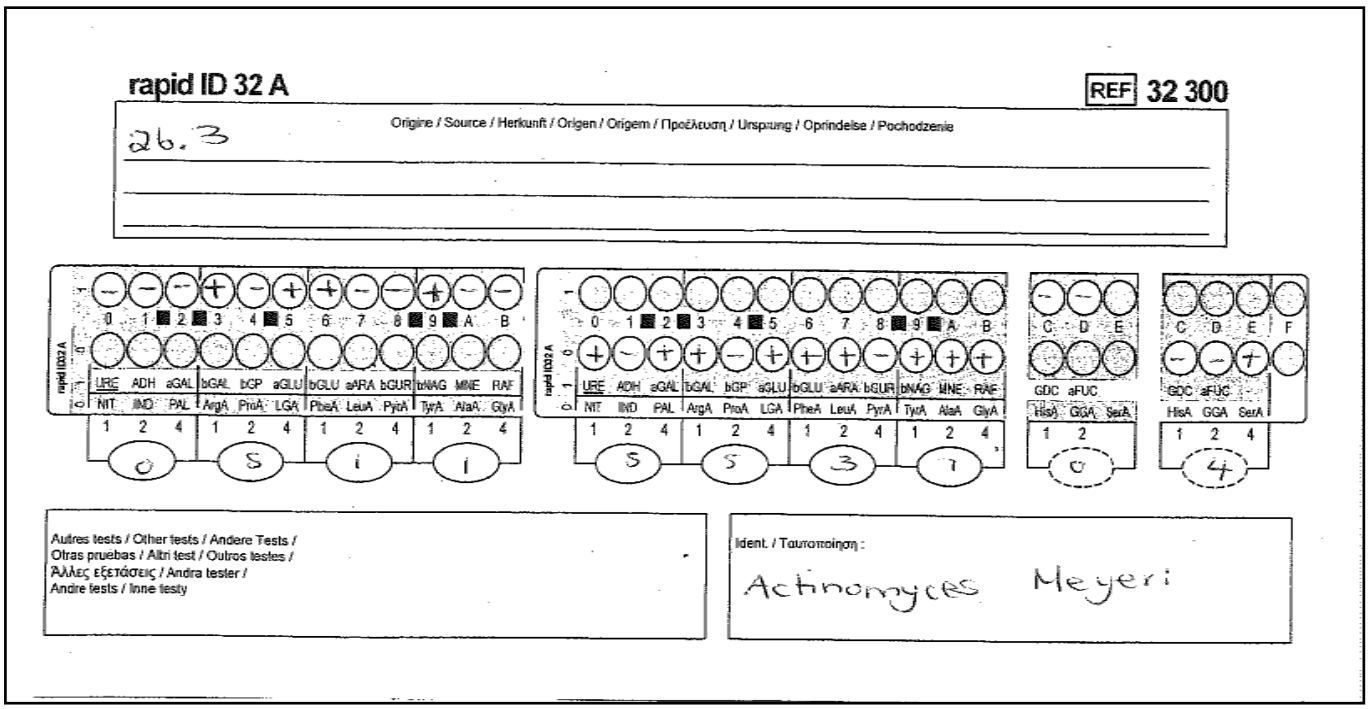


Figure 2.8 API Rapid 32 A numerial profile of *Actinomyces meyeri*

Table 2.1: Reading table for interpretation of the Rapid ID 32 A results (Biomérieux, La Balmes Les Grottes, France)

Cupule	Test	Active Ingredients	QTY (mg/cup.)	Reactions/ Enzymes	Result	
					Negative	Positive
1.0	URE	Urea	0.96	UREase	yellow	red
1.1	ADH	L-arginine	0.77	Arginine DiHydrolase		
1.2	αGAL	4-nitrophenyl-αD-galactopyranoside	0.026	α-GALactosidase	colorless	yellow
1.3	βGAL	4-nitrophenyl-βD-galactopyranoside	0.052	β-GALactosidase		
1.4	βGP	4-nitrophenyl-βD-galactopyranoside 6-phosphate-2CHA	0.034	β-GALactosidase 6 Phosphate		
1.5	αGLU	4-nitrophenyl-αD-glucopyranoside	0.026	α-GLUcosidase		
1.6	βGLU	4-nitrophenyl-βD-glucopyranoside	0.026	β-GLUcosidase		
1.7	αARA	4-nitrophenyl-αL-arabinofuropyranoside	0.024	α-ARAbinosidase		
1.8	βGUR	4-nitrophenyl-βD-glucuronide 4-nitrophenyl-N-acetyl-βD-glucosaminide	0.026	β-GlucURonidase		
1.9	βNAG		0.028	N-acetyl-β-Glucosaminidase		
1.A	MNE	D-mannose	056	MaNnosE fermentation	red	yellow-orange
1.B	RAF	D-raffinose	0.56	RAFfinose fermentation		
1.C	GDC	Glutamic acid	0.56	Glutamic acid DeCarboxylase	yellow-vert	blue
1.D	αFUC	4-nitrophenyl-αL-fucopyranoside	0.024	α-FUCosidase	colorless	yellow
0.0	NIT	Potassium nitrate	0.14	Reduction of NITrates	colorless	Red
0.1	IND	L-tryptophan	0.056	INDole production	colorless	pink
0.2	PAL	2-naphthyl-phosphate	0.04	Alkaline Phosphatase	colorless	purple
0.3	ArgA	L-arginine-β-naphythylamide	0.056	Arginine Arylamidase	colorless pale orange	orange
0.4	ProA	L-proline-β-naphythylamide	0.048	Proline Arylamidase		
0.5	LGA	L-leucyl-L-glycine- β-naphythylamide	0.052	Leucyl Glycine Arylamidase		
0.6	PheA	L-phenylalanine- β-naphythylamide	0.048	Phenyalanine Arylamidase		
0.7	LeuA	L-leucine- β-naphythylamide	0.052	Leucine Arylamidase		
0.8	PryA	Pyroglutamic acid β-naphythylamide	0.044	Pyroglutamic acid Arylamidase		
0.9	TryA	L-tyrosine- β-naphythylamide	0.052	Tyrosine Arylamidase		
0.A	AlaA	L-alanyl-L-alanin-β-naphythylamide	0.048	Alanine Arylamidase		
0.B	GlyA	L-glycine-β-naphythylamide	0.04	Glycine Arylamidase		
0.C	HisA	L-histidine-β-naphythylamide	0.048	Histidine Arylamidase		
0.D	GGA	L-glutamyl-L-glutamic acid β naphythylamide	0.068	Glutamyl Glutamic acid Arylamidase		
0.E	SerA	L-serine- β-naphythylamide	0.04	Serine Arylamidase		

2.5 Antimicrobial susceptibility

2.5.1 Disk Diffusion test

Bacterial colonies that grew on Amoxicillin blood agar plates but did not grow on Amoxicillin-clavulanic acid blood agar plates were presumed to be β-lactamase producing and subjected to antimicrobial susceptibility testing using the disk diffusion test. A loopful of the β-lactamase producing isolates was inoculated into 2 ml of saline (Diagnostic Media Products, South Africa) and adjusted to the density of a 0.5 Macfarland standard. The inoculum was then vortexed with a Vortex Genie 2 (Lasec │SA, South Africa) and a sterile cotton swab was dipped into the suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. Blood agar plates were inoculated by streaking the swab over the entire sterile agar surface to ensure an even distribution of inoculum.

Antimicrobial disks (Oxoid, United Kingdom) (Table 2.2) were placed onto the surface of the inoculated blood agar plate with a dispensing apparatus. The plates were inverted and incubated at 37 ºC for one week under anaerobic conditions in anaerobic jars. A vernier caliper was used to measure the diameters of the zones of inhibition, including the diameter of the disk. The zones were measured to the nearest whole millimeter. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2006) performance standards for antimicrobial disk susceptibility tests (Table 2.2).

Table 2.2: Antimicrobial agents and zone diameter measurements for disk diffusion test

Antimicrobial Agent	Disk Content µg	Zone Diameter Nearest whole mm		
		R	I	S
		≤		≥
Penicillin G	10	28	-	29
Ampicillin	10	28	-	29
Clindamycin	2	14	15-20	21
Trimethoprim-sulfamethoxazole	1.25/23.75	10	11-15	16
Chloramphenicol	30	12	13-17	18
Rifampicin	5	16	17-19	20
Linezolid	30	20	-	21
Quinupristin-dalfopristin	15	15	16-18	19
Fusidic acid	10	20	-	21
Vanomycin	30	10	11-13	14
Teicoplanin	30	10	11-13	14
Gentamicin	10	12	13-14	15
Erythromycin	15	13	14-22	23
Ciprofloxacin	5	15	16-20	21

2.5.2 Minimum Inhibitory Concentration test (MIC)

Minimum inhibitory concentrations (MICs) were performed using the microbroth dilution method according to CLSI Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria (CLSI, 2004). Seventeen β-lactamase producing bacterial cultures were revived and based on morphological identification they were subjected to antimicrobial susceptibility using the MIC test.

2.5.2.1Preparation of stock solutions and microtitre plates

Refer to Appendix 3 (Page 110) for stock solution preparation of Amoxycillin, Penicillin and Amoxicillin-clavulanic acid. Wells of microtiter plate were inoculated with two fold concentrations of antibiotics prepared in a growth medium. For amoxicillin and penicillin the starting concentration was 128µg/ml, and for Amoxicillin-Clavulanic acid (Augmentin) it was 64/32 µg/ml. A 100 µl of Tryptone broth was added to each well of the microtitre plate followed by the addition of the appropriately diluted antibiotic stock solution to the first well of each row in the microtitre plate. This resulted in a 1:2 dilution of the antibiotic to be tested. Using a multi-channel pipette (8 channels for 96 well microtitre plate) set to deliver a 100µl volume, the antibiotic broth mixture in the first row of wells was mixed. One hundred microlitres of the mixture in row one was transferred to row 2 of wells.

The pipette tips were discarded and new ones used to mix the solution in the second row of wells. The procedure was repeated until row 10 of well, once this row was mixed the remaining 100 µl in the pipette tips was discarded. Therefore each well (rows 1-10) contained a 100 µl mixture of antibiotic and tryptone broth with progressive doubling dilutions. Wells of row 11 and 12 contained a 100 µl of tryptone broth only as they were control wells. Table 2.3 illustrates an example of the amoxicillin-clavulanic acid microtitre plate. Plates were stacked and the topmost plate covered, they were then sealed in a plastic bag and stored in a freezer at -70 ºC.

Table 2.3 Amoxicillin-Clavulanic acid two-fold dilutions in microtitre plate

	1	2	3	4	5	6	7	8	9	10	11 (Negative control column)	12 (Positive control column)
A	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
B	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
C	64/32µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
D	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
E	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
F	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
G	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only
H	64/32 µg/ml	32/16 µg/ml	16/8 µg/ml	8/4 µg/ml	4/2 µg/ml	2/1 µg/ml	1/0.5 µg/ml	0.5/0.25 µg/ml	0.25/0.125 µg/ml	0.125/0.063 µg/ml	Tryptone Broth only	Tryptone Broth only

*Amoxicillin concentration is 2:1 to that of clavulanic acid

2.5.2.2Broth microdilution technique

Inoculation of microtitre plates requires a standardized inoculum to be delivered to each well. For each experiment fresh cultures were used. Cultures previously stored in storage media (2 % skim milk or Microbank™) were thawed and a loopful of culture was inoculated into two bottles of 10 ml tryptone broth. These bottles were incubated for 7 days under anaerobic conditions at 37 ºC. The β-lactamase-producing bacteria were then plated out onto non-selective blood agar and further incubated for 7 days anaerobically at 37 ºC. Whichever stock culture grew was used for the broth microdilution technique. Purification of cultures was verified based on morphology of the stock culture.

A microbiological loop was used to select isolated colonies from the blood agar plates and inoculated into 2 ml of saline and adjusted to a 0.5 Mcfarland turbidity standard using a turbidity reader. Within 15 minutes after the inoculum was standardized 0.5 ml of suspension was added to 4.5 ml of saline, this resulted in a 1:10 dilution yielding 10^7 CFU/ml. Five microlitres of this suspension was inoculated into the 100 µl antibiotic-broth mixture in each well from rows 1-10, and row 12, this resulted in the final test concentration of anaerobic bacteria being approximately 5×10^5 CFU/ml or 5×10^4 CFU/ml. Wells in row 12 served as positive controls, whereas wells in row 11 contained broth only and were thus the negative controls. Microtitre plates were stacked and incubated anaerobically in anaerobic jars containing an anaerobic gaspak (Davies diagnostic, South Africa) and an anaerobic indicator strip (Becton, Dickson and Company, USA) for 5 days at 37 ºC.

Bacteroides fragilis ATCC ® 25285 was used as a control strain for the MICs. Colony counts of inoculum suspension were performed to ensure that the final inoculum concentration obtained approximately 1×10^5 CFU/ml for *Bacteroides fragilis ATCC ® 25285.* This was obtained by removing 10 µl from the inoculated growth control well and diluting it into 10 ml saline. After mixing well a 100 µl aliquot was spread onto blood agar. The plates were incubated and the presence of approximately 100 colonies indicated an inoculum of 1×10^5 CFU/ml in the well. A purity check of the inoculum suspensions of the β-lactamase-producing bacteria was performed by subculturing an aliquot of the suspension in each well onto blood agar plates for simultaneous inoculation both anaerobically and aerobically. Interpretation of control strain results was performed using Table 2.5.

The MIC value of each antibiotic was determined by viewing the microtitre plates from the bottom using a viewing apparatus. The MIC breakpoints were read as the concentration where no growth or the most significant reduction of growth was observed. Interpretation of results was performed using Table 2.4

Table 2.4: Interpretative categories and Minimal Inhibitory Concentration (MIC) correlates (µg/ml)

Antimicrobial Agent	MIC (µg/ml)		
	Susceptible	Intermediate	Resistant
Amoxicillin-Clavulanic acid	≤ 4/2	8/4	≥ 16/8
Amoxicillin	≤ 0.5	1	≥ 2
Penicillin	≤ 0.5	1	≥ 2

Table 2.5: Acceptable ranges of MIC for *Bacteroides Fragilis ATCC ® 25285*for broth microdilution testing

Antimicrobial Agent	MIC range (µg/ml)
Amoxicillin-Clavulanic acid (2:1)	0.25/0.125 - 1/0.5
Penicillin	8-32
Amoxicillin	16-64

2.6 Molecular analysis

β-lactamase-producing isolates were screened for the presence of β-lactamase genes bla_{TEM} and bla_{cfxA} using PCR.

2.6.1 DNA extraction

For molecular analyses of the isolates, DNA was extracted using a technique described by Handal et al (2005) and stored. A loopful of culture was inoculated into a sterile Eppendorf tube containing 10 µl of 10× PCR buffer, 15 mM $MgCl_2$ (Qiagen, Maryland USA) and 90 µl of sterile distilled water. The inoculated buffer was boiled at 95 º C for 10 minutes, cooled on ice, and centrifuged using a micro centrifuge 5424 (Merck Chemicals Pty. Ltd, SA) at 5 000 rpm for 10 minutes. The supernatant was harvested and transferred into a sterile Eppendorf tube and stored at – 70 º C until required.

2.6.2 Polymerase Chain Reaction (PCR)

β-lactamase-producing bactera were screened for the presence of the main resistance β-lactamase genes bla_{TEM} and bla_{CfxA} which are generally found in periodontal pathogens (Fosse et al., 2002, Handal et al., 2005). These genes were amplified using

the PCR conditions and primers (Table 2.6 and 2.7) described by Handal et al. (Handal et al., 2005).

Table 2.6: Genes and Primers used in PCR reaction

Gene	´ - 3 ´)	Expected amplified product size
bla$_{TEM}$	GTATGGATCCTCAACATTTCCGTGTCG ACCAAAGCTTAATCAGTGAGGCA	1048 bp
bla$_{CfxA}$	GCAAGTGCAGTTTAAGATT GCTTTAGTTTGCATTTTCATC	831 bp

The supernatant (containing extracted DNA) was thawed and used as a template for PCR. DNA was amplified in a 25 µl reaction mixture containing 12.5 µl of 2 × PCR Master Mix (Fermentas Life Sciences), 2.5µl of sterile nuclease-free water (Fermentas Life Sciences), 5µl of 5µM primer (Inqaba biotec, South Africa), to which 5µl of template DNA was added. Samples were amplified in an iCycler thermal cycler (BIO-RAD, USA), the PCR conditions are summarized in Table 2.7.

Table 2.7: PCR programs used for the detection of β-lactamase genes

Gene	PCR program			Cycles
bla$_{TEM}$	Initial step	95 º C	5 min	1
	Denaturation	95 º C	1 min	30
	Annealing	55 º C	1 min	
	Extension	72 º C	1 min	1
	Final step	72 º C	5 min	
bla$_{CfxA}$	Initial step	94 º C	5 min	1
	Denaturation	94 º C	1 min	25
	Annealing	54 º C	1 min	
	Extension	72 º C	1 min 30 s	1
	Final step	72 º C	10 min	

During PCR, strictly regulated sterile conditions were followed to prevent contamination. Negative and positive controls were included with each batch of samples being analyzed. The positive control for the amplification of bla_{TEM} was *Escherichia Coli* 25746 (University of Copenhagen). A positive control for bla_{CfxA} could not be obtained. To generate a control, a few isolates that had tested positive for β-lactamase using the Nitrocefin Paper Disc Spot test were selected and amplified using PCR. The PCR products were viewed under UV light and the product which had the most intense DNA band at the expected size of 831 bp was sent for sequencing to Inqaba biotec (Pty) Ltd. The sequencing result was characterized using GenBank│EMBL- databases and confirmed that the bla_{cfxA} gene was present in the isolate. The *Prevotella intermedia* isolate containing bla_{cfxA} gene was then used as the positive control for the amplification of the bla_{cfxA} gene. For both PCR reactions the negative control consisted of sterile water instead of sample DNA.

The PCR products were separated alongside a mass DNA ladder(Fermentas Life Sciences, USA) through 1% agarose gels (Whitehead Scientific, South Africa) containing ethidium bromide by horizontal electrophoresis. The gels were visualized and the images analyzed and captured using the Universal Hood II system (BIO-RAD, USA).

2.6.3 Additional analysis of the Bla_{cfxA} gene

Amplified PCR products that had a positive result upon being visualized under ultraviolet light were sent for sequencing using the Sanger method, to Inqaba Biotec (Pty) Ltd. Once the sequences were retrieved from Inqaba Biotec, a cross-platform graphical DNA trace viewer and editor called Ridom TraceEdit was utilized to further

anaylze the sequences by editing incorrect base calls. Once edited the sequences were further characterized using GenBank │EMBL- databases.

2.7 Statistical Analysis

Descriptive statistics such as the means, standard deviations and medians were calculated to describe the data using the STATA statistical package (College Station, Texas, USA).

Chapter 3 Results

3.1 Demography

Forty eight patients participated in this study, over a period of 7 months. The mean age of the patients was 52 and the range 22 to 83 years of age. Fifty eight percent of the patients were female and 42% were male. The average pocket depth upon probing was 7 mm, with the range between 5 mm to 13 mm (Table 3.1).

Table 3.1: Demographical results of the study population

Patient number	Age	Gender	Tooth no. 1 Pocket depth (mm)	Tooth no. 2 Pocket depth (mm)
1	78	F	8	5
2	67	F	8	6
3	37	F	5	6
4	55	F	7	6
5	58	M	6	6
6	56	F	7	9
7	83	F	7	5
8	64	F	10	7
9	47	M	8	8
10	35	M	5	6
11	37	F	5	6
12	67	F	7	6
13	65	M	5	6
14	64	F	8	7
15	39	F	7	6
16	61	F	8	6
17	33	M	10	7
18	41	M	5	6
19	44	M	8	6
20	65	F	5	6
21	37	F	6	6
22	42	M	10	9
23	40	F	5	6
24	60	F	5	5
25	76	M	6	6
26	45	M	9	7
27	57	F	6	6
28	52	M	7	5
29	51	M	8	9
30	22	M	8	8
31	58	M	7	6

32	29	F	5	7
33	48	F	10	10
34	72	F	6	7
35	70	F	12	10
36	34	F	6	6
37	32	M	6	8
38	65	F	5	5
39	57	F	7	5
40	60	F	6	6
41	67	M	6	6
42	54	F	7	8
43	63	M	9	7
44	26	M	6	6
45	54	M	7	5
46	33	M	6	8
47	29	F	6	8
48	63	F	13	7
MEAN ± SD	52±15.1	F: 58%	7±1.87	6.60±1.31
		M: 42%	6.84±1.61	

3.2 Prevalence of β-lactamase-producing bacteria

Seventy five percent of patients attending Periodontology clinic at the Oral and Dental Hospital in Johannesburg carried on average two strains of β-lactamase-producing oral anaerobic bacteria, which constituted 10% of the total cultivable oral flora (Table 3.2). Of the 48 patients that participated in the study 36 patients carried β-lactamase-producing oral anaerobes. Eighty five strains of β-lactamase-producing bacteria were isolated from patients with chronic periodontitis. Complete results are shown in Appendix 2. The blood agar plate without any antimicrobial had the highest number of bacterial colonies, whereas the blood agar plate with augmentin had the least number of bacterial colonies. The mean total count of the control (blood agar only) plates amounted to 1.8×10^6 cfu/ml of sample. The counts of β-lactamase-producing species that grew on Amoxicillin-clavulanic acid plates were the lowest at 5.9×10^4 cfu/ml. Figures 3.1-3.3 illustrate growth of oral anaerobic microorganisms on blood

agar plates with and without antimicrobials. Growth of black pigmented *Prevotella intermedia* colonies were noted on the amoxicillin plate but not on the augmentin plate.

Table 3.2: The prevalence of β-lactamase-producing anaerobic oral bacteria in patients with chronic periodontitis (n=48)

Bacteria	Mean± Standard deviation
total-cfu/ml - control plates	$1.8\times10^6 \pm 2.3\times10^6$
total-cfu/ml - amoxicillin plates	$1.9\times10^5 \pm 5.3\times10^5$
total-cfu/ml – amoxicillin-clavulanic acid plates	$5.9\times10^4 \pm 1.5\times10^5$
Total no. of patients included	48
Total no. of patients with β-lactamase-producing species	36
Prevalence of β-lactamase-producing species	75 %
Number of β-lactamase strains isolated	85
Mean number of β-lactamase strains/patient	2
Mean β-lactamase spp. proportion of oral bacteria/patient	9.4%

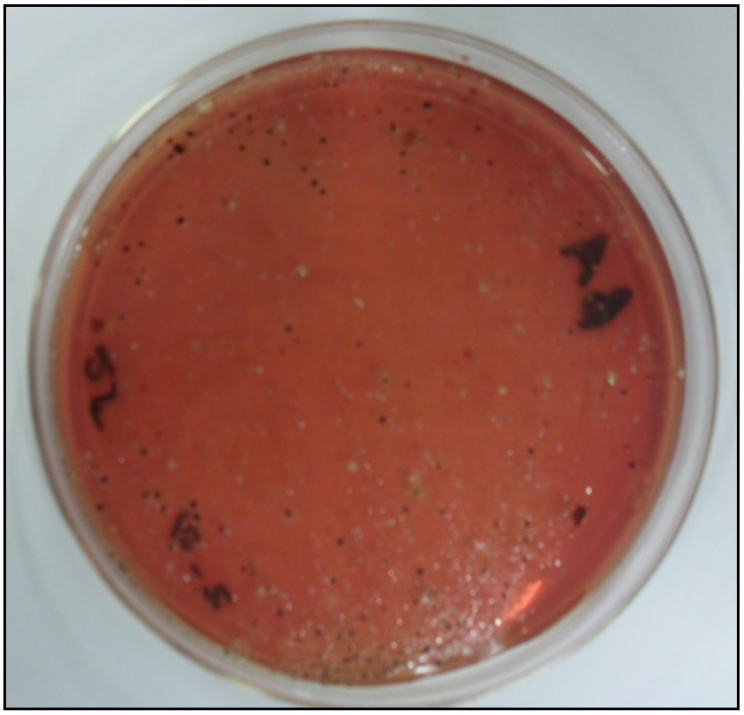


Figure 3.1 Growth of oral anaerobic bacteria on blood agar plate without β-lactam antibiotics

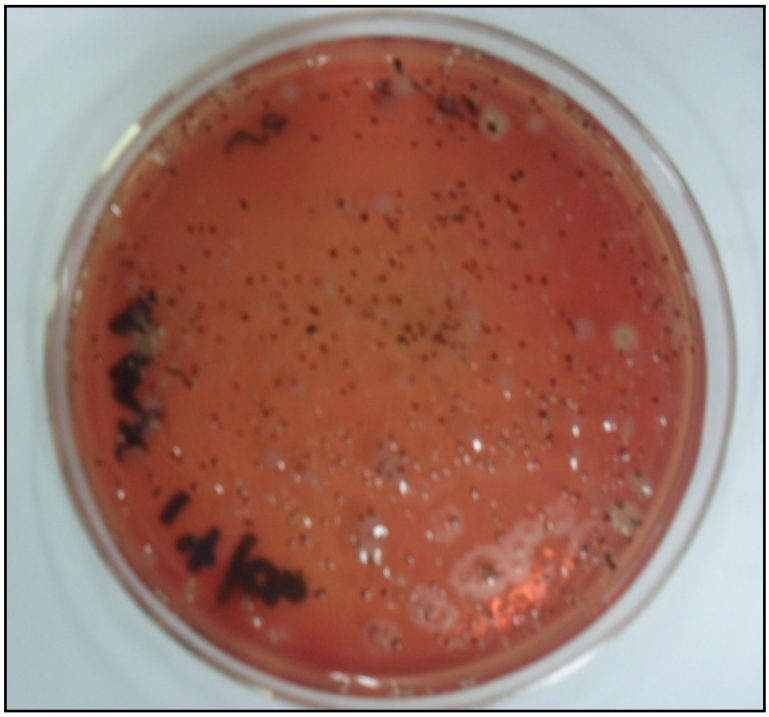


Figure 3.2 Growth of oral anaerobic bacteria on blood agar plate containing amoxicillin

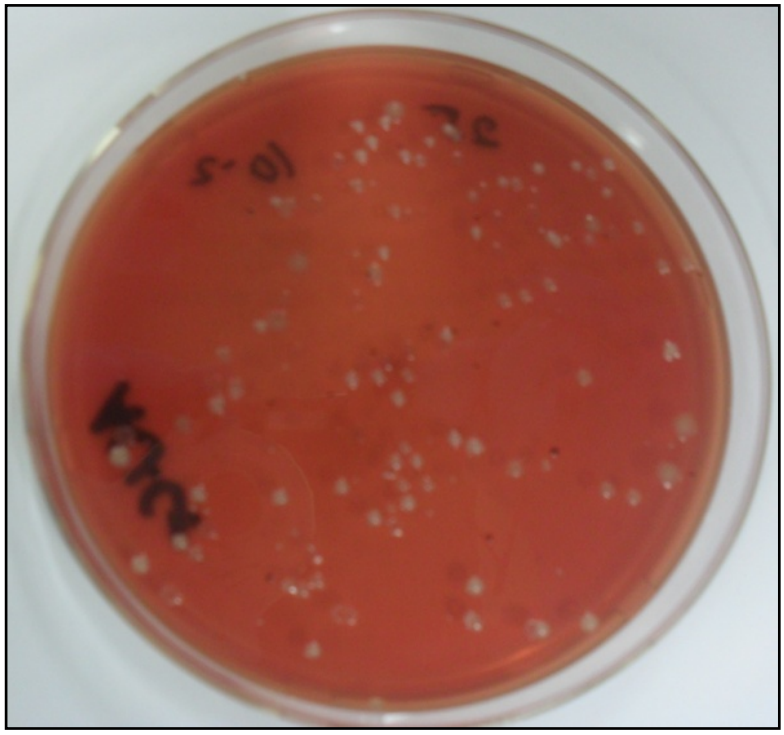


Figure 3.3 Growth of oral anaerobic bacteria on blood agar plate containing Amoxicillin-clavulanic acid

3.3 Identification and characterization of β-lactamase-producing oral anaerobic bacteria

The gram reaction of β-lactamase-producing oral anaerobic bacteria showed that the proportion of gram positive bacilli was higher than that of gram positive cocci, as seen in Table 3.3 and Figure 3.4. Of the 85 β-lactamase-producing species isolated from patients with chronic periodontitis 78 species (91.7%) were gram negative bacteria and 7 isolates (8.2%) were gram positive. Of the 91.7 % gram negative anaerobes, 89.4% were rod-shaped anaerobes whereas 2.35% were cocci shaped. Figures 3.5 to 3.7 illustrate gram stain reaction and morphology of *Porphyromonas gingivalis*, *Propionibacterium granulosum* and *Veillonella spp* isolated from patients.

Table 3.3: Gram reactions and morphology of β-lactamase-producing oral anaerobic bacteria

Gram reaction and morphology	**No. of Oral anaerobic β-lactamase-producing bacteria**	**% of Oral anaerobic β-lactamase-producing bacteria**
Negative bacilli	76	89.4
Negative cocci	2	2.35
Positive bacilli	6	7.07
Positive cocci	1	1.18
Total	**85**	**100**

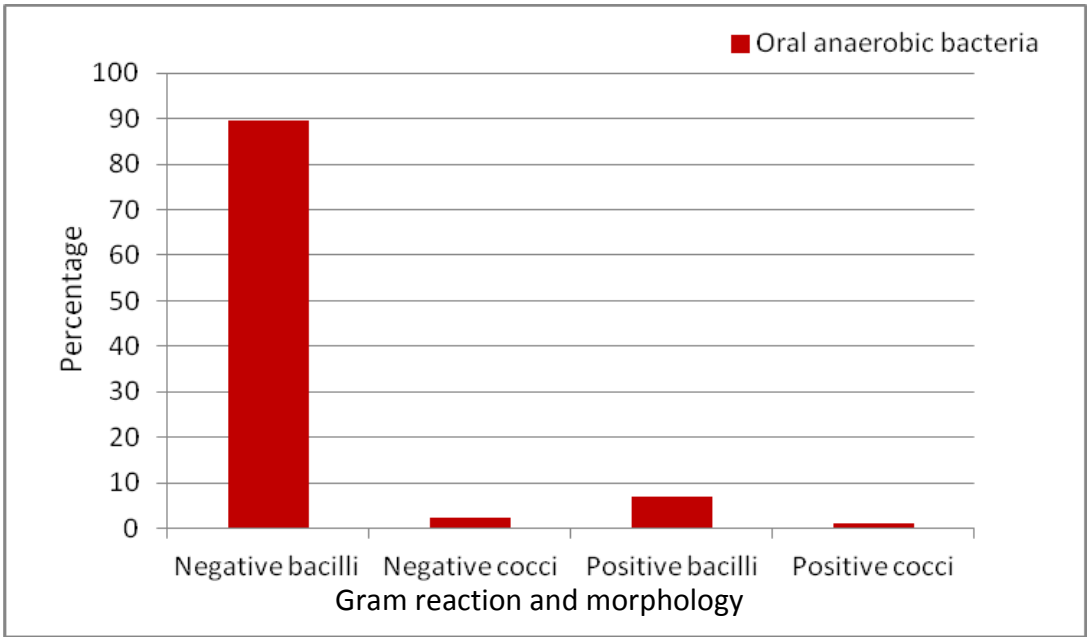


Figure 3.4 Gram reaction and morphology of β-lactamase-producing oral anaerobic bacteria

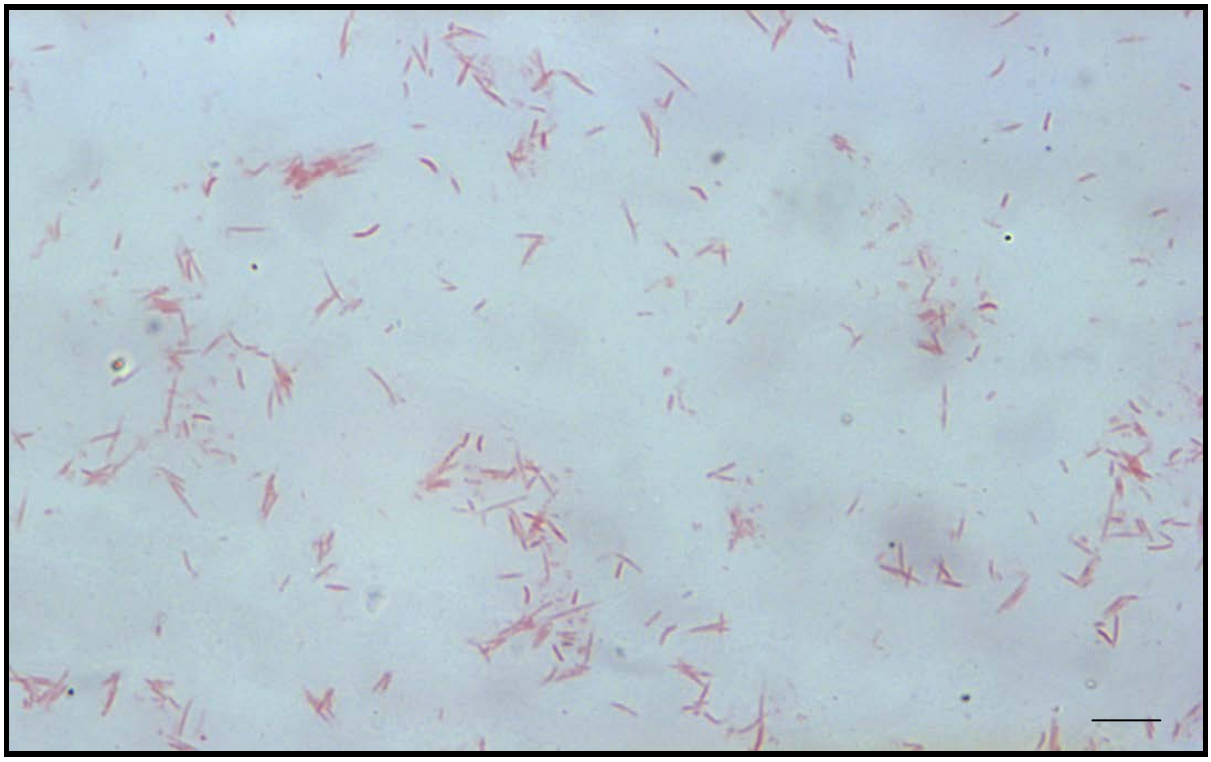


Figure 3.5 Gram negative rods of *Fusobacterium nucleatum* (Scale bar is 10 µm)

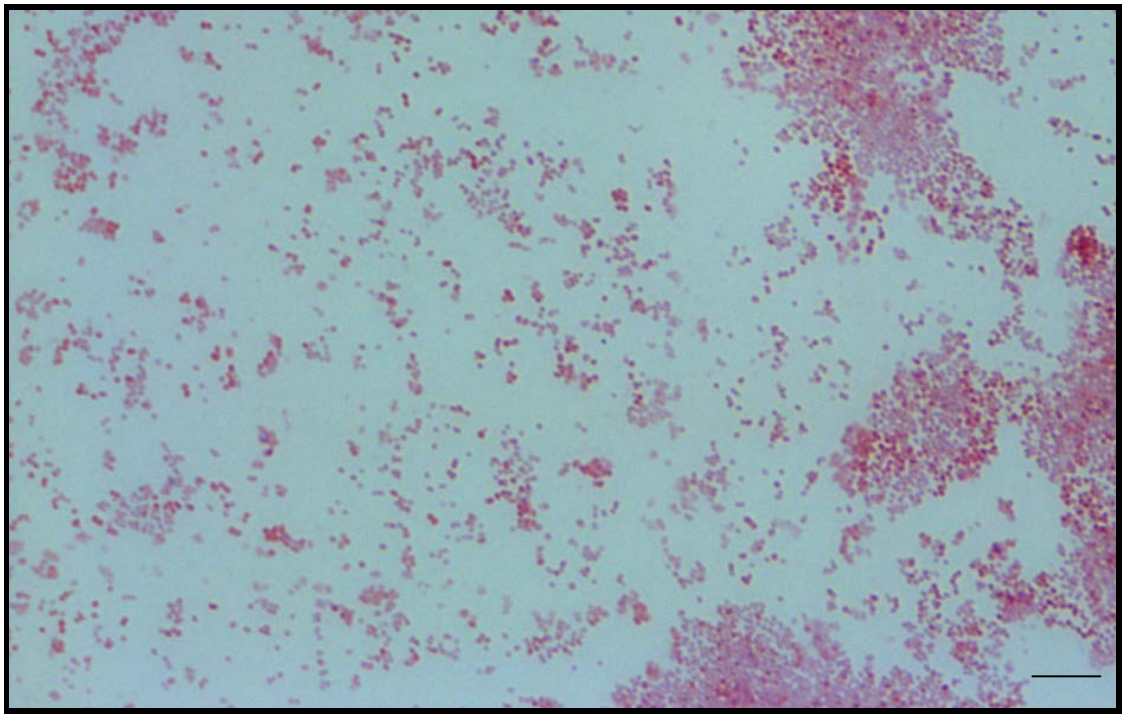


Figure 3.6 Gram negative cocci of *Veillonella spp.* (Scale bar is 10 µm)

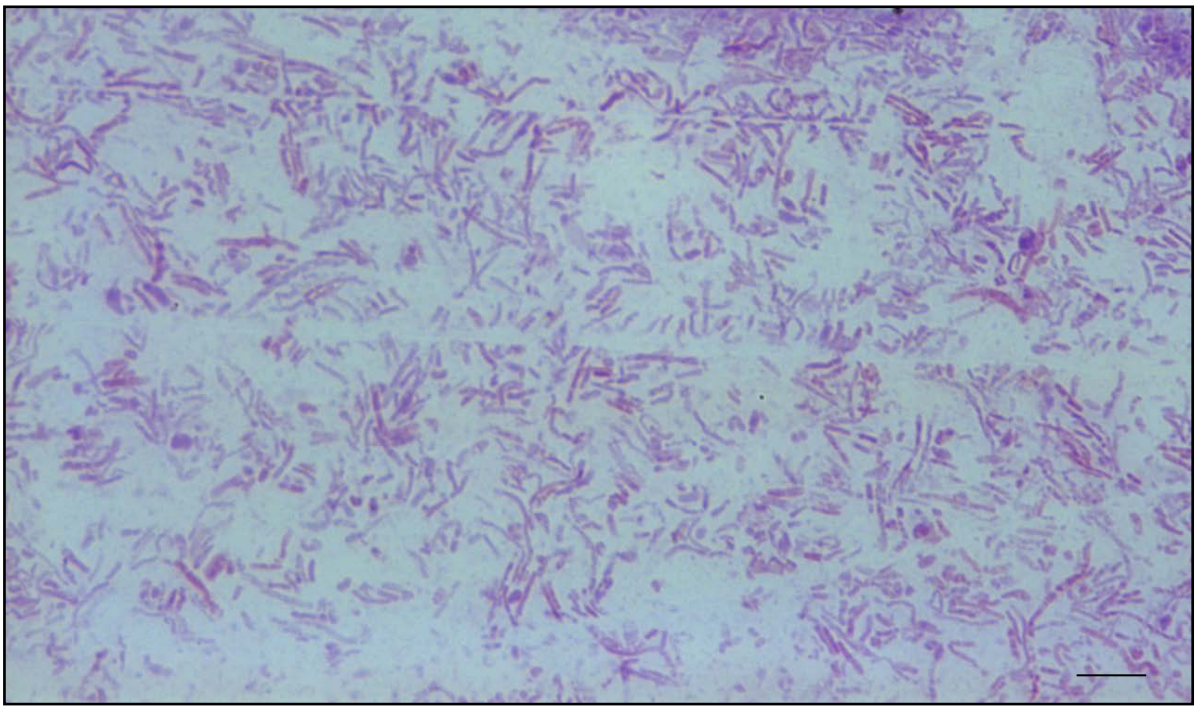


Figure 3.7 Rod-shaped gram positive *Propionibacterim granulosum* (Scale bar is 10 µm)

Table 3.4 indicates all the identified β-lactamase-producing bacteria from patients with chronic periodontitis that participated in this study. Forty nine of the 85 (58%) oral anaerobes were identified as *Prevotella* spp. *Prevotella oralis* was the most prevalent β-lactamase-producing microbe with 21 strains identified. Sixteen strains of *Prevotella intermedia* were also identified making the black pigmented the second most prevalent bacteria belonging to the *Prevotella* species. Sixteen strains of the 85 (18%) belonged to the Bacteroides group of bacteria with *Bacteroides capillosus* as the most predominant strain of the group. The least prevalent anaerobic microorganisms were identified as *Veillonella* spp, *Mobiluncus* spp, and *Actinomyces Meyeri*.

Table 3.4: Identification of β-lactamase-producing bacteria isolated from patients with chronic periodontitis

Genus	Species	Number of strains	Percentage of strains (%)
Prevotella		**49**	**58**
	P. oralis	21	
	P. intermedia	16	
	P. bivia	3	
	P. melaninogenica	5	
	P. buccae	2	
	P. denticola	1	
	P. buccalis	1	
Porphyromonas		**6**	**7**
	P. ginigivalis	2	
	P. endodontalis	4	
Bacteroides		**16**	**18.8**
	B. capillosus	7	
	B. ureolyticus	3	
	B. eggerthii	4	
	B. uniformis	1	
	B.merdae	1	
Fusobacteruim		**4**	**4.7**
	F. nucleatum	1	
	F. necrophorum	3	
Clostridium		**3**	**3.5**
	C. sordelli	1	
	C. perfringens	1	
	C. botulinum 2	1	
Propionobacterium		**3**	**3.5**
	P. granulosum	2	
	P. acnes	1	
***Veillonella* spp**		**2**	**2**
***Mobiluncus* spp**		**1**	**1**
Actinomyces Meyeri		**1**	**1**
Total		**85**	**100**

3.4 Antimicrobial susceptibility

3.4.1 Disk diffusion test

Selected colonies of all the β-lactamase producing cultures were subjected to antimicrobial susceptibility using a disk diffusion test (Figure 3.8 and 3.9). Zones of inhibition were measured to the nearest whole millimeter and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) performance standards for antimicrobial disk susceptibility tests (CLSI, 2006).

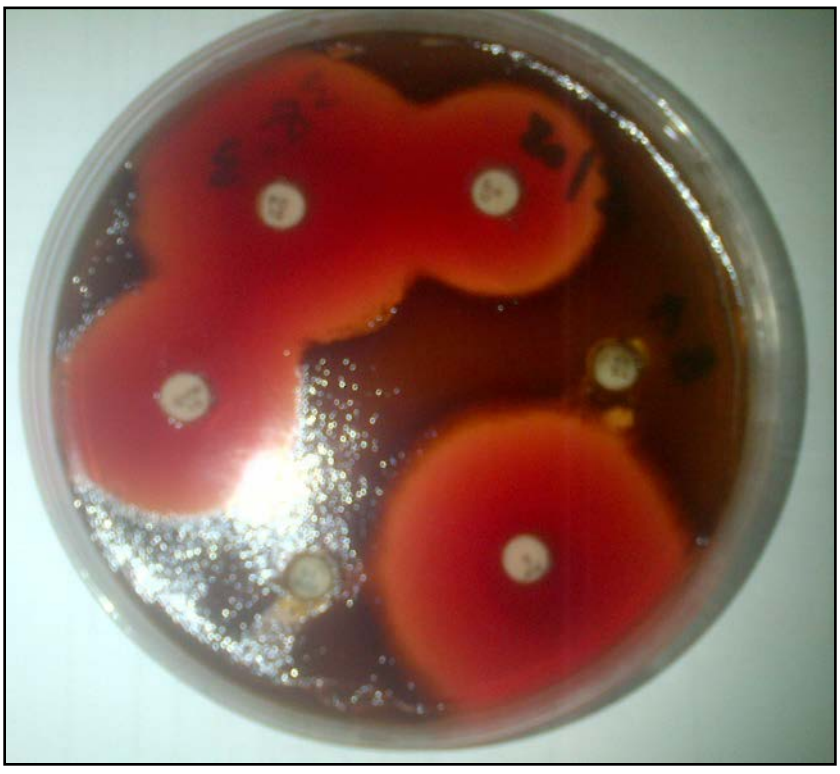


Figure 3.8 Disk diffusion test of *Prevotella intermedia* demonstrating clear zones of inhibition around ciprofloxalin, fusidic acid, rifampicin and quinupristin-dalfopristin.

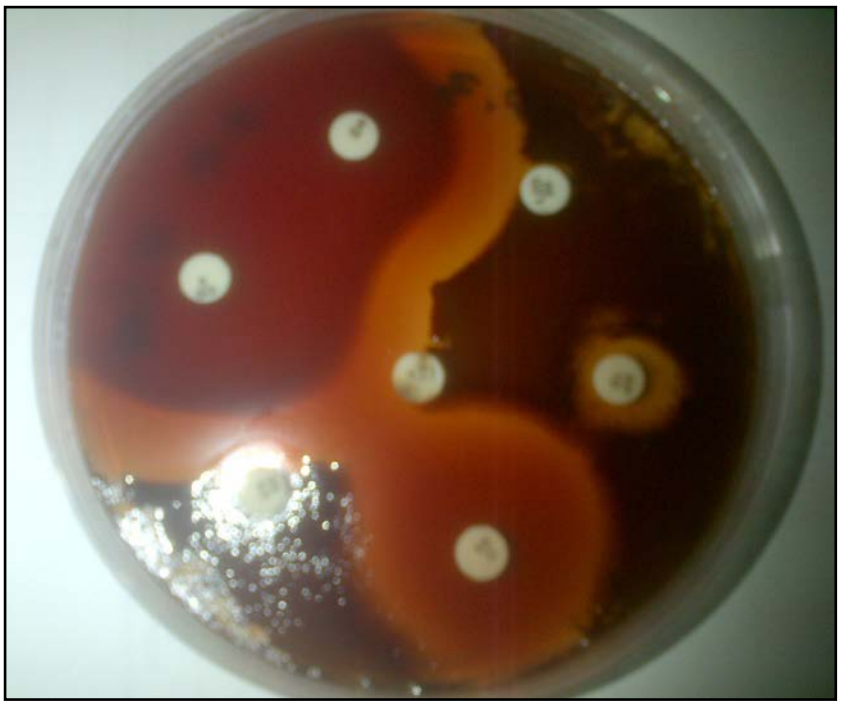
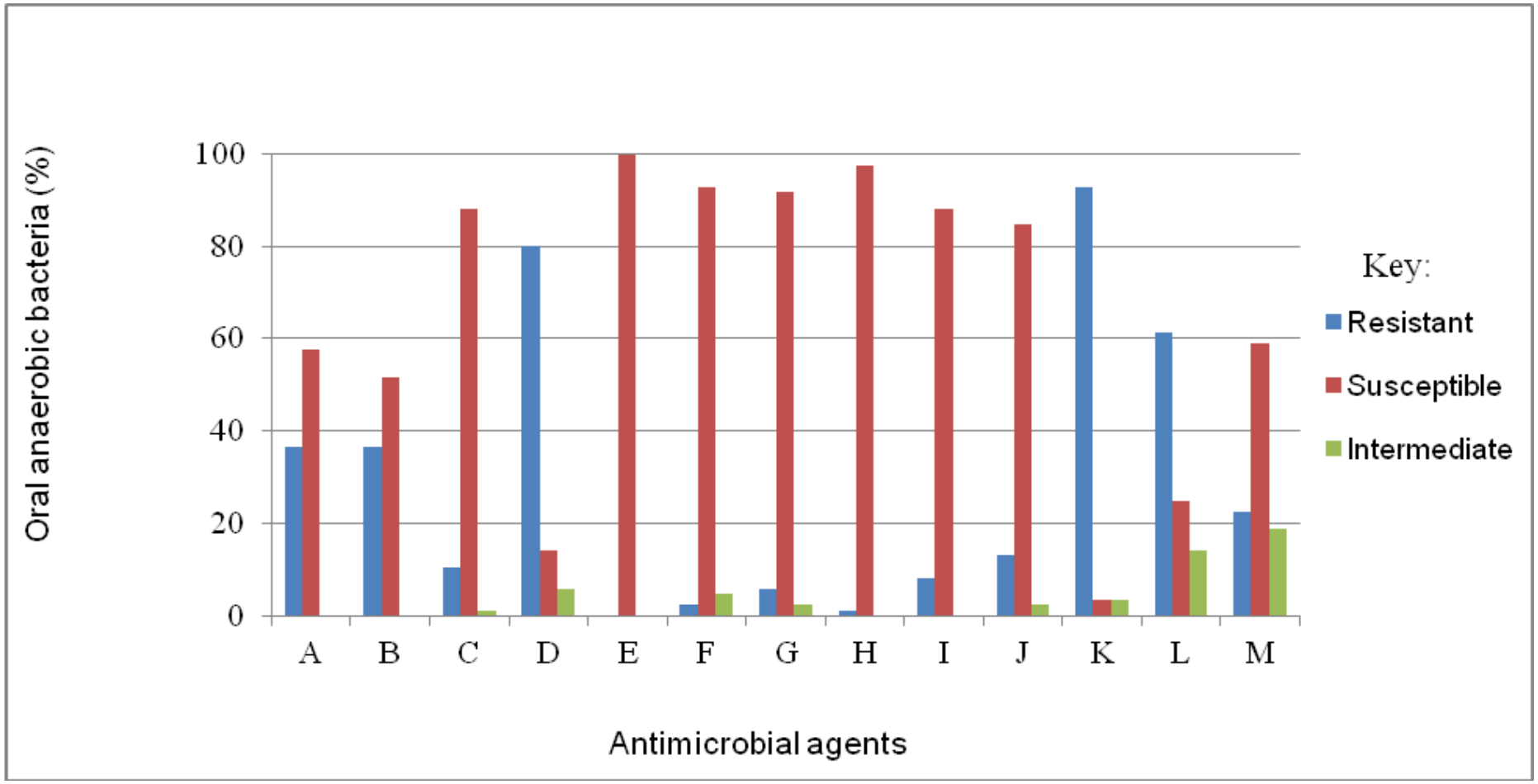


Figure 3.9 Disk diffusion test of *Prevotella intermedia* demonstrating clear zones of inhibition around antimicrobial agents clindamycin, chloramphenicol, ampicillin, and erythromycin.

A significant proportion of β-lactamase-producing oral anaerobes were susceptible to all the antimicrobial agents tested except for gentamicin and trimethoprim-sulfamethoxazole with only 3.5% and 14.1% of the bacteria susceptible respectively. None of the oral microbes demonstrated resistance to chloramphenicol and very few of them showed resistance to linezolid, rifampicin, and quinupristin-dalfopristin. Thirty one of the 85 strains (36.5%) demonstrated resistance to β-lactam antimicrobials. Table 3.5 and Figure 3.10 summarize the antimicrobial susceptibility disk diffusion results of the β-lactamase-producing oral anaerobic bacteria.

Table 3.5: Antimicrobial susceptibility (Disk Diffusion) of β-lactamase-producing oral anaerobic bacteria (n=85)

Antimicrobial agents	**Proportion of anaerobes resistant and susceptible to antimicrobial agents (%)**		
	Resistant	**Susceptible**	**Intermediate**
Penicillin	36.5	57.6	-
Ampicillin	36.5	51.8	-
Clindamycin	10.6	88.2	1.2
Trimethoprim-sulfamethoxazole	80.0	14.1	5.9
Chloramphenicol	0.0	100.0	0.0
Rifampicin	2.4	92.9	4.7
Quinupristin-dalfopristin	5.9	91.8	2.4
Linezolid	1.2	97.6	-
Fusidic acid	8.2	88.2	-
Erythromicin	12.9	84.7	2.4
Gentamicin	92.9	3.5	3.5
Vancomycin	61.2	24.7	14.1
Ciprofloxacin	22.4	58.8	18.8



A: Penicillin, B: Ampicillin, C: Clindamycin, D: Trimethoprim-sulfamethoxazole, E: Chloramphenicol, F: Rifampicin, G: Quinupristin-dalfopristin, H: Linezolid, I: Fusidic acid, J: Erythromicin, K: Gentamicin, L: Vancomycin, M: Ciprofloxacin

Figure 3.10 Antimicrobial susceptibility of β-lactamase-producing oral anaerobic bacteria

Of the 31 strains of β-lactamase-producing oral anaerobes that were resistant to both β-lactam antibiotics Penicillin and Ampicillin *Prevotella species* were predominantly resistant to the β-lactam antibiotics. Five of 16 *Bacteroides* spp and 3 of 6 *Porphyromonas* spp expressed resistance to penicillin whereas 6 of 16 Bacteroides spp and 2 of 6 P*orphyromonas* species demonstrated resistance to Ampicillin. All *Fusobacterium* strains were susceptible to penicillin and a single strain (*Fusobacterium necrophorum*) was resistant to ampicillin. Other oral anaerobes tested such as *Veillonella* spp, *Mobiluncus* spp and *Actinomyces* spp were susceptible to both ampicillin and penicillin (Table 3.6 and Figure 3.11).

Table 3.6: Proportion of β-lactamase-producing oral anaerobic bacteria resistant to β-lactam antibiotics (n = 31)

Species	**Resistant to Penicillin No. strains (%)**	**Resistant to Ampicillin No. strains (%)**
Prevotella spp (49)	21 (25)	20 (24)
Porphyromonas spp (6)	3 (4)	2 (2)
Bacteroides spp (16)	5 (6)	6 (7)
Fusobacterium spp (4)	0	1 (1)
Clostridium spp (3)	1 (1)	1 (1)
Propionobacterium spp (3)	1 (1)	1 (1)
Other (4)	0	0
Total 85	**31 (37)**	**31 (37)**

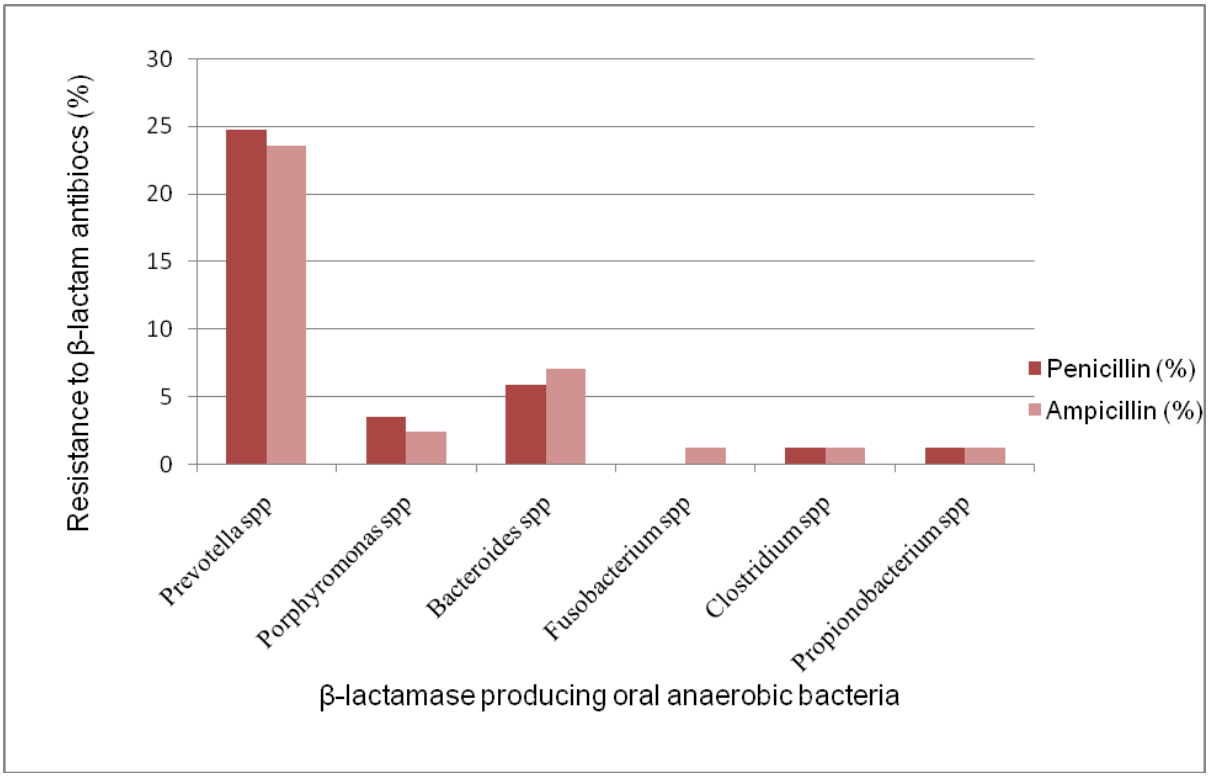


Figure 3.11 Proportion of β-lactamase-producing bacteria resistant to β-lactam antimicrobial agents

3.4.2 Minimum Inhibitory concentration

Out of 48 β-lactamase-producing oral anaerobes that carried the β-lactamase *cfxA* gene and had been stored in storage media, seventeen strains grew upon revival. These seventeen strains were tested for antimicrobial susceptibility using the MIC test. The MIC values (range and MIC_{50} and MIC_{90}) of amoxicillin, amoxicillin-clavulanic acid, and Penicillin are given in Table 3.7.

All the β-lactamase-producing microorganisms tested for amoxicillin-clavulanic acid sensitivity were sensitive to the antimicrobial with the exception of two strains; *Propionibacterium acnes* and *Bacteroides eggerthii.* Eleven of seventeen (65%) strains tested showed resistance to penicillin, of the eleven, five (29%) were *Bacteroides* species, four (24%) were *Prevotella* species and two (12%) *Propionibacterium s*pecies. Of the nine (53%) strains of β-lactamase-producing anaerobes that presented resistance to amoxicillin 29% (five of seventeen) belonged to the *Prevotella* group of species, 18% (three of seventeen) belonged to the *Bacteroides* group of species and a single strain of *Propionibacterium acnes*. The antimicrobial susceptibility (MIC) of the 17 bla_{cfxA}-producing bacteria is summarized in Table 3.11

Table 3.7: Minimum Inhibitory concentrations of β-lactam antibiotics against β-lactamase-producing oral anaerobic bacteria which carried *bla*$_{cfxA}$ gene (n=17).

β-lactamase-producing oral anaerobes		MIC (μg/ml)		
	n	Range	MIC_{50}	MIC_{90}
Prevotella melaninogenica	3			
Amoxicillin		0.25 - 2	0.25	2
*Amoxicillin-clavulanic acid		≤0.125/0.0625 - 0.5/0.25	0.25/0.125	0.5/0.25
Penicillin		0.125 - 1	0.25	1
Prevotella oralis	3			
Amoxicillin		4 - 16	4	16
Augmentin		0.25/0.125 - 4/2	2/4	4/2
Penicillin		8 - 64	8	64
Prevotella intermedia	1			
Amoxicillin		2	-	2
Augmentin		4/2	-	4
Penicillin		8	-	8
Bacteroides spp	8			
Amoxicillin		≤0.125 - 8	0.125	2
Augmentin		≤0.25/0.125 - 32/16	0.25/0.125	8/4
Penicillin		≤0.125 - >64	2	8
Propionibacterium spp	2			
Amoxicillin		0.125 - 64	0.125	64
Augmentin		≤0.125/0.0625 - 32/16	0.125/0.0625	32/16
Penicillin		4-64	4	64

* Amoxicillin-clavulanic acid concentration at a ratio 2:1

3.5. Molecular Analysis

3.5.1 Detection of β-lactamase genes

The eighty five strains of β-lactamase-producing oral anaerobes isolated from patients with chronic periodontitis were tested for the presence of common β-lactamase genes; β-lactamase *CfxA* gene (bla_{cfxA}) and β-lactamase *TEM* gene (bla_{TEM}). An amplicon of 831 bp was produced in 43 of the 85 strains, indicating that 51% of the β-lactamase-producing strains were positive for bla_{CfxA} (Figure 3.12). The *cfxA* gene was most frequently detected in *Prevotella* species with 21 of the 43 strains (49%) testing positive for the *cfxA* gene. Of the 43 strains, 12 strains (28%) belonged to *Bacteroides* species and 4 (9%) to *Porphyromonas* species as seen in Table 3.9. No non-specific products were observed in any of the reactions (Figure 3.12). No PCR product bands were observed at 1048 bp therefore none of the β-lactamase-producing oral anaerobes contained the bla_{TEM} gene.

Figure 3.12 Representative results of electrophoresis of PCR products from β-lactamase-producing oral anaerobes in the detection of *bla*cfxA gene. Lane M indicates the O'GeneRuler™ 50 bp DNA ladder molecular marker. Lanes 1 to 26, indicate β-lactamase-producing oral anaerobes, Lane 27 is the negative control and Lane 28 the Positive control. Images of other gel electrophoresis results are shown in Appendix 2.5.

Table 3.8 Detection of β-lactamase genes in 85 strains of β-lactamase-producing oral anaerobes isolated from patients with chronic periodontitis

Genus (n)	Species	No. of isolates tested	No. of strains with β-lactamase genes (%)	
			*bla*CfxA	*bla*TEM
Prevotella (49)	*P. intermedia*	15	4 (26.7)	0
	P. oralis	21	11 (52.4)	0
	P. melaninogenica	6	4 (66.7)	0
	P. denticola	1	0	0
	P. bivia	3	0	0
	P. buccae	2	2 (100)	0
	P. buccalis	1	0	0
Bacteroides (16)	*B. eggerthii*	4	3 (75)	0
	B. ureolyticus	3	1 (66.7)	0
	B. capillosus	7	6 (85.7)	0
	B. uniformis	1	1	0
	B. merdae	1	1	0
Porphyromonas (6)	*P. gingivalis*	2	1 (50)	0
	P. endodontalis	4	3 (75)	0
Fusobacterium (4)	*F. nucleatum*	1	0	0
	F. necrophorum	3	2 (66.7)	0
Veillonella (2)	*Veillonella spp.*	2	0	0
Propionibacterium (3)	*P. granulosum*	2	1 (50)	0
	P. acnes	1	1 (100)	0
Clostridium (3)	*C. sordelli*	1	1 (100)	0
	C. botulinum 2	1	0	0
	C. perfringens	1	1 (100)	0
Actinomyces (1)	*A. meyeri*	1	0	0
Mobiluncus (1)	*Mobiluncus spp*	1	0	0
Total 85		**85**	**43**	**0**

3.5.2 Additional analysis of *cfxA* gene

The PCR products of the 43 different *cfxA* positive β-lactamase isolates were sequenced. The results are shown in table 3.9. Thirty three sequences were 100 % identical to *cfxA2* GenBank Accession number AM940016 of *Bacteroides ovatus.* Of the 33 strains, 14 were *Prevotella* spp, 10 *Bacteroides* spp, 3 *Porphyromonas* spp and 2 *Clostridium* spp, *Fusobacterium* spp, and *Propionibacterium* spp. Three strains of *Prevotella oralis* and 1 strain of *Prevotella melaninogenica, Bacteroides capillosus, Bacteroides ureolyticus* contained the *cfxA3* gene which was 100% identical to *cfxA3* GenBank Accession number Ay860640 of *Capnocytophaga ochracea* plasmid Pcap MobA. The *cfxA6* gene was identified in 4 strains of β-lactamase-producing microorganisms, namely, *Prevotella melaninogenica, Prevotella intermedia, Prevotella oralis* and *Porphyromonas endodontalis.*

The above-mentioned oral anaerobes possessed the *cfxA6* gene showing 100% similarity with *Prevotella intermedia* partial *cfxA6* gene, GenBank Accession number FN3764261.

In Table 3.10 the antimicrobial sensitivity (disc diffusion) of 43 bacterial strains producing the bla_{cfxA} gene is illustrated. The antimicrobial susceptibility (MIC) of the 17 bla_{cfxA}-carrying bacteria is summarized in Table 3.11. Complete antimicrobial susceptibility results of the periodontal pathogens are shown in Appendix 2.4. Fifty nine percent of oral anaerobic bacteria that carried bla_{cfxA} genes were resistant to β-lactam antibiotics penicillin and amoxicillin which was reduced to 82% due to β-lactamase inhibitor e.g the combination augmentin. Forty-two out of eighty-five β-lactamase producing anaerobes did not carry the bla_{cfxA} gene, the antimicrobial sensitivity to β-lactam antibiotics of these bacteria was tested using the disc diffusion technique and the results are shown in Table 3.12. Table 3.13 shows summary of disc diffusion, MIC tests and the presence or absence of *cfxA* gene. When the *cfxA* gene was present 53% of the organisms were resistant to β-lactam antibiotics. When the

genes were absent, the disc diffusion test showed that 33% of the isolates were still resistant. A complete summary of all the results obtained is found in Appendix 2.1.

Table 3.9 The prevalence of *Bla*$_{CfxA}$ genes harboured by oral anaerobes isolated from periodontal pockets of patients with chronic periodontitis (n=43)

β-lactamase gene (43/85 strains)	**Genus (n=43)**	**No. of positive strains**	***Bla*$_{CfxA}$ genes**		
			***Bla*$_{CfxA2}$**	***Bla*$_{CfxA3}$**	***Bla*$_{CfxA6}$**
Bla$_{CfxA}$ *CfxA2:* 76.7 % *CfxA3:* 14 % *CfxA6* : 9.3	*Prevotella spp*	21	14	4	3
	Porphyromonas spp	4	3	-	1
	Bacteroides spp	12	10	2	-
	Fusobacterium spp	2	2	-	-
	Clostridium spp	2	2	-	-
	Propionobacterium spp	2	2	-	-
	Veillonella spp	0	-	-	-
	Actinomyces spp	0	-	-	-
	Mobiluncus spp	0	-	-	-
Bla$_{TEM}$	As above	0	-	-	-

Table 3.10 Anaerobic bacteria carrying the *Bla*$_{CfxA}$ gene and their Antimicrobial susceptibility the using Disc diffusion technique

Bacterial species (43)	**Gene**	**Ampicillin**	**Penicillin**
P. melaniniogenica (4)	CfxA3	R	R
	CfxA2	R	R
	CfxA6	S	S
	CfxA2	S	S
P. oralis (11)	CfxA2	R	R
	CfxA2	S	R
	CfxA2	S	R
	CfxA2	S	I
	CfxA3	R	R
	CfxA6	S	S
	CfxA3	R	R
	CfxA2	I	R
	CfxA2	R	R
	CfxA3	R	R
	CfxA2	S	S
P. intermedia (4)	CfxA2	S	R
	CfxA2	S	S
	CfxA2	S	S
	CfxA6	R	R
P. buccae (2)	CfxA2	R	R
	CfxA2	S	R
B. capillosus (6)	CfxA2	S	S
	CfxA2	S	S
	CfxA2	S	S
	CfxA2	R	R
	CfxA3	S	S
	CfxA2	S	S
B. ureolyticus	CfxA3	R	R
B. uniformis	CfxA2	S	S
B. eggerthii (4)	CfxA2	S	S
	CfxA2	R	R
	CfxA2	R	R
	CfxA2	R	S
P. gingivalis	CfxA2	R	R
P. endodontalis (3)	CfxA2	S	R
	CfxA6	S	R
	CfxA2	S	S
P. acnes	CfxA2	S	S
P. granulosum	CfxA2	R	R
F. necrophorum (2)	CfxA2	R	S
	CfxA2	S	S
C. perfringens	CfxA2	S	S
C. Sordelli	CfxA2	S	R
Total		R:39.53%, I:2.33%, S:58.14%	R:53.49%, I:2.32%, S:44.19%

Table 3.11 Anaerobic bacteria harbouring the *Bla*$_{CfxA}$ gene and their Antimicrobial susceptibility using MIC technique

Strain (n=17)	**Bla$_{CfxA}$ gene**	**Amoxicillin**	**Augmentin**	**Penicillin**
P. melaninogenica $_1$	*CfxA3*	0.125 (S)	0.25/0.0625 (S)	0.5 (I)
P. melaninogenica $_2$	*CfxA2*	0.125 (S)	0.0625/0.0312 (S)	<0.125 (S)
P. melaninogenica $_3$	*CfxA6*	1 (R)	0.25/0.125 (S)	0.125 (S)
P. oralis $_1$	*CfxA3*	8 (R)	0.125/0.0625 (S)	32 (R)
P. oralis $_2$	*CfxA3*	0.25 (R)	1/0.5 (S)	4 (R)
P. oralis $_3$	*CfxA2*	2 (R)	2/1 (S)	8 (R)
P. intermedia	*CfxA6*	2 (R)	4/2 (S)	8 (R)
B. capillosus $_1$	*CfxA2*	0.125 (S)	0.25/0.125 (S)	0.125 (S)
B. capillosus $_2$	*CfxA2*	1 (R)	1/0.5 (S)	1 (R)
B. capillosus $_3$	*CfxA2*	0.5 (S)	0.25/0.125 (S)	R
B. capillosus $_4$	*CfxA2*	1 (R)	0.125/0.0625 (S)	1 (R)
B. eggerthii $_1$	*CfxA2*	4 (R)	0.125/0.0625 S	<0.125 (S)
B. eggerthii $_2$	*CfxA2*	<0.125 (S)	16/8 (R)	<0.125 (S)
B. uniformis	*CfxA2*	0.125 (S)	4/2 (S)	4 (R)
B. ureolyticus	*CfxA3*	<0.125 (S)	0.5/0.25 (S)	<0.125 (S)
Prop. acnes	*CfxA2*	>64 (R)	16/8 (R)	>64 (R)
Prop. granulosum	*CfxA2*	<0.125 (S)	0.0625/0.0312 (S)	2 (R)
Total		S: 41.18% R: 58.82%	S: 82.24% R: 11.76%	S: 41.18% R: 58.82%

R: Resistant, S: Sensitive, I: Intermediate Sensitivity

Table 3.12 Antimicrobial susceptibility (disc diffusion technique) of β-lactamase producing anaerobes that did not carry the *Bla*$_{CfxA}$ gene

Bacterial species (n = 42)	**Ampicillin**	**Penicillin**
P. bivia (3)	I	I
	R	S
	R	S
P. intermedia (13)	S	I
	I	S
	R	R
	S	S
	S	S
	S	S
	S	S
	R	I
	R	R
	R	R
	S	R
	S	S
	S	S
P. oralis (10)	I	S
	I	R
	R	R
	S	R
	S	S
	R	S
	R	I
	R	S
	S	S
	S	S
P. denticola (1)	I	S
P. melaninogenica (1)	R	S
P. buccalis (1)	R	S
B. ureolyticus (2)	S	S
	S	S
B. merdae (1)	R	R
B. capillosus (1)	I	S
Veillonella spp (2)	I	I
	S	S
P. gingivalis (1)	R	S
P. endodontalis (1)	I	S
F. nucleatum (1)	S	S
F. necrophorum (1)	S	S
P. granulosum (1)	I	S
A. meyeri (1)	S	S
Mobiluncus (1)	S	S
Total	R: 33.33%, I:21.43%, S: 45.24%	R:19.05%, I: 11.90%, S: 69.05%

Table 3.13 Antimicrobial susceptibility of β-lactamase-producing anaerobic bacteria against β-lactam antibiotics

CfxA gene	No. of isolates resistant to β-lactam antibiotics (%)											
	Disc diffusion test (n=85)						Broth dilution test (n=17)					
	Ampicillin			Penicillin			Amoxicillin			Penicillin		
	R	I	S	R	I	S	R	I	S	R	I	S
Present (n=43)	17 (39.53)	1 (2.33)	25 (58.14)	23 (53.49)	1 (2.32)	19 (44.19)	9 (52.94)	0	8 (47.06)	10 (58.82)	1 (5.89)	6 (35.29)
Absent (n=42)	14 (33.33)	9 (21.43)	19 (45.24)	8 (19.05)	5 (11.90)	29 (69.05)	-	-	-	-	-	-

R: Resistant, S: Sensitive, I: Intermediate Sensitivity

Chapter 4 Discussion

Chronic periodontal disease is a chronic inflammatory disease that affects 70% - 80% of adults worldwide and is more prevalent in developing countries. It has been found that prevalence and severity increase with age (Pihlstrom et al., 2005). The mean age of patients in our study group was 52 years which is higher than has been found in similar investigations in European and Colombian populations (Herrera et al., 2000, Van Winkelhoff et al., 2002, Ardila et al., 2010). The male-female ratio was also similar to findings in the Dutch and Spanish populations in which the percentage of females suffering from chronic periodontitis was found to be more than 55% (Herrera et al., 2000). In our study 58% of patients were females. Although a study by Van Winkelhoff et al (2002) showed a mean pocket depth of 6.3, in our study the mean pocket depth was 6.8 mm which is generally found in studies conducted in patients with chronic periodontitis (Herrera et al., 2000).

4.1 Prevalence of β-lactamase-producing oral anaerobes

Over the years many oral anaerobic bacteria associated with chronic periodontal diseases have developed resistance to β-lactam antibiotics by virtue of their production of β-lactamase enzymes (Handal and Olsen, 2002). Not much is known about production of β-lactamase enzymes and drug resistance in oral anaerobic bacteria in South Africa. Results collected over a 7 month period in this study showed that 75% of patients carried β-lactamase-producing oral anaerobic bacteria, which has increased compared to the study conducted by Patel (2011) who reported prevalence of 69%. However it is lower than the prevalence reported in the Spanish population

(87%) but higher than the French and Dutch populations, which was 73% and 53% respectively (van Winkelhoff et al., 1997, Fosse et al., 1999, Herrera et al., 2000). The difference in this prevalence in different populations can be attributed to extensive drug use. Generally in developing countries the use of antibiotics is low and thus antibiotic resistance levels are generally lower, however in South Africa there are high levels of antibiotic resistance in pathogens other than oral in public sector hospitals (Essack, 2006). Antimicrobial surveillance stewardships are being implemented in major South African public and private hospitals to monitor antimicrobial sensitivity and development of drug resistance in major pathogens (Ramsey et al, 2013).

On average patients had two strains of β-lactamase-producing oral anaerobic bacteria, which constituted 10% of the total cultivable oral flora. β-lactamase is an extracellular enzyme released by these bacteria which renders penicillin inactive (Brook., 2009, Herrera et al., 2000). These 10% of β-lactamase producing bacteria can therefore protect coexisting non-β-lactamase-producing, penicillin sensitive bacteria in the periodontal pocket.

4.2 Types of β-lactamase-producing oral anaerobes

Eighty five isolates of β-lactamase-producing bacteria were derived from 48 patients with chronic periodontitis, which is different to the numbers obtained in The dutch population, in which 33 β-lactamase-producing strains were isolated from 30 patients (Van Winkelhoff et al., 2000). The majority of these bacteria were gram negative rods, which was expected because gram negative anaerobic bacteria are generally implicated in the periodontal diseases (Philstrom., 2005, Legg and Wilson., 1990).

Most of these bacteria produce proteinases and toxins which causes tissue damage (Legg and Wilson, 1990, Kim et al., 2011).

Prevotella spp. was the predominant genus isolated in our study, which coincides with other studies (Legg and Wilson, 1990, van Winkelhoff et al., 2000, Patel, 2011, Herrera et al., 2000). Socransky et al (1998) suggested an association between deep periodontal pockets, attachment loss and the presence of *P. intermedia* and *P. gingivalis.* A deep periodontal pocket harbors more plaque, large amounts of orange and red microbial species and is likely to produce more gingival crevicular fluid than a shallow pocket (Haffajee et al., 2008). The mean pocket depth in our study group was 6.84 mm which is considered a deep pocket and therefore the presence of an orange complex species *Prevotella species* can be explained.

In a study by Ali et al (1994) *P. intermedia* was always detected in the presence of fellow orange complex species *F. nucleatum* from deep periodontal pockets from patients with chronic periodontitis. However that was not the case in this study, only a single strain of *F. nucleatum* was isolated and it was not in the presence of *P. intermedia* in the periodontal pocket. Only 19 % of the isolated strains were *P. intermedia*, this proportion is lower than has been isolated in the Dutch and American populations, in which 26% and 52% of the isolated bacteria were *P. intermedia* (van Winkelhoff et al., 1997, Appelbaum et al., 1990). *P. intermedia* is well known as the greatest producer of β-lactamase enzymes (Kuriyama et al., 2007, Handal et al., 2005).

The genus *Prevotella* comprises of a wide and diverse group of anaerobic bacteria that cause oral infections (Nadkarni et al., 2012). *Prevotella melaninogenica* has been isolated from patients with chronic periodontitis in a study in Australia (Nadkarni et al., 2012). These species of *Prevotella* including *Prevotella oralis clones* and *Prevotella oris* were consistently isolated from the subgingival pockets of patients (Nadkarni et al., 2012)**.** Forty-nine isolates of *Prevotella species* were isolated in this study, these species included *P. melaninogenica* (5), *P. buccae* (1), *P. oralis* (21), *P. denticola* (1) *and P. buccalis* (1). These results show the diversity of the genus *Prevotella* that produces β-lactamase, in patients with periodontal diseases (Table 3.4). *P. buccae* and *P. buccalis* are considered not to be periodontal pathogens, as very low numbers of these bacteria have been isolated from studies (van Winkelhoff et al., 1997). However these bacteria might be able to protect β-lactam susceptible bacteria when they release the enzyme into the periodontal pocket environment and transfer responsible genes to other sensitive bacteria.

Porphyromonasgingavalis is a member of the microbial red complex and has been found to dominate in deep periodontal pockets (Socransky et al., 1998, Haffajee et al., 2008). In a study by Benrachadi et al (2012) in Morocco on patients with chronic periodontitis, *P. gingivalis* was found to be more prevalent than *P. intermedia*, which is usually highly associated with periodontal diseases. In contrast in our investigation, a very low percentage (2%) of *Porphyromonas species* were isolated (Table 3.4). This is an increase from the study by Patel (2011) who did not isolate any *Porphyromonas spp.* from patients with chronic periodontitis. *Porphyromonas spp.* is an aggressive organisms and causes extensive tissue damage causing very deep periodontal pockets. Five patients carried these organisms, out of which only one patient had a pocket

depth of 5 mm, the others had depths ranging from 8 to 10 mm and this explains the presence of these organisms in these patients.

Fusobacterium species is a member of the orange complex of bacteria that are detected early in disease development of periodontitis (Nadkarni et al., 2012).
These bacteria are associated with deep periodontal pockets and often with members of the red complex such as *Porphyromonas species*. A single strain of *F. nucleatum* was isolated from a patient with a mean pocket depth of 8 mm. β-lactamase-producing *Fusobacterium species* are not common in South African patients, as they were not isolated in a preliminary study by Patel (2011) and only a single strain was isolated in this study.

Gram negative *Bacteroides spp*. have been associated with periodontal lesions (Nonnenmacher et al., 2001). *B. ureolyticus* and *B. forsythus* are implicated in periodontal, root canal and other oropharyngeal infections (Falagas and Siakavellas, 2000). As seen in Table 3.4, 18.8% of oral bacteria isolated from patients were *Bacteroides spp*., this makes this species the second most prevalent species of β-lactamase-producing periodontal pathogens. There has also been an increase in the number of *Bacteroides spp.* isolated in comparison to results obtained by Patel (2011). In our study we found *B. eggerthii* (4), *B. ureolyticus* (3), *B. capillosus* (7), Patel (2011) isolated (3) *B. eggerthii*, (2) *B. ureolyticus* and a single *B. buccae* strain.

Veillonella are gram negative cocci that are part of the normal flora of the mouth, vagina and small intestines of certain people. *Veillonella* species are part of the predominant anaerobes in patients with poor oral hygiene. Kumar et al (2005) found

that *Veillonella spp* were associated with periodontal health, as higher numbers of this bacterium were isolated from healthy patients compare to those with periodontal disease. In addition, they are generally not associated with tissue destruction.
In this study only two strains of *Veillonella* species were isolated that produced the β-lactamase enzyme (Table 3.4).

Propionibacterium species are members of the normal microbial flora of the skin and mouth. *P. granulosum* has been isolated from the subgingival plaque from shallow periodontal pockets of patients after head and neck irradiation for the treatment of nasopharyngeal carcinoma (Leung et al., 1998) and a single strain was isolated in this study. A single strain of *Mobiluncus spp* was isolated from the periodontal pockets of a patient with pocket depths of 6 mm. *Mobiluncus* spp are gram positive cocci which are highly associated with bacterial vaginosis (Spiegel, 1987, Nyirjesy et al., 2007, Schwebke et al., 1996). These bacteria have also been isolated from patients with oral lichen planus (Bornstein et al., 2008). *Mobiluncus* spp produced β-lactamase, although these bacteria are usually susceptible to β-lactam antibiotics (Spiegel, 1987).

Periodontal pathogens such as *Bacteroides forsythus* and *Campylobacter rectus* were not identified in our investigation. This could be due to the difficulty of cultivating these bacteria in vitro. The oral cavity is the most colonized site in the human body containing between 500 and 600 different species of bacteria (Kazor et al, 2003, Paster et al, 2001). However, only half of them are culturable (Pater et al, 2001). A fraction of bacteria will be isolated from a single plaque sample at a particular time. Therefore, a fraction of β-lactamase-producing bacteria were isolated. Although this study did not isolate gram positive commensals such as streptococci and lactobacilli,

gram positive bacteria are known to produce relatively large amounts of β-lactamase and secrete it into the environment thus protecting other bacteria from β-lactam antibiotics even though the number of gram positive bacteria are low (Soars et al, 2010).

4.3 Antimicrobial susceptibility of β-lactamase producing oral anaerobes

Levels of antibiotic resistance are high in South Africa. Although the statistics are not known, the country is recognized as one of the world leaders in the prevalence of gram negative organisms with resistance to β-lactam antibiotics (Johnston, 2012).

We investigated the antimicrobial resistance of the periodontal pathogens isolated from patients with chronic periodontitis. There is little information available in scientific literature regarding the level of resistance of these oral anaerobes in Southern Africa.

4.3.1 *Porphyromonas species*

P. gingivalis was susceptible to the tested antibiotics amoxicillin-clavulanic acid, clindamycin and chloramphenicol. This result is in accordance with other studies that show that this bacterium is susceptible to these antibiotics (Kulik et al., 2008, van Winkelhoff et al., 2005, Kuriyama et al., 2007). However the *P. gingivalis* isolates also expressed resistance to β-lactam antibiotics ampicillin and penicillin, and other antibiotics such as erythromycin. This resistance can spread because *P. gingivalis* is capable of conjugal transfer of chromosomal and plasmid DNA which would provide an effectual way to transfer resistance determinants to other anaerobic bacteria in the

periodontal pocket (Tribble et al., 2007). *P. endodontalis* isolates were found to be highly susceptible to ampicillin, chloramphenicol, clindamycin, and erythromycin antibiotics, as was also found by a previous study by van Winkelhoff et al (1992). This result indicates an apparent difference in the susceptibility pattern between *P. gingivalis* and *P. endodontalis*.

4.3.2 *Fusobacterium species*

All but a single strain of *Fusobacterium spp*. were susceptible to amino-penicillin antibiotics in this study. This sensitivity to penicillin is not uncommon amongst *Fusobacteria* with β-lactamase production being the main resistance mechanism when it is found (Hecht, 2006). In The Netherlands *Fusobacterium nucleatum* isolates were found to be 100% susceptible to penicillin (van Winkelhoff et al., 2005) , as has been found in the South African population of patients with chronic periodontitis.

The single strain of *Fusobacteria* that produced resistance to ampicillin may have been carrying an ampicillin resistance gene that conveys resistance to ampicillin. A study by Lakhssassi et al (2005) found *Fusobacterium nucleatum* to be susceptible to ampicillin, amoxicillin and augmentin. Studies in European and South American populations have also found that *Fusobacterium* species exhibited good susceptibility to a wide range of antibiotics such as clindamycin, augmentin, and erythromycin (van Winkelhoff et al., 2005, Jacinto et al., 2008).

Bla$_{CfxA}$-type β-lactamases have been found in β-lactamase-producing strains and could be carried on transposons in association with tetracycline and erythromycin resistance genes (Giraud-Morin and Fosse, 2003). Carriage of different resistance

genes on transposons could result in multi-drug resistant bacteria, however we did not test oral anaerobes for the presence of tetracycline and erythromycin genes.

4.3.3 *Prevotella species*

All the β-lactamase positive strains of *Prevotella spp.* were susceptible to amoxicillin-clavulanic acid as has been reported by other investigators (Behra-Miellet et al., 2003, van Winkelhoff et al., 2000, Mosca et al., 2007, Kuriyama et al., 2007). Antibiotics such as amoxicillin-clavulanic acid (a β-lactamase inhibitor) and clindamycin are generally regarded as highly effective antibiotics against *Prevotella* species (van Winkelhoff et al., 2000, Lakhssassi et al., 2005). Various studies including this study have shown very low levels of resistance of β-lactamase-producing oral anaerobes to clindamycin (Aldridge et al., 2001, van Winkelhoff et al., 2000, Ardila et al., 2010).

In a study by Kuriyama et al (2007), β-lactamase production was found in all amoxicillin resistant strains and β-lactamase production was detected in 48 % of the amoxicillin susceptible strains. These amoxicillin-susceptible strains exhibited relatively high MIC's for amoxicillin. These findings suggest that the production of β-lactamases is the principle mechanism of amoxicillin resistance amongst *Prevotella spp*. In our study 28% of the *Prevotella spp*. isolated from patients with chronic periodontitis were resistant to amoxicillin, these findings are lower than those found in Colombia, Spain and The Netherlands (van Winkelhoff et al., 2000, Ardila et al., 2010). Van Winkelhoff et al (2000) showed that 82.6% and 37% of β-lactamase-producing oral anaerobes in the Spanish and Dutch population were resistant to penicillin.

In Bulgaria resistance to penicillin has been found to be 60.6%, these findings are comparable to findings from Greece (69.0%) and The United States of America (57.0%), however in contrast, our findings of penicillin resistance of *Prevotella spp* are below 25% which are similar to elsewhere (Papaparaskevas et al., 2008, Ednie and Appelbaum, 2009, Boyanova et al., 2010).

Isolates of *P. intermedia* were found to be highly susceptible to amoxicillin-clavulanic acid, clindamycin and erythromycin, these results are in agreement with previous investigations that studied the sensitivity of this bacterium to amoxicillin-clavulanic acid (van Winkelhoff et al., 2000, Kulik et al., 2008). *P. intermedia* is well known to produce β-lactamase enzymes (Kuriyama et al., 2007, Handal et al., 2005).
Lakhssassi et al (2005) showed that *P. intermedia* is the greatest producer of β-lactamases amongst oral anaerobes tested in their study. The enzyme production ability of these bacteria may partly explain the resistance of this bacterium and other *Prevotella spp*. to penicillin and amoxicillin and high levels of susceptibility to amoxicillin-clavulanic acid, due to the presence of β-lactamase inhibitor.

In this study none of the *P. melaninogenica* strains were resistant to clindamycin, however Behra-Miellet et al (2003) found a single strain resistant to clindamycin. In recent years, a steady increase in penicillin resistance in *P. melaninogenica* has been noted, a study in the Spanish population found that 18.2% and 9.1% of *P. melaninogenica* were resistant to amoxicillin and clindamycin respectively (Maestre et al., 2007). However in the South African population *P. melaninogenica* species were susceptible to β-lactam antibiotics penicillin and amoxicillin, with only two strains showing resistance to penicillin and a single strain resistant to amoxicillin.

Prevotella oralis isolates presented resistance to β-lactam antibiotics amoxicillin and penicillin, whereas 52% of the strains presented resistance to penicillin when tested using the disk diffusion method. A previous study found that 70 % of the *P. oralis* strains were resistant to penicillin and 33% resistant to clindamycin, in our study only 9.5% (2 of 21) of the *P.oralis* bacteria were resistant to clindamycin, thus resistance is still low in this population (Papaparaskevas et al., 2008).

4.3.4 *Bacteroides species*

In this investigation some isolates of the genus Bacteroides were resistant to amoxicillin, penicillin, and ampicillin. A single strain of *Bacteroides eggerthii* expressed resistance to amoxicillin-claulanic acid. *B. eggerthii* and *Bacteroides uniformis* are members of the *Bacteroides fragilis* group of bacteria and were isolated from patients with chronic periodontitis. These *Bacteroides* species are known to play a role in human infectious diseases as they exhibit multiple mechanisms of resistance to antimicrobial agents, especially many β-lactam antibiotics (Aldridge, 1993).
All members of the *Bacteroides fragilis* group produce β-lacatmase enzymes (Rasmussen et al., 1997, Falagas and Siakavellas, 2000).

Alridge et al (2001) reported 86% resistance of *B. uniformis* to penicillin and 76% to clindamycin. In 1993 a study by Alridge et al. reported 16% resistance of *B. uniformis* to clindamycin, and suggested that imipenem to be the most active penicillin amongst other penicillins against *B. uniformis* strains. In Greece 98% of the *B. uniformis* bacteria isolated from oral cavities of patients with odontogenic infections were resistant to penicillin (Papaparaskevas et al., 2008). In our study the single *B.*

uniformis isolate was found to be susceptible to β-lactam antibiotics and clindamycin but showed resistance to erythromycin.

Resistance of the *B. fragilis* group of bacteria to amoxicillin-clavulanic acid has been found in a study conducted in Spain (Betriu et al., 2005). The species of the *B. fragilis* group that were found to be resistant to amoxicillin-clavulanic acid were *B. uniformis*, *B. erggerthii*, *B. merdae*. The proportion of bacteria that was reported to be resistant to amoxicillin clavulanic acid was less than 19% of the *Bacteroides* species isolated from patients (Betriu et al., 2005).

This data indicates that anaerobic bacteria that are clinically important can vary widely in their antimicrobial sensitivity (Aldridge et al., 2001). The level of resistance of the bacteria to the antimicrobial agents varies from country to country because of the different use of antibiotics. Less than 50% of β-lactamase producing periodontal pathogens in the South African population were found to be resistant to β-lactam antibiotics, therefore if patients do not respond to β-lactam antibiotics alternative antimicrobial agents should be administered to them as β-lactamase-producing anaerobes may be present in their periodontal pockets.

4.4 Detection of β-lactamase-genes

The habitation of β-lactamase genes within mobile genetic elements such as plasmids or transposons allows for transfer of these resistance genes between distantly related bacteria within the periodontal pocket (Williams, 1999, Wilke et al., 2005, Handal et al., 2005). The β-lactamase-producing oral anaerobes isolated from patients with

chronic periodontitis were tested for the presence of common β-lactamase genes; β-lactamase *CfxA* gene ($Bla_{\text{CfxA)}}$ and β-lactamase *TEM* gene (Bla_{TEM}). Plasmid mediated β-lactamases genes are present in many species of gram negative bacteria, and the most common of these is the *TEM*-type enzyme (Lacroix and Walker, 1992).

However similarly to a results obtained by Handal et al (2005) the Bla_{TEM} resistance gene was not isolated from any of the β-lactamase-producing oral anaerobes. In a study by Rosenau et al (2000) the $Bla_{TEM\text{-}17}$ gene was found in *Capnocytophaga species* isolated from blood, they proposed that the all capnocytophaga strains carry Bla_{TEM} genes. The present study did not isolate any capnocytophaga strains nor identify *the* Bla_{TEM} gene.

Bla_{CfxA} genes are known to be present in oral anaerobes that produce β-lactamase.

A high prevalence of the Bla_{CfxA} gene was found in *Prevotella* species from the subgingival plaque of South African patients. These findings are similar to the results obtained in the American, French and Norwegian population (Handal et al., 2005, Giraud-Morin and Fosse, 2003).

There are various chromosome-encoding and plasmid mediated genes that result in the production of β-lactamase. These genes include *TEM*, *OHA*, *CF*, *cepA* and *cblA*. *Prevotella spp.* rarely harbor *TEM*, *AmpC*, *CF* genes. But a few strains have been found to contain *cepA* and *cblA* genes which encode for the production of the β-lactamase enzyme (Iwahara et al., 2006). Therefore β-lactamase producing bacteria that did not have the *CfxA* gene present, but were resistant to β-lactam antibiotics by virtue of their enzyme production could have been utilizing other genes for enzyme production.

4.5 Analysis of Bla_{cfxA} gene

Bla_{CfxA} genes are highly prevalent in *Prevotella species* and *Capnocytophaga species* isolated from periodontal pockets (Fosse et al., 2002, Handal et al., 2005).

Of the 85 β-lactamase producing strains, *Bla_{CfxA}* was identified in 43. *Prevotella spp* had the highest prevalence of these genes followed by *Bacteroides spp*, in which 12 strains produced the *Bla_{CfxA}* gene. *Bla_{CfxA}* has been identified in *Bacteroides spp.*, isolated from periodontal pockets of patients infected with chronic periodontitis, these bacteria have been shown to transfer the gene amongst the species (Fosse et al., 2002). This transference amongst the species has been found to be associated with the conjugative transposon Tn 4555 (García et al., 2008). Tn 4555 is a non-autonomous conjugative transposon which is associated with *Bla_{CfxA}* and is involved in the horizontal transfer of the β-lactamase gene amongst periodontal pathogens (García et al., 2008).

Other genetic elements can also contribute to the transposition of β-lactamase genes, as a sequence tag from Tn4351 (which is normally associated with erythromycin resistance) was detected in the genomic context upstream of *CfxA* (García et al., 2008). Garcia et al (2008) also suggested that the β-lactamase genes that have been described in *Bacteroides spp.* have different degrees of sequence diversity therefore it could be related to their transference pathways. Studies of these transposons suggest that they play a significant role in the spread of drug resistance (Arzese et al., 2000).

4.5.1 CfxA genes

The *CfxA2* gene was present in 33 strains of β-lactamase producing oral anaerobes, this finding makes this gene the most prevalent β-lactamase gene in periodontal pockets of the South African population attending the Wits oral health sciences dental clinic affected with chronic periodontal disease. Our results of a high prevalence of *CfxA2* are similar to those found in America and Norway (Handal et al., 2003, Handal et al., 2005). A study by Giraud-Morin et al (2003) suggested that the *CfxA/CfxA2* type partition of the β-lactamase-producing strains could be related to the geographical origin as the *CfxA2* type predominates in North America and *CfxA* predominates in France. The *CfxA2* gene shares >98% identity with the *CfxA* gene. A previous study revealed a high prevalence of *CfxA/CfxA2* in *Prevotella* spp. isolated from patients diagnosed with periodontitis, however they did not identify the type of *CfxA* gene that was isolated from the *Prevotella spp* (Fosse et al., 2002, Giraud-Morin and Fosse, 2003).

The *CfxA3* gene differs from *CfxA2* by possessing an aspartic acid instead of a tyrosine at the position 239 of the nucleotide and differs from *CfxA* by possessing glutamic acid instead of lysine at position 272 of the nucleotide. Jolivet-Gougeon (2004) isolated the *CfxA3* gene from a beta-lactam resistant clinical strain of *Capnocytophaga ochracea* (E201) and found that the *CfxA3* gene was located on a plasmid which carried a mobilizable trasnposon (Jolivet-Gougeon, 2004). In our study the *CfxA3* gene was isolated from 6 strains of which 4 belonged to *Prevotella spp.*, and 2 to *Bacteroides* spp.

Periodontal pathogens in which *CfxA3* was isolated from including *Capnocytophaga spp* by Jolivet-Gougeon (2004), belong to the *Bacteroidetes* phylum of bacteria, and thus this finding suggests that the *CfxA3* gene could be prevalent amongst groups of bacteria which are resistant to β-lactams and belong to this phylum (Wolfgang *et al.*, 2010).

CfxA6 was isolated from species belonging to the families *Prevotellaceae* and *Porphyromonadoceae,* these families belong to the *Bacteriodales* order of bacteria (Wolfgang *et al*., 2012). Therefore *CfxA6* may be specific to the *Bacteroidales* order of bacteria which are classified as periodontal pathogens. However, the *CfxA2* gene was isolated in bacteria belonging to various phylums including *Fusobacteria, Firmicutes and Actinobacteria,* this indicates the spread of the *CfxA2* gene, and thus the spread of resistance to β-lactam anitibiotics between distantly related bacteria (Wolfgang *et al*., 2012, Goodfellow *et al*., 2012)

B-lacatamse genes play an important role in the progression of periodontal disease (Kinane, 2003). As these resistance genes are recurrently found on plasmids they could give rise to multi-drug resistant strains of periodontal pathogens (Jolivet-Gougeon, 2003).

4.6 Periodontal pathogens, their transmission and role in other infections

In addition to their primary site of isolation (periodontal pocket), periodontal pathogens have been isolated in other oral sites such as the tonsils, root canals, saliva,

peritonsillar abscesses, deep neck infections and extraoral sites such as the brain and lungs (Mättö et al,. 1997, Paquette, 2002, Bidault et al., 2007, Veloo et al., 2012). Predominant anaerobes that have been isolated in peritonsillar, retropharyngeal, and lateral pharyngeal abscesses include *Prevotella*, *Fusobacterium* and *Porphyromonas* species (Brook., 2004). Some of these bacteria were isolated from our patients harbouring resistance genes and producing β-lactamase enzymes. Untreated abscesses can rupture into the pharynx resulting in aspiration and they can become potentially life-threatening (Brook., 2004). Antimicrobial therapy can reduce abscess formation if treatment is administered at an early stage and if strains that are causing the infection are not resistant to the antibiotic (Brook., 2004).

Anaerobes are frequently isolated from blood in bacteremia cases resulting from endodontic therapy. Dissemination of periodontal pathogens into the bloodstream is also common during dental procedures, and microorganisms from the infected sites may reach the heart, lungs and peripheral blood capillary system (Li et al, 2000). Distribution of oral anaerobes to distant sites of the body occurs especially in immuno-compromised patients such as those that are suffering from diabetes, HIV malignancies or rheumatoid arthritis (Li et al, 2000).

Due to the high numbers of gram negative bacteria in the periodontal disease state, individuals could be predisposed to cardiovascular disease (Li et al, 2000). Numerous proposed mechanisms exist in which oral anaerobic bacteria may trigger pathways leading to cardiovascular disease. For example *P. gingivalis* can induce platelet aggregation which leads to thrombus formation (Li et al, 2000). Atherosclerotic plaques are commonly infected with the oral anaerobe *P. gingivalis* (Li et al, 2000).

Horizontal transmission of periodontal pathogens such as *P. gingivalis* has been found between spouses and the transmission range for *P. gingivalis* is 30% to 75% (Van Winkelhoff and Boutaga, 2005).Therefore it seems as if periodontal pathogens are transmitted between spouses and this transmission results in the recipient spouse having periodontitis (Asikainen et al., 1997, Van Winkelhoff and Boutaga, 2005). Mother to child as well as care-givers to child transmission has also been established (Asikainen et al, 1997). Dental units also have a potential to transmit oral pathogens from patient to patient if infection control measures are not applied (Montebugnoli et al, 2004). These studies suggest that resistant oral bacteria can be transmitted from person to person and become a problem in serious illnesses.

Horizontal and vertical transmission of periodontal pathogens may be controlled by periodontal treatment involving the elimination of the pathogen (Van Winkelhoff and Boutaga, 2005). However if resistant pathogens are present it is necessary for the dentist to re-call the patient and check if the treatment given is effective in eliminating the periodontal pathogens and if it is not, then they should prescribe an alternative antibiotic. Failing to do so will result in the spread of resistance pathogens between family members and spouses of patients with chronic peridontitis.

4.7 Gene transfer and oral bacteria

The oral cavity is the most colonized site in the human body containing between 500 and 600 different species of bacteria (Kazor et al, 2003, Paster et al, 2001). These bacteria live in a biofilm which protects them against antimicrobial compounds. However, this environment is highly stressful and competitive for some bacteria, therefore many oral bacteria adapt to genetic transfer. Recent metagenomic and

bioinformatic studies have confirmed that oral bacteria play a major role in horizontal gene transfer (Liu et al, 2012, Smillie et al, 2011). For example, extensive genetic variation has been seen in *P. gingivalis* (Tribble et al, 2007). It improves their chance of survival, increases virulence, changes metabolism and alters drug resistance. Generally genetic transfer can occur through transformation, transduction and conjugation.

Studies have shown environmental DNA (eDNA) released from dead lysed oral bacteria as well as extraoral bacteria in the dental plaque which facilitates transformation (Hannan et al, 2010). This eDNA survive even after 24 hours in the presence of saliva (Mercer et al, 1999). It has been shown that transformation frequencies increases in the biofilms grown cells compared to the planktonic cells (Li et al, 2001).

In addition, a highly mobile Tn916 like genetic element transposon has also been found in many oral bacteria such as *Streptococci, F. nucleatum, Eubacterium, Veillonella* and *Actinobacillus*. These transposons facilitate conjugation. Sex pheromones that induce mating have been detected in oral *streptococci* (Vickerman et al, 2010). Both plasmid and chromosomal-borne transfer of antibiotic resistance have been shown in oral bacteria (Roe et al, 1995, Guiney et al, 1990, Lancaster et al, 2004).

Another mechanism of gene transfer is through membrane vesicles (MV) that are released by many gram negative bacteria including bacteria in the dental plaque. These membrane vesicles package periplasmic components including genetic

elements and store them extracellularly which allows them to fuse into surfaces of other species transferring information. MVs are very small and therefore they have easy access to unreachable areas (Olsen et al, 2013). Although some laboratory studies have shown phage facilitated genetic transfer, transduction in oral bacteria (Willi et al, 1997), there is not sufficient evidence to show transduction in oral bacteria. Exposure of oral biofilms to antibiotics can alter the bacterial composition and changes the antibiotic resistance profile of the biofilm (Ready et al, 2002). In addition, sub-lethal concentrations of antibiotics promote the transfer of resistance genes (Showsh and Andrews, 1992).

The literature in this section highlights the importance of the presence of resistance genes in the oral bacterial community even if two species of organisms per patient carry them as shown in our study. The transfer of these genes to other bacteria is possible.

The dental community in the UK accounts for 7% of all community prescription of antibiotics. Figures for South Africa are not available but there is a need for better education in the dental community with regards to antibiotic resistance, the usage, surveillance programs and the use of diagnostic services including susceptibility testing to prevent the ever rising of antimicrobial resistance worldwide (Sweeney et al, 2004).

Chapter 5 Conclusions, future research and limitations

5.1 Conclusions

A high prevalence of β-lactamase-producing anaerobic bacteria (75%) was found in South African patients diagnosed with chronic periodontitis. These patients carried on average two strains of β-lactamase-producing oral anaerobic bacteria, which constituted 10% of the total cultivable oral flora. Thirty one of the 85 strains (36.5%) demonstrated resistance to β-lactam antimicrobials. *Prevotella* species were found to be the most prevalent oral bacteria in this population. Fifty one percent of these β-lactamase-producing oral anaerobic bacteria carried the $Bla_{CfxA\ (CfxA2,\ CfxA3,\ CfxA6)}$ gene. However, none of them carried $Bla_{TEM.}$ The Bla_{CfxA} gene may have been responsible for the resistance to β-lactam antibiotics because the resistance to β-lactam antibiotics was 58% in these bacteria.

Although this finding of β-lactamase-producing anaerobic bacteria was relatively low (10% of oral flora), these bacteria are able to cause antibiotic failure or disease recurrence as they release the β-lactamase enzyme into the surrounding environment. In addition, horizontal gene transfer may occur from β-lactamase-producing anaerobic bacteria to other non-producers. β-lactam antibiotics should still remain the first choice of treatment for patients with periodontal disease, however alternate antimicrobial agents should be considered in patients who do not respond to β-lactam antibiotics. High prevalence of β-lactamase-producing bacteria suggests that education among dental community, and surveillance programs with routine diagnostic susceptibility testing are required.

5.2 Future research

- Surveillence studies are important for monitoring levels of antibiotic resistance within the oral pathogens and commensals. These bacteria do cause some serious extraoral infections.
- *CfxA* genes are transported on transposons in combination with other resistance genes such as *cep A, cblA*, *tetQ* and *ermF* giving rise to multi-drug resistance strains therefore further investigations are required into this simultaneous transportation and occurrence of other drug resistance.
- Further studies are also required to characterize the *CfxA3* and *CfxA6* genes associated with periodontal pathogens carrying the genes and their spread to other oral bacteria.
- Molecular techniques could be developed to detect presence of β-lactamase genes from pathological samples. Iwahara et al (2006) reported a high performance of real-time PCR in detecting *CfxA* and *CfxA2* in clinical samples of dentoalveolar infections, this molecular method could thus provide a rapid clinical test for the detection of these resistance genes in patients and aid in the selection of antibiotic therapy.
- Quorum sensing or cell-to-cell signaling also influences diverse gene expression including virulence and antibiotic resistance. Research can focus on a unique approach targeting virulence rather than the actual organisms which will suppress the development of drug resistance.

5.3 Limitations

- Collection of subgingival plaque and pocket debri is not a routine procedure. Samples were collected by the student and processed purely for the research purpose. Since the culture media required for the study are not commercially available. All the media used in this study were prepared by the student. In addition, due to the long incubation time period (one week at a time) not many samples could be included in the study.
- Bacterial samples were stored in skim milk and microbank tubes with beads, however the number of samples that were recovered upon attempts to revive the samples were only 17. Anaerobes are hard to revive once they have been frozen, thus resulting in the low numbers that were recovered for the MIC study.
- Patients attending the Dental school at the University of the Witwatersrand were asked to participate in this study. A larger sample size would have been more of a representation of the country's population, but as funds were limited, patients could not be sampled across dental schools in South Africa.

- In vitro susceptibility testing has considerable variation in laboratory media and conditions of testing as well as interpretative criteria used by different laboratories although CLSI guidelines have been implemented.

- The CLSI guidelines that have been implemented for the disk diffusion antimicrobial susceptibility test are normally used for aerobic bacteria. Oxygen toxicity plays a role in the ability of anaerobic bacteria to move from lag-phase to exponential-phase of growth, this may lead to lack of

reproducible results.however in this study we applied these guidelines to interpret antimicrobial susceptibility results of anaerobic bacteria. The results of anaerobic disk diffusion tests performed either in the presence of some oxygen or in a complete anaerobic condition have been found similar (Johnson et al., 1995)

- Previous exposure to antibiotics are important in the development of drug resistance but this data was not available because patients could not tell me, some records were either missing or they were incomplete.

Chapter 7 Appendices

Appendix 1

1.1 Consent form

SUBJECT INFORMATION SHEET Revised
For verbal consent

Good Day,

How are you?

I am Dr M Patel from Oral Microbiology. My colleague and I are doing a study on germs that occur in our mouth and cause sicknesses.

These germs cause sores in our mouth and sometimes we have to take antibiotics to cure it. Penicillin and tetracycline are often used for our mouth. In many parts of the world these oral germs have become resistant to penicillin. Which means patients with resistant germs will not get better with penicillin. We would like to know if there are resistant germs in patients attending our clinics (South Africa).

In order to study, we would like to collect a sample from the gap between your gums and teeth. This may cause slight pain or the gum may bleed slightly for a day. However this will not cause any harm. The sample will be processed into a laboratory. I may not be present at the time of collection of sample, but my colleagues will read this consent and explain the procedure to you.

You may or may not participate it is entirely up to you. What you decide will not affect your treatment. If you agree to participate you may withdraw from the study at any time without affecting your treatment. The sample will be collected once only during your normal visit. There is no direct advantage of this procedure to you however once all the results from many patients are put together, we will know if we have developed penicillin resistance in South Africa or not and everybody will benefit from the knowledge.

Your sample will be given a number and will be processed under a number. Your name will not appear anywhere on the results or on any publications. This study has been through University ethics committee. Should you have any problems please contact Prof P. Cleaton-Jones at 011 717-1234

Patient's name:	Investigator's name:
Date:	Date:
Signature:	Signature:

1.2 Ethics certificate

M110112M110112

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Mrudula Patel

CLEARANCE CERTIFICATE	M110112
PROJECT	Prevalence of Beta-Lactamase-Producing Anaerobes in South African Patients
INVESTIGATORS	Dr Mrudula Patel.
DEPARTMENT	Clinical Microbiology & Infectious Diseases
DATE CONSIDERED	28/01/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

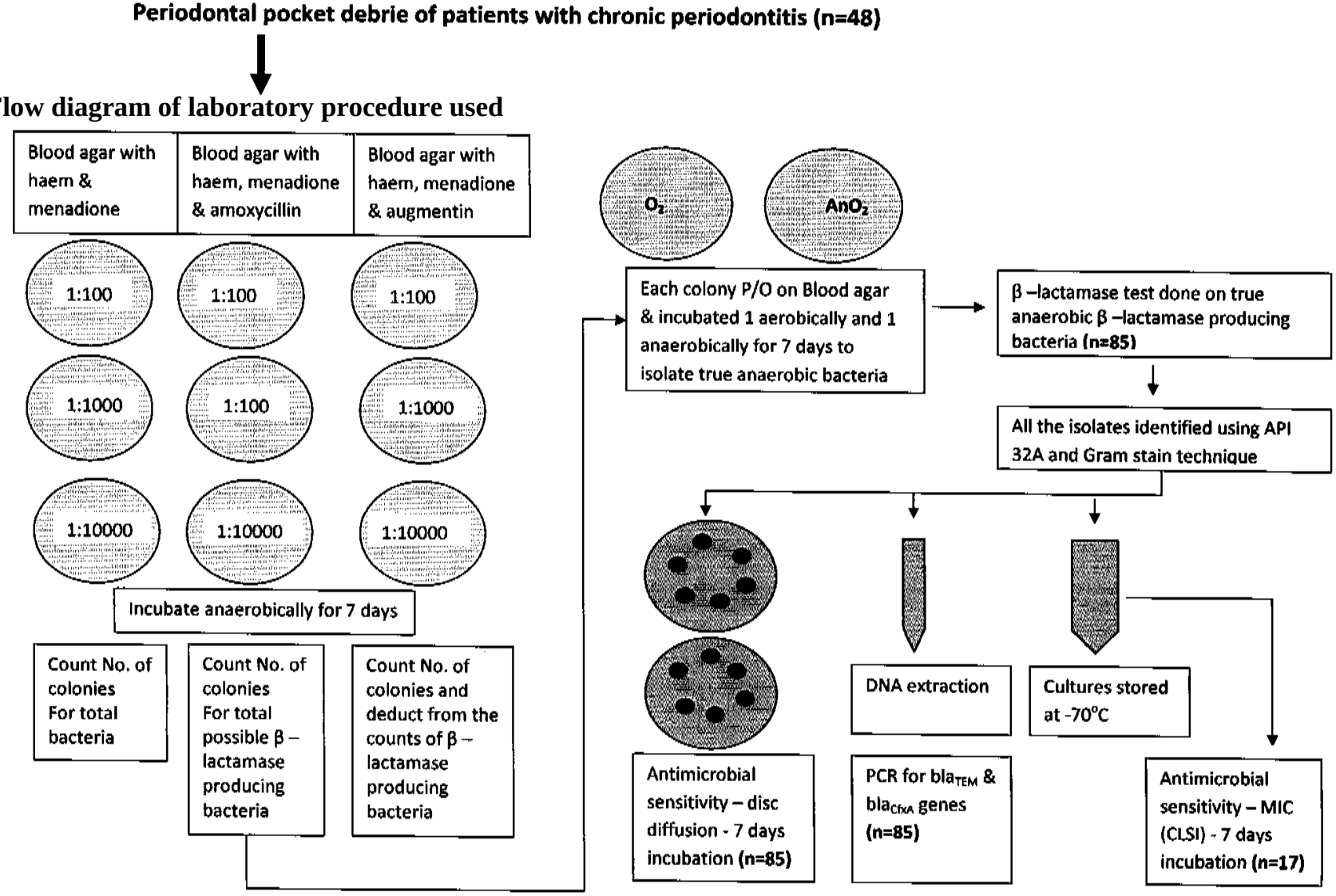
DATE 28/01/2011 CHAIRPERSON .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr M Patel

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

1.3 Flow diagram of laboratory procedure used



Appendix 2

2.1 Summary of results

Prevalence of β-lactamase-producing anaerobic oral bacteria (n=48 patients) →

Prevalence of β-lactamase species: **75 %**
No. of β-lactamase strains: 85 **(GNB:76,GNC:2,GPB:6,GPC:1)**
No. of β-lactamase strains/patient: **2**
Mean β-lactamase spp.
proportion of oral bacteria/patient: **9.4%**

β-lactamase-producing bacteria (n=85 isolates) →

Prevotella (49), Porphyromonas (6)
Bacteroides (16), Fusobacteruim (4)
Clostridium (3), Propionobacterium (3)
Veillonella (1), Mobiluncus (1), *Actinomyces Meyeri* (1)

Resistance to β-lactam antibiotics →

(disc diffusion test)

	Resistant to Penicillin No. strains (%)	**Resistant to Ampicillin No. strains (%)**
Prevotella spp	21 (25)	20 (24)
Porphyromonas spp	3 (4)	2 (2)
Bacteroides spp	5 (6)	6 (7)
Fusobacterium spp	0	1 (1)
Clostridium spp	1 (1)	1 (1)
Propionobacterium	1 (1)	1 (1)
Other	0	0
Total	31 (37)	31 (37)

Prevalence of β-lactamase genes →

(n=85)

β-lactamase gene (43/85 strains)	Genus (n=43)	No. of positive strains	Bla_{CfxA} genes		
			Bla_{CfxA2}	Bla_{CfxA3}	Bla_{CfxA6}
Bla_{CfxA} *CfxA2:* 76.7% *CfxA3:* 14 % *CfxA6* : 9.3	*Prevotella spp*	21	14	4	3
	Porphyromonas spp	4	3	-	1
	Bacteroides spp	12	10	2	-
	Fusobacterium spp	2	2	-	-
	Clostridium spp	2	2	-	-
	Propionobacterium spp	2	2	-	-
	Veillonella spp	0	-	-	-
	Actinomyces spp	0	-	-	-
	Mobiluncus spp	0	-	-	-
Bla_{TEM}	As above	0	-	-	-

Antimicrobial sensitivity and *cfxA* gene →

CfxA gene	No. of isolates resistant to β-lactam antibiotics (%)											
	Disc diffusion test (n=85)						Broth dilution test (n=17)					
	Ampicillin			Penicillin			Amoxicillin			Penicillin		
	R	I	S	R	I	S	R	I	S	R	I	S
Present (n=43)	17 (39.53)	1 (2.33)	25 (58.14)	23 (53.49)	1 (2.32)	19 (44.19)	9 (52.94)	0	8 (47.06)	10 (58.82)	1 (5.89)	6 (35.29)
Absent (n=42)	14 (33.33)	9 (21.43)	19 (45.24)	8 (19.05)	5 (11.90)	29 (69.05)	-	-	-	-	-	-

R: Resistant, S: Sensitive, I: Intermediate Sensitivity

2.2 Demography and total bacterial counts per sample

Patient number	Age	Gender	Pocket depth 1 (mm)	Pocket depth 2 (mm)	Blood agar only plate (cfu/sample)	Blood agar and amoxicillin plate (cfu/sample)	Blood agar and augmentin plate (cfu/sample)
1	78	F	8	5	2496000	16900	1300
2	67	F	8	6	33300	500	700
3	37	F	5	6	19300	1700	5800
4	55	F	7	6	16200	0	0
5	58	M	6	6	43600	100	0
6	56	F	7	9	72800	40000	0
7	83	F	7	5	760000	2600	17200
8	64	F	10	7	368000	95000	13000
9	47	M	8	8	404000	143000	776000
10	35	M	5	6	1560000	2000	0
11	37	F	5	6	132000	6100	1000
12	67	F	7	6	186400	56800	18400
13	65	M	5	6	34400	1100	800
14	64	F	8	7	74400	13100	2200
15	39	F	7	6	96000	92800	63200
16	61	F	8	6	560000	220000	0
17	33	M	10	7	159000	5400	0
18	41	M	5	6	124800	3300	0
19	44	M	8	6	1480000	99000	93000
20	65	F	5	6	680000	560000	470000
21	37	F	6	5	2610000	230000	140000
22	42	M	10	9	728000	10000	4000
23	40	F	5	6	1104000	40000	13000

24	60	F	5	5	2370000	430000	120000
25	76	M	6	6	1880000	720000	90000
26	45	M	9	7	2320000	220000	60000
27	57	F	6	6	5840000	930000	630000
28	52	M	7	5	530000	20000	10000
29	51	M	8	9	9380000	3520000	90000
30	22	M	8	8	1200000	380000	40000
31	58	M	7	6	6640000	120000	0
32	29	F	5	7	620000	40000	0
33	48	F	10	10	686000	5000	0
34	72	F	6	7	5120000	250000	30000
35	70	F	12	10	728000	12000	1000
36	34	F	6	6	912000	39000	3000
37	32	M	6	8	1120000	24000	10000
38	65	F	5	5	4160000	320000	20000
39	57	F	7	5	1760000	9000	0
40	60	F	6	6	39000	1200	4000
41	67	M	6	6	141000	15000	4000
42	54	F	7	8	896000	73000	31000
43	63	M	9	7	2960000	140000	40000
44	26	M	6	6	1034000	15000	2000
45	54	M	7	5	704000	78000	12000
46	33	M	6	8	7350000	30000	10000
47	29	F	6	8	7350000	30000	1000
48	63	F	13	7	5440000	36000	5000

2.3 Species isolated per patient

Patient number	Bacterial Species 1	Bacterial species 2	Bacterial species 3	Bacterial species 4	Bacterial species 5	Bacterial species 6
1	*Prevotella bivia*	*Prevotella oralis*	*Bacteriodes capillosus*	*Prevotella melaninogenica*		
2	Yeast					
3	Grew on both amx and aug					
4	No growth on antibiotic plates					
5	Facultative					
6	Aerobic					
7	Grew on both amx and aug					
8	*Porphyromonas endodontalis*					
9	*Prevotella intermedia*	*Fusobacterium nucleatum*	*Bacteriodes ureolyticus*			
10	*Prevotella oralis*	*Prevotella oralis*				
11	*Fusobacterium necrophorum*	*Propionibacterium granulosum*	*Prevotella oralis*	*Prevotella denticola*		
12	*Prevotella Melaninogenica*					
13	*Prevotella oralis*					
14	*Bacteriodes Ureolyticus*	*Prevotella oralis*	*Fusobacterium necrophorum*	*Clostridium botulinum 2*		
15	Grew on both amx and aug					
16	*Bacteroides eggerthii*	*Prevotella buccae*	*Clostridium sordelli*			
17	*Prevotella intermedia*	*Porphyromonas endodontalis*				
18	*Bacteroides eggerthii*	*Prevotella intermedia*				
19	Grew on both amx and aug					
20	*Prevotella oralis*					
21	Facultative					
22	*Veillonella spp*					
23	*Fusobacterium necrophorum*					
24	*Porphyromonas endodontalis*	*Prevotella oralis*	*Propionibacterium acnes*	*Prevotella oralis*	*Prevotella intermedia*	
25	*Prevotella Intermedia*	*Propionibacterium granulosum*				

26	*Porphyromonas gingivalis*	*Bacteriodes eggerthii*	*Actinomyces meyeri*			
27	*Prevotella Intermedia*	*Bacteriodes capillosus*				
28	*Bacteriodes capillosus*					
29	*Prevotella Intermedia*	*Prevotella intermedia*	*Prevotella oralis*			
30	*Prevotella oralis*	*Veillonella spp*	*Bacteriodes uniformis*			
31	*Prevotella oralis*	*Clostridium perfringens*				
32	*Prevotella Melaninogenica*	*Prevotella oralis*				
33	*Prevotella bivia*	*Porphyromonas gingivalis*	*Prevotella bivia*	*Prevotella oralis*		
34	*Prevotella buccae*	*Prevotella Melaninogenica*	*Prevotella intermedia*	*Prevotella intermedia*	*Bacteriodes capillosus*	*Prevotella intermedia*
35	*Bacteriodes capillosus*	*Prevotella oralis*				
36	*Prevotella oralis*	*Prevotella oralis*	*Prevotella oralis*	*Prevotella intermedia*	*Mobiluncus spp*	
37	*Porphyromonas endodontalis*	*Bacteriodes capillosus*				
38	*Prevotella intermedia*	*Prevotella oralis*	*Prevotella intermedia*			
39	*Bacteriodes ureolyticus*					
40	Grew on both amx and aug					
41	Facultative					
42	*Bacteroides Merdae*	*Prevotella oralis*				
43	*Prevotella intermedia*	*Prevotella intermedia*				
44	*Prevotella buccalis*					
45	Facultative					
46	*Prevotella melaninogenica*	*Prevotella oralis*				
47	*Prevotella intermedia*	*Prevotella oralis*				
48	*Bacteroides capillosus*	*Prevotella buccae*				

2.4 Antimicrobial susceptibility of bacterial samples

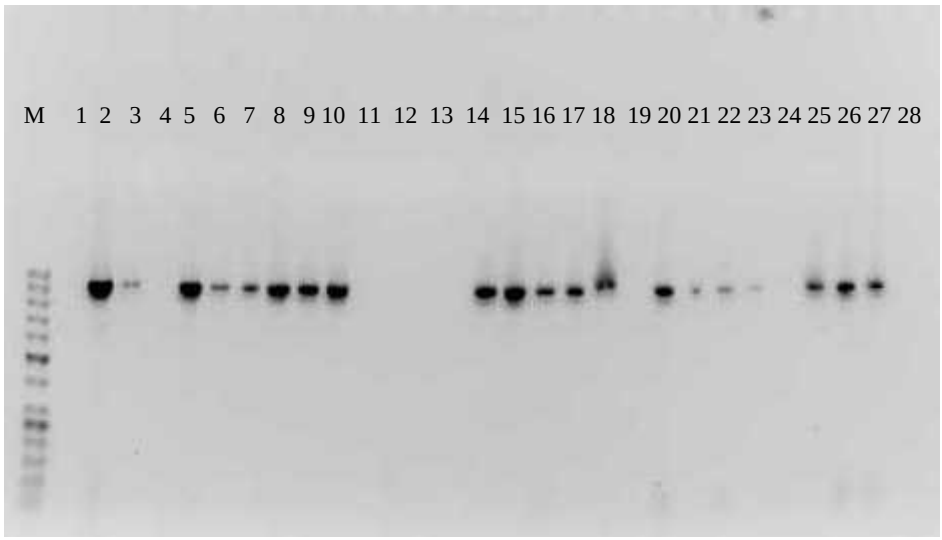
Patient number	Sample number	API ID	Antimicrobial susceptibility											
			CD	TS	C	RP	SYN	LZD	FC	AP	P	VA	GM	E
1	1.2	*Prevotella bivia*	S	R	S	S	S	S	S	I	I	S	R	S
	1.3	*Prevotella oralis*	S	S	S	S	I	S	S	R	R	R	R	S
	1.4	*Bacteriodes capillosus*	S	I	S	S	S	S	S	S	S	R	R	S
	1.5	*Prevotella melaninogenica*	S	S	S	S	S	S	S	R	R	R	R	S
2	2	Yeast												
3	3	Grew on both amx and aug												
4	4	No growth on antibiotic plates												
5	5	Facultative												
6	6	Aerobic												
7	7	Grew on both amx and aug												
8	8	*Porphyromonas endodontalis*	S	R	S	S	S	S	S	S	R	I	R	S
9	9.1	*Prevotella intermedia*	S	R	S	S	S	S	S	S	I	R	R	S
	9.2	*Fusobacterium nucleatum*	R	R	S	I	R	S	R	S	S	R	R	R
	9.4	*Bacteriodes Ureolyticus*	R	R	S	S	S	S	S	R	R	I	R	S
10	10.1	*Prevotella oralis*	S	R	S	S	S	I	S	S	R	I	R	S
	10.3	*Prevotella oralis*	I	R	S	S	S	S	R	S	R	S	R	S
11	11.1	*Fusobacterium necrophorum*	S	R	S	S	S	S	S	R	S	R	R	S
	11.2	*Propionibacterium granulosum*	S	S	S	S	S	S	S	I	S	S	R	S
	11.3	*Prevotella Oralis*	R	R	S	S	S	S	I	I	S	R	R	S
	11.4	*Prevotella denticola*	R	R	S	S	S	S	S	I	S	R	R	S
12	12	*Prevotella melaninogenica*	S	R	S	S	S	S	S	R	R	R	R	S
13	13	*Prevotella oralis*	S	R	S	S	S	S	S	S	I	R	R	S
14	14.1	*Bacteriodes Ureolyticus*	S	S	S	S	S	S	S	S	S	R	R	S
	14.2	*Prevotella oralis*	S	I	S	S	S	S	S	I	R	R	I	S
	14.3	*Fusobacterium necrophorum*	S	R	S	S	S	S	R	S	S	I	R	S

	14.4	*Clostridium botulinum 2*	S	S	S	S	S	S	S	R	S	I	S	S
15	15	Grew on both amx and aug												
16	16.1	*Bacteroides eggerthii*	R	S	S	S	S	S	S	S	S	I	I	S
	16.2	*Bacteroides eggerthii*	S	R	S	S	S	S	S	R	R	R	R	S
	16.4	*Clostridium sordelli*	S	R	S	S	S	S	S	S	R	R	R	S
17	17.1	*Prevotella intermedia*	S	R	S	S	S	S	S	S	R	R	R	S
	17.2	*Porphyromonas endodontalis*	S	S	S	S	S	S	S	S	R	R	R	S
18	18.1	*Bacteroides eggerthii*	S	R	S	S	S	S	S	R	R	R	R	S
	18.2	*Prevotella intermedia*	S	R	S	S	S	S	S	S	S	R	R	S
19	19	Grew on both amx and aug												
20	20	*Prevotella oralis*	S	R	S	S	S	S	I	R	R	I	R	I
21	21	Facultative												
22	22	*Veillonella spp*	S	R	S	I	R	S	R	I	I	R	R	R
23	23	*Fusobacterium necrophorum*	S	R	S	S	S	S	S	S	S	R	R	S
24	24.1	*Porphyromonas endodontalis*	S	R	S	S	S	S	S	S	S	R	R	S
	24.2	*Prevotella oralis*	S	R	S	S	S	S	S	S	S	R	R	S
	24.3	*Propionibacterium acnes*	S	R	S	S	S	S	S	S	S	R	R	S
	24.5	*Prevotella Oralis*	S	R	S	S	S	S	S	R	R	R	R	S
25	25.1	*Prevotella Intermedia*	S	R	S	S	S	S	S	I	S	R	R	S
	25.2	*Propionibacterium granulosum*	S	S	S	S	S	S	S	R	R	R	R	S
26	26.1	*Porphyromonas gingivalis*	R	S	S	S	S	S	S	R	R	R	R	R
	26.2	*Bacteriodes eggerthii*	S	S	S	S	S	S	S	R	S	S	R	S
	26.3	*Actinomyces meyeri*	S	S	S	S	S	S	S	S	S	S	R	S
27	27.1	*Prevotella Intermedia*	S	R	S	S	I	S	S	S	S	R	S	S
	27.2	*Bacteriodes capillosus*	S	R	S	I	S	S	S	S	S	R	R	R
28	28	*Bacteriodes capillosus*	S	R	S	S	S	S	S	S	S	R	R	R
29	29.1	*Prevotella intermedia*	R	R	S	S	S	S	S	R	R	R	R	S
	29.2	*Prevotella intermedia*	S	R	S	S	S	S	S	R	R	R	R	S
	29.3	*Prevotella oralis*	S	R	S	S	S	S	S	R	R	I	R	S
30	30.1	*Prevotella oralis*	S	R	S	S	S	S	S	I	R	S	S	S
	30.2	*Veillonella spp*	S	R	S	S	R	S	R	S	S	R	R	R
	30.3	*Bacteriodes uniformis*	S	R	S	S	S	S	S	S	S	R	R	R

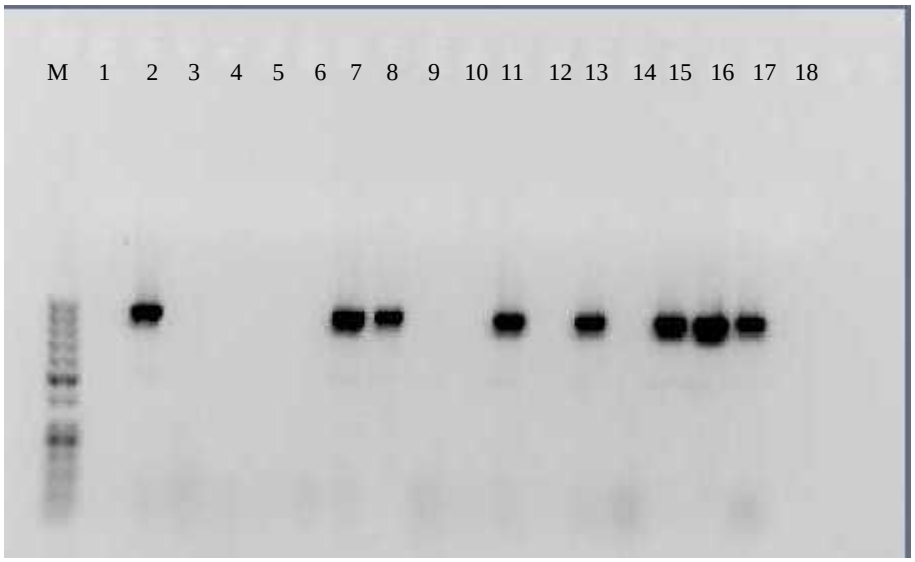
31	31.1	*Prevotella oralis*	S	R	S	S	R	S	R	S	R	R	R	R
	31.3	*Clostridium perfringens*	S	S	S	S	S	S	S	S	S	S	R	S
32	32.1	*Prevotella Melaninogenica*	S	R	S	R	S	S	S	S	S	S	R	S
	32.2	*Prevotella oralis*	S	R	S	S	S	S	S	S	S	R	R	S
33	33.1	*Prevotella bivia*	S	R	S	S	S	S	S	R	S	R	R	S
	33.2	*Porphyromonas gingivalis*	S	R	S	S	S	S	S	R	S	R	R	S
	33.3	*Prevotella bivia*	S	R	S	S	S	S	S	R	S	R	R	S
	33.4	*Prevotella oralis*	S	R	S	S	S	S	S	R	S	R	R	S
34	34.1	*Prevotella buccae*	S	R	S	S	S	S	S	R	R	R	R	R
	34.2	*Prevotella melaninogenica*	S	R	S	S	S	S	S	S	S	I	R	I
	34.3	*Prevotella intermedia*	S	I	S	S	S	S	S	S	S	S	I	S
	34.4	*Prevotella intermedia*	S	R	S	S	S	S	S	S	S	S	R	S
	34.5	*Bacteriodes capillosus*	S	R	S	S	S	S	S	S	S	S	R	S
	34.6	*Prevotella Intermedia*	S	R	S	S	S	S	S	S	S	I	R	S
35	35.1	*Bacteriodes capillosus*	S	I	S	R	S	S	S	R	R	R	R	S
	35.2	*Prevotella oralis*	S	I	S	S	S	S	S	R	R	S	R	S
36	36.1	*Prevotella oralis*	S	R	S	S	R	S	S	R	R	R	R	S
	36.2	*Prevotella oralis*	S	R	S	S	S	S	S	R	I	R	R	S
	36.3	*Prevotella Intermedia*	S	R	S	S	S	S	S	S	S	R	R	S
	36.4	*Mobiluncus spp*	S	R	S	S	S	S	S	S	S	S	R	S
37	37.1	*Porphyromonas endodontalis*	S	R	S	S	S	S	S	I	S	I	R	S
	37.2	*Bacteriodes capillosus*	S	R	S	S	S	S	I	I	S	I	R	S
38	38.1	*Prevotella intermedia*	S	R	S	S	S	S	S	R	R	R	R	S
	38.2	*Prevotella oralis*	S	R	S	S	S	S	S	R	S	R	R	S
	38.3	*Prevotella intermedia*	S	R	S	S	S	R	S	R	R	R	R	S
39	39	*Bacteriodes ureolyticus*	S	R	S	S	S	S	S	S	S	R	R	S
40	40	Grew on both amx and aug												
41	41	Facultative												
42	42.1	*Bacteroides Merdae*	R	R	S	S	S	S	R	R	R	S	R	R
	42.2	*Prevotella oralis*	R	R	S	S	S	S	S	S	S	S	R	R
43	43.1	*Prevotella intermedia*	S	R	S	S	S	S	S	S	R	S	R	S
	43.2	*Prevotella intermedia*	S	R	S	S	S	S	S	S	S	S	R	S

44	44	*Prevotella buccalis*	S	R	S	I	S	S	S	R	S	R	R	S
45	45	Facultative												
46	46.1	*Prevotella melaninogenica*	S	R	S	S	S	S	S	R	S	R	R	S
	46.2	*Prevotella oralis*	S	R	S	S	S	S	S	S	S	S	R	S
47	47.1	*Prevotella intermedia*	S	R	S	S	S	S	S	S	S	S	R	S
	47.2	*Prevotella oralis*	S	R	S	S	S	S	S	S	S	S	R	S
48	48.1	*Bacteroides capillosus*	S	R	S	S	S	S	S	S	S	R	R	S
	48.2	*Prevotella buccae*	S	R	S	S	S	S	S	S	R	S	R	S

2.5 Gel electrophoresis results of PCR products from β-lactamase-producing oral anaerobes



Lane M indicates the O'GeneRuler™ 50 bp DNA ladder molecular marker. Lanes 1 to 26, indicate β-lactamase-producing oral anaerobes, Lane 27 is the Positive control and Lane 28 the negative control.



Lane M indicates the O'GeneRuler™ 50 bp DNA ladder molecular marker. Lanes 1 to 16, indicate β-lactamase-producing oral anaerobes, Lane 17 is the Positive control and Lane 18 the negative control.

Appendix 3

3.1 Composition and preparation of media

1% Agarose gel

0.1 g	Seakom® LE Agarose (Lonza, USA)
100ml	1× Tris-borate-EDTA (TBE) Buffer
5µl	Ethidium Bromide

Ethidium bromide was added to a 1% agarose gel made up Agarose and TBE buffer. The gel was left to cool down for 2 minutes, poured into a moulding apparatus, a comb placed into the notch to create sample wells, and the gel left to solidify for 15 minutes forming a gel 'slab'.

Amoxicillin (Stock solution for MIC test)

129 g	Amoxicillin (Smithkline Beecham)
100 ml	Phosphate buffer, pH6.0

Amoxicillin powder was added to Phosphate buffer, vortexed and dispensed into appropriate sterile vials, sealed and frozen at ≥ 60˚C.

Blood agar

39 g	Columbia agar (Oxoid Ltd, UK)
5 g	Sterile defibrinated blood
5mg	Haemin
1mg	Menadione
1000 ml	Distilled water

Columbia agar base was dissolved in a 1000 ml of distilled water. It was sterilized by autoclaving at 151b and 121 °C for 10 minutes. It was allowed to cool to 50 °C and 5 % sterile defibrinated blood was added. It was poured into petri dishes, allowed to set and refrigerated until use.

Blood agar with Amoxicillin

39 g	Columbia agar (Oxoid Ltd)
5 g	Sterile defibrinated blood
5mg	Haemin

1mg	Menadione
3mg	Amoxicillin (Smithkline Beecham)
1000 ml	Distilled water

Columbia agar base was dissolved in a 1000 ml of distilled water. It was sterilized by autoclaving at 151b and 121 °C for 10 minutes. The agar base was allowed to cool to 50 °C and 3mg of Amoxicillin and 5 % sterile defibrinated blood was added. It was poured into petri dishes and they were refrigerated until use.

Blood agar with amoxicillin and clavulanic acid

39 g	Columbia agar (Oxoid Ltd)
5 g	Sterile defibrinated blood
5mg	Haemin
1mg	Menadione
3 mg	Amoxicillin (Smithkline Beecham)
0.75 mg	Clavulanic acid (Smithkline Beecham)
1000 ml	Distilled water

Columbia agar base was dissolved in a 1000 ml of distilled water. It was sterilized by autoclaving at 151b and 121 °C for 10 minutes. The agar base was allowed to cool to 50 °C and 3mg of Amoxicillin, 0.75 mg of Clavulanic acid and 5 % sterile defibrinated blood was added. It was poured into petri dishes and they were refrigerated until use.

Clavulanic acid (Stock solution for MIC test)

76 g	Clavulanic acid (Smithkline Beecham)
50 ml	Phosphate buffer, pH6.0

Clavulanic acid powder was added to Phosphate buffer to create a stock solution with a concentration of 1280 µg/ml and dispensed into appropriate sterile vials, sealed and frozen at ≥ 60˚C.

Fusi Form Medium

37 g	Brain heart infusion (Biolab Diagnostics Pty. Ltd, SA)
3 g	Yeast extract
2 g	Soluble starch
1000 ml	Distilled water
pH 7.6	

Medium was dissolved in water and autoclaved at 151b and 121 °C for 15 minutes.

Haemin

0.5 g	Haemin
10 ml	1N NaOH
90 ml	Distilled water

Haemin and Sodium hydroxide were dissolved in distilled water and autoclaved at 151b and 121 °C for 15 minutes.

Penicillin (Stock solution for MIC test)

125 g	Penicillin
100 ml	Distilled water

Penicillin powder was suspended in water, vortexed and dispensed into appropriate vials. These were stored in a freezer at ≥ 60 ˚C.

Phosphate buffered saline

4.2 g	Sodium Chloride
0.078 g	Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)
0.64 g	Sodium hydrogen phosphate ($NaHPO_4$)
500 ml	Distilled water

These were suspended in water and autoclaved at 151b and 121 °C for 15 minutes.

Reduced Transport fluid

7.5 ml	K_2HPO_4
1 ml	0.1 M EDTA
0.5 ml	Na_2CO_3
0.12 g	Sodium chloride
0.12 g	$(NH4)_2SO_4$

0.06 g	KH_2PO_4
0.025 g	$MgSO_4$
2 ml	Fresh dithiothreitol
81.4 ml	Distilled water

Reagents were mixed with distilled water, filter sterilized with a 0.22µm filter and pre-reduced by being placed in an anaerobic environment for 24 hours. The fluid was then dispensed into vials and refrigerated until use.

Tryptone Broth

12.5 g	Tryptone powder (Biolab Diagnostics Pty. Ltd, SA)
7.5 g	Yeast extract
5 g	Sucrose
0.5 ml	Haemin
0.5 ml	Menadione
500 ml	Distilled water
pH 7.0	

Medium was dissolved in water and autoclaved at 151b and 121 °C for 15 minutes.

The Broth was was dispensed into microtitre plates and refrigerated until use.

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