ANTIBIOTIC RESISTANCE IN ANAEROBIC BACTERIA

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ABSTRACT

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Anaerobic bacteria are important human pathogens capable of causing serious debilitating infections ranging from abscesses to life threatening infections and warrant more attention than they are currently receiving. Anaerobes are often present in mixed infections in association with other anaerobes/facultative anaerobes and aerobic bacteria and it is imperative to administer correct antimicrobial therapy *ab initio*. Antibiotic resistance development in anaerobic bacteria has a tremendous impact on selection of effective antimicrobial agents for empiric therapy. ß-Lactam antibiotics are frequently used and have for many years been the first choice in the treatment and prophylaxis of anaerobic and mixed aerobic/anaerobic infections. Current knowledge of ß-lactam resistance progression and resistance mechanisms in anaerobic bacteria is, however, limited. Metronidazole is often used empirically against suspected anaerobic infections, but anaerobes can no longer be considered to be universally susceptible as resistance has been noted in strains of *Bacteroides fragilis*, clostridia and peptostreptococci.

The objectives of the study were to: 1) assess antibiotic susceptibilities of anaerobic bacteria isolated in the Bloemfontein area to antimicrobial agents currently employed in empiric treatment, 2) compare the *in vitro* activity of currently employed antibiotics with new antimicrobial agents, and 3) conduct studies on antibiotic resistance development.

Anaerobic bacteria were isolated from clinically significant infections from April 1996 to March 1997 from the Universitas and Pelonomi Hospitals, Bloemfontein. Infection sites traced for 302 of 378 of the isolates, were from blood, brain abscesses, liver abscesses, lung infection/abscesses, eustachian infection/sepsis, neoplasms, bone fracture/infection, post-operative/amputation sepsis, gunshot/stab wound infection/

sepsis, genital tract isolates, general abscesses, and intestinal tract infections. Isolates were identified in the routine diagnostic laboratory by presumptive and preliminary methods and results confirmed by the Rapid ID32A identification system. Minimum inhibitory concentrations (MICs) were determined by the National Committee for Clinical Laboratory Standards (NCCLS) agar dilution method for the following 18 antimicrobial agents: six ß-lactams (amoxicillin, ampicillin, penicillin, piperacillin, cefoxitin, cefepime and cefpirome), two carbapenems (imipenem and meropenem), metronidazole, clindamycin, chloramphenicol, ciprofloxacin, trovafloxacin, vancomycin, dalfopristin/quinupristin, linezolid and loracarbef. Screening for ß-lactamase production was performed by employing nitrocefin and inhibition of ß-lactamases determined using amoxicillin/clavulanic acid combination. For detection of carbapenemase/metallo-ß-lactamase production a biological assay was performed; cell extracts and imipenem being added to agar seeded with *E. coli* ATCC 25922. Detection of metallo-ß-lactamase genes was undertaken with primers directed to *cfiA, cphA* and bla_{IMP} genes. PBP profiles and penicillin affinities were determined by labelling with [^{3}H]penicillin, separation of proteins by SDS-PAGE and visualisation after fluorography. In PBP competition studies whole cell samples were initially preincubated with imipenem, piperacillin or ampicillin at different concentrations and post-labelled with [^{3}H]penicillin, followed by SDS-PAGE and fluorography.

Metronidazole MICs of 64 isolates were correlated with inhibitory concentrations (ICs) obtained with two batches of Etest strips (range 0.006 - 32 µg/ml and 0.016 - 256 µg/ml). Membrane proteins of parental and metronidazole mutant strains of a *Veillonella* sp. and *Peptostreptococcus prevotii* were separated by SDS-PAGE and profiles compared. The prevalence of *rdxA* genes was investigated in 16 anaerobic/ facultative anaerobic bacteria with metronidazole MICs ≥ 1 µg/ml employing two sets of primers and fragments of approximately 937 bp and 491 bp sequenced. The prevalence of *nim* genes were investigated in 64 anaerobic/facultative anaerobic isolates with metronidazole MICs ≥ 0.5 µg/ml using a pair of universal *nim* gene primers. Amplification was performed at two annealing temperatures (52°C & 62°C) and fragments at approximately 458 bp recorded as presumptive positives and sequenced. Positive strains were subjected to plasmid extraction.

MICs indicated overall susceptibility of Gram-positive anaerobic isolates to be higher than for the Gram-negative isolates. Reduced susceptibility to penicillin (MICs

≥ 1 µg/ml) was found in 20 *Peptostreptococcus* strains and seven non-perfringens *Clostridium* spp. ß-Lactamases hydrolysing both penicillins and cephalosporins were demonstrated in all *Bacteroides* and *Prevotella* isolates with ampicillin MICs >4 µg/ml. Only 8% *Peptostreptococcus* spp. were resistant to piperacillin in contrast to 41% *Bacteroides* spp. and 68% *Veillonella* spp. *Veillonella* spp. exhibited selective ß-lactam resistance to piperacillin. Cefoxitin showed excellent activity against both Gram-positive and Gram-negative isolates, except for *Bacteroides* and *Fusobacterium* species. The majority of Gram-positive isolates were susceptible to cefepime and cefpirome, whereas < 50% *Bacteroides* spp. and < 70% *Prevotella* spp. were susceptible. High-level resistance to imipenem/meropenem (MICs >128 µg/ml) was seen for 13/37 *Fusobacterium* spp. Two *P. magnus* isolates were resistant to metronidazole (MIC >128 µg/ml), three *C. perfringens* strains showed reduced susceptibility (MICs 4-8 µg/ml), while two *Prevotella* spp. had metronidazole MICs of 32 µg/ml. Eighty five percent of all isolates were susceptible to clindamycin. Dalfopristin/quinupristin exhibited excellent activity throughout the Gram-positive bacterial spectrum with only one *Peptostreptococcus* sp. showing reduced susceptibility (MIC 8 µg/ml), but poor activity against *B. fragilis* group isolates. Trovafloxacin was effective against all the Gram-positive anaerobes except for two *P. anaerobius* strains (MICs 8 µg/ml), and demonstrated superior activity to ciprofloxacin against the Gram-negative isolates. Overall, chloramphenicol was the most effective antibiotic, with only two *Clostridium* spp. being resistant (MICs 16 µg/ml).

PCR products of predicted size of *cfiA* genes were found in two strains of *B. vulgatus,* a *B. capillosus* and a *P. loescheii* strain and of *cphA* genes in three *B. fragilis* strains and a strain of *P. loescheii.* None of the PCR products on sequencing, however, were seen to be positive for *cfiA* or *cphA* genes. No bla_{IMP} genes were amplified. PBP profiles were analysed with respect to identification based on the API Rapid ID 32A system. Seven major groups of fusobacteria PBPs could be identified, but although PBP profile/API agreement was evident for the majority of *F. mortiferum* strains, the API system did not lend itself to reliable identification of fusobacteria. PBP profiles were seen to distinguish species/subspecies of *Clostridium* species other than *C. perfringens*, while comparison with the API identification method showed some correlation, but not with most of the species investigated. Comparing PBP profiles of nine *Veillonella* spp. assigned three groups of species/subspecies. Commercial identification systems appeared not to be as reliable as promoted. The

differentiation of anaerobic bacterial species by PBP profiling could certainly assist in situations of therapeutic failure.

For *F. mortiferum* the PBP with the lowest affinity for penicillin and imipenem was the highest-molecular-weight PBP, 74 kDa. The PBP profile of an imipenem-resistant variant of *F. varium* (MIC >128 µg/ml) demonstrated an additional PBP (±69 kDa) when compared to that of the parental strain (apparent MIC 0.5 µg/ml). In two *C. tertium* strains, a *C. sporogenes* and a *C. bifermentans* strain low-molecular-weight (< 50 kDa) PBPs exhibited reduced affinity towards penicillin. In *Veillonella* isolates a PBP (66 kDa) that possessed the highest affinity for penicillin, was seen to exhibit the lowest affinity for piperacillin.

Regression analysis revealed good correlation between metronidazole agar dilution MIC and Etest IC values. In the clinical setting, metronidazole Etests provide an important role in the susceptibility testing of anaerobes. Inducing metronidazole resistance in a *P. prevotii* and a *Veillonella* strain produced mutants with only a two-fold increase in metronidazole MICs increase, yet alterations to several membrane proteins were apparent. Such findings, as were also found with PBP analysis conducted in this study, complexes interpretation as to how antibiotic resistance has developed in anaerobic bacteria.

Nim genes were demonstrated in 14/64 strains (MICs ≥ 0.5 µg/ml) and identified as *NimA* genes in five propionibacteria, a *P. bivia*, a *C. bifermentans* and an *A. odontolyticus* strain, with *nimB* genes identified in five isolates of *B. fragilis* and a *P. magnus* strain. Sequence divergence was < 4% from the respective documented *nimA* and *nimB* gene sequences. Although the origin of *nim* genes is unknown, predominance of *nimA* genes in facultative anaerobes, propionibacteria, may indicate a transferable *nimA* gene source in anaerobic environments.

To avert the clinical problem of untreatable anaerobic infections, it is necessary to continuously monitor for the emergence of antibiotic-resistant strains, conduct investigations into how resistance has developed, and understand conditions that foster inter- and intra-dissemination of resistance genes among anaerobic bacteria.

DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Ph.D. at the University of the Orange Free State, Bloemfontein. It has not been submitted for any degree or any examination at any other university.

Maria Magdalena Theron

28th day of September 2000.

DEDICATION

Aan Pa en Ma

... Met liefde

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ABBREVIATIONS

AMP	Ampicillin
AMX	Amoxicillin
API	Analytical Profile Index
ATCC	American Type Culture Collection
bp	basepair(s)
CFU	Colony forming units
CPI	Cefepime
CPO	Cefpirome
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ESBL	Extended-spectrum ß-lactamase
FOX	Cefoxitin
HIV	Human Immunodeficiency Virus
IC	Inhibitory concentration
IMP	Imipenem
IS	Insertion sequence
kb	Kilobases
kDa	Kilodalton
MER	Meropenem
MIC	Minimum inhibitory concentration
MWM	Molecular-weight marker
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced form)
NCCLS	National Committee for Clinical Laboratory Standards
NI	Nitroimidazole
OD	Optical density
OMP	Outer membrane protein
PBP	Penicillin-binding protein
*p*CMB	*p*-Chloromercuribenzoic acid
PCR	Polymerase chain reaction
PIP	Piperacillin
rRNA	Ribosomal ribonucleic acid
RSA	Republic of South Africa

SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TAE	Tris EDTA
UK	United Kingdom
USA	United States of America
UV	Ultra violet
VPI	Virginia Polytechnic Institute

CHAPTER 1

INTRODUCTION

1.1 ANAEROBIC INFECTIONS

In South Africa there is a disastrous incidence of trauma and most of the population is at risk. Recently it has been estimated that the annual mortality rate of homicide in South Africa is six times greater than in the USA (Van der Spuy, 1996). Furthermore the traffic death rate is approximately ten times that of the USA, Britain and the Scandinavian countries (Van der Spuy, 1996). These factors together with several other diverse causes, gunshot wounds, stab wounds and burns resulting from unrest related incidents such as ideological conflicts, taxi violence or faction fights, are responsible for injuries that can predispose to anaerobic infections. Additional factors for anaerobic infections may also include angiopathy, haemostasis, suppressed immune system and existing aerobic infection (Davies Diagnostics, 1999).

Anaerobes constitute a major portion of normal microbial flora and are found on the skin, in the nasopharynx, gastrointestinal tract/rectum and genital tract. Should they be displaced from these sites where they occur in a balanced microenvironment, anaerobes will proceed to initiate disease. Consequently anaerobic bacteria represent a large group of human and animal pathogens that are capable of causing a diversity of infections, ranging from minor cutaneous infections and abscesses, to serious life-threatening septicaemia (Odelson *et al.*, 1987; Appelbaum, 1992). Anaerobes present as normal flora are afforded ample opportunity for infecting adjacent areas such as those in the oral cavity/oropharynx often being associated with lung infections (Finegold & Sutter, 1986).

Anaerobes are commonly found in conjunction with aerobic and/or facultative anaerobic bacteria, but in favourable anaerobic environmental conditions, even from within these mixed infections, anaerobes may proliferate to become the major

infecting agent (Appelbaum, 1992). Such polymicrobial aerobic/anaerobic bacterial infections occur frequently and in most anatomic sites, the anaerobic bacteria in such situations being described as relatively aerotolerant (Nichols & Smith, 1993). It is also thought that in mixed infections, aerobic bacteria that precede infection by anaerobic bacteria, cause a decrease in the oxidation-reduction necessary for initiating anaerobic infection (Ajiki *et al.*, 1991). The aetiology of these infections is endogenous as the normal microbial flora that colonises the various mucosal surfaces of the body can be isolated from infections after trauma to these membranes. Proliferation to adjacent tissues and normally sterile sites may then ensue. Infections such as brain abscesses and aspiration pneumonia can be purely anaerobic in aetiology (Aldridge, 1995).

Certain clinical signs, symptoms and conditions are characteristic of anaerobic infections. This may include foul-smelling discharge, necrotic tissue, gangrene, gas in tissues or in discharges, endocarditis with negative routine blood cultures, infection following human or animal bites or clinical conditions predisposing to anaerobic infection (septic abortion, gastrointestinal surgery and other surgical procedures and diseases) (Finegold & Sutter, 1986). The high incidence of trauma in South Africa has already been established and the introduction of foreign material (soil, clothing, metal) enhances anaerobic bacterial growth, while gut spills into the peritoneal cavity from penetrating abdominal wounds may be lethal. Traumatised tissue can often become deprived of its blood (and oxygen) supply so that anaerobic conditions will develop, creating a milieu for proliferation of anaerobes that may have been introduced into wounds. Aerobic and anaerobic bacteria normally found in human endogenous gastrointestinal microflora are the most frequent isolates from both postoperative incisional infections and intra-abdominal infections (Nichols & Smith, 1993). If these infections are not contained, e.g. ulceration providing a portal of entry to regional lymphatics, ruptured appendix/ surgery/ penetrating trauma, anaerobes can invade the peritoneum with susequent septicaemia or peritonitis, often with serious complications (Nichols & Smith, 1993). Large numbers of anaerobes are also present in the vagina, which may predispose to vaginal discharge and pelvic infection (Croco *et al.*, 1994; Aldridge, 1995). Anaerobic bacteria have been identified as the main agents in many clinically significant infections, especially when host defences are lowered by processes such as surgery, malignancy, malnutrition and trauma and now more recently HIV (Appelbaum, 1992; Coronado *et al.*, 1996).

Most people go through life without suffering a clinically significant anaerobic infection, which is remarkable when considering the enormity of the anaerobe population that surrounds us. Whereas anaerobes are present in soil, manure, decaying matter and hot springs, only a small number of the anaerobic species have been involved in pathogenic processes in humans or animals. Anaerobic bacteria are seldom involved in epidemics and secondary infections are rarely, if ever, caused by anaerobes transmitted from person to person, making it difficult to believe that the primary role of anaerobes is in illness (Bokkenheuser, 1993).

Infections developing during the first 48 hours after surgery are characteristically caused by either anaerobic clostridia or aerobic ß-haemolytic streptococci (Nichols & Smith, 1993). Dramatic clinical presentations may include profound systemic toxicity and rapid local advancement of the infection that will often involve all layers of the body wall. High mortality rates (60-80%) can be expected unless a rapid diagnosis is made from the clinical presentation and the preliminary results from the laboratory (Nichols & Smith, 1993).

Some of the most potent exotoxins known to man are produced by anaerobic bacteria (Odelson *et al.*, 1987). Toxin production, together with proteolytic enzymes which have the capacity to decompose proteins, are rendering some anaerobic bacteria exceptionally virulent. The anaerobes (like the aerobes) also produce enzymes that can cause cellular destruction (e.g. collagenases, hyaluronidases), abscess formation and antimicrobial destruction (ß-lactamases) (Aldridge, 1995).

Although anaerobic bacteria have been increasingly recognised as important pathogens in a variety of diseases, studies have yielded conflicting results about the incidence of anaerobic bacteraemia (Peraino, Cross & Goldstein, 1993; Goldstein, 1996). Although there have been reports of a decline in the incidence of anaerobic bacteraemia over the last two decades, there is also mention of an increase in the risk among the elderly and patients with haematological diseases (Peraino, Cross & Goldstein, 1993; Goldstein, 1996). The decline may be attributed to improvements in the prevention, recognition and treatment of anaerobic infections, especially surgical wound infections.

Enormous progress has been made in the knowledge of anaerobic bacteria and their role in disease, but there are still many problems and controversies that need be addressed. The clinical relevance of anaerobic bacteriology is a major contentious issue as it is not always possible for a microbiologist with limited resources to perform reliable and detailed studies of anaerobic bacteriology (Finegold, 1990). Neither has the when and how to perform susceptibility testing been clarified (Finegold, 1990). There is also inadequate information on the role of certain newly described or reclassified anaerobes in disease processes, normal sites of carriage, as well as antimicrobial susceptibility profiles (Finegold & Jousimies-Somer, 1997).

1.2 ANAEROBIC BACTERIA

1.2.1 Gram-negative genera

The Gram-negative anaerobic bacilli are the most commonly encountered anaerobes in clinical infections (Finegold, 1995). Important genera include *Bacteroides, Prevotella, Porphyromonas, Fusobacterium* and to a lesser extent *Bilophila, Actinobacillus, Capnocytophaga* and *Leptotrichia* (Finegold, 1995). Microscopically *Bacteroides* spp. can be seen as pleomorphic rods with rounded cells (*B. fragilis*, *B. vulgatus* and *B. thetaiotaomicron*), straight rods with rounded ends (*B. distasonis*) or oval cells with rounded ends (*B. ovatus*) (Howard & Keiser, 1994). Colonies of *Bacteroides* species are characteristically circular, smooth and shiny and except for some *B. fragilis* strains that may be slightly ß-haemolytic, are generally non-haemolytic (Howard & Keiser, 1994). *Prevotella* species are coccobacillary rods with typical circular, shiny, nonhaemolytic colonies (Howard & Keiser, 1994). Some species like *P. disiens* may show light orange to pink fluorescence on blood agar, while *P. melaninogenica* has dark pigmented colonies that fluoresce red under UV light. Fusobacteria on Gram-stain appear microscopically as pale, pleomorphic rods that may be filamentous with round bodies (*F. mortiferum* and *F. necrophorum*), coccoid (*F. varium*) or long and slender with pointed ends (*F. nucleatum*) (Howard & Keiser, 1994). Colony morphology of fusobacteria shows a "bread-crumb" appearance and on blood agar *F. necrophorum* colonies have a ground-glass appearance (Howard & Keiser, 1994). The most commonly encountered *Fusobacterium* sp. is *F. nucleatum* (Howard & Keiser, 1994)

The only Gram-negative coccus considered at present to be of clinical importance is *Veillonella* species (Gillespie, 1994). Microscopically *Veillonella* can be seen as diplococci in masses and short chains. The colonies are smooth, grayish white and may show red fluorescence under UV light (Howard & Keiser, 1994).

Although a variety of anaerobic bacteria may be isolated from clinical material/infections, the *B. fragilis* group is regarded as the most important anaerobe. When *Bacteroides* species are present, the prognosis is believed to be poorer, as they produce virulence factors and high-levels of a variety of ß-lactamases, which if not taken into account can seriously compromise initial therapy (Aldridge, 1995). One of the virulence factors associated with *B. fragilis* is a capsular polysaccharide that is capable of depressing circulating serum antibodies (Sommers & Shulman, 1992). *Bacteroides* species are not known to produce classic endotoxaemia, but an endotoxin of lower potency may be present in septic shock (Finegold & Sutter, 1986). It is believed that there is a greater incidence of thrombophlebitis in anaerobic bacterial disease and that certain strains of *Bacteroides* secrete a heparinase, which may enhance clotting (Sommers & Shulman, 1992). The intestinal *Bacteroides* have also been specifically investigated biochemically because of a potential role in complex carbohydrate degradation (Odelson *et al.*, 1987; Salyers, Shoemaker & Guthrie, 1987) and some species have been found to produce mutagenic compounds which may be responsible for some colon cancers (Wilkins & van Tassell, 1983; Odelson *et al.*, 1987).

The fusobacteria possess a potent endotoxin and there are also strains that produce heparinase, as does *Bacteroides*, to enhance clotting (Finegold & Sutter, 1986; Sommers & Shulman, 1992). *Veillonella* species seem to be the only Gram-negative anaerobic cocci recovered from human clinical material with any frequency, but are rarely found in pure culture. Pathogenic factors associated with the *Veillonella* species are unknown, but predisposing conditions associated with the recovery of *Veillonella* are previous surgery, malignancy, steroid therapy, the presence of a foreign body and also immunodeficiency (Wren, 1996).

1.2.2 Gram-positive genera

Gram-positive anaerobes collectively may be responsible for 30 - 40% of anaerobic bacteria isolated and belong to a large and somewhat heterogeneous taxonomic group (Canard *et al.*, 1992). Important Gram-positive bacilli include the spore-forming clostridia, the non-spore-forming *Eubacterium, Mobiluncus, Bifidobacterium* and the facultative anaerobic *Actinomyces* and *Propionibacterium* species (Sommers & Shulman, 1992; Howard & Keiser, 1994; Gillespie, 1994). The clostridia vary with regard to oxygen requirements and Gram stain reaction. *C. tertium* and *C. histolyticum* are aerotolerant, while *C. novyi* and *C. haemolyticum* are strict anaerobes (Howard & Keiser, 1994). Characteristically the clostridia are Gram-positive straight rods, usually larger than other microorganisms. Oval spores can be seen as either terminal or subterminal, depending on the species (Howard & Keiser, 1994). On blood agar, colonies may vary from circular to irregular to swarming. Most of the species are ß-haemolytic and *C. perfringens* can be distinguished by a double zone of ß-haemolysis (inner zone of complete haemolysis caused by theta toxin and outer zone of incomplete haemolysis caused by alpha toxin) (Howard & Keiser, 1994).

Pathogenicity in clostridia has been attributed mainly to the release of highly destructive exotoxins that can cause devastating clinical conditions. These deadly toxins constitute the basis for dividing the species into serotypes (Canard *et al.*, 1992). *C. perfringens* produces at least 10 separate toxins (Sommers & Shulman, 1992). One of the most severe infections and perhaps most feared, is clostridial myonecrosis or "gas gangrene", caused by *C. perfringens, C. histolyticum, C. septicum, C. novyi* and *C. bifermentans*, usually in concert (Finegold & Sutter, 1986; Sommers & Shulman, 1992; Gillespie, 1994). This infection occurs after contamination of an injury or a surgical wound (Finegold & Sutter, 1986). Patients with gas gangrene have a poor prognosis. *C. tetani* on the other hand, is on its own harmless as it is not an invasive organism, but it produces a neurotoxin that acts on the central nervous system, reducing central inhibitory activity (Finegold & Sutter, 1986). If the patient is not immunised immediately and treatment of the infection is not adequate, the fatality of generalised tetanus is approximately 50% (Jawetz, Melnick & Adelberg, 1987). Clostridia may be present in a variety of clinical specimens for which the pathogenic potential of a given isolate is frequently

determined by the causative species necessitating prompt and accurate identification (Alexander *et al.*, 1995).

The majority of anaerobic Gram-positive cocci isolated from clinical specimens belong to the genus *Peptostreptococcus*. The most common species classified under *Peptostreptococcus* are *P. magnus, P. anaerobius* and *P. asaccharolyticus* (Howard & Keiser, 1994). *Peptostreptococcus* spp. vary from small to large Gram-positive cocci with convex grey to white colonies, sometimes α-haemolytic on blood agar. The only species in the genus *Peptococcus* is *P. niger* and is a rare clinical isolate, readily identified by black colonies on blood-containing agar (Howard & Keiser, 1994).

1.2.3 Anaerobic bacteria: spectrum of infections

In cases of infection from which multiple anaerobes are isolated, there is a need to establish whether certain strains are more pathogenic than others. Studies that may help answer such questions involve quantitative microbiology and detection of pathogenic markers such as endotoxins, exotoxins, virulent enzymes and fatty acids (Sapico & Aldridge, 1993).

B. fragilis, often in association with *C. perfringens*, has an advantage over other bacteria in causing infection in the liver or biliary tract as it is capable of growth in high concentrations of bile (Sommers & Shulman, 1992). Other specific diseases predominantly caused by *B. fragilis* are anaerobic cellulitis and secondarily infected vascular gangrene (Finegold & Sutter, 1986).

Fusobacteria have been implicated in endocarditis and in association with species of *Bacteroides* and peptostreptococci have been reported to be responsible for most brain abscesses (Finegold & Sutter, 1983). Most female genital tract infections, including classic sexually transmitted diseases tend to be polymicrobial in nature. Commonly found in these infections are *Peptostreptococcus* and *Bacteroides* spp. (Ng & Dillon, 1991). Clostridia due to their ubiquitous nature are causative agents of a variety of infections that may be unrelated with different clinical manifestations. These include pseudomembranous colitis, botulism, tetanus, soft tissue infections frequently with muscle invasion (gas gangrene and cellulitis) and food poisoning

(Onderdonk & Allen, 1995). A very dramatic infection is caused when clostridia invade the uterus (post-abortal) (Finegold & Sutter, 1986). Many clostridial diseases are serious and life threatening and are all caused by exotoxins produced by the clostridia (Onderdonk & Allen, 1995).

Anaerobic cocci are also recovered from a wide spectrum of infections, posing the ever present threat for serious disease such as bacteraemia (Wren, 1996). Peptostreptococci are not usually differentiated to species level when isolated from polymicrobic infections because they are frequently considered either as contaminants or as less significant pathogens than *Bacteroides* (Ng & Dillon, 1991). Although peptostreptococci are generally considered opportunistic or synergistic pathogens, they have been isolated in pure cultures from soft tissue infections and have been implicated in female genital tract infections such as pelvic inflammatory disease and bacterial vaginosis. The importance of these indigenous cervico-vaginal floras in health and in disease has therefore become increasingly recognised (Ng & Dillon, 1991). Part of the problem in ascertaining the pathogenic potential of *Peptostreptococcus* spp. concerns the identification and taxonomic classification of clinical isolates (Ng & Dillon, 1991; Ng *et al.*, 1994; Murdoch *et al.*, 1998).

1.3 ISOLATION AND IDENTIFICATION

When considering laboratory diagnosis of anaerobic infections, three questions are often raised: 1) What is the clinical relevance of anaerobic bacteriology? 2) How can the microbiologist, with limited and/or decreasing resources, perform reliable, detailed studies of anaerobic bacteriology? 3) When and how should susceptibility testing be performed? (Finegold, 1990). Close and frequent communication with clinicians is of utmost importance (Rosenblatt, 1997).

Three crucial factors may affect the successful recovery of anaerobes from clinical specimens: 1) specimen collection and transportation procedures, 2) the anaerobic incubation system used, and 3) the quality and selection of the primary isolation media. Anaerobic transport systems are necessary because incorrect collection and transport can cause changes to occur in the microbial population of a clinical specimen (Mangels, 1994). Important to remember is that oxygen is toxic to

anaerobes and while some laboratories may use less demanding transport systems that are adequate in recovering the *B. fragilis* group or other aerotolerant anaerobes from abdominal sources, it may not be possible to recover more oxygen-intolerant anaerobes from sources such as head, neck, pelvic, chest and brain abscesses (Mangels, 1994). Even though several methods are available for transporting anaerobic specimens, it remains one of the weakest links in the chain from specimen collection to isolation and identification. Should the technical expertise of a laboratory be inadequate, isolation of relatively non-fastidious anaerobes such as *B. fragilis* and *C. perfringens* can cause other more demanding anaerobes to be overlooked (Finegold & Sutter, 1986).

A further problem in isolating a full complement of anaerobes from an infected site is that of specimen collection as not all specimens are suited for culture (Finegold & Sutter 1986; Howard & Keiser, 1994). Collection of normally sterile material, such as blood and spinal fluid, requires only the usual precautions of skin decontamination. However, most anaerobes associated with infections are also present on mucous membranes or other areas of the body as part of the indigenous flora and the anaerobes at these sites are frequently so numerous that a clinical specimen can easily be contaminated by only a small portion of normal flora, causing seriously misleading culture results (Finegold & Sutter, 1986). Anaerobic infections of the lung pose a real challenge to the clinician since specialised techniques are required for the collection of uncontaminated specimens via the upper airways, where anaerobes represent the dominant component of the flora (Bartlett, 1993). This can hamper laboratory diagnosis of patients who do not have empyema or bacteraemia. Uncontaminated specimens that are valid for anaerobic culture include pleural fluid, trans-tracheal aspirates, trans-thoracic aspirates and specimens obtained at thoracotomy (Bartlett, 1993). Routine culturing of blood for anaerobes is still used in many institutions because of the unpredictable clinical sources of some bacteraemias and the improved yields of both anaerobes and some streptococci when anaerobic blood culture systems are used (Rosenblatt, 1997).

As a variety of different anaerobic species have been implicated in infections (section 1.2) isolation and identification is of paramount importance. Comprehensive identification is necessary for: 1) consistency in a prognosis, 2) assessing the impact of an isolate on epidemiology and infection control, 3) recognising a possible cryptic

underlying disease process and 4) increasing knowledge about the relation of virulence properties of species to the infectious processes (Baron & Allen, 1993). Special media and growth conditions are required for the isolation and identification of anaerobic bacteria, contributing to higher cost per identification than for aerobic isolates. New and more rapid methods are being employed to streamline the identification process, yet confirmation to the species level is often difficult (Whaley *et al.*, 1995). The cost of detailed anaerobic bacteriology is also high because it is labour intensive; for economic reasons, some laboratories that are capable of producing good, definitive work are therefore compelled to perform "less-than-thorough" anaerobic bacteriology (Finegold & Wexler, 1988).

The identification method that is regarded as the most reliable and comprehensive is the Virginia Polytechnic Institute (VPI) technique, referred to as the "Gold Standard" in the identification of anaerobic bacteria (Holdeman, Cato & Moore, 1977). This method is, however, extremely labour intensive, expensive and needs expertise to perform. In addition to the routine identification procedures normally performed in a diagnostic laboratory, the VPI method is an intensively executed system consisting of gas chromatography for the detection of alcohol and volatile acid products and a very large series of biochemical tests. For this reason most laboratories have come to rely on more convenient, simpler and more rapid methods of identification. Such methods may either consist of presumptive and preliminary grouping with Gram-stain information, plate morphology and several spot and disk tests or a variety of preformed-enzyme tests (Mangels, 1998). Commercially available identification systems that have been miniaturised, such as the API system Rapid ID32A (bioMérieux, France), are able to provide results within 4 hours and use non-growth-dependent reactions. These systems do provide practical alternatives for the identification of many anaerobes in clinical laboratories, although comparative evaluations with the VPI method have revealed discrepancies in the identification of some anaerobes including certain *Clostridium* species (Alexander *et al.*, 1995). In one study, of three rapid identification kits, none could be employed as the sole method for identifying *Clostridium* species and all three systems had to be supplemented with additional tests for the complete identification of many selected anaerobes (Alexander *et al.*, 1995).

Anaerobic bacteria that warrant identification are: the *B. fragilis* group because of their virulence and resistance to many antimicrobial agents; *Clostridium* species known to be resistant to antibiotics, more specifically *C. septicum* that is associated with gastrointestinal malignancy; *C. perfringens*, known to be a cause of potentially serious infection; anaerobic cocci that may be resistant to metronidazole and clindamycin and fusobacteria that may also be virulent and resistant to clindamycin and penicillin (Citron & Appelbaum, 1993). A constant source of discouragement to clinical microbiologists is the absence of clear guidelines for species characterisation and the role of many anaerobic bacteria in health and disease (Watt & Jack, 1997).

Taxonomy of anaerobic bacteria is in a stage that can only be described as dynamic turbulence. The recent transition to a phylogenically-based taxonomic system is expected to continue to yield new information, resulting in the relocation of old species as well as the placement of new species (Jousimies-Somer, 1997). Phenotypic characteristics, comprising cellular fatty acid composition and metabolic reactions producing enzyme profiles, are being replaced with genetic approaches. Nucleic acid-based methods include the determination of G + C content, DNA-DNA hybridisation and sequencing of 16S and 23S rRNA (Jousimies-Somer, 1997). The 16S rRNA sequencing method in particular enables taxonomic classification of bacteria according to their phylogenetic relatedness (Jousimies-Somer, 1997).

Examples of noteworthy genus shifts have been made in the genus *Bacteroides*. Former *Bacteroides* spp. that were bile-sensitive and moderately saccharolytic (e.g. *B. bivius, B. buccae, B. denticola*) have now been reclassified in the genus *Prevotella* and former *Bacteroides* spp. that were assacharolytic (*B. asaccharolyticus, B. endodontalis* and *B. gingivalis*) are now members of the new genus *Porphyromonas* (Howard & Keiser, 1994). In 1983 Ezaki *et al.* (1983) described the transfer of *Peptococcus indolicus, Peptococcus asaccharolyticus, Peptococcus prevotii* and *Peptococcus magnus* to the genus *Peptostreptococcus,* leaving only one species *P. niger* remaining in the genus *Peptococcus.* When *Arachnia propionica* was reclassified as *Propionibacterium propionica*, the genus *Arachnia* totally fell away, as it was the only type species (Howard & Keiser, 1994). The classification of obligate anaerobic cocci of clinical interest is currently in a state of confusion as different authorities place these bacteria into different species and even different

genera, while some workers allow inclusion of micro-aerophilic or CO_2-requiring strains (Watt & Jack, 1997).

Improvements and implementation of new methodologies, including techniques for sample collection, transportation and culture can only increase the recognition that anaerobes deserve within the context of playing an important role in a variety of infections. Availability of commercial identification kits have, although not providing definitive classification, simplified the identification of anaerobes to enable a diagnostic input within a reasonable time frame (Schieven *et al.*, 1995).

1.4 TREATMENT

It may take considerable time to isolate anaerobic bacteria from clinical specimens in pure culture to perform even a preliminary identification and determine antibiotic susceptibilities. It is therefore imperative until laboratory data is available, to initiate empiric therapy when anaerobic infections are suspected. Another important consideration of empiric therapy is that the distribution of clinically important anaerobes can vary according to anatomical site (Aldridge, 1995). As surgical infections are nearly always polymicrobial, initial therapeutic choices have to be made empirically (Nagy, Werner & Heizmann, 1990). In such cases treatment should be based on knowledge of the nature of the infectious process, the micro-organisms usually encountered in similar infections, preliminary results from the laboratory, the severity of the infection, as well as current susceptibility patterns in the specific setting in which the physician practices (Finegold & Wexler, 1988; Finegold, 1997).

Initially the empiric administration of antimicrobial agents that are effective against both components of a mixed aerobic and anaerobic infection is required for appropriate management of infection, together with surgical interventions where necessary (Brook, 1991). Internists and surgeons alike have also come to rely on drainage procedures or débridement in determining the outcome of many anaerobic infections, thereby incorrectly de-emphasising the importance of knowledge of individual pathogens and leaving antimicrobial therapy considered adjunctive to intervention and not directed at specific virulent organisms (Wilson & Huh, 1997).

Many variables, apart from the therapy administered, may affect the outcome of mixed infections. These include the 1) site of infection, 2) type and severity of underlying disease, 3) nutritional status of the patient, 4) operative procedure and 5) presence of other pathogens (Snydman *et al.*, 1992). A delay in response to treatment and a tendency for loculated collections to form in anaerobic empyemas render these infections difficult to manage and many or most of the affected patients require open thoracotomy or decortication. Anaerobic pulmonary infections, however, generally respond well to antibiotic treatment combined with adequate drainage of empyemas (Bartlett, 1993). Important factors in the treatment of intra-abdominal sepsis are early diagnosis and prompt surgical intervention with antibiotic treatment initiated as soon as diagnosis is made and continued through the surgical operative procedure into the postoperative period (Nichols & Smith, 1993). Again the activity spectrum of chosen antibiotics must include both colonic aerobes and anaerobes (Nichols & Smith, 1993). The clinical response to antibiotic treatment of intra-abdominal sepsis may sometimes be difficult to assess as some patients may respond with no or inappropriate antimicrobial therapy, with or without surgical intervention (Finegold, 1990).

Included in recommended antimicrobial regimes for treatment of and prophylaxis for the anaerobic infections are metronidazole, clindamycin, ß-lactam agents plus ß-lactamase inhibitors, carbapenems, and quinolones (Nord, 1996). One of the most frequently prescribed antibiotics for many years, is metronidazole. Resistance is rarely encountered, although high dosages may be necessary to eradicate some pathogens (Poulet, Duffaut & Lodter, 1999).

Carbapenems such as imipenem and meropenem are also active antibiotics with the ability to inhibit many *B. fragilis, B. thetaiotaomicron* and *C. difficile* strains that have developed resistance to cefoxitin or clindamycin (Skeikh, Pitkin & Nadler, 1993). The carbapenems are broad-spectrum antimicrobial agents active against a wide variety of anaerobic and aerobic pathogens confirming their utility as effective empiric monotherapeutic agents for a variety of mixed aerobic and anaerobic infections. They are able to penetrate through the outer membranes of Gram-negative bacteria and possess excellent stability to the Class C and A serine ß-lactamases, including extended spectrum ß-lactamases (ESBLs) (Bellido *et al.*, 1990; Skeikh, Pitkin & Nadler, 1993; Yang, Bhachech & Bush, 1995).

Most of the quinolones such as ciprofloxacin, ofloxacin, pefloxacin, enoxacin and lomefloxacin have only mediocre activity against anaerobes and even less activity against members of the *B. fragilis* group (Wexler, Molitoris & Finegold, 1993; Aldridge, Ashcraft & Bowman, 1997; Spangler, Jacobs & Appelbaum, 1997; Ednie, Jacobs & Appelbaum, 1998). The newer quinolones and fluoroquinolones (sparfloxacin, grepafloxacin, levofloxacin, gatifloxacin and trovafloxacin) undergoing evaluation, have markedly improved activity against anaerobes in general, and the *B. fragilis* group in particular (Wexler, Molitoris & Finegold, 1993; Wexler *et al.*, 1994; Wexler *et al.*, 1996; Aldridge, Ashcraft & Bowman, 1997; Rasmussen, Bush & Tally, 1997; Ednie, Jacobs & Appelbaum, 1998). The fluoroquinolones, having broad antibacterial spectra as well as favourable pharmacokinetics, raise the possibility of their increasing use for therapy of mixed infections involving anaerobes (Wexler, Molitoris & Finegold, 1992; Wexler *et al.*, 1994).

Many antimicrobial agents with known anaerobic activity are available, but emerging development of antibiotic resistance remains poorly investigated (Zabransky, 1989).

1.5 SUSCEPTIBILITY TESTING

In the past it was believed that susceptibility testing of anaerobes should only be considered under very limited circumstances: 1) when an anaerobe was found as the sole organism in a selected specimen, 2) on isolates from blood and other normally sterile body fluids and 3) on surgical tissues and needle aspirates from any closed abscess, the central nervous system, lower respiratory tract, abdomen, pelvis, bone and joints - assuming the specimen had been correctly collected and transported to the laboratory (Zabransky, 1989; Rosenblatt, 1997; Wexler, Molitoris & Molitoris, 1997). Susceptibility testing of anaerobes has recently been expanded to encompass the following: 1) validation of empiric therapy in local communities and individual hospitals; 2) monitoring of susceptibility patterns at regional, national and international levels; 3) activity studies on new antibiotics; 4) severe infection and critically ill patients, 5) failure of empiric therapy, 6) species associated with resistance; 7) in instances where little data is available on a specific specie; 8) anaerobes that are regarded as the most virulent and most resistant to antimicrobial

agents; 9) persistent or recurrent infections; 10) in cases of long term therapy; and 11) for antibiotics crucial for successful treatment (Schieven *et al.*, 1995; Finegold, 1997; Davies Diagnostics, 1999). Because of the increasing unpredictability of susceptibility patterns and the desire on the part of clinicians to be aware of all susceptibility results for patients who are critically ill, timely testing should be available for anaerobes isolated from sites of importance (Rosenblatt, 1997).

On a medicolegal basis routine culturing and susceptibility testing whenever anaerobic infection is suspected may be rationalised, avoiding problems in defending poor clinical outcome on grounds that culture and susceptibility testing are not done routinely (Wilson & Huh, 1997). From a practical standpoint, results of susceptibility tests are generally not available until the patient has responded or failed to respond to empiric treatment, but in the long term, surveillance of resistance patterns of bacteria do facilitate a more accurate initial therapeutic approach (Finegold, 1990). Overall, from a clinical perspective it is considered important to determine susceptibilities, especially for the pathogens *Bacteroides, Fusobacterium, Peptostreptococcus* and *Clostridium* species that present with unpredictable antibiograms (Finegold & Wexler, 1988). *In vitro* susceptibility testing is only one step in the evaluation of the potential efficacy of antimicrobial agents against anaerobes as *in vivo* efficacy forms an integral part of the assessment process (Brook, 1991). Standardisation of techniques, choice of breakpoints, inability to use recent clinical isolates, too few test strains, non-representative species choices, clustering at the breakpoints and the need for clinical correlation are just some of the problems encountered in obtaining sound susceptibility data (Finegold & Wexler, 1988).

It was evident for some time that rapid methods such as agar disk diffusion or disk broth susceptibility testing were far from satisfactory, these methods having now been rejected by the National Committee of Clinical Laboratory Standards (NCCLS, 1993; Wexler, Molitoris & Molitoris, 1997). The disk-elution method used by many laboratories was no longer recommended by the NCCLS in view of inaccuracy in testing the activity of ß-lactam antibiotics (Wüst & Hardegger, 1995). It was found in the agar disk diffusion method that: 1) zone size was affected when inocula of anaerobic bacteria were exposed to oxygen during the set-up procedure, and 2) the gradient profile around the disk was seen to change over time and that a large

inhibition zone would simply reflect slow growth (Bolmström, 1993; Johnson, Thatcher & Cox, 1995). NCCLS susceptibility testing of anaerobes has, however, been of limited use in directing initial therapy as results may not be available to clinicians for 3-5 days (NCCLS, 1993). This has resulted in general bacteriology laboratories not routinely performing susceptibility testing of anaerobic bacteria. It has therefore become necessary for reference laboratories to conduct periodic surveys to detect major changes in susceptibility profiles and to provide susceptibility patterns useful for rational empiric therapy (Bourgault *et al.*, 1986). Reports published by reliable large research centres can serve as guide-lines for clinicians to initiate appropriate therapy (Baron & Citron, 1997), however, susceptibility data vary temporally, geographically, even differing within hospitals in the same city (Wilson & Huh, 1997).

The NCCLS currently recommends three different methods for testing the susceptibility of anaerobic bacteria with the standard reference method being an agar dilution procedure using Wilkins Chalgren agar. Two alternative methods are an agar dilution technique using Brucella blood agar or a microdilution procedure using a broth version of Wilkins Chalgren medium, but it is important to determine whether these three procedures actually produce identical test results with each antimicrobial agent tested against specific anaerobes (Bary *et al.*, 1996). Likewise there is no simple method for testing individual isolates against a large battery of antimicrobial agents. Even though results with the microdilution procedure are not always in full agreement with the reference agar dilution method, the microdilution procedure has become the most practical method for use in clinical laboratories. The microdilution endpoints are usually one dilution lower than agar dilutions with some anaerobes giving trailing or hard-to-read endpoints or poor growth (Rosenblatt & Brook, 1993; Zabransky & Dinuzzo, 1994). Although not viewed as practical in the clinical laboratory, the agar dilution method is currently considered the gold standard (Rosenblatt & Brook, 1993; Wren, 1996). There is also concern about the raising of susceptibility breakpoints without new information on improved efficacy of the particular antimicrobial agents; establishment of intermediate categories seems preferable (Rosenblatt & Brook, 1993).

The reference methods used for antimicrobial susceptibility testing of anaerobes are cumbersome and can not be readily applied to the clinical situation. Etest stable

gradient technology has shown promise for routine use in the real-time and for epidemiological screening of antibiotic susceptibility of anaerobic bacteria (Brazier, Hall & Duerden, 1992; Eschenbach, 1993; Bolmström, 1993; Croco *et al.*, 1994; Croco *et al.*, 1995). The Etest has emerged as the most practical, quantitatively accurate, alternative procedure for clinical microbiology laboratories (Croco *et al.*, 1995). It has been extensively evaluated for *Bacteroides* and *Clostridium* species and good correlation with the agar dilution method was generally seen (Sanchez & Jones, 1992; Wexler, 1996). The Etest is simple to use in that it is very similar to the disk-diffusion technique, is easy to read, suitable for a wide range of anaerobes and susceptibility testing of individual organisms or antibiotics can be performed without waste (Citron *et al.*, 1991; Schieven *et al.*, 1995). The Etest holds promise as being accurate and flexible enough for use in most clinical laboratories (Sanchez & Jones, 1992; Rosenblatt & Gustafson, 1995). There have, however, been some instances of disagreement between methods, principally due to differences in innoculum, media and/or supplement additions (Sanchez & Jones, 1992). The one major drawback of the Etest is its high cost, placing it out of reach of laboratories and/or communities with limited financial resources.

Concerning the pathological nature of anaerobes there is a surprising lack of information relating to correlation between *in vitro/in vivo* findings and ultimately clinical outcome. The outcome of polymicrobial infections is often extremely difficult to predict (Snydman *et al.* 1992; Pankuch, Jacobs & Appelbaum, 1993). Reasons may include methodological problems with susceptibility testing, the type and spectrum of anaerobes tested and pharmacokinetic factors (Pankuch, Jacobs & Appelbaum, 1993). Therapeutic factors that may affect correlation are: a) speed of implementation, b) pharmacology (dosage, blood brain barrier, serum levels, etc.), and c) activity reduced by enzymes, low Eh or pH (abscesses) (Davies Diagnostics, 1999). The large bacterial population found in anaerobic abscesses, for example, raises important questions concerning the validity of common methods of *in vitro* susceptibility testing of anaerobes, which usually employ small inocula (Brook, 1991). This difference in bacterial numbers may explain the frequent dichotomy between *in vitro* activity and *in vivo* efficacy, especially with the ß-lactam antibiotics (Brook, 1991).

The results of susceptibility tests are influenced by many factors, which must be standardised for reproducibility. They include the growth phase of the inoculum, the incubation time and temperature, the age, composition, cation content, and depth of the test medium and the manner in which the results are interpreted (Johnson, Thatcher & Cox, 1995). Strict anaerobiosis is critical to ensure accurate metronidazole susceptibility testing and this environmental failure has been reported as the most likely source of false-resistant results (Cormican, Erwin & Jones, 1996).

Susceptibility results, especially with resistant isolates or infections involving strains with MICs near the breakpoint, may predict therapeutic failure as it has been found that prognosis and outcome of anaerobic infections were improved when physicians utilised specific anaerobic susceptibility data provided within a clinically relevant time frame (Bourgault, Harkness & Rosenblatt, 1978). Consequently, there has been a clinical need, even among non-teaching hospital physicians, to have susceptibility data available for specific anaerobic bacteria isolated, despite all the relevant problems and controversies (Goldstein, Citron & Goldman, 1992).

1.6 ANTIBIOTIC RESISTANCE

Anaerobic bacteria, as with aerobic bacteria, have developed the full range of resistance strategies from production of hydrolysing/modifying enzymes, decreased target sensitivity, development of efflux systems to modification of the diffusion barrier (Frère, 1995; Laraki *et al.*, 1999). Emergence of resistance in anaerobic bacteria is increasingly being documented with distinct differences in resistance patterns being related to antibiotic-prescribing regimens in individual hospitals (Rosenblatt & Brook, 1993; Rasmussen, Bush & Tally, 1997). As a result this has impacted on the selection of antimicrobial agents for empiric therapy (Rasmussen, Bush & Tally, 1997).

1.6.1 Resistance to ß-lactam and carbapenem antibiotics

ß-Lactam antibiotics have long been the mainstays of therapy for anaerobic infections (Johnson, 1993). However, susceptibility varies depending on the ß-

lactam and the bacterial species to be targeted. In the USA, increasing resistance and consequent variation in clinical efficacy have made it mandatory to periodically review the *in vitro* activity of ß-lactam agents against anaerobes (Johnson, 1993).

Penicillin-binding proteins (PBPs) are enzymes required for the biosynthesis of bacterial cell walls as they catalyse the final stages of polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan (Zhao *et al.*, 1999). The function of PBPs in the terminal stage of cell wall synthesis is, therefore, essential for bacterial growth and wall maintenance (Rasmussen, Bush & Tally, 1997). For high level ß-lactam resistance to develop, reduction in ß-lactam affinity of essential PBPs is necessary. In *B. fragilis*, three PBPs have been described (Botta, Privitera & Menozzi, 1983). PBP1 possesses the lowest affinity for ß-lactam antibiotics, however, it primarily binds imipenem which results in cell lysis (Nord & Hedberg, 1990). PBP2, in contrast, was found to have the highest affinity for ß-lactam antibiotics, also efficiently binding cefoxitin and imipenem (Nord & Hedberg, 1990). PBP3 has intermediate affinity for ß-lactam antibiotics, and PBP4 binds primarily clavulanic acid and imipenem (Nord & Hedberg, 1990). Changes in the affinity of PBP1 and PBP2 in laboratory-derived mutants have been correlated with a decrease in susceptibility to cefoxitin (Piddock & Wise, 1987).

In *Clostridium perfringens* at least six PBPs with molecular weights ranging from 42,000 to 100,000 have been demonstrated (Murphy, Barza & Park, 1981). Most of the ß-lactam antibiotics show affinity for PBP3 and PBP4, suggesting that these proteins are the major killing targets (Nord & Hedberg, 1990). The majority of clostridia remain susceptible to penicillin G and amoxicillin (Johnson, 1993), however, the occasional resistant *C. ramosum, C. clostridioforme* or *C. innocuum* isolate may also be encountered (Johnson, 1993). Resistance to penicillin in *C. perfringens* has been found to be mediated by decreased penicillin-binding affinity of the largest PBP (PBP1) (Williamson, 1983). PBP affinity studies on *P. anaerobius* confirmed PBPs 1 and 2 (low affinity PBPs) to be involved in penicillin resistance (Chalkley & Sooka, 1994). Imipenem was found to bind more effectively than cefoxitin to all the PBPs demonstrated in a strain of *P. magnus* (Chalkley & Sooka, 1994). Species specific PBP profiles were seen with *P. magnus*, *P. anaerobius,* and *P. prevotii* suggesting profiling could be useful in differentiating peptostreptococcal species/subspecies (Chalkley & Sooka, 1994).

The most prevalent mechanism of high-level resistance to ß-lactam antibiotics is ß-lactamase production (Laraki *et al.*, 1999). Bacteria are often, because of antibiotic pressure, selected to produce two or more chromosomal encoded or plasmid-mediated ß-lactamases active against a wide range of ß-lactam compounds (Felici *et al.*, 1995). More than 200 different ß-lactamases have been described and characterised with their catalytic mechanisms involving either an active-site serine residue (serine ß-lactamase) or a divalent transition metal ion (metallo-ß-lactamase) (Laraki *et al.*, 1999).

ß-Lactamase production in anaerobic bacteria has been extensively investigated during recent years, being especially prevalent in species of *Bacteroides* (Hedberg *et al.*, 1992). Seventy-five to 100% of strains in the *B. fragilis* group have been reported to be ß-lactamase producers (Hedberg *et al.*, 1992). A survey performed in South Africa (Van der Westhuyzen & Chalkley, 1992) revealed that ß-lactamases were produced by 99% of the *Bacteroides* strains isolated during 1988-1989 in the Johannesburg, Baragwanath and Hillbrow Hospital laboratories. This percentage is higher than that reported from Australia (61%) (Munro, 1989) and the USA (75%) (Wexler, 1987).

Gram-negative bacilli in the genera *Prevotella, Porphyromonas* and *Fusobacterium* have increasingly been found to produce ß-lactamases (Goldstein, 1996; Ednie, Jacobs & Appelbaum, 1998). In 1990 it was estimated that up to 40-50% of fusobacteria in the USA isolated from human sources were resistant to ß-lactam antibiotics (Appelbaum, Spangler & Jacobs, 1990; Jang & Hirsh, 1994). Anderson & Sykes (1973), on characterising a ß-lactamase from a strain of *B. fragilis*, found the enzyme to be a non-inducible cephalosporinase and that production correlated with high levels of ß-lactam resistance. It has now also been determined that ß-lactamases of *Bacteroides* spp. are seldom inducible (Nord & Hedberg, 1990). In the *B. fragilis* group, ß-lactamases are constitutive, localised in the periplasmic space (with a close association with the bacterial cell envelope), have primarily cephalosporinase activities and isoelectric point (pI) values in the acid range (Nord & Hedberg, 1990; Wexler, Molitoris & Finegold, 1994). ß-Lactamases of *B. fragilis* are sensitive to inhibition by cloxacillin, cefoxitin, *p*-chloromercuribenzoate (PCMB), and

sulbactam (Nord & Hedberg, 1990). ß-Lactamases in the non-*fragilis* group of *Bacteroides* and *F. nucleatum* are primarily penicillinases (Johnson, 1993).

ß-Lactamases from *Clostridium* species are rare, but have been described in *C. butyricum, C. ramosum, C. chauvoeii* and *C. clostridioforme*, all being found to be penicillinases and inducible by ß-lactams (Nord & Hedberg, 1990; Appelbaum, 1992; Van der Westhuyzen & Chalkley, 1992; Appelbaum *et al.*, 1994; Rasmussen, Bush & Tally, 1997). One ß-lactamase of *C. butyricum* that has been fully characterised was found to be inhibited by clavulanic acid and sulbactam, partially by *p*CMB, but not by cloxacillin or cefoxitin, whereas enzymes of *C. ramosum* were inhibited by cefoxitin and *p*CMB but not by cloxacillin (Nord & Hedberg, 1990). Preliminary studies also revealed that ß-lactamases from *C. clostridioforme* were not inhibited by cefoxitin, cloxacillin or *p*CMB (Appelbaum *et al.*, 1994). A ß-lactamase from one clinical isolate of *C. clostridioforme* that was poorly inhibited by clavulanic acid, sulbactam and tazobactam was only inactivated by the penem BRL 42715, C6-(N1-methyl-1,2,3-triazolylmethylene) penem, which is an inhibitor of a broad range of bacterial ß-lactamases (Coleman *et al.*, 1989; Appelbaum *et al.*, 1994). Although most clostridial ß-lactamases are considered chromosomal-mediated, plasmid-mediated ß-lactamases have been found in *C. ramosum* (Nord & Hedberg, 1990).

Reports are indicating that ß-lactamase inhibitors may extend the usefulness of ß-lactam agents with marginal activity against anaerobes especially the *B. fragilis* group (Wexler, Molitoris & Finegold, 1991). It has been proposed for Gram-negative anaerobes, that the widespread use of ß-lactamase-stable antibiotics and ß-lactams in combination with ß-lactamase inhibitors may lead to adaptation, which will result in increasing changes in permeability characteristics (Wexler, 1997). Decreased permeability is already often regarded, in association with an increase in ß-lactamase production, as the major contributor to resistance development in Gram-negative bacteria (Rasmussen, Bush & Tally, 1997).

Isolates of *B. fragilis* capable of hydrolysing carbapenems are occasionally encountered. Typically, these carbapenemases are zinc-dependent, metallo-ß-lactamases produced by the *cfiA* gene and display a high affinity for a broad spectrum of ß-lactam compounds (Edwards, Hawkyard & Hashmi, 1998). The degree of expression of the *cfiA* gene may vary, with high metallo-ß-lactamase production

leading to high-level carbapenem resistance and low production usually associated with strains classified as susceptible (imipenem MIC < 8 µg/ml).

Metallo-ß-lactamases are also often produced in combination with group 1 cephalosporinases (Bush, 1998). As the majority of the carbapenem-hydrolysing ß-lactamase genes are chromosomally encoded, dissemination has been slow, but it is anticipated that plasmid-mediated genes will continue to spread (Rasmussen & Bush, 1997). The first plasmid-mediated metallo-ß-lactamase, from *Pseudomonas aeruginosa* was reported in 1991 and soon after this a transferable metallo-ß-lactamase gene was reported from *B. fragilis* (Watanabe *et al.*, 1991; Bandoh *et al.*, 1992). It is now possible for these enzymes from formerly single isolates to be transmitted in a promiscuous manner. The metallo-ß-lactamases need to be examined more thoroughly with regard to their production and function in an attempt to determine whether these enzymes will be of critical clinical concern to the infectious diseases community (Bush, 1998). Metallo-ß-lactamases are poorly inhibited by commercially available ß-lactamase inhibitors, clavulanic acid, sulbactam and/or tazobactam (Rasmussen & Bush, 1997). Fortunately some derivatives of monobactams are totally stable against the metallo-ß-lactamases making them potential candidates for treatment of anaerobic infections (Osano *et al.*, 1994).

1.6.2 Resistance to metronidazole

Metronidazole is a nitroimidazole that is widely used for the treatment of anaerobic infections (Dachs, Abratt & Woods, 1995). The inhibitory effect of metronidazole was found to be similar to that of oxygen when phenylalanine and leucine metabolism by *P. anaerobius* under both anaerobic and aerobic conditions was investigated (Hamid *et al.*, 1997). It was demonstrated that the metronidazole sensitive steps required anaerobic conditions as they were inhibited by oxygen, while the metronidazole tolerant steps were tolerant of oxygen (Hamid *et al.*, 1997). The bactericidal effect of metronidazole appears to be dependent on its being converted to a reduced intermediate, which then interferes with the transfer of electrons resulting in metabolic inhibition (Hamid *et al.*, 1997). Although relatively little is known about its mode of action *in vivo*, *in vitro* experiments have shown that on reduction a reactive intermediate compound is formed that is able to interact and cause breakage of

single and double stranded DNA (Figure 1.1). The nature of this DNA damage in living bacterial cells has not been fully elucidated and although interaction between the activated form of metronidazole and DNA is the most likely cause of toxicity, other cellular targets such as proteins, ribosomes and certain cell surface components have been suggested (Dachs, Abratt & Woods, 1995).

The nitroimidazole group of agents is remarkable for its spectrum of activity against Gram-positive and Gram-negative anaerobic bacteria and despite its use for more than 30 years, the incidence of resistance in anaerobes is still very low (Edwards, 1993). In 1978 the first metronidazole resistant *B. fragilis* was reported (Ingham *et al.*, 1978). The mechanism of metronidazole-resistance has not been elucidated, but studies have ruled out the involvement of efflux or reduced drug penetration (Rasmussen, Bush & Tally, 1997). Metronidazole inactivating enzymes have not been described in anaerobes although one such enzyme has been suggested to be produced by *Enterococcus faecalis* (Nagy & Földes, 1991).

The development of full resistance to metronidazole is a lengthy process and is accompanied by a concomitant decrease and eventual abolition of the activity of the pyruvate:ferredoxin oxidoreductase system (Figure 1.1) (Edwards, 1993; Edwards, 1993). It was proposed that the rate of entry of nitroimidazole into the cell would be dependent upon the rate of reduction of the nitro group. A decrease in the activity of nitro-reductase would therefore lead to decreased uptake of the drug (Edwards, 1993). Correlation in this respect has been found between the level of pyruvate:ferredoxin oxidoreductase activity in obligate anaerobes, the degree of susceptibility of the organism and the rate of metronidazole uptake into the cell (Edwards, 1993; Edwards, 1993). Theories will remain speculative until the oxygen tension and *in situ* uptake rates of metronidazole are known (Smith & Edwards, 1995).

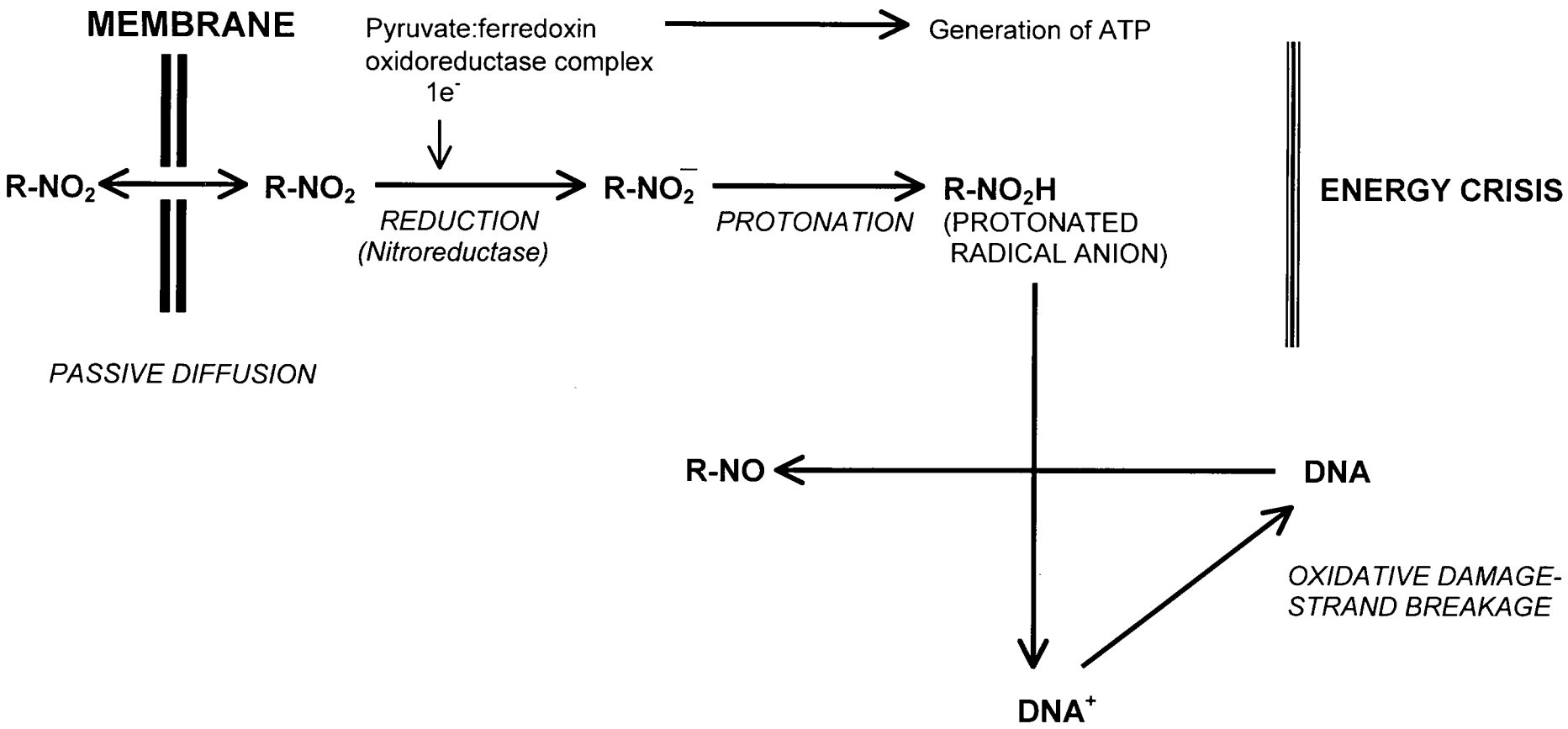


Figure 1.1: A simplified representation of the speculative pathway following proposed actions of metronidazole (Modified from Edwards, 1993).

In reports from France by Reysset *et al.* (1992) and Reysset, Haggoud & Sebald (1993) presumptive metronidazole-resistance genes have been cloned and characterised, but the mechanism by which these genes conferred resistance to metronidazole and other 5-nitroimidazoles was not clear (Salyers & Shoemaker, 1996). In a molecular study conducted in 1996 it was demonstrated that the moderate resistance phenotype of clinical strains was in all cases associated with the presence of a 5-NI resistance gene (*nim*) (Trinh & Reysset, 1996). Four related *nim* genes have now been identified in *Bacteroides* species, of which three were found to be located on low-copy-numbered mobilisable plasmids: pIP417 (7.7 kb, *nimA*) from *B. vulgatus* BV-17, pIP419 (10 kb, *nimC*) from *B. thetaiotaomicron* BT-13, and pIP421 (7.3 kb, *nimD*) from *B. fragilis* BF-F239. The fourth gene (*nimB*) was mapped to the chromosome of *B. fragilis* BF-8 (Haggoud, Reysset & Sebald, 1992). The majority of both plasmid-borne and chromosomal located *nim* genes were shown to be transferable by conjugation (Reysset, Haggoud & Sebald, 1993; Trinh & Reysset, 1996). Mobilization of the *nim* genes is therefore thought to account for the spread of 5-NI resistance among at least some Gram-negative anaerobic bacteria (Trinh & Reysset, 1996). In any event, the possibility of future widespread metronidazole resistance must be seriously considered. As the presence of moderate metronidazole resistance is sometimes difficult to detect by conventional susceptibility testing, monitoring the spread of *nim* genes has been recommended for clinically important anaerobic pathogens (Trinh & Reysset, 1996).

1.6.3 Resistance to quinolones

DNA gyrase is a primary target of the quinolones with resistance mediated by alterations in the target DNA gyrase and alterations that appear to decrease permeability of the quinolone (Hooper & Wolfson, 1991). It is by mutations in both the *gyrA* and *gyrB* genes that resistance to quinolones is conferred (Hooper & Wolfson, 1991). In order to reach their intracellular target, the quinolones must traverse the membranes of bacteria (Hooper & Wolfson, 1991). As quinolones differ in hydrophobicity, different interactions with the inner and outer membranes may occur, causing a decrease or increase in permeability depending on their structure (Hooper & Wolfson, 1991). Despite high activity against many bacteria, the quinolones and fluoroquinolones were for years considered to be poorly effective

against most anaerobic bacteria (Nord, 1996; Rasmussen, Bush & Tally, 1997). Recent quinolones, in particular grepafloxacin, trovafloxacin and clinafloxacin have been shown to exhibit improved and interesting *in vitro* activity (Nord, 1996). In 1994 Wexler, Molitoris & Finegold, in the USA, reported grepafloxacin to have substantially better activity against *B. fragilis* than ciprofloxacin or fleroxacin, while in 1996 Hecht & Wexler found grepafloxacin to have moderate activity against the anaerobes and clinafloxacin and trovafloxacin the fluoroquinolones that had the highest activity against the *B. fragilis* group. In another study in the USA, Barry & Fuchs (1997) described grepafloxacin as having superior activity to ciprofloxacin, ofloxacin and fleroxacin against the Gram-positive cocci. As fluoroquinolones are now increasingly used to treat anaerobic infections, it is only reasonable to expect resistance to this group of antibiotics to emerge (Salyers & Shoemaker, 1996). A reservation to the use of quinolones as treatment of anaerobic infections is that under anaerobic conditions the quinolones are bacteriostatic and not bactericidal (Rasmussen, Bush & Tally, 1997).

1.6.4 Resistance to tetracycline

The first antibiotics to which anaerobes developed widespread resistance were the tetracyclines with the result that they are no longer considered useful for empiric treatment of suspected anaerobic infections (Rosenblatt & Brook, 1993). The three tetracycline resistance mechanisms known are ribosome protection, tetracycline efflux and tetracycline modification, of which the ribosome protection type of resistance appears to be the most widespread in nature (Nikolich, Shoemaker & Salyers, 1992). In 1992 a new ribosome protecting tetracycline resistance gene (*tetQ*) was designated which is now found in virtually all clinical isolates of *Bacteroides* species (Salyers & Shoemaker, 1996). Mating experiments and the high similarity of the *tetQ* genes among the *Prevotella* and *Bacteroides* genera support the theory of horizontal exchange of antibiotic resistance determinants on conjugative transposons among bacteria, not only between different species but from different ecological niches and animal hosts (Walker & Bueno, 1997). Tetracycline resistance mediated by the *tetQ* gene is both inducible and transferable (Rasmussen, Bush & Tally, 1997). Two tetracycline resistant genes, *tetA*(P) and *tetB*(P) have also recently been identified in *C. perfringens* (Rasmussen, Bush & Tally, 1997). The *tetA*(P) and

tetB(P) genes form an operon encoding two unrelated proteins causing tetracycline resistance that is mediated by two different mechanisms. The *tetA*(P) mediates active tetracycline efflux, whereas *tetB*(P) is related to the ribosomal protection family of tetracycline resistance determinants (Sloan *et al.*, 1994).

1.6.5 Resistance to clindamycin

The activity of clindamycin involves binding of the antibiotic to the 50S ribosomal sub-unit, resulting in the inhibition of protein biosynthesis (Rosenblatt, 1991). Clindamycin has in the past been proposed as one of the most effective antimicrobial agents used for anaerobic infections, but increases in resistance have been reported in *Peptostreptococcus* spp. (Sanchez, Jones & Croco, 1992) and in the USA, the percentage of *B. fragilis* strains resistant to clindamycin rose from 8-14% from 1990 to 1994 (Labbé *et al*, 1999). In a recent study a significant increase in resistance rates to clindamycin among *B. fragilis* group strains in the last 13 years was reported in Canada (Labbé *et al,* 1999). In several other countries there have been similar trends in levels of clindamycin resistance and in Korea, in 1994, resistance to clindamycin for the *B. fragilis, B. thetaiotaomicron* and other *Bacteroides* spp. was 38, 45.5 and 60% respectively (Labbé *et al*, 1999). In 1996 20-35% *C. perfringens* and 15-25% *B. fragilis* group were reported from US hospitals as being resistant (Finegold & Wexler, 1996; Wexler, Molitoris & Molitoris, 1997). In general, the rate of clindamycin resistance in *Bacteroides* species remains low and this antibiotic is still considered to be effective in the treatment of infections due to *Bacteroides* and in 1997 the percentage *B. fragilis* isolates resistant was reported as 15% in the USA (Rosenblatt, 1991; Citron & Appleman, 1997; Rasmussen, Bush & Tally, 1997). However, in 1997 a frequency of 25% resistance to clindamycin was reported in southern Europe and Japan (Rasmussen, Bush & Tally, 1997). Three mechanisms of clindamycin resistance have been proposed: 1) inactivation of the antibiotic, 2) altered permeability and 3) altered target site, being the ribosome (Rasmussen, Bush & Tally, 1997). Conjugal transfer of clindamycin resistance was first reported in 1979 (Privitera, Dublanchet & Sebald, 1979; Tally *et al.*, 1979; Welch, Jones & Macrina, 1979) and was shown to be plasmid-mediated, but resistance to clindamycin has also been documented to be chromosomally encoded (Rasmussen, Bush & Tally, 1997). In several cases resistance to clindamycin has been linked with

resistance to tetracycline (Mays *et al.* 1982; Shoemaker, Guthrie & Salyers, 1985; Shoemaker, Barber & Salyers, 1989).

1.6.6 Permeability and efflux

The bacterial cell is surrounded by an asymmetric bilayer, which not only controls through channel-forming porin proteins the influx and efflux of respectively nutrients and metabolic products, but also constitutes a permeation barrier against certain antibiotics and other noxious molecules (Bellido, Pechere & Hancock, 1991). The outer membrane especially plays an important role in the physiology of Gram-negative bacteria and is essentially composed of proteins associated with an external lipopolysaccharide- and internal phospholipid-monolayer (Bellido, Pechere & Hancock, 1991). The ability to cross the outer membrane is a prerequisite for antibacterial agents to have any effect on Gram-negative cells (Bellido, Pechere & Hancock, 1991). In conjunction with affinity for target PBPs and resistance to ß-lactamases, reduced permeability is an important codeterminant of ß-lactam antibiotic efficacy. For this reason quantitative estimations of outer membrane permeability are crucial in the design of antibiotic structure (Johnson, 1993). The role of permeability barriers in antibiotic resistance development in anaerobes has been approached only indirectly, as research on outer membrane proteins (OMP)s of anaerobic bacteria with regard to their role in dynamic cell processes and characterisation of pore-forming molecules, has been hampered by lack of knowledge about the structure and functional characteristics (Wexler, Getty & Fisher, 1992). Little information is available about porin molecules in anaerobes, but they have been described in *B. fragilis, Porphyromonas* spp. and *Fusobacterium* (Wexler, 1997). In studies performed by Wexler, Getty & Fisher (1992) clinical isolates of *Bacteroides* spp. that were resistant to cefoxitin revealed OMP changes, an apparent absence of a 49-50 kDa protein and reduced affinity of PBPs for cefoxitin. Wexler *et al,* (1992) were also the first group to report porin activity in a *Bacteroides* OMP fraction as well as in a heat-modifiable OMP in *Bacteroides* spp.

Many different bacteria (including the anaerobes) are able to pump out antibiotics, adding another dimension to the many methods of resistance already known. Efflux is a major resistance mechanism, especially if it occurs in combination with

enzymatic attack on the antimicrobial agent or with alteration of the target site (Williams, 1996). Efflux systems are seen with ß-lactams, aminoglycosides and tetracyclines, when access of antibiotics to target sites is prevented in part by a membrane-associated, energy-driven efflux mechanism (Nikaido, 1994). Increase in the genetic expression of efflux transporters occurs with repression of the OmpF porin synthesis (Williams, 1996). Increased transcription of the MarA regulatory protein represses OmpF production, which in turn reduces the number of large channels in the membrane preventing passage of antibiotics into the cell (Williams, 1996).

Although treatment failures of anaerobic infections are still considered relatively uncommon, multiply resistant strains of *Bacteroides* and other anaerobes can easily become a serious problem (Salyers & Shoemaker, 1996). Among the clinically important anaerobes, the *B. fragilis* group are most prominent because of high virulence and their ability to develop resistance to many antimicrobial agents, especially ß-lactams (Aldridge, 1995). In addition, the fibrotic capsule of *Bacteroides* is detrimental to the activity of many antimicrobial agents as it interferes with the penetration of the agent and the presence of binding proteins may impair activity (Brook, 1991).

Informed selection of antimicrobial therapy and the design of new antimicrobial agents are highly dependent on the understanding of resistance mechanisms. It is equally important to appreciate variations in resistance and to relate these variations to mechanisms of resistance, as susceptibility patterns vary notably among different anaerobes with respect to individual strains and to the different classes of antimicrobial agents (Rasmussen, Bush & Tally, 1997). Surveys of anaerobic susceptibility have shown regional variations which, coupled with developing multiple resistance makes a totally reliable empiric therapy difficult or impossible (Goldstein, 1996). An issue of concern arising in connection with antibiotic resistance development/acquisition in anaerobes, is that they are prominent members of human commensal microflora and hence in a position to serve as reservoirs of antibiotic-resistance genes (Salyers & Shoemaker, 1996).

Problems are evident from the outset concerning all facets of monitoring and controlling anaerobic associated infections: 1) isolation/identification is time

consuming, expensive and not necessarily reliable, making it extremely difficult to follow the progression of infection if cure is not effected and 2) anaerobes can no longer be considered universally susceptible to antimicrobial agents as resistance is developing gradually in some species and appearing sporadically throughout other species, seriously compromising empiric therapy. There is a need to improve identification and susceptibility testing of anaerobes, characterise resistance genes and assess new anti-anaerobic agents.

1.7 OBJECTIVES

The objectives of the study were to: 1) assess antibiotic susceptibilities of anaerobic bacteria isolated in the Bloemfontein area to antimicrobial agents currently employed in empiric treatment, 2) compare the *in vitro* activity of currently employed antibiotics with new antimicrobial agents, and 3) conduct studies on antibiotic resistance development.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

Anaerobic bacteria were isolated from clinically significant infections from April 1996 to March 1997 from Universitas and Pelonomi Hospitals, Bloemfontein. Infection sites could be traced for 302 of 378 of the isolates as follows: blood 5; brain abscesses 7; liver abscesses 3; lung infection/ abscesses 30; eustachian infection/ sepsis 11; neoplasms 10; bone fracture/infection 30; post-operative/amputation sepsis 11; gunshot/ stab wound infection/sepsis 10; genital tract isolates 40; general abscesses 75; and intestinal tract infection 70. Isolates were stored at -70°C in Microbank cryotubes (Pro-Lab Diagnostics, Round Rock Texas).

2.2 IDENTIFICATION

Isolates were identified in the routine diagnostic laboratory by employing presumptive and preliminary methods as described by Mangels (1998), consisting of Gram-staining, plate morphology and various biochemical and antibiotic disk tests. These results were confirmed by using the Rapid ID32A identification system (bioMérieux, France). The Rapid ID32A comprises 29 test cupules that contain an array of dehydrated test substrates. Isolates were inoculated onto KH-agar [brain heart infusion agar (Oxoid, Unipath Ltd., Basingstoke, England) supplemented with vitamin K (10 mg/l), haemin (0.5 g/l), L-cystine (0.5 g/l) and yeast extract (5 g/l)] plates. After 48h incubation at 37°C cells were suspended in 0.85% NaCl to a turbidity equivalent to a McFarland 4 standard (approximately 1.2 x 10^9 cells/ml) and distributed into the cupules on the Rapid ID32A strips. After 4 h aerobic incubation at 37°C appropriate

reagents were added to designated cupules. Colour reactions were read and recorded to produce an eight or 10 (if using the additional two tests included for low discrimination profiles) digit profile number. The profile numbers were then converted to assigned species according to the Analytical Profile Index (bioMérieux) or forwarded to P Cahill (Separation Scientific, Johannesburg, RSA) for interpretation using the more comprehensive anaerobe data base.

2.3 MIC DETERMINATIONS

2.3.1 Agar dilution method

Minimum inhibitory concentrations (MICs) of 18 antimicrobial agents were determined by the National Committee for Clinical Laboratory Standards (NCCLS) agar dilution method (NCCLS, 1993). For the *Clostridium, Bacteroides* and some *Prevotella* species, Wilkins Chalgren agar (Mast Diagnostics, UK) was used. Wilkins Chalgren agar was supplemented with 5% lysed horse blood to enhance growth of fastidious bacteria: *Peptostreptococcus*, *Porphyromonas*, *Fusobacterium* and *Veillonella* species as well as strains of *Prevotella intermedia* and *Prevotella buccae*. Laboratory standard powders were obtained from various companies for the following antibiotics: amoxicillin and ampicillin (SmithKline Beecham Laboratories, UK), penicillin and metronidazole (Sigma Chemical Co., MO, USA), piperacillin (Lederle, NY, USA), cefoxitin and imipenem (Merck Research Laboratories, NJ, USA), cefepime (Bristol-Myers Squibb, NJ, USA), cefpirome (Hoechst Marion Roussel, Italy), meropenem (Zeneca, UK), linezolid (a synthetic oxazolidonone-class antibiotic) and clindamycin (Pharmacia & Upjohn Inc., MI, USA), chloramphenicol (Parke-Davis, MI, USA), ciprofloxacin (Bayer AG, Germany), trovafloxacin (Pfizer, CT, USA), vancomycin and loracarbef (Eli Lilly & Co., IN, USA) and dalfopristin/ quinupristin (dalf/quin) (Rhône-Poulenc Rorer, PA, USA).

The inoculum was prepared by the direct suspension of colonies from KH-agar plate cultures incubated for 18 h (or 48 h for slower growing isolates), into Brucella broth (Difco Laboratories, Detroit, MI, USA). The cell suspensions were inoculated onto the surface of plates containing different antibiotic dilutions using a multipoint inoculator (Mast Laboratories, Merseyside, UK) to deliver 1 x 10^5 CFU/spot. MICs

were read after 48 h incubation at 35°C in an anaerobic environment generated by AnaeroGen gaspaks (Oxoid, Basingstoke, UK). An aerobically incubated control plate was included. The MIC was recorded as the lowest concentration of antibiotic that inhibited growth, disregarding one or two colonies or a trailing haze of growth. Control strains *Bacteroides fragilis* ATCC 25285 and *Eubacterium lentum* ATCC 43055 were included in each series. Approved and tentative NCCLS susceptible breakpoints (NCCLS, 1993) or preliminary breakpoints as suggested by respective manufacturers were used. Breakpoints for linezolid and loracarbef were not available.

2.3.2 ß-Lactamase production

Screening for ß-lactamase production was performed by employing nitrocefin (Oxoid). The freeze-dried nitrocefin was rehydrated in the solution provided by the manufacturer and 50 µl of this suspension placed on a filter paper. Cells were mixed directly onto the moist nitrocefin on the filter paper and left up to 1 h for any colour reaction to occur. A change from yellow to red indicated the production of ß-lactamase.

2.3.3 Inhibition of ß-lactamases

Inhibition of ß-lactamases was determined using amoxicillin (range 0.06 - 128 µg/ml) and clavulanic acid (SmithKline Beecham, UK) at two fixed concentrations (1 µg/ml and 4 µg/ml). Two concentrations of clavulanic acid were employed, as all the strains investigated grew at 1 µg/ml, while some strains were inhibited at 4 µg/ml, which is the level that can be attained following oral therapy. The agar dilution method as described in section 2.3.1 was used.

2.3.4 Metronidazole inhibitory concentration (IC) determinations employing the Etest

Metronidazole Etest strips were obtained from AB Biodisk (Solna, Sweden) and tests were performed according to the manufacturer's specifications. Two batches of Etest

strips were employed containing different concentration ranges, the first covered the range 0.006 - 32 µg/ml and a second product introduced in 1997 to accommodate the testing of all anaerobic species, ranged from 0.016 - 256 µg/ml. Wilkins Chalgren agar plates were poured to a depth of 4 mm and for fastidious bacteria (section 2.3.1) the agar was supplemented with 5% lysed horse blood. For MIC and IC comparisons the same culture and agar composition was used for both determinations. The inoculum for Etests was prepared by suspending cells from KH-agar plates into Brucella broth to a turbidity of ± McFarland 1 standard (1 x 10^8 CFU/ml). The entire surface of the plate was inoculated by dipping a sterile swab into the cell suspension and by using a circular plating machine (Mast Laboratories) an even distribution of cells was achieved. The non-porous plastic Etest strips were applied to dry inoculated plates, as there is an immediate release of the antibiotic from the carrier surface into the agar matrix. Plates were placed in an incubator within 5 min and after incubation at 35°C. At 24 and 48 h, the IC value was read from the scale where the inhibition ellipse edge intersected the strip and the results correlated with the MICs (section 2.3.1).

2.4 GENETIC ANALYSIS OF RESISTANCE DETERMINANTS

All primers used in the study were synthesised by the Department of Biochemistry, University of Cape Town, South Africa. PCR amplification was performed in a Gene Amp PCR System 9600 (Perkin Elmer, Conn, USA).

2.4.1 Cell lysis

A quarter plate of confluent growth from a KH-agar plate was suspended in 250 µl of 50 mM Tris-HCl, 10 mM EDTA buffer, pH 7.5. To the cell suspension 1.25 µl lysozyme (10 mg/ml) was added and the mixture left for 20 min at 25°C. For Gram-positive species, lysis was enhanced by the addition of 10 µg of mutanolysin. To the suspension, 250 µl of 2% TritonX-100 in 50 mM Tris-HCl, 10 mM EDTA buffer, pH 8 with 1.25 µl proteinase K (5 mg/ml) was added and incubation continued for 30 min at 37°C. Lysates were stored at 4°C until required for further analysis.

2.4.2 Agarose gel electrophoresis

PCR products (25 µl or 100 µl) were mixed with 2-8 µl of 10 x TAE buffer and 1-4 µl of bromophenol blue (0.25%). PCR mixtures were applied to 1-1.5% agarose gels (NuSieve; FMC BioProducts, Rockland, USA) and products separated by electrophoresis for 2-4 h at 90 V using 1 x TAE running buffer (4.84 g/l Tris, 0.37 g/l EDTA, pH 8). The products were visualised by staining with ethidium bromide and photographed under UV illumination (λ 312 nm). A 100 bp DNA ladder (Gibco BRL, Life Technologies Ltd., Paisley, UK) was included in each run.

2.4.3 DNA extraction

PCR products (100 µl) were separated by agarose gel electrophoresis (section 2.4.2) on 1% gels and bands of predicted sizes cut from the gels with minimum exposure to UV illumination. The DNA was extracted from the gel slices using a Nucleon GX extraction kit (Amersham, Buckinghamshire, England) by incubation in sodium perchlorate (for extracting fragments <500 bp) or sodium iodide (for fragments ≥500 bp) at 55°C for 5 min. Resin (15 µl) was then added and the samples incubated at room temperature for 1 min. After centrifugation in a microcentrifuge at top speed (16 000 x *g*) for 30 sec, the pellet was resuspended in 1 ml Washing Solution, followed by a 1:1 dilution with 99% ethanol. The sample was again centrifuged at top speed for 30 sec and the supernatant discarded. The pellet was incubated at 55°C (tube with open lid) until all the alcohol had evaporated. Sterile distilled H_2O (10 µl) was added to the pellet after 1 min incubation at room temperature. After centrifugation for 30 sec the supernatant containing the DNA was carefully removed to a clean tube. The final step was repeated to yield approximately 20 µl of DNA sample.

2.4.4 Sequencing

Antibiotic resistant gene regions that had undergone DNA extraction (section 2.4.3) were sequenced. Automated sequencing was performed in both directions employing a Thermo Sequenase Dye Terminator Cycle Sequencing Kit (Amersham).

According to manufacturer's specifications, a sample (20 µl) was prepared for PCR consisting of 8 µl sequencing reagent pre-mix, 1 µl primer (20 pmol) and 11 µl DNA template (125 ng). Cycling consisted of a denaturation step of 1 min at 96°C, followed by 30 cycles: 96°C for 30 sec, 45°C for 15 sec and 60°C for 4 min. After the PCR process, 7 µl of 7.5 M ammonium acetate and 70 µl of 100% cold (-20°C) ethanol were added to each reaction tube, mixed and placed on ice to precipitate the DNA. After centrifugation in a microcentrifuge at 14 000 x *g* for 16 min at room temperature the supernatant was removed. The pellet was washed with 300 µl 70% cold (-20°C) ethanol and centrifuged for a further 2 min. After removing the supernatant, the pellet was dried at 60°C for 3 min and resuspended in 3 µl loading dye. Reaction mixtures for sequencing of presumptive *nim* genes were analysed by the Department of Microbiology and Biochemistry, University of the Orange Free State, on a Sequence Navigator gel and computer analysis was performed with ABI PRISM, Model 377 (Perkin Elmer). Sequencing analysis of reaction mixtures for the *cfiA, cphA* and *rdxA* genes were performed by the Department of Haematology and Cell Biology, University of the Orange Free State with ABI PRISM, Model 310 Genetic Analyzer (Perkin Elmer) and computer analysis by employing Data Collection Software (Sequence Navigator).

2.4.5 Detection of nim genes

The primers and amplification conditions as described by Trinh & Reysset (1996) were used. The primers were those designed for the universal amplification of known *nim* genes to produce a PCR product of 458 bp: *NIM*-3: 5' ATGTTCAGAGAA-ATGCGGCGTAAGCG and *NIM*-5: 5' GCTTCCTTGCCTGTCATGTGCTC. Annealing temperatures of 52°C and 62°C were used as it was not known whether South African strain variation would limit *nim* gene detection. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was conducted as follows: one cycle of 5 min at 96°C, cooling to 52/62°C, at which temperature 0.5 units of *taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies, Leatherhead, England) was added, followed by 5 min at 72°C. Thirty two cycles were then performed; denaturation for 1 min at 94°C, annealing for 2 min at 52/62°C and extension for 2 min at 72°C with a

final extension step of 5 min at 72°C. PCR products from control strains *B. vulgatus* BV-17 and *B. fragilis* BF-8 were also used as references in assisting *nim* gene size correlation (Trinh & Reysset, 1996). The presence of fragments at approximately 458 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive *nim* genes compared to those of documented *nimA, B, C* and *D* genes (Trinh & Reysset, 1996).

2.4.6 Detection of rdxA genes:

2.4.6.1 Using Mtz6EF and MtzRBgl primers:

Primers as described by Goodwin *et al.* (1998) and amplification conditions as described by Jenks, Ferrero & Labigne (1999) were employed. The primers were *Mtz6EF*: 5' TGAATTCGAGCATGGGGCAG and *MtzRBgl*: 5' AGCAGGAGCATCAG-ATAGATCTGA. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was conducted as follows: one cycle of 5 min at 96°C, cooling to 48°C at which temperature 0.5 units of *taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) was added, followed by 5 min at 72°C. Thirty cycles were then performed; denaturation for 2 min at 95°C, annealing for 2 min at 48°C, and extension for 2 min at 72°C with a final extension step of 10 min at 72°C. PCR products were separated on 1.5% agarose gels (section 2.4.2) and fragments of approximately 937 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive *rdxA* genes compared to the documented *rdxA* gene (Goodwin *et. al.*, 1998).

2.4.6.2 Alternative detection of the rdxA gene:

The primers and amplification conditions (section 2.4.6.1) as described by Jenks, Ferrero & Labigne (1999) were employed. The primers were: *rdxA* 3: 5' CGTTAGG-GATTTTATTGTATGCTA and *rdxA* 5: 5' CCCCACAGCGATATAGCATTGCTC. PCR products were separated on 1.5% agarose gels (section 2.4.2) and fragments of approximately 491 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive *rdxA* genes compared to the documented *rdxA* gene (Goodwin *et. al.*, 1998).

2.4.7 Detection of genes encoding for metallo-ß-lactamases:

2.4.7.1 CfiA gene

The primers and amplification conditions as described by Khushi *et. al.* (1996) were followed. The primers used were *cfiA* 3: 5' CAGAAAAGCGTAAAAATA and *cfiA* 5: 5' TCGTGAA-GTTTCGGTATC. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was conducted as follows: one cycle of 5 min at 96°C, cooling to 42°C at which temperature 0.5 units of *taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) was added, followed by 5 min at 72°C. Twenty cycles were then performed; denaturation for 1 min at 94°C, annealing for 1 min at 42°C, and extension for 1 min at 72°C with a final extension step of 5 min at 72°C. PCR products were separated on 1.5% agarose gels (section 2.4.2) and fragments of approximately 747 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive *cfiA* genes compared to those of the documented *cfiA* gene (Thompson & Malamy, 1990).

2.4.7.2 Bla_{IMP} gene

The primers and amplification conditions as described by Senda *et. al.* (1996) were used. The primers were bla_{IMP} 3: 5' CTACCGCAGCAGAGTCTTTG and bla_{IMP} 5: 5' AACCAGTTTTGCCTTACCAT. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was conducted as follows: one cycle of 5 min at 96°C, cooling to 55°C at which temperature 0.5 units of *taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) was added, followed by 5 min at 72°C. Thirty cycles were then performed; denaturation for 1 min at 94°C, annealing for one min at 55°C, and extension for 1 min 30 sec at 72°C with a final extension step of 5 min at 72°C. PCR products were separated on 1.5% agarose gels (section 2.4.2) and fragments approximately 587 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive bla_{IMP} genes compared to those of the documented bla_{IMP} gene (Arakawa *et. al.*, 1995).

2.4.7.3 CphA gene

The primers and amplification conditions as described by Villadres *et al.* (1996) were used. The primers used were *#CP-Nde*/F: 5' GCGAGGGAGCCATATGATGAAAG-GTTGGATGAAGT and *#CP-Bam/R*: 5' CGGCTGCCGGGATCCGGCTTATGACT-GGGGTGCGGCC. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was conducted as follows: one cycle of 5 min at 96°C, cooling to 52°C at which temperature 0.5 units of *taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) was added, followed by 5 min at 72°C. Thirty eight cycles were then performed; denaturation for 15 sec at 94°C, annealing for 1 min at 52°C, and extension for 30 sec at 72°C with a final extension step of 5 min at 72°C. PCR products were separated on 1.5% agarose gels (section 2.4.2) and fragments of approximately 769 bp were recorded as presumptive positives. Sequencing was performed as described in section 2.4.4 and sequences of presumptive *cphA* genes compared to those of the documented *cphA* gene (Massida, Rossolini & Satta, 1991).

2.5 PLASMID ANALYSIS

Plasmids were extracted using a Wizard Miniprep DNA purification system (Promega, Madison, WI, USA) in accordance with the protocol provided. Overnight growth from half a KH-agar plate was resuspended in 200 µl of Cell Resuspension Solution to which 200 µl of Cell Lysis Solution was added. The suspension was mixed thoroughly (by tube inversion) and left at room temperature until the suspension became clear. Neutralisation Solution (200 µl) was added and the suspension mixed again by inverting the tube. After centrifugation at 16 000 x *g* for 5 min, the supernatant was transferred to a clean microcentrifuge tube and 1 ml of the DNA Purification Resin added. One Wizard Minicolumn was prepared for each miniprep. The plunger of a 3 ml syringe was removed and the syringe barrel attached to the Luer-lock extension of each Minicolumn. The Minicolumn/syringe barrel assembly was then inserted into a vacuum manifold. The resin/DNA mixture was carefully pipetted into the syringe barrel and a vacuum applied to draw the mixture into the Minicolumn. The vacuum was then broken and 2 ml of Column Wash Solution added

to the syringe barrel. The vacuum was then reapplied to draw the wash solution through the Minicolumn. The resin with bound DNA was then dried by a continuous vacuum-pull for an additional 1-2 min. The Minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and centrifuged at 16 000 x *g* for 20 sec in order to remove any residual Column Wash Solution. After transferring the Minicolumn to a further clean microcentrifuge tube, 50 µl of distilled H_2O (25°C) was applied to the Minicolumn which was left for 1 min at 25°C. The DNA was eluted from the Minicolumn by centrifugation at 16 000 x *g* for 20 sec. The plasmid preparations (20 µl) were separated on 0.7% agarose gels using 1 x TAE buffer for 1 h at 85 V and sized approximately employing a supercoiled DNA ladder, range 2-16.2 kb (Promega, Madison, USA) and a known 39 kb tetracycline-resistance plasmid of *Neisseria gonorrhoeae*.

2.6 METRONIDAZOLE-RESISTANT STRAINS

2.6.1 Selection of mutant strains

A *Veillonella* species isolate (metronidazole MIC 4 µg/ml) and a *Peptostreptococcus prevotii* strain (metronidazole MIC 4 µg/ml) were induced by subculturing on Mueller-Hinton agar (Difco Laboratories) containing increasing concentrations of metronidazole (4, 8 & 16 µg/ml), to select for mutant strains with increased MICs. Mutants were selected on 4 µg/ml.

2.6.2 Membrane protein analysis

Inner and outer membrane extracts of the parental strain of *Veillonella* species (MIC 4 µg/ml) and mutant (MIC 8 µg/ml), and the parental strain of *P. prevotii* (MIC 4 µg/ml) and mutant (MIC 8 µg/ml) were prepared as described by Livermore & Williams (1996). Confluent growth from 6-8 KH-plates was suspended in 25 ml of 0.14 M mercapto-ethanol in 0.1 M phosphate buffer, pH 7. The cell suspension was centrifuged at 5 000 x *g* for 10 min, washed with 0.1 M phosphate buffer, pH 7 and resuspended in 20 ml of the same buffer. Cells were then disrupted in a sonifier (Branson Sonic Power Company, Danbury, Conn., USA) on ice (5 x 30 sec) and

centrifuged again at 5 000 x *g* for 10 min at 4°C to remove cell debris. To 13.75 ml of the sonicate, 1.25 ml of 20% sarkosyl was added and the solution incubated at 25°C for 20 min. Membranes were harvested after centrifugation at 100 000 x *g* for 30 min at 15°C. The supernatant containing mainly membrane proteins and the resuspended pellet (in 1 ml phosphate buffered saline) consisting of mainly outer membrane proteins (Livermore & Williams, 1996), were stored at -70°C. Membrane fractions were separated on SDS-PAGE gels, 12% and 6% acrylamide separating and stacking gels respectively. Electrophoresis was performed in continuous system reservoir buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3). A constant current of 25 mA per gel was applied for the initial hour followed by 4½ h at a constant current of 35 mA per gel. Gels were stained with 0.1% Coomassie Blue R-250 in fixative, 40% methanol and 10% methanol, for 1 h and membrane protein bands from respective parental and mutant strains compared. A Molecular weight marker (Rainbow mix, Amersham) was included in each gel run.

2.7 PENICILLIN-BINDING PROTEINS (PBPS)

2.7.1 PBP profile analysis

2.7.1.1 Membranes

Membranes were prepared for PBP studies as described by Livermore & Williams (1996). Confluent growth on 6 KH-plates was suspended in 10 ml 0.14 M mercapto-ethanol in 0.1 M phosphate buffer, pH 7. The cells were centrifuged at 5 000 x *g* for 10 min and resuspended in the same buffer. Cells were then disrupted by sonication (5 x 30 sec) (Branson Sonic Power Company) and centrifuged at 5 000 x *g* for 10 min at 4°C to remove cell debris. After centrifugation at 100 000 x *g* for 60 min at 4°C, the membrane component was washed and resuspended in 2 ml 0.01 M phosphate buffer, pH 7. Aliquots of membranes were stored at -70°C. Protein concentration was determined (section 2.7.1.3) and membrane fractions of approximately 50 µg labelled with 5 µl [^{3}H]benzylpenicillin (17.3 µCi/mmol, Amersham) at 30°C for 10 min for large gels (160 x 180 mm) and 2 µl [^{3}H]benzylpenicillin for small gels (72 x 100 mm). A Hoefer SE 600 gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) connected to a cooling system and a Mini-PROTEAN system

(Bio-Rad Laboratories, München, Germany) was used. To the labelled membrane solution 10 µl sarkosyl (20%) was added and the mixture incubated at 30° for 10 min. Sample buffer (0.5 M Tris-HCl, 4% SDS, 20% glycerol 0.2 M 2-mercaptoethanol, 1% bromophenol blue, pH 6.8) was then added (30 µl for large gels, comb size 25 x 8 x 1.5 mm and 10 µl for small gels, comb size 5 x 13 x 1.5 mm). Samples were boiled for 3 min at ± 96°C, held on ice during loading and PBPs separated by SDS-PAGE. The large gels were run at a constant current of 25 mA per gel initially and after 1 h this was increased to 35 mA per gel for 4-4½ h. The small gels were run at a constant voltage of 200 V for 50 min. The gel composition was 4% and 8% acrylamide in the stacking and separating gels respectively, using gel spacers of 1.5 mm. A protein molecular weight marker (Rainbow ^{TM}, [^{14}C]-labelled, Amersham) was included in each gel run.

After electrophoresis the SDS-PAGE gels were fixed with 7% acetic acid for 1 h to overnight on a rotary shaker (Heidolph, Germany) at minimum speed. The acetic acid was discarded and replaced with Amplify (Amersham) (sufficient to cover gels) for 1-2 h. Gels were dried under vacuum on a SE 1160 Slab Gel Dryer (Hoefer Scientific Instruments) at 60°C for 1½ h. Dried gels were exposed to preflashed Hyperfilm-MP (Amersham) at -70°C for periods of one week to one month before developing the fluorograms.

2.7.1.2 Whole cells

Confluent growth from KH-agar plates was suspended in Brucella broth to a turbidity of ± 5 McFarland standard. The cell suspensions (1 ml) were then distributed into several microcentrifuge tubes per strain, centrifuged at 16 000 x *g* and the pellets stored at -20°C. On labelling, the pellets were resuspended in 0.02 M sodium phosphate buffer, pH 7 + 0.2% Triton X-100 (25 µl for large gels, 10 µl for small gels) with 10 µl and 4 µl lysozyme (1 mg/ml) added respectively. For Gram-positive bacteria mutanolysin at a final concentration of 0.1 mg/ml was added. The samples were then incubated at 37°C for 30 min (tubes flicked every 5 min to enhance lysis). After incubation [^{3}H]benzylpenicillin (17.3 µCi/mmol, Amersham) was added to each sample (5 µl for large gels and 2 µl for small gels). Gel preparation, electrophoresis and fluorography were performed as described above for membranes (section 2.7.1.1).

2.7.1.3 Protein concentration determination

Protein concentrations of the membrane fractions for PBP studies were determined by using the Bio-Rad Standard Assay Procedure (Bio-Rad Laboratories). A range of bovine serum albumin concentrations (14, 28, 56, 84, 112 and 140 µg protein) were prepared. To the standard protein dilution and membrane preparations (100 µl) 5 ml of diluted dye reagent was added. After 5 min to 1 h, sample OD_{595} was measured spectrophotometrically. A linear plot of the OD_{595} of the protein standards was constructed and unknown concentrations of the test samples determined.

2.7.2 PBP affinity studies

2.7.2.1 Membrane preparations

Affinity studies were carried out on small gels requiring approximately 30 µg of membrane protein (section 2.7.1.1). Membranes were labelled with increasing concentrations of [^{3}H]benzylpenicillin: 0.01 µl, 0.1 µl, 0.5 µl and 2 µl at 30°C for 10 min. After labelling, 7 µl solubilisation buffer (0.5 M Tris, 20% SDS, pH 6.8) was added and labelled membrane solutions incubated at 30°C for 10 min. Sample buffer was added and electrophoresis/fluorography performed as described in section 2.7.1.1.

2.7.2.2 Whole cells

Cells were harvested and lysed as described in section 2.7.1.2. Concentrations of [^{3}H]benzylpenicillin added were 5 µl, 1 µl, 0.5 µl, 0.05 µl and 0.01 µl. After labelling at 30°C for 30 min, 30 µl of 2 x sample buffer was added. Electrophoresis and fluorography was performed as described in section 2.7.1.1.

2.7.3 PBP Competition studies

2.7.3.1 Veillonella spp.

Competition studies were conducted by initially preincubating whole cell preparations (section 2.7.1.2) of *Veillonella* spp. with piperacillin and ampicillin at concentrations 1, 2, 5 and 10 µg/ml, for 10 min at 37°C. Samples were then post-labelled with 2 µCi

[^{3}H] benzylpenicillin at 37°C for 10 min. PBPs were separated by SDS-PAGE using a small gel system and visualised after fluorography (section 2.7.1.1).

2.7.3.2 Fusobacterium spp.

Competition studies were conducted by initially preincubating whole cell preparations (section 2.7.1.2) of *Fusobacterium* spp. with imipenem at concentrations of 0.5, 1, 2, 5 and 10 µg/ml and post-labelled with 5 µl [^{3}H]benzylpenicillin at 37°C for 10 min. PBPs were separated by SDS-PAGE using a large system and visualised after fluorography (section 2.7.1.1).

2.8 BIOASSAY METHOD FOR DETECTION OF METALLO-ß-LACTAMASES

A method described by Edwards, Hawkyard & Hashmi (1998) was used to screen for the production of metallo-ß-lactamases. Confluent growth from 3 KH-agar plates was suspended in 5 ml 0.1 M phosphate buffer, pH 7. The suspensions were sonicated (Branson Sonic Power Company) on ice (6 x 30 sec) and sonicates centrifuged at 10 000 x *g* for 20 min. The supernatant was freeze dried on a Virtis Freezemobile (The Virtis Company, Inc., NY) and concentrated to provide four-fold and 10-fold concentrations of the crude enzyme extracts. Mueller-Hinton agar (Difco Laboratories) was seeded with an indicator strain *Escherichia coli* ATCC 25922 (1 ml of an overnight nutrient broth culture diluted to a turbidity of 20 Klett per 200 ml agar). Wells (7 mm diameter) were cut into the agar and filled with 30 µl of the concentrated cell extracts, 10 µl imipenem (final concentration 5 µg/ml) and 10 µl 0.01 M phosphate buffer, pH 7.0. Control wells contained only imipenem and phosphate buffer. In a second set of wells the phosphate buffer was replaced with EDTA (final concentration 2 mM), which would inhibit the activity of any metallo-ß-lactamase present. Plates were then incubated aerobically at 37°C for 24 h and zones of inhibition measured with callipers. Zone sizes that resulted from the anaerobes under investigation were compared with that of a high-level imipenem-resistant *Stenotrophomonas maltophilia* strain (Imipenem MIC > 128 µg/ml).

CHAPTER 3

COMPARATIVE ACTIVITIES OF EIGHTEEN ANTIMICROBIAL AGENTS

3.1 INTRODUCTION

The clinical importance of anaerobic bacteria is emphasised by the infections they cause, which include brain abscesses, endocarditis, osteomyelitis, joint infections, infections of prosthetic devices and vascular grafts and refractory or recurrent bacteraemia (Davies Diagnostics, 1999). Less is often known about anaerobic bacteria than aerobic or facultative bacteria, but their participation in human health and well-being should not be underestimated as high mortality and morbidity rates are often associated with anaerobic infections (Rasmussen, Bush & Tally, 1997). Due to unavoidable delays in obtaining a definitive report from the microbiology laboratory in cases such as suspected anaerobic bacteraemia, clinicians are compelled to initiate therapy empirically (Finegold, 1990; Goldstein, 1996). Knowledge of the "usual" bacteriology of various types of anaerobic infection and how this may be modified by pathophysiological processes in the host or by prior therapy can assist clinicians in the formulation of a logical empiric treatment approach (Finegold, 1990).

Empirical therapy directed at anaerobic bacteria can be seriously compromised, since it has been shown that resistance develops gradually in some species and appears sporadically throughout different species (Finegold, 1990; Pankuch *et al.*, 1993; Rosenblatt & Brook, 1993; Spangler, Jacobs & Appelbaum, 1994). There is the need for reliable susceptibility testing methods for anaerobic bacteria extending from small hospital laboratories to large research centres (Davies Diagnostics, 1999). As anaerobes are often present in mixed infections, antimicrobial choice should include agents with activity against both anaerobic and aerobic bacteria (Brook, 1991; Aldridge, 1995). Administration of correct anti-anaerobic therapy *ab initio* can

only be achieved if one is cognisant of the antimicrobial susceptibility of anaerobes in the local environment.

Antimicrobial susceptibility patterns are no longer predictable and can be different in individual hospitals (Davies Diagnostics, 1999). The principal uses for susceptibility testing of anaerobic bacteria are to: 1) assist in the management of infection in individual patients, 2) monitor susceptibility patterns periodically in local communities and individual hospitals, 3) monitor susceptibility patterns periodically in various centres world-wide, and 4) determine the susceptibility to newly introduced antimicrobial agents (Finegold, 1997).

3.2 RESULTS AND DISCUSSION

Site location from laboratory reports could be traced for 302/378 of the isolates. A breakdown of anaerobes from the various infection sites showed the following: blood 5, brain abscesses 7, liver abscesses 3, lung infection/abscesses 30, eustachian infection/sepsis 11, neoplasms 10, bone fracture/infection 30, post-operative/amputation sepsis 11, gunshot/stab wound infection/sepsis 10, genital tract isolates 40, general abscesses 75 and infection of the intestinal tract 70 isolates. Gram-positive bacteria comprised 108 *Clostridium* and 99 *Peptostreptococcus* species and the Gram-negative bacteria 39 *Bacteroides*, 57 *Prevotella*, 7 *Porphyromonas*, 37 *Fusobacterium* and 31 *Veillonella* spp. From the available information pertaining to isolation site, seven anaerobes from brain abscesses were quite diverse, comprising two *Fusobacterium necrophorum* strains, one *Fusobacterium nucleatum*, one *Bacteroides fragilis*, one *Prevotella loescheii*, one *Prevotella buccae* and one *Peptostreptococcus prevotii* strain. From the liver, one *Peptostreptococcus micros* and two *Clostridium perfringens* strains were identified. Of note, eighteen of the 30 anaerobic bacteria isolated from lung infections were *Veillonella* spp. One *B. fragilis*, three *C. perfringens* and one *C. paraputrificum* strain were isolated from blood.

The results of the agar-dilution susceptibility tests and comparative antimicrobial activities against for the different anaerobic species investigated are given in Table 3.1. Reduced susceptibility to penicillin (MICs $\geq$ 1 µg/ml) was evident in 20 *Peptostreptococcus* strains and seven non-perfringens *Clostridium* species tested. On comparing results obtained for Gram-positive anaerobes with a study performed in South Africa in 1992 (Van der Westhuyzen & Chalkley, 1992), resistance to penicillin appears to be declining. This could possibly be due to the retraction of treatment with penicillin as first choice for *Neisseria gonorrhoeae, Streptococcus pneumoniae* and prophylaxis for nosocomial infections in South African hospitals. In 1996, Bowker *et al.* (1996) found the majority of *C. perfringens* and *Clostridium* spp., but none of the *Peptostreptococcus* isolates in the UK to be resistant to penicillin.

ß-Lactamases hydrolysing both penicillins and cephalosporins were demonstrated in all *Bacteroides* and *Prevotella* isolates, but amoxicillin in combination with clavulanic

acid (4 µg/ml) was effective against 30/31 *Bacteroides* and *Prevotella* isolates. Intermediate resistance was maintained by one *B. fragilis* strain (MIC 8 µg/ml). In 1996 Bowker *et al.* (1996) found *Bacteroides* spp. with amoxicillin/clavulanic acid MICs 32 µg/ml, but ß-lactamase production was not indicated. In 1992, 37% of ß-lactamase producing *Bacteroides* spp. investigated in Johannesburg were resistant to amoxicillin/clavulanic acid (Van der Westhuyzen & Chalkley, 1992), whereas in the present study and that of Koch *et al.* (1998) on strains from Cape Town, the percentage of *B. fragilis* strains resistant to amoxicillin/clavulanic acid was low, ≤ 2%.

Piperacillin exhibited good activity against the Gram-positive isolates with only eight strains (8%) of *Peptostreptococcus* species being resistant (MICs ≥ 64 µg/ml). The Gram-negative anaerobic species, however, were less susceptible with resistance being demonstrated in 41% of strains identified as *Bacteroides* species. Findings by Aldridge & Johnson (1997) on anaerobes isolated in the USA revealed similar piperacillin activities with no strains of clostridia being resistant, but lower levels of resistance to piperacillin were again found in strains *Bacteroides* strains (≤ 16% resistant). In a study performed in the USA on strains collected from 1989-1994 (Spangler, Jacobs & Appelbaum, 1994), resistance to piperacillin was equally evident in Gram-negative anaerobic bacteria, with 50% of the *B. fragilis* group isolates being resistant, yet no resistance was evident in the Gram-positive strains examined. Selective ß-lactam resistance to piperacillin alone was observed in 21/31 (68%) *Veillonella* spp., a finding similar to that from Australia as reported by Mendes, Gordon & Mitchell (1997) where piperacillin/tazobactam was highly active against most strains other than *Veillonella.* The origin of 15 of the 21 piperacillin-resistant *Veillonella* isolates in the current study could be traced, i.e. 13 from lung infections, one from an open bone fracture and one from septic abdominal tissue.

Table 3.1: Activities of antimicrobial agents against anaerobe groups.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	Range
Peptostreptococcus anaerobius: (59)	Amoxicillin	73	0.25	32	≤0.06 - 64
	Ampicillin	85	0.125	16	≤0.06 - 32
	Penicillin	71	≤0.06	8	≤0.06 - 16
	Piperacillin	92	0.25	32	≤0.06 - >128
	Cefoxitin	100	0.5	16	0.125- 16
	Cefepime	97	0.5	16	0.5 - 64
	Cefpirome	100	0.25	8	0.25 - 16
	Imipenem	100	≤0.06	2	≤0.06 - 4
	Meropenem	100	0.25	4	≤0.06 - 4
	Metronidazole	100	0.5	1	≤0.06 - 8
	Clindamycin	98	≤0.06	0.5	≤0.06 - >128
	Chloramphenicol	100	1	1	0.5 - 2
	Ciprofloxacin	97	1	1	0.25 - 64
	Trovafloxacin	97	≤0.06	≤0.06	≤0.06 - 8
	Vancomycin	100	0.25	0.5	0.125- 1
	Dalf/quin	100	0.25	0.25	0.125- 1
	Linezolid		0.5	1	0.25 - 128
	Loracarbef		2	128	0.5 - >128
Peptostreptococcus magnus: (14)	Amoxicillin	100	0.125	0.25	≤0.06 - 0.25
	Ampicillin	100	0.125	0.25	≤0.06 - 0.25
	Penicillin	100	≤0.06	≤0.06	≤0.06 - 0.125
	Piperacillin	100	1	2	≤0.06 - 2
	Cefoxitin	100	0.25	0.25	≤0.06 - 0.5
	Cefepime	100	8	16	0.25 - 16
	Cefpirome	100	4	4	0.125- 8
	Imipenem	100	≤0.06	≤0.06	≤0.06
	Meropenem	100	≤0.06	0.125	≤0.06 - 0.25
	Metronidazole	86	0.5	>128	0.125- >128
	Clindamycin	93	1	2	≤0.06 - 64
	Chloramphenicol	100	2	4	1 - 4
	Ciprofloxacin	100	0.5	1	0.125- 1
	Trovafloxacin	100	0.125	0.5	≤0.06 - 1
	Vancomycin	100	0.25	0.5	0.25 - 0.5
	Dalf/quin	100	0.5	0.5	0.125- 1
	Linezolid		1	2	1 - 2
	Loracarbef		2	4	0.125- 8
Peptostreptococcus spp.[a]: (26)	Amoxicillin	92	0.125	0.5	≤0.06 - 32
	Ampicillin	96	0.125	1	≤0.06 - 32
	Penicillin	88	≤0.06	1	≤0.06 - 16
	Piperacillin	96	0.5	16	≤0.06 - 64
	Cefoxitin	100	0.25	2	≤0.06 - 16
	Cefepime	92	2	16	0.25 - 64
	Cefpirome	96	1	16	≤0.06 - 64
	Imipenem	100	≤0.06	0.125	≤0.06 - 4
	Meropenem	100	≤0.06	0.25	≤0.06 - 4
	Metronidazole	100	0.5	1	≤0.06 - 4
	Clindamycin	96	0.125	2	≤0.06 - >128
	Chloramphenicol	100	1	2	0.5 - 4
	Ciprofloxacin	92	2	2	0.125- 8
	Trovafloxacin	100	0.25	0.5	≤0.06 - 1
	Vancomycin	92	0.25	0.5	≤0.06 - 8
	Dalf/quin	96	0.5	1	≤0.06 - 8
	Linezolid		0.5	2	0.25 - 4
	Loracarbef		1	8	≤0.06 - 64

Table 3.1: Continued.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	Range
Clostridium perfringens: (72)	Amoxicillin	100	≤0.06	0.125	≤0.06 - 0.125
	Ampicillin	100	0.125	0.25	≤0.06 - 0.5
	Penicillin	100	0.125	0.25	≤0.06 - 0.5
	Piperacillin	100	0.25	0.5	≤0.06 - 0.5
	Cefoxitin	100	1	1	0.125- 2
	Cefepime	100	1	2	0.125- 2
	Cefpirome	100	1	2	≤0.06 - 4
	Imipenem	100	0.125	0.25	≤0.06 - 0.25
	Meropenem	100	≤0.06	≤0.06	≤0.06 - 0.125
	Metronidazole	100	1	2	0.25 - 8
	Clindamycin	86	0.125	4	≤0.06 - 16
	Chloramphenicol	100	4	4	2 - 4
	Ciprofloxacin	99	1	1	0.25 - 4
	Trovafloxacin	100	0.125	0.25	≤0.06 - 0.5
	Vancomycin	100	0.5	1	0.25 - 1
	Dalf/quin	100	0.5	0.5	0.125- 1
	Linezolid		2	2	1 - 2
	Loracarbef		1	2	≤0.06 - 16
Clostridium spp.[b]: (36)	Amoxicillin	100	0.25	1	≤0.06 - 2
	Ampicillin	100	0.25	1	≤0.06 - 2
	Penicillin	83	0.125	1	≤0.06 - 2
	Piperacillin	100	1	16	≤0.06 - 32
	Cefoxitin	89	1	4	≤0.06 - >128
	Cefepime	78	4	>128	≤0.06 - >128
	Cefpirome	78	1	128	≤0.06 - >128
	Imipenem	97	0.25	2	≤0.06 - 8
	Meropenem	100	0.125	1	≤0.06 - 2
	Metronidazole	100	0.5	1	≤0.06 - 2
	Clindamycin	67	0.5	32	≤0.06 - >128
	Chloramphenicol	94	2	4	≤0.06 - 16
	Ciprofloxacin	64	1	8	≤0.06 - 16
	Trovafloxacin	100	0.25	0.5	≤0.06 - 1
	Vancomycin	94	0.5	2	≤0.06 - 8
	Dalf/quin	97	0.5	1	≤0.06 - 2
	Linezolid		2	4	0.5 - 8
	Loracarbef		2	16	0.5 - 16
Bacteroides fragilis group[c]: (32)	Amoxicillin	16	32	>128	1 - >128
	Ampicillin	19	32	>128	2 - >128
	Piperacillin	61	32	>128	1 - >128
	Cefoxitin	68	16	32	4 - 64
	Cefepime	29	128	>128	16 - >128
	Cefpirome	16	64	>128	16 - >128
	Imipenem	100	0.25	1	≤0.06 - 4
	Meropenem	100	0.25	4	0.125- 4
	Metronidazole	100	0.5	2	0.25 - 4
	Clindamycin	71	2	>128	≤0.06 - >128
	Chloramphenicol	100	4	4	1 - 8
	Ciprofloxacin	6	8	128	0.125- >128
	Trovafloxacin	90	0.125	2	≤0.06 - 8
	Vancomycin	0	32	128	8 - >128
	Dalf/quin	35	2	8	0.5 - 32
	Linezolid		4	4	2 - 8
	Loracarbef		>128	>128	16 - >128

Table 3.1: Continued.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC (µg/ml) MIC_{50}	MIC_{90}	Range
Bacteroides spp.[d]: (7)[#]	Amoxicillin	29	32	-	≤0.06 - >128
	Ampicillin	29	32	-	≤0.06 - >128
	Piperacillin	57	16	-	≤0.5 - >128
	Cefoxitin	57	16	-	≤0.06 - >128
	Cefepime	43	64	-	≤0.5 - >128
	Cefpirome	29	64	-	≤0.5 - >128
	Imipenem	100	0.25	-	≤0.06 - 2
	Meropenem	100	0.125	-	≤0.06 - 2
	Metronidazole	100	0.5	-	0.25 - 1
	Clindamycin	57	1	-	≤0.06 - >128
	Chloramphenicol	100	4	-	1 - 4
	Ciprofloxacin	43	4	-	1 - 16
	Trovafloxacin	100	0.25	-	0.25 - 0.5
	Vancomycin	43	16	-	0.5 - 128
	Dalf/quin	57	1	-	0.25 - 4
	Linezolid		2	-	1 - 8
	Loracarbef		>128	-	≤0.06 - >128
Prevotella loescheii: (33)	Amoxicillin	21	16	32	≤0.5 - >128
	Ampicillin	24	16	32	≤0.5 - >128
	Piperacillin	85	8	64	≤0.5 - >128
	Cefoxitin	82	8	32	≤0.5 - 64
	Cefepime	55	32	>128	≤0.5 - >128
	Cefpirome	30	32	>128	1 - >128
	Imipenem	100	0.25	1	≤0.06 - 4
	Meropenem	100	0.125	2	≤0.06 - 4
	Metronidazole	100	1	1	0.125- 1
	Clindamycin	85	1	4	≤0.06 - >128
	Chloramphenicol	100	4	4	1 - 8
	Ciprofloxacin	6	4	32	1 - 64
	Trovafloxacin	97	0.125	0.5	≤0.06 - 4
	Vancomycin	0	16	64	8 - >128
	Dalf/quin	39	2	4	0.25 - 16
	Linezolid		4	8	2 - 8
	Loracarbef		>128	>128	16 - >128
Prevotella bivia: (7)[#]	Amoxicillin	63	2	-	≤0.5 - 32
	Ampicillin	63	2	-	≤0.5 - 32
	Piperacillin	100	4	-	≤0.5 - 32
	Cefoxitin	100	4	-	1 - 16
	Cefepime	63	32	-	2 - 128
	Cefpirome	63	16	-	2 - 128
	Imipenem	100	≤0.06	-	≤0.06 - 0.125
	Meropenem	100	0.125	-	≤0.06 - 0.125
	Metronidazole	100	1	-	0.125- 4
	Clindamycin	100	≤0.06	-	≤0.06 - 1
	Chloramphenicol	100	2	-	0.125- 4
	Ciprofloxacin	38	8	-	0.25 - 16
	Trovafloxacin	100	1	-	≤0.06 - 1
	Vancomycin	0	32	-	8 - >128
	Dalf/quin	88	0.25	-	≤0.06 - 2
	Linezolid		2	-	1 - 32
	Loracarbef		16	-	2 - >128

Table 3.1: Continued.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	Range
Prevotella buccae: (7)[#]	Amoxicillin	57	≤0.5	-	≤0.5 - >128
	Ampicillin	43	16	-	≤0.5 - >128
	Piperacillin	86	2	-	1 - >128
	Cefoxitin	86	4	-	1 - 32
	Cefepime	57	16	-	4 - >128
	Cefpirome	57	2	-	2 - >128
	Imipenem	100	≤0.06	-	≤0.06 - 1
	Meropenem	100	0.125	-	≤0.06 - 1
	Metronidazole	100	0.5	-	0.5 - 4
	Clindamycin	86	≤0.06	-	≤0.06 - 4
	Chloramphenicol	100	2	-	2 - 4
	Ciprofloxacin	57	2	-	2 - 4
	Trovafloxacin	100	0.5	-	0.5 - 1
	Vancomycin	0	64	-	32 - 128
	Dalf/quin	71	1	-	0.5 - 2
	Linezolid		2	-	2 - 4
	Loracarbef		64	-	1 - >128
Prevotella spp.[e]: (9)[#]	Amoxicillin	78	2	-	≤0.5 - 32
	Ampicillin	89	4	-	≤0.5 - 16
	Piperacillin	89	2	-	≤0.5 - 64
	Cefoxitin	100	2	-	≤0.5 - 8
	Cefepime	78	16	-	1 - 128
	Cefpirome	67	4	-	≤0.5 - 128
	Imipenem	100	≤0.06	-	≤0.06 - 0.25
	Meropenem	100	≤0.06	-	≤0.06 - 0.25
	Metronidazole	89	1	-	0.25 - 32
	Clindamycin	100	≤0.06	-	≤0.06 - 0.125
	Chloramphenicol	100	2	-	1 - 2
	Ciprofloxacin	89	1	-	1 - 16
	Trovafloxacin	100	0.5	-	≤0.06 - 1
	Vancomycin	0	64	-	16 - >128
	Dalf/quin	44	2		≤0.06 - 2
	Linezolid		2	-	2 - >128
	Loracarbef		8	-	≤0.06 - >128
Porphyromonas spp.[f]: (7)[#]	Amoxicillin	86	≤0.5	-	≤0.5 - 128
	Ampicillin	86	≤0.5	-	≤0.5 - 128
	Piperacillin	86	≤0.5	-	≤0.5 - 128
	Cefoxitin	100	≤0.5	-	≤0.5 - 2
	Cefepime	100	1	-	≤0.5 - 16
	Cefpirome	71	≤0.5	-	≤0.5 - 32
	Imipenem	100	≤0.06	-	≤0.06 - 0.125
	Meropenem	100	≤0.06	-	≤0.06 - 0.25
	Metronidazole	86	≤0.06	-	≤0.06 - 32
	Clindamycin	86	≤0.06	-	≤0.06 - 16
	Chloramphenicol	100	2	-	1 - 4
	Ciprofloxacin	100	1	-	0.25 - 1
	Trovafloxacin	100	0.125	-	≤0.06 - 0.25
	Vancomycin	86	2	-	0.25 - 4
	Dalf/quin	100	0.25	-	0.25 - 0.5
	Linezolid		2	-	1 - 2
	Loracarbef		1	-	≤0.06 - 8

Table 3.1: Continued.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC(μg/ml)		
			MIC_{50}	MIC_{90}	Range
Fusobacterium mortiferum: (13)	Amoxicillin	31	32	>128	≤0.06 - 128
	Ampicillin	23	>128	>128	≤0.06 - >128
	Piperacillin	54	32	>128	≤0.06 - >128
	Cefoxitin	62	8	>128	≤0.06 - >128
	Cefepime	8	>128	>128	≤0.06 - >128
	Cefpirome	8	>128	>128	≤0.06 - >128
	Imipenem	31	64	>128	≤0.06 - >128
	Meropenem	31	64	>128	≤0.06 - >128
	Metronidazole	100	0.25	2	≤0.06 - 4
	Clindamycin	62	≤0.06	16	≤0.06 - 16
	Chloramphenicol	100	1	4	0.25 - 4
	Ciprofloxacin	69	1	4	0.25 - 8
	Trovafloxacin	100	0.5	1	≤0.06 - 2
	Vancomycin	0	>128	>128	16 - >128
	Dalf/quin	8	8	>128	≤0.06 - >128
	Linezolid		8	>128	0.5 - >128
	Loracarbef		>128	>128	≤0.06 - >128
Fusobacterium necrophorum: (12)	Amoxicillin	75	≤0.06	>128	≤0.06 - >128
	Ampicillin	75	≤0.06	>128	≤0.06 - >128
	Piperacillin	83	≤0.06	>128	≤0.06 - >128
	Cefoxitin	83	≤0.06	>128	≤0.06 - >128
	Cefepime	83	0.125	>128	≤0.06 - >128
	Cefpirome	83	0.25	>128	≤0.06 - >128
	Imipenem	83	≤0.06	>128	≤0.06 - >128
	Meropenem	83	≤0.06	>128	≤0.06 - >128
	Metronidazole	100	0.125	0.25	≤0.06 - 0.5
	Clindamycin	92	≤0.06	0.5	≤0.06 - 4
	Chloramphenicol	100	1	2	0.5 - 4
	Ciprofloxacin	83	1	4	0.25 - 8
	Trovafloxacin	100	0.25	1	0.125- 1
	Vancomycin	0	128	>128	16 - >128
	Dalf/quin	67	0.5	4	0.25 - >128
	Linezolid		8	32	4 - 32
	Loracarbef		≤0.06	>128	≤0.06 - >128
Fusobacterium nucleatum: (8)[#]	Amoxicillin	100	≤0.06	-	≤0.06
	Ampicillin	100	≤0.06	-	≤0.06
	Piperacillin	100	≤0.06	-	≤0.06
	Cefoxitin	100	≤0.06	-	≤0.06 - 0.25
	Cefepime	100	0.125	-	≤0.06 - 2
	Cefpirome	100	0.125	-	≤0.06 - 4
	Imipenem	100	≤0.06	-	≤0.06
	Meropenem	100	≤0.06	-	≤0.06
	Metronidazole	100	≤0.06	-	≤0.06 - 0.25
	Clindamycin	100	≤0.06	-	≤0.06 - 0.25
	Chloramphenicol	100	1	-	1 - 2
	Ciprofloxacin	100	0.5	-	0.25 - 1
	Trovafloxacin	100	0.125	-	0.125- 0.5
	Vancomycin	0	64	-	64 - >128
	Dalf/quin	75	1	-	≤0.06 - 2
	Linezolid		8	-	4 - 8
	Loracarbef		0.125	-	0.125- 1

Table 3.1: Continued.

Organism (n)	Antimicrobial agent	Percent susceptible at breakpoint*	MIC (µg/ml) MIC_{50}	MIC_{90}	Range
Fusobacterium varium: (4)#◎	Amoxicillin	-	-	-	0.125; >128; 0.125; >128
	Ampicillin	-	-	-	0.25; >128; 16; 0.125
	Piperacillin	-	-	-	1; >128; >128; 8
	Cefoxitin	-	-	-	4; >128;128;1
	Cefepime	-	-	-	32; >128; >128;16
	Cefpirome	-	-	-	8; >128; >128; 4
	Imipenem	-	-	-	8; >128; >128; 0.125
	Meropenem	-	-	-	0.5; >128; >128; 0.5
	Metronidazole	-	-	-	0.125; 0.25; 0.5; 0.5
	Clindamycin	-	-	-	8; 4; 16; 4
	Chloramphenicol	-	-	-	8; 2; 2; 8
	Ciprofloxacin	-	-	-	32; 4; 8; 0.5
	Trovafloxacin	-	-	-	2; 1; 2; 4
	Vancomycin	-	-	-	>128; >128; 128; 8
	Dalf/quin	-	-	-	1; >128; >128; 4
	Linezolid	-	-	-	32; 32; 32; 0.25
	Loracarbef	-	-	-	0.5; >128; >128; 0.25
Veillonella spp: (31)	Amoxicillin	94	1	4	≤0.06 - >128
	Ampicillin	94	1	8	≤0.06 - >128
	Piperacillin	32	128	>128	≤0.06 - >128
	Cefoxitin	97	8	16	≤0.06 - 32
	Cefepime	94	8	16	≤0.06 - >128
	Cefpirome	94	4	8	≤0.06 - >128
	Imipenem	100	0.25	1	≤0.06 - 2
	Meropenem	100	0.5	2	≤0.06 - 4
	Metronidazole	100	1	4	0.125- 4
	Clindamycin	100	≤0.06	0.125	≤0.06 - 0.125
	Chloramphenicol	100	1	2	0.125- 2
	Ciprofloxacin	100	0.125	0.125	≤0.06 - 0.5
	Trovafloxacin	100	0.25	0.5	≤0.06 - 1
	Vancomycin	0	>128	>128	64 - >128
	Dalf/quin	3	16	32	0.5 - 32
	Linezolid		64	>128	0.5 - >128
	Loracarbef		8	16	0.125- >128
All Isolates: (378)	Amoxicillin	74	0.25	32	≤0.06 - >128
	Ampicillin	76	0.25	32	≤0.06 - >128
	Penicillin (n=207)	87	0.125	1	≤0.06 - >128
	Piperacillin	84	1	128	≤0.06 - >128
	Cefoxitin	91	1	16	≤0.06 - >128
	Cefepime	88	4	64	≤0.06 - >128
	Cefpirome	76	2	>128	≤0.06 - >128
	Imipenem	96	0.125	2	≤0.06 - >128
	Meropenem	96	0.125	2	≤0.06 - >128
	Metronidazole	98	0.5	2	≤0.06 - >128
	Clindamycin	85	0.125	4	≤0.06 - >128
	Chloramphenicol	99.5	2	4	≤0.06 - 16
	Ciprofloxacin	74	1	8	≤0.06 - >128
	Trovafloxacin	98	0.125	0.5	≤0.06 - 8
	Vancomycin	56	1	>128	≤0.06 - >128
	Dalf/quin	72	0.5	8	≤0.06 - >128
	Linezolid		2	32	0.25 - >128
	Loracarbef		4	>128	≤0.06 - >128

a. *P. asaccharolyticus* (7), *P. indolicus* (2), *P. micros* (3), *P. prevotii* (10), *Peptostreptococcus* spp. (4).
b. *C. bifermentans* (4), *C. difficile* (1), *C. glycolicum* (1), *C. histolyticum* (1), *C. innocuum* (1), *C. paraputrificum* (1), *C. sordelli* (4), *Clostridium sporogenes* (6), *Clostridium subterminale* (1), *Clostridium tertium* (8), *Clostridium spp.* (8).
c. *B. fragilis* (26), *B. vulgatus* (6).

d. *B. capillosus* (3), *B. caccae* (1), *B. ureolyticus* (3).
e. *P. denticola* (1), *P. disiens* (1), *P. intermedia* (4), *P. melaninogenica* (1), *P. oralis* (2).
f. *P. asaccharolytica* (1), *P. endodontalis* (6).

* Susceptibility breakpoints (µg/ml): amoxicillin 4, ampicillin 8, penicillin 0.5, piperacillin 32, cefoxitin 16, cefepime 32, cefpirome 16, imipenem 4, meropenem 4, metronidazole 8, clindamycin 2, chloramphenicol 8, ciprofloxacin 2, trovafloxacin 2, vancomycin 4, dalf/quin 1, linezolid (not available), loracarbef (not available).
\# No MIC_{90}s were reported if the number of isolates were < 10.
◎ No MIC_{50}s or MIC_{90}s are reported if the number of isolates tested were ≤ 4. Individual MIC values for each strain are reported in the MIC range column.

Cefoxitin exhibited better activity against the Gram-positive anaerobic bacteria than the Gram-negative bacteria studied, with only three *Clostridium* species other than *C. perfringens* resistant (MICs 32 - >128 µg/ml). One of these *Clostridium* species was isolated from a stomach infection and a *C. glycolicum* was identified from septic thigh muscle tissue. Decreased activity to cefoxitin was noted for all *Bacteroides* and *Fusobacterium* species (except *F. nucleatum*), susceptibility ranging from 57- 68%; with strains of *Prevotella, Porphyromonas* and *Veillonella* tending to be more susceptible (82-100%). Of all the anaerobes investigated, 91% were susceptible to cefoxitin, which is similar to a report from the USA by Spangler, Jacobs & Appelbaum (1994) in which 90% of all the isolates tested were susceptible. High-level cefoxitin resistance (MIC >128 µg/ml) was seen for a non-ß-lactamase producing *C. glycolicum* strain isolated during the present study. This strain is discussed further in Chapter 4. In 1997, in two separate studies conducted in the USA, decreased susceptibility to cefoxitin among *Clostridium* spp. was also found; 21% resistance in Louisiana, USA (MIC range 0.25 - 64 µg/ml) and 9% resistance in California (MIC range 0.5 - 64 µg/ml) (Aldridge & Johnson, 1997; Citron & Appleman, 1997).

With the exception of ten non-perfringens *Clostridium* isolates, the Gram-positive anaerobic bacteria were susceptible to cefepime and cefpirome. This was, however, not the case for the Gram-negative bacteria, in particular the *Bacteroides* spp. with susceptibility to cefepime < 50% and to cefpirome < 30%. *Prevotella* spp. susceptibility to cefpirome ranged from 30-67% and to cefepime from 55-78%. In Japan by 1993, resistance to cefepime and cefpirome was increasing as < 50% of *B. fragilis* isolates were found to be susceptible to both cephalosporins (Mine *et al.*, 1993).

The majority of Gram-positive anaerobic strains investigated were susceptible to imipenem/meropenem. This was similar to findings of other investigators in the UK, USA and Sweden (Sheikh, Pitkin & Nadler, 1993; Bowker *et al.*, 1996; Nord 1996). High-level resistance to imipenem/meropenem (MIC >128 µg/ml) was seen in nine *F. mortiferum* (from stomach perforations, peri-anal abscesses, peripheral oedema and abscesses unknown), two *F. necrophorum* (from open bone fracture wounds) and two *F. varium* isolates (from pus and tissue samples from septicaemic patients). A corresponding resistance of fusobacteria against imipenem was also observed in three studies performed in the USA in 1981, 1983 and 1985, with MICs as high as

256 µg/ml (Rolfe & Finegold, 1981; Owens & Finegold, 1983; Wexler & Finegold, 1985). *F. mortiferum* and *F. varium* are not encountered commonly but do pose a problem, as they have been seen to be relatively resistant to many antimicrobial agents, especially ß-lactams (Finegold, 1995). According to Tunér & Nord (1993) only 50% *F. varium* and 75% *F. mortiferum* strains in Europe were susceptible to ß-lactam antibiotics. As early as 1985 Wexler & Finegold (1985) found that 23% of the *F. mortiferum* and *F. varium* strains isolated in Los Angeles, California were highly resistant (MICs >128 µg/ml) to imipenem. The fusobacteria exhibiting high-level carbapenem resistance were investigated further (Chapter 4).

Three *C. perfringens* strains (two of which were isolated from septic bone fractures and one from the peritoneum) showed metronidazole MICs 4-8 µg/ml, being within two dilutions of the susceptibility breakpoint (MIC 16 µg/ml). Although reports from the UK in 1996 and the USA in 1997/1998 only mentioned reduced metronidazole susceptibility in *C. perfringens* strains with MICs ranging from 2 µg/ml to 4 µg/ml (Bowker *et al.*, 1996; Citron & Appleman, 1997; Ednie, Jacobs & Appelbaum, 1998), Spangler, Jacobs & Appelbaum (1994) in 1994 had reported strains of *C. perfringens* from the USA with metronidazole MICs 8 µg/ml. Two *P. magnus* isolates (one from a tissue biopsy from the foot and the other from an unknown origin) were resistant to metronidazole (MIC >128 µg/ml). In 1993 in the USA, metronidazole-resistant *P. micros* strains (MIC >128 µg/ml) were described and in 1994, 20% of *Peptostreptococcus* spp. isolated in Pennsylvania and Ohio, USA were found to be resistant to metronidazole (Sheikh, Pitkin & Nadler, 1993; Spangler, Jacobs & Appelbaum, 1994). In a further study undertaken by Spangler, Jacobs & Appelbaum (1997) a *P. magnus* strain was also described as metronidazole-resistant. In 1997, a study based in California, USA, found 30% *Peptostreptococcus* spp. were resistant to metronidazole (Citron & Appleman, 1997). However, in 1998, in a study comparing activities of new fluoroquinolones with older antibiotics in Pennsylvania and Ohio, USA, no resistance to metronidazole was found in the peptostreptococci investigated (Ednie, Jacobs & Appelbaum, 1998). In studies conducted in the UK in 1996, metronidazole resistance was not evident (Bowker *et al.*, 1996).

Two *Prevotella* strains, a *P. asaccharolytica* (isolated from a knee abscess) and a *P. melaninogenica* (isolated from a female genital tract infection) were resistant to metronidazole (MIC 32 µg/ml). Similar decreased susceptibility was found for

Prevotella spp. in the USA by Spangler, Jacobs & Appelbaum (1994) and Ednie, Jacobs & Appelbaum (1998) with metronidazole MICs 8-16 µg/ml being recorded. However, other investigations from Sweden in 1993, the UK in 1996 and the USA in 1996/1997 have shown overall susceptibility of *Prevotella* spp. to metronidazole (Nord, 1993; Bowker *et al.*, 1996; Wexler *et al.*, 1996; Aldridge, Ashcraft & Bowman, 1997; Citron & Appleman, 1997). Metronidazole resistance, especially among *Peptostreptococcus* and *Bacteroides/Prevotella* species appears to vary both regionally and temporally making it important that metronidazole resistance development/acquisition be regularly monitored (see Chapter 5).

Of all the anaerobes investigated 85% were susceptible to clindamycin. There was no apparent difference between Gram-positive Gram-negative anaerobic bacteria, but the highest level of resistance was observed in the *Bacteroides* spp. other than *B. fragilis* (43%). In two studies done in the USA in 1997, similar results were found, with susceptibility of *B. fragilis* strains 85% and 90% respectively, while for strains of *Bacteroides* other than *B. fragilis*, susceptibility ranged from 20-60% and 57-100% (Citron & Appleman, 1997; Aldridge & Johnson, 1997). In the same two studies it was found that the Gram-positive anaerobic bacteria were generally susceptible to clindamycin, 86-100%.

Dalfopristin/quinupristin (dalf/quin) exhibited excellent activity throughout the Gram-positive bacterial spectrum investigated, with only one *Peptostreptococcus* sp., isolated from a lung abscess, showing reduced susceptibility (MIC 8 µg/ml). Poor activity was found against the Gram-negative bacteria, with susceptibility ranging from 35% for *B. fragilis* group isolates, to 39% for *P. loescheii*, with only 3% of the *Veillonella* spp. susceptible. In 1993 in the USA, Appelbaum, Spangler & Jacobs (1993) found one strain of *C. innocuum* resistant to dalf/quin, but in contrast to the current study, they found all Gram-negative anaerobes to be susceptible. Baquero *et al.* (1993) found in a collaborative European study conducted in 1993 that 97.6 % *Clostridium* spp. were susceptible to dalf/quin, but reported a rather high MIC_{90} (32 µg/ml) for the *B. fragilis* group of 32 µg/ml. In 1997 in France, Bouanchaud (1997) described all *C. perfringens* isolates as being susceptible and *Bacteroides* spp. with decreased susceptibility (MICs 4-8 µg/ml), while Betriu *et al.* (1999) reported from Spain, on isolates collected in 1997, that all *B. fragilis* strains were susceptible to dalf/quin (MIC $\leq$ 2 µg/ml). Koch, Derby & Abratt (1998) reported 12%

of *Peptostreptococcus* species dalf/quin-resistant in a study done in South Africa in 1998. On average dalf/quin performed very well against the Gram-positive anaerobes in the present study, with comparable or even better activity against *Clostridium* species than clindamycin and/or imipenem.

With the exception of two *P. anaerobius* strains (MICs 8 µg/ml), trovafloxacin was effective against all the Gram-positive anaerobic bacteria examined. Hecht & Wexler (1996) in the USA found that the trovafloxacin MIC_{90} values for *Peptostreptococcus* spp. ranged from ranging from 0.25 to 0.5 µg/ml. In the same year Wexler *et al.*, (1996) in the USA reported that MICs of trovafloxacin were 4 µg/ml for 3% of peptostreptococcal isolates and 4-8 µg/ml for 7% *Clostridium* spp. In three other studies conducted in the USA in 1994, 1997 and 1998 resistance was also detected among the *Peptostreptococcus* and *Clostridium* species (Spangler, Jacobs & Appelbaum, 1994; Citron & Appleman, 1997; Ednie, Jacobs & Appelbaum, 1998). Trovafloxacin MICs of 8 µg/ml were also found in *C. perfringens* by Bowker *et al.* (1996) in 1996 in the UK, but in this study all *Peptostreptococcus* spp. were susceptible. In the present study, trovafloxacin demonstrated superior activity to ciprofloxacin against the Gram-negative isolates examined with only three *B. fragilis* (from urethra, tubo-ovarian-appendix complex and a gut resection), one *P. loescheii* (isolated from a stomach infection) and a *F. varium* (from a lower leg tissue specimen) showing MICs of trovafloxacin above the susceptibility breakpoint (≤ 2 µg/ml). Several other comparisons from the USA have also shown trovafloxacin to exhibit far better activity against the Gram-negative anaerobes (MICs ≤ 4 µg/ml) than ciprofloxacin, for which MICs of 64 - > 128 µg/ml were often found (Spangler, Jacobs & Appelbaum, 1994; Bowker *et al.*, 1996; Wexler *et al.*, 1996; Citron & Appleman, 1997; Ednie, Jacobs & Appelbaum, 1998). However, in 1997 in the USA, Citron & Appleman (1997) found decreased susceptibility to trovafloxacin among *Fusobacterium* spp. (MICs 4 µg/ml) and in 1998 in the USA Ednie, Jacobs & Appelbaum (1998), found one *B. thetaiotaomicron* (MIC 32 µg/ml), one *F. varium* (MIC 16 µg/ml) and *Prevotella* spp. (MIC 4 µg/ml) to be resistant to trovafloxacin. Trovafloxacin is, however, currently withdrawn from general clinical use due to adverse liver effects.

Chloramphenicol was the most effective antibiotic tested, with only two *Clostridium* spp. seen to be resistant (MICs 16 µg/ml). Excellent activity of chloramphenicol

against anaerobic bacteria has been generally reported throughout the world (Patey *et al.*, 1994; García-Rodríguez, García-Sánchez & Muñoz-Bellido, 1995; Wexler *et al.*, 1996; Rasmussen, Bush & Tally, 1997), which may reflect its infrequent use due to side-effects such as aplastic anaemia and bone marrow suppression.

Current knowledge of antibiotic resistance progression in anaerobic bacteria isolated in South Africa is limited, yet they can no longer be considered universally susceptible to antimicrobial agents. The present study has emphasised the requirement of periodic susceptibility testing of anaerobic bacteria in conjunction with speciation. For the treatment of mixed aerobe/anaerobe infections the current trend is towards monotherapy (Wexler, Molitoris & Finegold, 1993; Aldridge, 1995). The results indicate that antimicrobial agents such as the carbapenems (imipenem/ meropenem) and quinolones are of value for the treatment of polymicrobial infections. Despite reduced efficacy of dalf/quin against some Gram-negative isolates (*Bacteroides* spp., *Prevotella loescheii*, *F. mortiferum*, *F. varium* and *Veillonella* spp.), dalf/quin proved to be excellent against the Gram-positive anaerobic bacteria and could be considered for therapy in mixed aerobe/anaerobe Gram-positive bacterial infections.

Certainly of major clinical importance in the South African setting are the anaerobic bacteria isolated from normally sterile sites, especially the brain, liver, lungs and blood. Forty trauma related injuries were noted with a variety of anaerobes identified from gunshot and stab wounds as well as bone and fracture-related infections. Current therapeutic regimens for central nervous system infections include chloramphenicol, all the strains isolated from brain abscesses being susceptible to chloramphenicol. For infections of unknown aetiology, empirical therapy in Bloemfontein state hospitals would be clindamycin or cefoxitin or amoxicillin/ clavulanic acid.

To maintain effective empiric therapeutic regimens against anaerobic infections, it is imperative to monitor susceptibility data as antimicrobial resistance is constantly emerging and proactive investigation of the potential of new anti-anaerobic agents is mandatory. In the present study, as in a previous study conducted in Bloemfontein, anaerobes that were identified as requiring specific diagnostic attention regarding susceptibility testing were the non-perfringens clostridia and peptostreptococci

(Chalkley, Krüger & Botha, 1997). Additionally the current findings also recognise *Veillonella* and fusobacteria as being problem genera concerning resistance to ß-lactam/carbapenem antimicrobial agents.

CHAPTER 4

ß-LACTAM/CARBAPENEM RESISTANCE

4.1 INTRODUCTION

ß-Lactam antibiotics (penicillins/cephalosporins) and the carbapenems are frequently used and have for many years been the first choice in the treatment and prophylaxis of anaerobic and mixed aerobic/anaerobic infections. The majority of anaerobic bacteria have remained susceptible to this group of antibiotics, but during the last several years reports have indicated an increasing number of anaerobic bacteria that are becoming resistant (Nord & Hedberg, 1990). Susceptibility to these agents, however, varies depending on the specific structure of the antibiotic and the types of resistance mechanisms that have been developed or acquired (Johnson, 1993). Although ß-lactam resistance may be mediated by three distinct mechanisms, the two of major importance contributing to resistance are 1) the production of ß-lactamases and 2) alteration of the number and/or type of penicillin-binding proteins (PBPs) (Rasmussen, Bush & Tally, 1993).

Penicillin is still recommended for the treatment of the majority of Gram-positive anaerobic infections, but is ineffective against Gram-negative anaerobes that produce ß-lactamases, especially *Bacteroides* spp. Piperacillin has maintained efficacy, although it is inactivated by chromosomal class A ß-lactamases produced by anaerobes, particularly those of the *B. fragilis* group (Livermore, 1995; Rasmussen, Bush & Tally, 1997). For this reason and in order to enhance the spectrum of activity, piperacillin is invariably given in combination with a ß-lactamase inhibitor, tazobactam (Jacobs *et al.*, 1993).

Many ß-lactamases from the *B. fragilis* group can be inhibited by the classical ß-lactamase inhibitors (Rasmussen, Bush & Tally, 1997). Several of these group 2e cephalosporinases have now been sequenced and confirmed to belong to the

molecular class A serine cephalosporinases (Rasmussen, Bush & Tally, 1997). These chromosomal class A ß-lactamases are virtually ubiquitous in strains of the *B. fragilis* group (Livermore, 1995). *B. fragilis* can, however, also produce class B ß-lactamases group 3 that are capable of inactivating cefoxitin and imipenem (Nord & Hedberg, 1990; Khushi *et al.,* 1996; Rasmussen, Bush & Tally, 1997). The non-*fragilis* species of *Bacteroides* produce ß-lactamases that are mainly penicillinases (Nord & Hedberg, 1990). The ß-lactamases of *Bacteroides* spp. or *Fusobacterium* spp., contrary to *Clostridium* spp., are seldom inducible (Nord & Hedberg, 1990; Rasmussen, Bush & Tally, 1997). *Fusobacterium* spp. and *Clostridium* spp. produce penicillinases rather than cephalosporinases that exhibit the characteristics of enzymes belonging to molecular class D ß-lactamases, the group 2d cloxacillin-hydrolysing enzymes (Nord & Hedberg, 1990; Rasmussen, Bush & Tally, 1997). A ß-lactamase from *F. nucleatum* has been classified as group 2a (Nord & Hedberg, 1990). In *Prevotella intermedia* a ß-lactamase was characterised as broad-spectrum with greater activity against cephalosporins than penicillins, but because of its unusual substrate profile did not readily conform to any of the Bush classifications (Bush, 1989; Walker & Bueno, 1997).

The carbapenems have excellent antimicrobial activity against a broad range of bacterial species, yet increasing resistance to imipenem in particular was reported in 1992 by Bandoh *et al.* (1992). In 1994, *B. fragilis* isolates exhibiting slightly elevated imipenem MICs, but still considered susceptible, were described in various locations worldwide (Rasmussen *et al.*, 1994). Carbapenems differ from penicillins and cephalosporins in having a carbon atom at position 1 of the bicyclic ring and a double bond between positions 2 and 3. Such a structure serves to extend the antibacterial spectrum and enhances the stability of the ß-lactam bond to hydrolysis by ß-lactamases (Yang, Bhachech & Bush, 1995). High activity of imipenem against *B. fragilis* is associated with ß-lactamase stability, ability to penetrate the cell, and high affinity for PBPs (Edwards & Greenwood, 1996). Until recently resistance to imipenem in anaerobes other than *B. fragilis*, occurred infrequently, if at all (Johnson, 1993). However, Wexler & Finegold (1985) noted imipenem resistance in some strains of *Fusobacterium*. Reduced affinity for PBPs or permeability barriers are more likely reasons for imipenem resistance in *B. fragilis*, but neither altered PBPs nor OMPs have been identified as contributing factors (Edwards & Greenwood, 1996). Resistance of the *B. fragilis* group to carbapenems has been attributed to two

metallo-ß-lactamases (Hurlbut, Cuchural & Tally, 1990). Fortunately high-level resistance to imipenem in *B. fragilis* is still rare as production of the metallo-ß-lactamases in these anaerobes usually give rise to MICs that range from 2-4 µg/ml (Edwards & Greenwood, 1996). The metallo-ß-lactamases belong to the class B ß-lactamase group (Osano *et al.*, 1994), having a primary structure quite different from those of the class A and class C serine enzymes in that they possess ZN^{++} at the active site (Osano *et al.*, 1994; Bush, 1998). It has been suggested that metallo-ß-lactamases were present before the widespread use of carbapenems (Khushi *et al.*, 1996). In situations where metallo-ß-lactamase producing anaerobic bacteria are present, the *in vivo* efficacy of ß-lactam antibiotics against other co-infecting bacteria is also decreased (Ajiki *et al.*, 1991). This raises the question as to why therapy of mixed bacterial infections, that include these highly resistant ß-lactamase-producing bacteria, should consist of antibiotics that are stable to hydrolysis (Ajiki *et al.*, 1991). The exact mechanism of ß-lactam hydrolysis by metallo-ß-lactamases remains only partially understood, and structural studies are required to further clarify their activity (Senda *et al.*, 1996).

The metallo-ß-lactamase of *B. fragilis* lies on a relatively small plasmid of 13.6 kb that appears to be self-transmissible (Rasmussen & Bush, 1997). In clinical isolates of *B. fragilis* the CfiA-type metallo-ß-lactamase appears to be the most common (Khushi *et al.*, 1996). These metallo-ß-lactamases have a broader substrate profile than any serine ß-lactamase and are able to hydrolyse penicillins, cephalosporins, and carbapenems (Khushi *et al.*, 1996). An even more important aspect is that they are not inhibited by any commercially available ß-lactamase inhibitors (Khushi *et al.*, 1996). Hybridisation analysis has indicated the presence of *cfiA* genes in 2.2% of *B. fragilis* clinical isolates of which the majority are not actively expressed, rendering these *B. fragilis* isolates silent "time bombs" from which carbapenem-resistant isolates may emerge (Rasmussen *et al.*, 1994).

The *cphA* gene has been described to encode a CphA metallo-ß-lactamase that is classified under the molecular class B metallo-ß-lactamases (Rossolini, Walsh & Amicosante, 1996; Villadares *et al.*, 1996). This enzyme also shares a degree of similarity to the CfiA, a class A metallo-ß-lactamase of *B. fragilis* (Massidda, Rossolini & Satta, 1991). The substrate specificity of the CphA enzyme is extremely narrow with carbapenems, and to a lesser extent ampicillin, being effectively hydrolysed

(Rossolini, Walsh & Amicosante, 1996). Current findings also suggest this as only slightly elevated carbapenem MICs (4 µg/ml) have been noted, with no remarkable decrease in susceptibility to other ß-lactam agents, in anaerobes possessing *cphA* genes (Rasmussen *et al.*, 1994; Rossolini, Walsh & Amicosante, 1996; Bush, 1998).

The bla_{IMP} gene encodes a metallo-ß-lactamase IMP-1 that is often present in clinical isolates of *Pseudomonas aeruginosa* and *Serratia marcescens* and that exhibit low-level carbapenem resistance (MIC ≤ 4 µg/ml) (Osano *et al.*, 1994; Senda *et al.*, 1996; Laraki *et al.*, 1999). Although the bla_{IMP} gene has not been described in anaerobic bacteria, it is known to be the most clinically threatening of the metallo-ß-lactamase genes as it is capable of rapid dissemination (Laraki *et al.* 1999).

PBPs are membrane-bound enzymes that are required for the biosynthesis of the bacterial cell wall. They catalyse the final steps of the polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan, an essential component of the bacterial cell wall (Zhao *et al.*, 1999). PBPs are members of the superfamily of penicilloyl serine transferases and are targets of ß-lactam antibiotics in that they interact with ß-lactam antibiotics via the formation of a (covalent) serine ester-linked penicilloyl complex (Hakenbeck *et al.*, 1994; Zhao *et al.*, 1999). The low-molecular-weight PBPs (carboxypeptidases) have been generally found and described as non-essential for maintaining cell wall integrity and in strains that develop resistance to penicillin these PBPs bind penicillin to the same extent as in susceptible strains (Bryan & Godfrey, 1991; Spratt, 1994). The high-molecular-weight PBPs have transpeptidase or transpeptidase/carboxypeptidase activity (Bryan & Godfrey, 1991). They tend to possess a lower affinity for penicillin than low-molecular-weight PBPs, even in susceptible isolates and are essential for maintaining cell wall integrity and preventing lysis/cell death (Spratt, 1977). In strains that have developed resistance, one or more of these high-molecular-weight PBPs are seen to alter their binding affinity for penicillin (Spratt, 1994).

In *B. fragilis* four PBPs have been described: PBP1 has been shown to exhibit the lowest affinity for ß-lactam antibiotics and primarily binds to imipenem; PBP2 has the highest affinity for ß-lactam antibiotics; PBP3 exhibits intermediate affinity for ß-lactam antibiotics; PBP4 binds primarily clavulanic acid and imipenem (Botta, Privitera & Menozzi, 1983; Georgopapadakou, Smith & Sykes, 1983; Nord &

Hedberg, 1990). In strains of *B. fragilis* with reduced susceptibility to imipenem (not attributed to metallo-ß-lactamase activity), Edwards & Greenwood (1996) found a low-molecular-weight PBP of 40 kDa that was not present in susceptible strains. Furthermore, in a competition study, imipenem was able to displace benzylpenicillin from proteins of molecular weight 60 to 100 kDa. This led to the assumption that in addition to the presence of a 40kDa PBP, reduced affinity of imipenem for PBPs of high-molecular-weight may also play a part in resistance development (Edwards & Greenwood, 1996).

PBPs were seen to be species specific (Tunér *et al.*, 1996) when PBPs of *F. nucleatum*, *F. mortiferum*, *F. varium* and *F. necrophorum* were compared. Most species had 5-6 PBPs (40-100 kDa) with cefotaxime showing greatest affinity for PBPs 1 and 4 and aztreonam for PBP4 (Tunér *et al.*, 1996).

In *C. perfringens* six PBPs have been demonstrated, these being similar in size to those found in aerobic bacteria (Nord, 1986). Resistance to penicillin in *C. perfringens* has been shown to be mediated by a decreased affinity of the largest PBP, i.e. PBP1 (Williamson, 1983). PBPs 2b and 4b were seen to show similar affinity characteristics to PBP1, although increasing resistance could not be correlated with alterations to these PBPs (Chalkley & Van der Westhuyzen, 1993). The low-molecular-weight PBPs 5 and 6 (carboxypeptidases) were seen to exhibit the highest affinity for penicillin (Williamson, 1983; Chalkley & Van der Westhuyzen, 1993). PBP profiling may be used as a control in validifying the identification of *Clostridium* species as PBPs 5 and 6 of *C. perfringens* were seen to be species specific (Chalkley & Van der Westhuyzen, 1993). Chalkley & Sooka (1994) found that in a *P. anaerobius* isolate, cefoxitin and imipenem bound efficiently to PBP 2, and there was also strong binding of imipenem to PBP 1. Imipenem was also found to bind more effectively than cefoxitin to all four PBPs demonstrated in a *P. magnus* strain (Chalkley & Sooka, 1994).

On analysing ß-lactam/carbapenem resistance (susceptibility data Chapter 3) the study focused on four major areas: 1) the possible role of metallo-ß-lactamases in decreased susceptibility to imipenem in *Bacteroides* spp., *Prevotella* spp. and *Fusobacterium* spp., 2) high-level imipenem resistance in *Fusobacterium* species, 3) PBP alterations in clostridia and 4) piperacillin resistance in *Veillonella* species.

4.2 RESULTS AND DISCUSSION

4.2.1 Investigations into metallo-ß-lactamase genes in Bacteroides spp., Prevotella spp. and Fusobacterium spp.

A bioassay was performed to detect carbapenem inactivating ß-lactamases or metallo-ß-lactamases from eight strains with imipenem MICs 0.5-4 µg/ml (Table 4.1). As seen in Table 4.1 three distinct trends in zone sizes were evident for the six *Bacteroides* species and two *P. loescheii* strains investigated. Three *Bacteroides* isolates, *B. vulgatus* B39 (imipenem MIC 0.5 µg/ml), *B. vulgatus* B64 (imipenem MIC 1 µg/ml), and *B. capillosus* B38 (imipenem MIC 2 µg/ml), did not show any noticeable change in zone size between the control imipenem, the crude cell extract + imipenem or the crude cell extract + imipenem + EDTA. EDTA is a metal ion chelator and reduction of zone size in the presence of EDTA would indicate the presence of metallo-ß-lactamase. For *B. fragilis* B82 (imipenem MIC 0.5 µg/ml), *B. fragilis* B97 (imipenem MIC 1 µg/ml), *P. loescheii* B71 (imipenem MIC 2 µg/ml), and *P. loescheii* B54 (imipenem MIC 4 µg/ml) no zone was seen around the crude cell extract + imipenem and a small zone was detected around the crude cell extract + EDTA, indicating partial inhibition of a possible metallo-ß-lactamase. A *B. fragilis* strain B121 (imipenem MIC 4 µg/ml) had a notably smaller zone around the crude cell extract plus imipenem than the control imipenem zone, but there was no increase in zone size around the well to which EDTA was added. Even though the full characteristics of a metallo-ß-lactamase were not evident from the bioassay, a carbapenem hydrolysing enzyme (low levels) appeared to be present in two *B. fragilis* and two *P. loescheii* strains.

No noteworthy change in zone size could be demonstrated by the bioassay with ten *Fusobacterium* strains (imipenem MICs 16 - > 128 µg/ml), indicating a total absence of enzymatic action against imipenem.

Table 4.1 **Bioassay results performed on eight *Bacteroides/ Prevotella* spp. strains with varying susceptibilities to imipenem.**
The imipenem control zone size was 25.4 mm (no enzyme preparation added).

Crude enzyme preparation	Zone size (mm)		Imipenem
	No EDTA	With EDTA	MIC (µg/ml)
B39 *B. vulgatus*	24.8	24.8	0.5
B64 *B. vulgatus*	26.0	26.0	1
B38 *B. capillosus*	25.1	25.1	2
B71 *P. loescheii*	0	17.1	2
B54 *P. loescheii*	0	19.6	4
B82 *B. fragilis*	0	18.0	0.5
B97 *B. fragilis*	0	14.7	1
B121 *B. fragilis*	13.9	15.5	4

PCR amplification with *cfiA* gene primers produced a fragment of predicted size (± 747 bp) in two strains of *B. vulgatus,* B39 and B64 (imipenem MIC 0.5 and 1 µg/ml), a *B. capillosus* B38 strain (imipenem MIC 2 µg/ml) and a *P. loescheii* strain B71 with an imipenem MIC 2 µg/ml (Figures 4.1 and 4.2). Sequences obtained for these fragments, however, did not correspond to that of the documented *cfiA* gene sequence. Only the *P. loescheii* strain (B71) gave some indication of carbapenemase activity in the bioassay (Table 4.1). PCR amplification with *cphA* gene primers produced a fragment of predicted size (± 764 bp) in three *B. fragilis* strains B82, B97 and B121 (imipenem MICs 0.5, 1 and 4 µg/ml) and a *P. loescheii* strain B54 (imipenem MIC 4 µg/ml) (Figures 4.3 and 4.4). Again, sequencing did not correspond to the expected sequence of the *cphA* gene. The *P. loescheii* strain (B54) and two of the *B. fragilis* strains (B82 and B97) were seen to exhibit metallo-ß-lactamase activity, while the third *B. fragilis* strain (B121) showed carbapenemase activity, but no metallo-ß-lactamase activity on the bioassay plates (Table 4.1). None of the *Fusobacterium* isolates produced amplification products to suggest the presence of the *cfiA* gene or the *cphA* gene (Figures 4.1 and 4.3). Universal primers were employed to ensure that strains had been adequately lysed for subsequent PCR *cfiA* and *cphA* amplification (Figure A1, Appendix).

All the anaerobic isolates that were screened for *cfiA* and *cphA* genes were also subjected to bla_{IMP} gene amplification. No PCR products of predicted bla_{IMP} size (±587 bp) were found in any of the *Bacteroides, Prevotella* or *Fusobacterium* strains investigated (Figure 4.5).

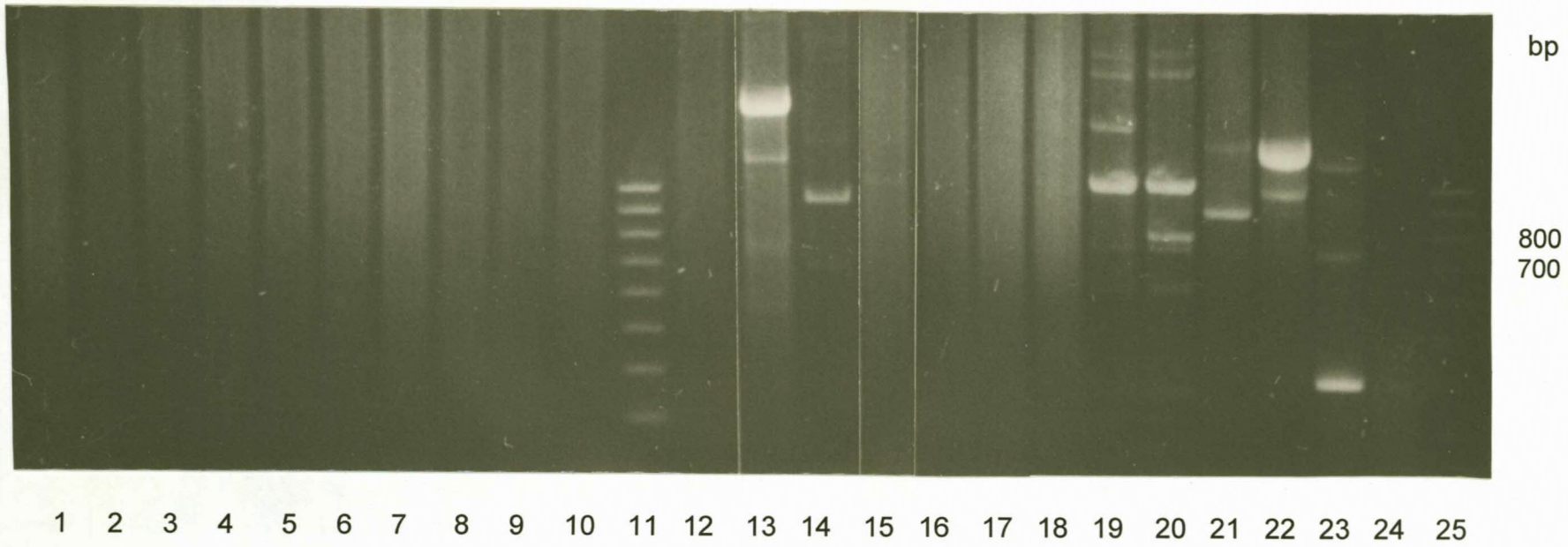


Figure 4.1: PCR products of the 23 isolates tested for the presence of the *cfiA* gene (±747 bp). MICs (µg/ml) of imipenem (IMP) and meropenem (MER) respectively are given in brackets. Lanes: **1**, B30 *F. mortiferum* (16, 16); **2**, B51 *F. mortiferum* (64, 64); **3**, F19 *F. mortiferum* (>128, >128); **4**, F23 *F. mortiferum* (>128, >128); **5**, F28 *F. mortiferum* (>128, >128); **6**, F35 *F. necrophorum* (>128, >128); **7**, F39 *F. varium* (>128, >128); **8**, F44 *F. varium* (>128, >128); **9**, F47 *F. mortiferum* (>128, >128); **10**, F49 *F. mortiferum* (64, >128); **11**, MWM; **12**, B38 *B. capillosus* (2, 2); **13**, B64 *B. vulgatus* (1, 0.25); **14**, B70 *B. vulgatus* (1, 1); **15**, B121 *B. fragilis* (4, 4); **16**, B6 *P. bivia* (2, 4); **17**, B12 *P. loescheii* (1, 2); **18**, B54 *P. loescheii* (4, 4); **19**, B71 *P. loescheii* (2, 4); **20**, B39 *B. vulgatus* (0.5, 0.5); **21**, B120 *B. fragilis* (0.5, 0.25); **22**, B124 *B. vulgatus* (0.5, 0.25); **23**, B128 *B. fragilis* (0.5, 0.25); **24**, B131 *B. fragilis* (0.5, 0.5); **25**, MWM. The PCR products from strains *B. capillosus* B38 (lane 12); *B. vulgatus* B64 (lane 13); *P. loescheii* B71 (lane19) and *B. vulgatus* B39 (lane 20) were selected for sequencing.

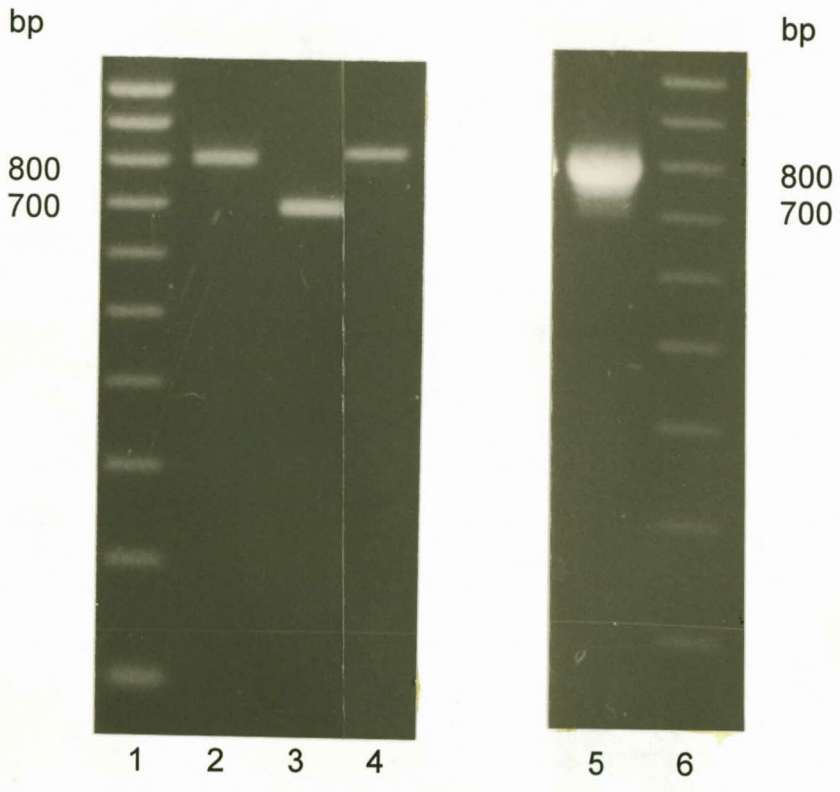


Figure 4.2: DNA purification of fragments with predicted size 747 bp (from Fig. 4.1). MICs (µg/ml) of imipenem and meropenem respectively are given in brackets. Lanes: **1**, MWM; **2**, B38 *B. capillosus* (2, 2); **3**, B39 *B. vulgatus* (0.5, 0.5); **4**, B71 *P. loescheii* (2, 4); **5**, B64 *B. vulgatus* (1, 0.5); **6**, MWM.

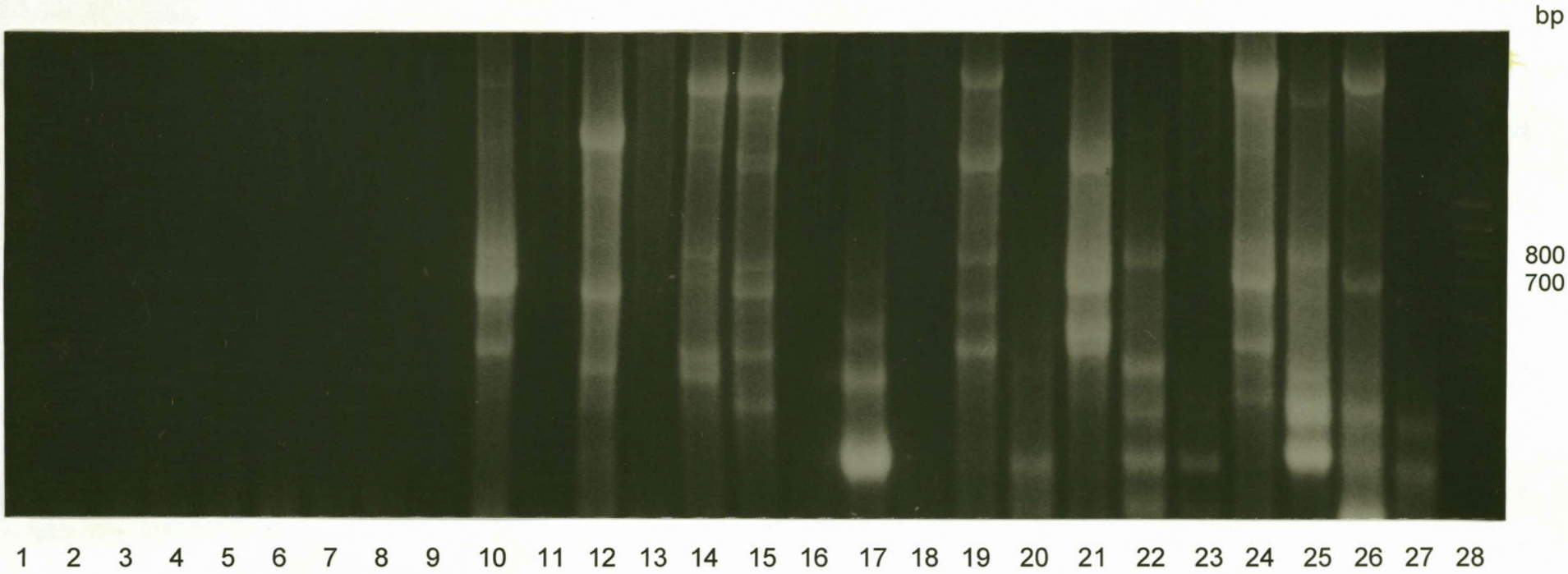


Figure 4.3: PCR products of the 27 isolates tested for the presence of the *cphA* gene (±764 bp). MICs (µg/ml) of imipenem (IMP) and meropenem (MER) respectively are given in brackets. Lanes: **1**, B51 *F. mortiferum* (64, 64); **2**, F19 *F. mortiferum* (>128, >128); **3**, F23 *F. mortiferum* (>128, >128); **4**, F28 *F. mortiferum* (>128, >128); **5**, F35 *F. necrophorum* (>128, >128); **6**, F39 *F. varium* (>128, >128); **7**, F44 *F. varium* (>128, >128); **8**, F47 *F. mortiferum* (>128, >128); **9**, F49 *F. mortiferum* (64, >128); **10**, B6 *P. bivia* (2, 4); **11**, B12 *P. loescheii* (1, 2); **12**, B38 *B. capillosus* (2, 2); **13**, B39 *B. vulgatus* (0.5, 0.5); **14**, B53 *B. fragilis* (2, 4); **15**, B54 *P. loescheii* (4, 4); **16**, B64 *B. vulgatus* (1, 0.25); **17**, B70 *B. vulgatus* (1, 1); **18**, B71 *P. loescheii* (2, 4); **19**, B82 *B. fragilis* (0.5, 4); **20**, B90 *B. fragilis* (0.5, 0.5); **21**, B97 *B. fragilis* (1, 4); **22**, B110 *B. fragilis* (0.5, 0.5); **23**, B120 *B. fragilis* (0.5, 0.25); **24**, B121 *B. fragilis* (4, 4); **25**, B124 *B. vulgatus* (0.5, 0.25); **26**, B128 *B. fragilis* (0.5, 0.25); **27**, B131 *B. fragilis* (0.5, 0.5); **28**, MWM. The PCR products from strains *P. loescheii* B54 (lane 15); *B. fragilis* B82 (lane 19); *B. fragilis* B97 (lane 21) and *B. fragilis* B121 (lane 24) were selected for sequencing.

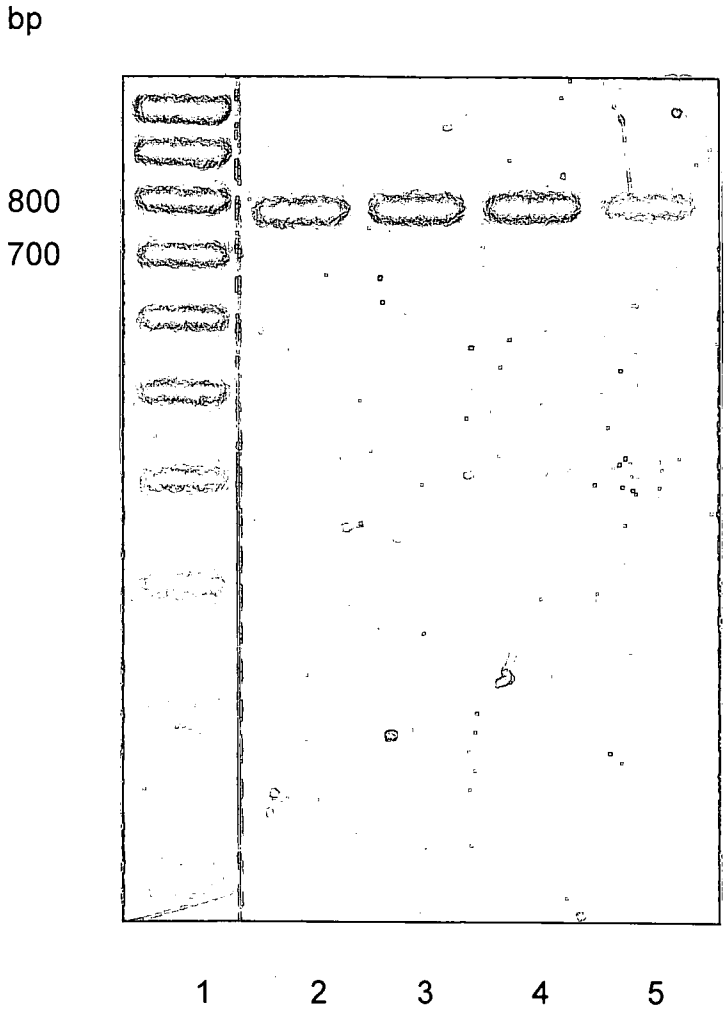


Figure 4.4: DNA purification of fragments with predicted size 764 bp (from Fig. 4.3). MICs (µg/ml) of imipenem and meropenem respectively are given in brackets. Lanes: **1**, MWM; **2**, B54 *P. loescheii* (4, 4); **3**, B82 *B. fragilis* (0.5, 4); **4**, B97 *B. fragilis* (1, 4); **5**, B121 *B. fragilis* (4, 4).

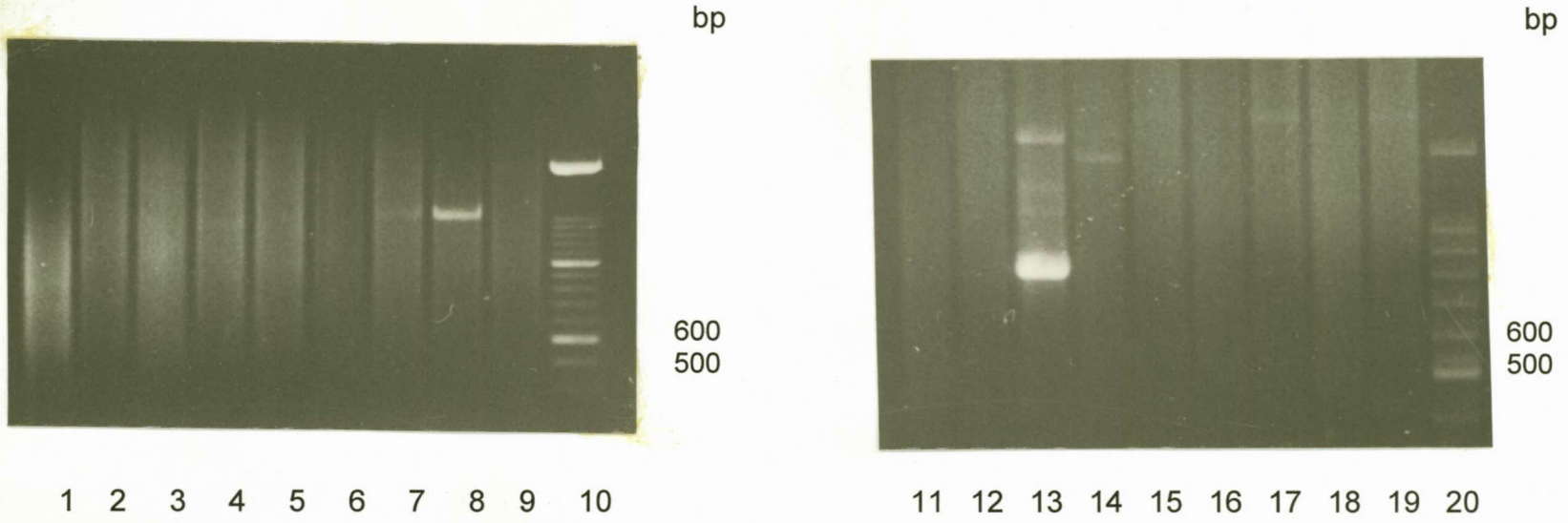


Figure 4.5: PCR products of the 18 isolates tested for the presence of the *bla*$_{IMP}$ gene (587 bp). MICs (µg/ml) of imipenem and meropenem respectively are given in brackets. Lanes: **1**, B51 *F. mortiferum* (64, 64); **2**, F19 *F. mortiferum* (>128, >128); **3**, F23 *F. mortiferum* (>128, >128); **4**, F28 *F. mortiferum* (>128, >128); **5**, F35 *F. necrophorum* (>128, >128); **6**, F39 *F. varium* (>128, >128); **7**, F44 *F. varium* (>128, >128); **8**, F47 *F. mortiferum* (>128, >128); **9**, F49 *F. mortiferum* (64, >128); **10**, MWM; **11**, B38 *B. capillosus* (2, 2); **12**, B53 *B. fragilis* (2, 4); **13**, B64 *B. vulgatus* (1, 0.25); **14**, B70 *B. vulgatus* (1, 1); **15**, B82 *B. fragilis* (0.5, 4); **16**, B90 *B. fragilis* (0.5, 0.5); **17**, B97 *B. fragilis* (1, 4); **18**, B110 *B. fragilis* (0.5, 0.5); **19**, B121 *B. fragilis* (4, 4); **20**, MWM.

Although screening for *cfiA, cphA* and bla_{IMP} genes in selected anaerobic bacteria failed to reveal definite results, early recognition of metallo-ß-lactamase-producing strains is imperative. Clinical strains susceptible to the vast majority of ß-lactams can convert to high-level ß-lactam resistance, including resistance to the carbapenems, by simply increasing the level of expression of "nearly" silent metallo-ß-lactamase genes. This effect has been observed in the laboratory in a one-step mutational event with clinical *B. fragilis* isolates, which harboured a metallo-ß-lactamase gene, but only expressed very low or undetectable levels of the enzyme (Rasmussen *et al.*, 1994). Although it remains to be seen whether indiscriminate use of carbapenems, will result in an increase in the prevalence of strains producing carbapenemase/ metallo-ß-lactamases, a more detailed understanding of the mechanism of action of these enzymes is important for effective future drug design.

4.2.2 Imipenem resistance in Fusobacterium spp.

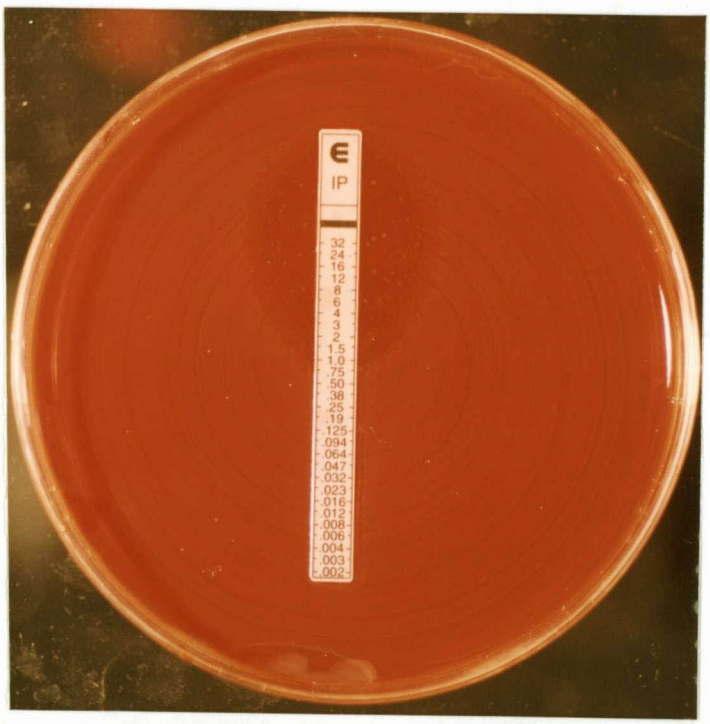
ß-Lactam and carbapenem MIC data for 13/37 fusobacteria (35%) that were resistant to imipenem (MICs $\geq$ 16 µg/ml, Chapter 3) are shown in Table 4.2. The *Fusobacterium* isolates resistant to imipenem tested negative for the production of ß-lactamases/metallo-ß-lactamases and attempts to identify a carbapenem inactivating enzyme gene were also fruitless (Section 4.2.1). In a study by Tunér *et al.* (1996) it was found that the most resistant *Fusobacterium* strains, especially *F. varium* and *F. mortiferum,* did not produce ß-lactamases. Imipenem Etest ICs performed on the 13 carbapenem resistant *Fusobacterium* spp. revealed interesting ellipses of "inhibition" (Figure 4.6). *Fusobacterium* spp. are known to form a haze of growth within the zone of inhibition that should be ignored (Wexler & Finegold, 1985; Johnson *et al.*, 1989). This haze has been described as cell-wall-defective variants of *Fusobacterium* spp. that can remain viable in cefoxitin concentrations of up to 16 000 µg/ml (Johnson *et al.*, 1989). These cell-wall-defective forms reverted to parental morphology after two passages on drug-free medium, raising questions as to the clinical relevance/importance of such strains (Johnson *et al.*, 1989). In Figure 4.6 the growth within the inhibition zones generated by the Etest was substantial and not just a haze. Investigation of this growth by Gram staining and microscopy, however, showed definite changes in morphology and increases in the size of the cells, possibly indicating such absence of the cell wall.

Table 4.2: **MICs of ß-lactam agents for 13 *Fusobacterium* spp. strains (summarised from Chapter 3).**

FUSOBACTERIUM strains	MIC (µg/ml)							
	PENICILLINS			CEPHALOSPORINS			CARBAPENEMS	
	PIP	AMP	AMX	FOX	CPI	CPO	IMP	MER
F29 *F. mortiferum*	16	1	1	8	>128	>128	**16**	**16**
F30 *F. mortiferum*	32	>128	16	8	>128	>128	**16**	**16**
F51 *F. mortiferum*	8	64	1	16	>128	>128	**64**	**64**
F49 *F. mortiferum*	32	16	8	2	>128	>128	**64**	**>128**
F47 *F. mortiferum*	1	8	4	4	>128	>128	**128**	**>128**
F19 *F. mortiferum*	>128	>128	>128	>128	>128	64	**>128**	**>128**
F23 *F. mortiferum*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F26 *F. mortiferum*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F28 *F. mortiferum*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F35 *F. necrophorum*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F36 *F. necrophorum*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F39 *F. varium*	>128	>128	>128	>128	>128	>128	**>128**	**>128**
F44 *F. varium*	>128	16	>128	128	>128	>128	**>128**	**>128**

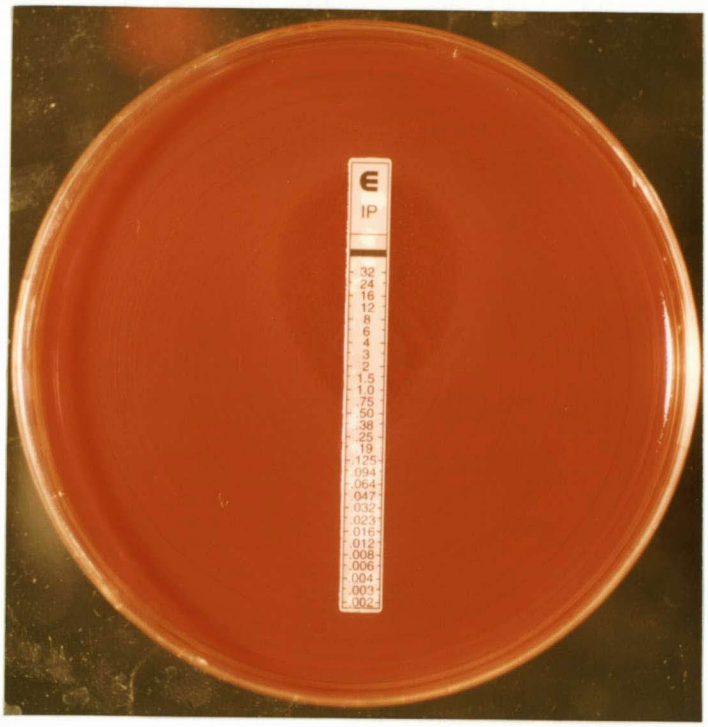
- PIP = piperacillin, AMP = ampicillin, AMX = amoxicillin, FOX = cefoxitin, CPI = cefepime, CPO = cefpirome, IMP = imipenem, MER = meropenem.
- Carbapenem resistance breakpoint $\geq$ 16 µg/ml.

F19 *F. mortiferum* (>128)

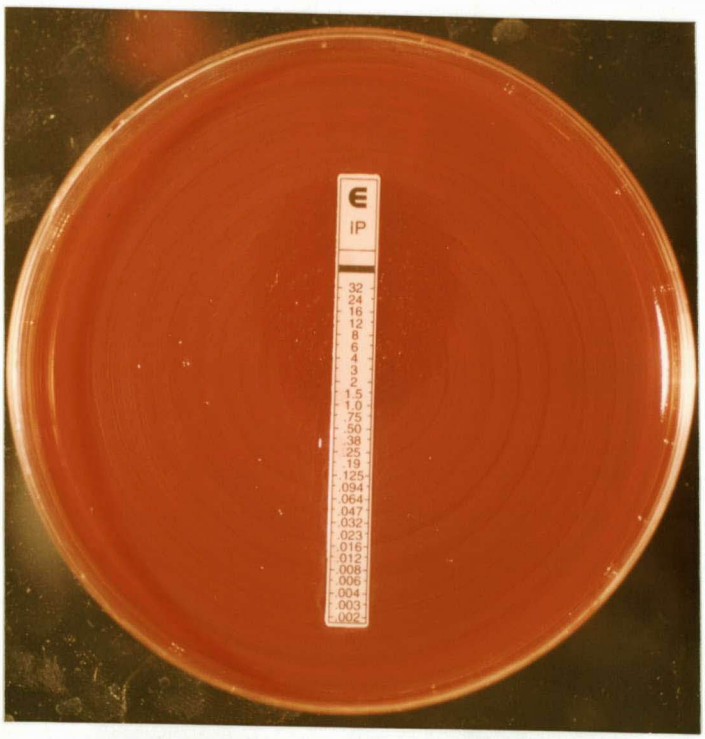


F23 *F. mortiferum* (>128)

Figure 4.6: Etest results on fusobacteria that exhibited "confluent" growth within the ellipse of inhibition. Imipenem MICs (µg/ml) (from Table 4.2) are given in brackets.

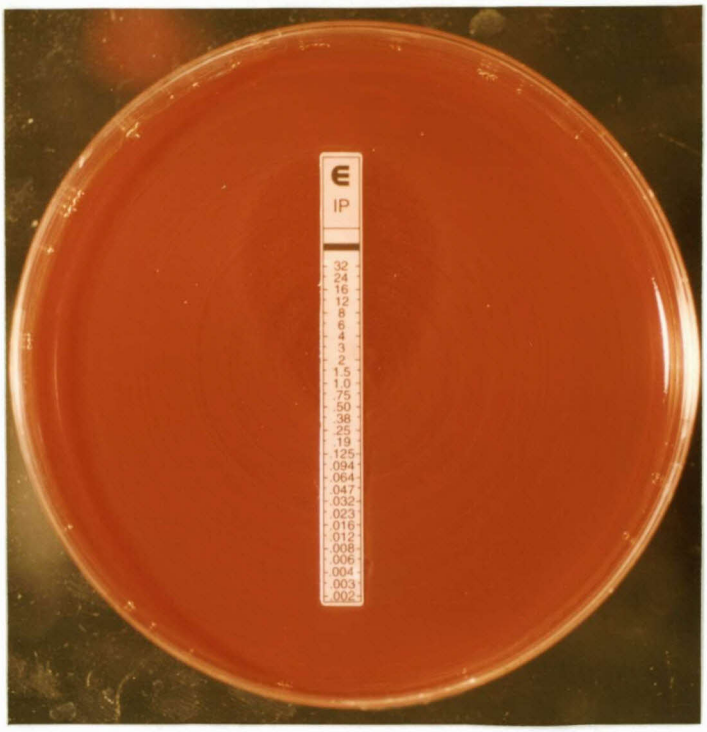


F28 *F. mortiferum* (>128)

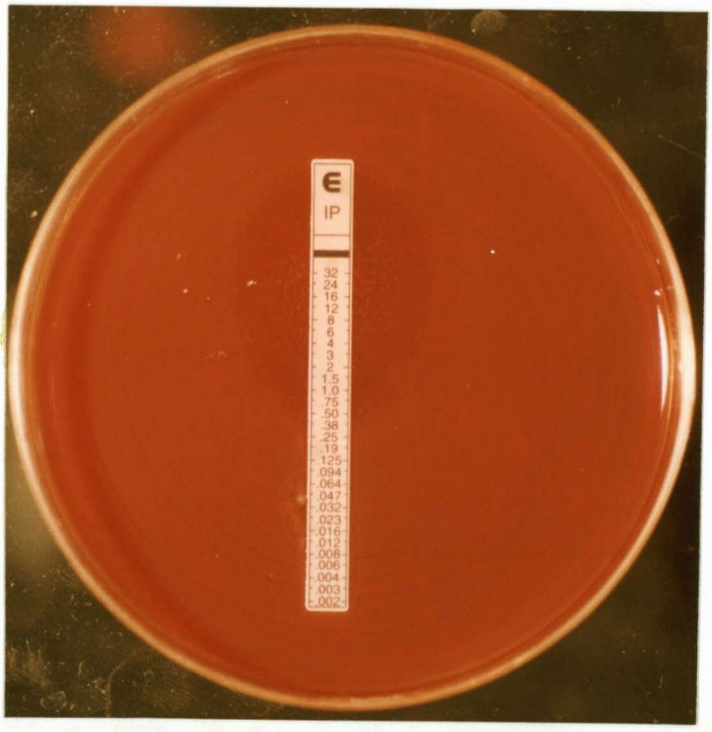


F35 *F. necrophorum* (>128)

Figure 4.6: (Continued)



F39 *F. varium* (>128)



F44 *F. varium* (>128)

Figure 4.6: (Continued)

Results of PBP affinity and imipenem competition studies performed on two *F. mortiferum* strains (Figure 4.7a & b) are described in Table 4.3. In *F. mortiferum* strain F28 (imipenem MIC >128 µg/ml) strong penicillin binding was seen for PBP4 (43 kDa) and although binding to PBP5 (32 kDa) was weaker, there was no reduction in affinity as the concentration of [^{3}H]penicillin was reduced (Figure 4.7a). The low affinity PBPs were PBPs 1 and 2 (74 & 69 kDa). Similarly, affinity studies on *F. mortiferum* strain B51 (imipenem MIC 64 µg/ml) showed that the lowest affinity PBP was PBP1 (74 kDa) (Figure 4.7b). It would be necessary to perform further [^{3}H]penicillin dilutions in the range 0.5-0.05 µCi to confirm the affinity order for the other PBPs observed in the strain. In the case of imipenem low affinity PBPs that appear to be involved in imipenem resistance in *F. mortiferum,* were PBP1 in strain F28 and PBPs 1, 3 and 4 in strain B51 (Figures 4.7a & b). It is evident that although the two *F. mortiferum* strains showed different PBP profiles, PBP1 (74 kDa) in both cases exhibited the least affinity for penicillin and imipenem is a strong candidate for the development of resistance to ß-lactam and carbapenem antibiotics.

The PBP profile of an imipenem resistant variant (MIC >128 µg/ml) of *F. varium* parental strain F39 (apparent MIC 0.5 - >128 µg/ml), selected from substantial growth (Figure 4.6) within the zone of inhibition produced by an imipenem Etest, was compared to that of the parental strain (Figure 4.8). The variant was seen to produce a PBP at approximately 69 kDa that was absent from the parental strain. With this exception, the variant had the same API and PBP profile to that of the parental strain. Such an extra PBP may well correlate with decreased susceptibility to ß-lactam antibiotics as has been reported by Tunér *et al.* (1996).

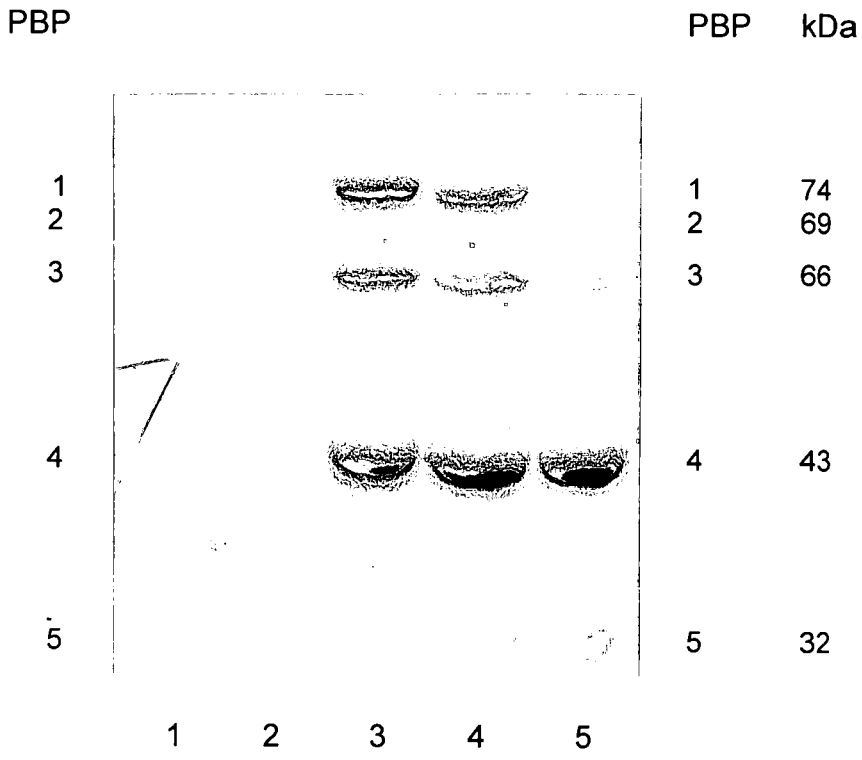


Figure 4.7a: PBP affinity and imipenem competition studies on *F. mortiferum* F28 (imipenem MIC >128 µg/ml). LANES **1** & **2**: Whole cell lysates incubated with dilutions of imipenem and post labelled with [^{3}H]penicillin. Lane **1**, 1 µg/ml imipenem; lane **2**, 0.5 µg/ml imipenem. LANES **3-5**: Whole cell lysates incubated with ten-fold dilutions of [^{3}H]penicillin. Lane **3**, 5 µCi; **4**, 0.5 µCi; **5**, 0.05 µCi.

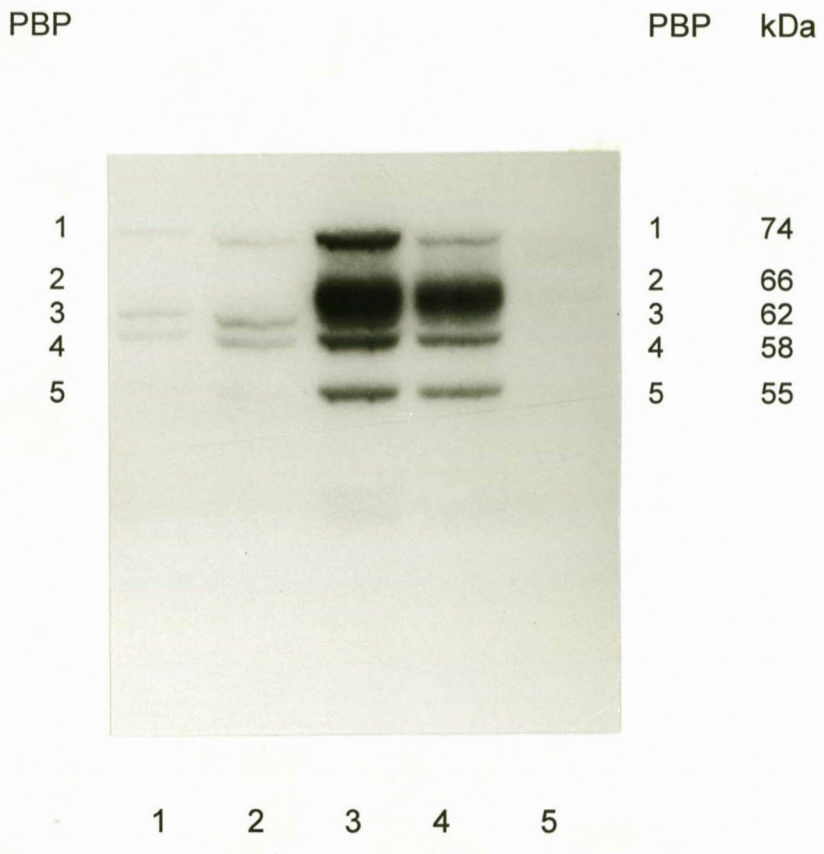


Figure 4.7b: PBP affinity and imipenem competition studies on *F. mortiferum* B51 (imipenem MIC 64 µg/ml). LANES: **1** & **2**: Whole cell lysates incubated with dilutions of imipenem and post labelled with [^{3}H]penicillin. Lane **1**, 1 µg/ml imipenem; lane **2**, 0.5 µg/ml imipenem. LANES: **3-5**: Whole cell lysates incubated with ten-fold dilutions of [^{3}H]penicillin. Lane **3**, 5 µCi; **4**, 0.5 µCi; **5**, 0.05 µCi.

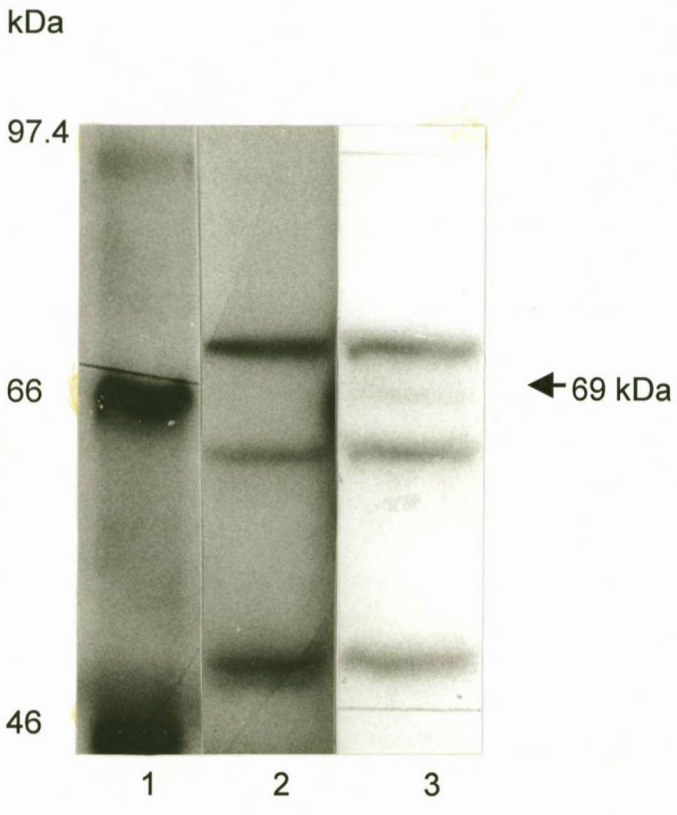


Figure 4.8: Comparison of PBP profiles of a parental *F. varium* strain F39 (imipenem apparent MIC 0.5->128 µg/ml) and an Etest subpopulation selected from within the zone of inhibition. Lanes: **1**, MWM; **2**, parental population; **3**, subpopulation (variant).

Table 4.3: Interpretation of penicillin and imipenem affinities for PBPs of *F. mortiferum* strains F28 and B51.

STRAIN	Figure	PBPs (kDa)							
		74	69	66	62	58	55	43	32
***F. mortiferum* F28 (>128)**							++		
Penicillin affinity	4.7a	+	+	-	-	-	++	++++	+++
Imipenem affinity	4.7a	+	+++	+++			++	+++	+++
***F. mortiferum* B51 (64)**									
Penicillin affinity	4.7b	+	-	++++	++++	++++	++	-	-
Imipenem affinity	4.7b	++		++++	+	+	++	-	-

Imipenem MICs (µg/ml) are given in brackets

++++ & +++ strong affinity

++ & + weak affinity

Seven major PBP groups could be identified (Figure 4.9) when the PBP profiles of 12 *Fusobacterium* strains were compared. These PBP groups were then correlated with species as identified by the Rapid ID32A system (Table 4.4). Three *F. mortiferum* strains (B51, F47 and F49) with identical profiles were assigned to Group Ia, while a fourth strain (B50) for which low-molecular-weight PBP bands were not, evident was placed in Group Ib (Table 4.4). The remaining three *F. mortiferum* strains (F23, F19 and F27) were each representative of different groups (Groups II, III and IV; Table 4.4). The *F. varium* strain was classified in Group Va. However, a subgroup Vb comprising *F. necrophorum* strain F35 and a *F. mortiferum* strain F28, had similar profiles. The remaining two *F. necrophorum* strains F36 and F15 had individual profiles (Groups VI and VII). The PBPs of *Fusobacterium* spp. have not been studied extensively. The first fusobacterial PBP patterns described by Tunér *et al.* (1996) were species specific, ranging in molecular-weight from 100 kDa to 40 kDa.

There appears to be some PBP/API profile agreement for the majority of *F. mortiferum* strains investigated in this study. Subspecies were, however, evident when employing PBP profiling (Groups I-IV). With PBP Group V, 3 API species (*varium, necrophorum* and *mortiferum*) were seen, complicating identification comparisons employing the two methodologies. The VPI procedure could perhaps verify the discrepancies, but nevertheless, the API system does not appear to lend to an overall reliable identification of fusobacteria.

An accurate identification of fusobacteria is of importance not only for taxonomic reasons but also for empiric treatment regimens. Antibiotic selection is difficult as susceptibility varies widely for different species and strain typing can aid in situations of therapeutic failures (George *et al.*, 1981; Bolstad, Jensen & Bakken, 1996). It has been suggested by Tunér *et al.* (1996) that description and characterisation of PBPs of fusobacteria is a necessary first step to investigating their potential role in antimicrobial resistance. The present study, although only at a preliminary stage, would endorse this view.

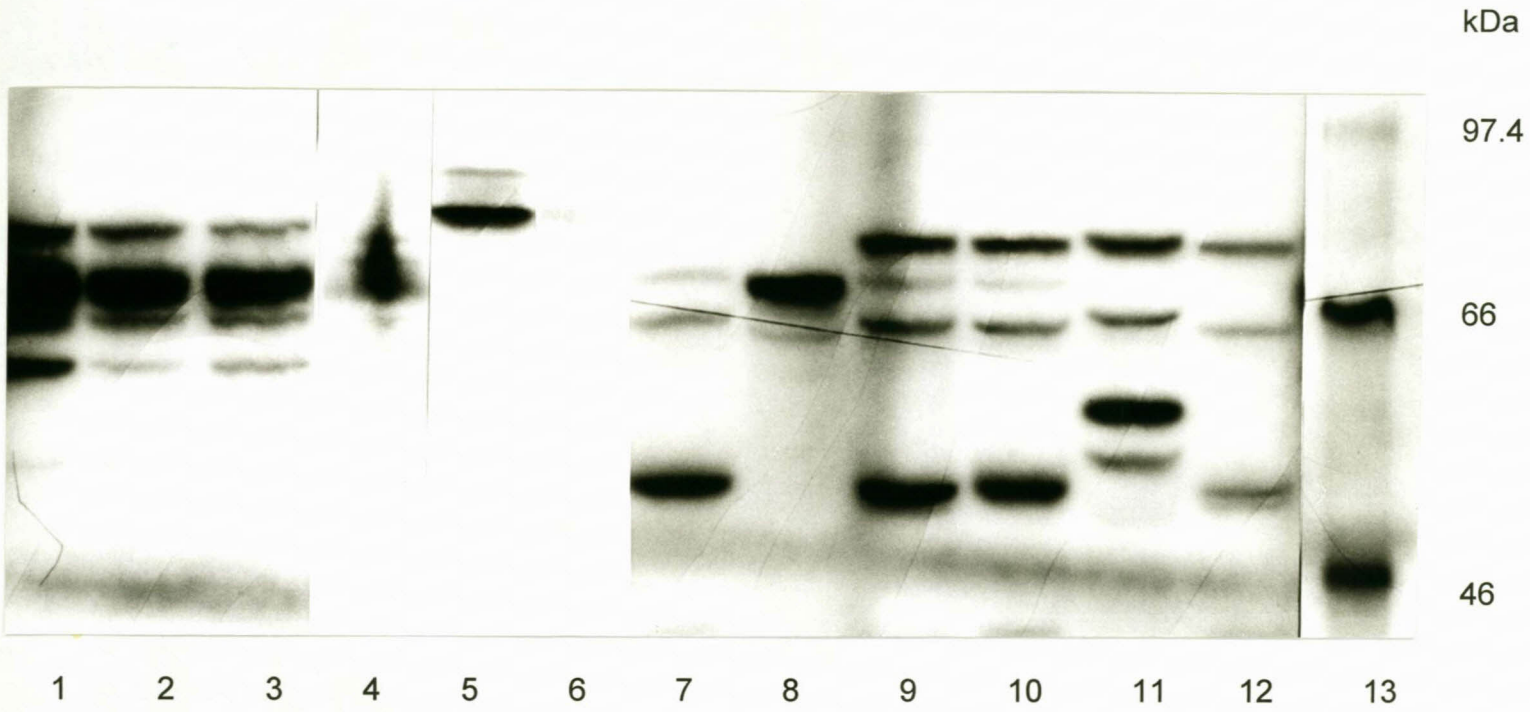


Figure 4.9: PBP profile comparisons of *Fusobacterium* spp.: Lanes: **1**, strain F47 (Group Ia); **2**, strain F49 (Group Ia); **3**, strain B51 (Group Ia); **4**, strain B30 (Group Ib); **5**, strain F15 (Group VII); **6**, strain F23 (Group II); **7**, strain F19 (Group IV); **8**, strain F27 (Group III); **9**, strain F28 (Group Vb); **10**, strain F35 (Group Vb); **11**, strain F36 (Group VI); **12**, strain F39 (Group Va); **13**, MWM. PBP Groups were assigned after profiling (Table 4.4).

Table 4.4: API identification vs. PBP profiles of *Fusobacterium* spp.:

Strain	Identification Rapid ID32A	Penicillin-Binding Proteins: MW (kDa)*											PBP Group
		94	82	74	69	66	62	58	55	52	50	43	
F47	*F. mortiferum*	-	-	+	-	+	+	-	-	-	+	+	Ia
F49	*F. mortiferum*	-	-	+	-	+	+	-	-	-	+	+	
B51	*F. mortiferum*	-	-	+	-	+	+	-	-	-	+	+	
B30	*F. mortiferum*	-	-	+	-	+	+	-	-	-	-	-	Ib
F23	*F. mortiferum*	-	+	-	-	-	-	+	-	-	+	-	II
F27	*F. mortiferum*	-	-	-	-	+	+	+	-	-	-	-	III
F19	*F. mortiferum*	-	-	-	-	+	+	-	-	+	-	-	IV
F39	*F. varium*	-	-	+	-	-	-	+	-	-	+	+	Va
F35	*F. necrophorum*	-	-	+	+	-	-	+	-	-	+	+	Vb
F28	*F. mortiferum*	-	-	+	+	-	-	+	-	-	+	+	
F36	*F. necrophorum*	-	-	+	-	-	+	-	+	+	-	-	VI
F15	*F. necrophorum*	+	+	-	-	+	-	-	-	-	-	-	VII

*PBP MWs were determined from Figure 4.9

4.2.3 Penicillin-binding proteins of the clostridia.

PBP profiles of 17 representative *Clostridium* isolates are illustrated in Figure 4.10. PBP profiles of the *Clostridium* spp. other than *C. perfringens* were not similar to those of *C. perfringens* (Figure 4.10). In Table 4.5 PBP profiles of the full compliment of *Clostridium* spp. other than *C. perfringens* examined (28 strains), are compared with API identification obtained with the Rapid ID32A system. The PBP profiles of seven *C. sordelli* strains were similar, all possessing strong PBP bands of approximately 74, 62 and 44 kDa with minor bands indicating the presence of three *C. sordelli* subgroups. For the *C. sordelli* isolates investigated, the API system appeared to be of value for species identification. Seven strains of *C. tertium* (penicillin MIC range $\leq$ 0.06 - 1 µg/ml) were seen to share two major bands (51 & 29 kDa), but two strains lacked strong bands at 48 and 39 kDa, perhaps again indicating the presence of two subgroups. Six *C. sporogenes* strains were distributed into two groups, comprising two and four strains with dissimilar profiles. A *C. septicum* strain shared major PBP bands of 47, & 43 kDa with two strains of *C. sporogenes*. The two *C. glycolicum* strains did not exhibit matching profiles, while individual strains of *C. histolyticum, C. ramosum, C. paraputrificum* and two *C. butyricum* strains with identical banding patterns, possessed unique profiles.

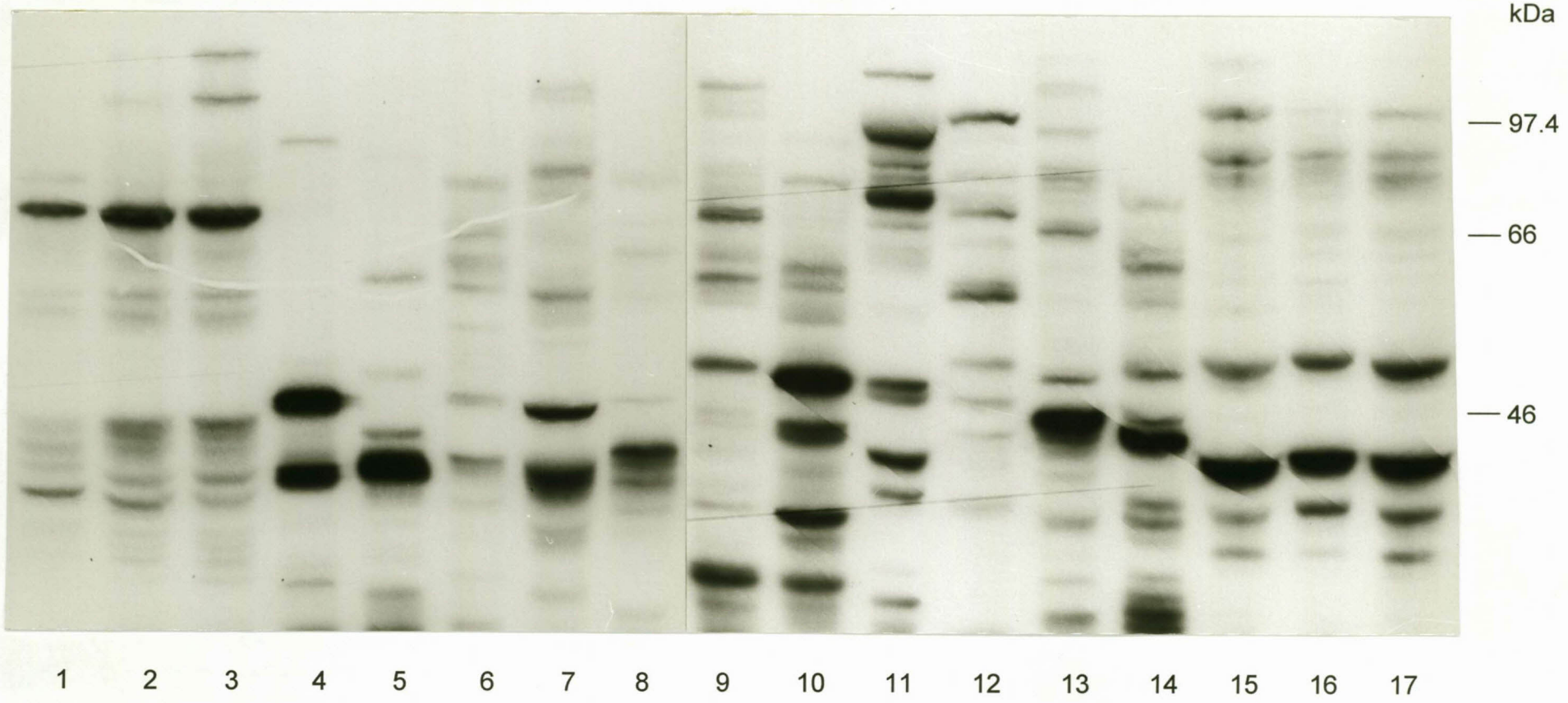


Figure 4.10: PBP profiles of representative *Clostridium* spp. Penicillin MICs (µg/ml) are given in brackets. Lanes: **1**, C146 *C. sordelli* (≤0.06); **2**, C115 *C. sordelli* (≤0.06); **3**, C93 *C. sordelli* (≤0.06); **4**, C211 *C. septicum* (≤0.06); **5**, C197 *C. glycolicum* (2); **6**, F7 *C. glycolicum* (≤0.06); **7**, C112 *C. sporogenes* (≤0.06); **8**, C165 *C. sporogenes* (0.125); **9**, C160 *C. tertium* (1); **10**, C121 *C. tertium* (1); **11**, C204 *C. histolyticum* (≤0.06); **12**, C64 *C. ramosum* (0.25); **13**, C205 *C. butyricum* (0.125); **14**, C189 *C. paraputrificum* (0.25); **15**, C150 *C. perfringens* (≤0.06); **16**, C139 *C. perfringens* (≤0.06); **17**, C158 *C. perfringens* (≤0.06).

Table 4.5: API identification vs. PBP profiles in non-*C. perfringens Clostridium* spp.:

Rapid ID32A (no. isolates)	Rep. strain	Penicillin-binding proteins: MW (kDa)																														
		116	112	106	102	99	97	91	83	80	76	74	71	70	69	66	62	53	51	48	47	44	43	41	39	38	36	35	34	32	29	27	26
C. sordelli (3)	C146	-	-	-	-	-	-	+	-	-	-	+++	-	-	-	-	+	-	-	-	-	+	-	+	+	-	++	-	-	-	-	-	-
C. sordelli (2)	C115	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	++	-	-	-	-	++	-	+	-	+	-	+	-	-	-	-	-
C. sordelli (2)	C93	++	-	-	++	-	-	-	-	-	-	+++	-	-	-	-	++	-	-	-	-	++	-	+	-	+	-	-	-	-	-	-	-
C. tertium (2)	C160	-	-	++	-	-	-	-	-	-	-	++	+	-	++	-	-		++	-	+	+	-	-	+	-	-	-	+	-	+++	-	+
C. tertium (5)	C121	-	-	-	-	-	-	+	-	-	-	-	-	++	-	+	+	-	+++	+++	-	-	-	-	+++	+	-	-	+	+	+++	-	-
C. septicum (1)	C211	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+++	-	+++	-	-	-	-	-	-	-	-	+	-
C. glycolicum (1)	C197	-	-	-		-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+++	-	-	-	-	-	-	-	++	-	+	-
C. glycolicum (1)	F7	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+
C. sporogenes (2)	C112	-	-	-	+	-	++	-	-	-	-	-	-	-	-	-	+	-	-	-	+++	-	+++	-		-	-	-	+	+	-	+	-
C. sporogenes (4)	C165	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+++	++	++	-	-	-	+	-	-	-	-	+
C. histolyticum (1)	C204	-	+++	-	-	-	+++	+	+++	+	-	-	-	-	-	-	-	-	++	++	-	+++	-	++	-	-	-	-	-	-	++	+	-
C. ramosum (1)	C64	-	-	-	-	++	++	-	-	-	-	+	-	-	++	-	++	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-
C. butyricum (2)	C205	-	-	+	-	-	+	+	-	++	-	-	-	-	+	-	-	-	+	+++	+++	+	-	-	+	-	-	-	+	-	++	-	++
C. paraputrificum (1)	C189	-	-	-	-	-	-	-	+	-	+	-	+	++	-	-	+	-	-	++	+++	-	-	-	++	++	+	-	+	++	+++	+++	-

Table compiled from Figure 4.10

+ & ++ Weak PBP bands

+++ Strong bands

C. sordelli and *C. tertium* identification by the Rapid ID32A system showed some correlation with PBP profiles, but for the majority of *Clostridium* species there was only limited conformability. This substantiates criticism levelled at commercial identification systems used for species identification of *Clostridium* spp. that they are not so reliable as promoted. Because of Gram-stain variability, lack of spores and atypical colonial morphology, identification of *Clostridium* species is often difficult (Alexander *et al.*, 1995). In a study comparing the identification of clinical isolates of *Clostridium* spp. it was found that none of three commercially available identification kits, the Rapid ANA II, AnIDent and ATB 32A, could be used as the sole method for identification and that all three systems should be supplemented with additional tests for the complete identification of selected anaerobes (Alexander *et al.*, 1995). It was also found that *Clostridium* species had various levels of resistance to ß-lactams, complicating the identification even further (Alexander *et al.*, 1995). Similar studies recommend additional methods of identification, such as rapid chromogenic identification panels (Presumpto Plates) and classic methods, i.e. VPI gas-liquid chromatography and prereduced anaerobic biochemical reactions (Goldstein, Citron & Goldman, 1992; Whaley *et al.*, 1995; Baron & Citron, 1997).

There is a need for the accurate identification of *Clostridium* isolates without the use of labour intensive VPI methodology (Alexander *et al.*, 1995). Infections with *Clostridium* species, including the non-*Clostridium perfringens* group, are increasingly encountered, especially when host defences are impaired (Parker & Smith, 1993). This emphasises the need for a reliable method for "correct" definitive identification of *Clostridium* spp. As susceptibility testing is frequently not performed, species-level identification has also been considered to provide valuable guidelines for therapy (Citron & Appelbaum, 1993). Certain clostridia have been associated with gastro-intestinal malignancies that necessitate full identification criteria (Citron & Appelbaum, 1993). PBP profiling may well be used as a control reference in the validification of identification results and may assist in the constructive differentiation of *Clostridium* species.

To investigate penicillin-binding affinity in *Clostridium* species, three strains were selected from the most common PBP profiles observed. The strains investigated

were *C. tertium* C121 (penicillin MIC 1 µg/ml), *C. tertium* C122 (penicillin MIC ≤0.06 µg/ml), *C. sporogenes* C112 (penicillin MIC ≤0.06 µg/ml). A fourth isolate, *C. bifermentans* C138 (penicillin MIC 0.125 µg/ml), was also included. It can be seen that for both *C. tertium* strains, PBPs 3 and 4 (49 and 41 kDa) exhibited the highest binding potential for penicillin, yet also reduced affinity, as varying concentrations of [^{3}H]penicillin were added (Figure 4.11a). Low affinity was evident with PBP 1d, 1f, 3b and 4 (73, 66, 46 and 41 kDa) in strain C121 and 1a, 1e and 3 (91, 69 and 49 kDa) in strain C122 (Figure 4.11a). On comparing PBP affinities obtained from *C. tertium* strains, C122 (MIC ≤0.06 µg/ml) with those of strains C121 (MIC 1 µg/ml) there was no apparent difference in a specific PBP that corresponded to the increased resistance expressed by strain C121.

In a *C. sporogenes* strain C112 (penicillin MIC ≤0.06 µg/ml), the PBPs exhibiting high penicillin-binding were PBPs 1a, 2f and 3 (111, 58 and 49 kDa) (Figure 4.11b). Low penicillin-binding, yet little to no reduction in affinity, was demonstrated for PBPs 2a and 2c (83 and 76 kDa). PBP 4a (41 kDa) showed the least affinity for penicillin when exposed to decreasing concentrations of [^{3}H]penicillin in *C. sporogenes*.

For the *C. bifermentans* strain C138 (penicillin MIC 0.125 µg/ml) the PBPs showing high binding were PBPs 2d (71 kDa), 2e (60 kDa) and 4b (39 kDa). Low penicillin-binding affinity was seen for PBPs 4a and 4b (41 and 39 kDa), the two low-molecular-weight PBPs that were demonstrated in this *C. bifermentans* strain.

The varied PBP penicillin affinities in the small number of *Clostridium* isolates studied (four, of which two were of the same species) do not allow reliable predictions to be made as to how ß-lactam resistance has developed in this genus. Of note, PBPs of *C. tertium, C sporogenes* and *C. bifermentans* that consistently exhibited reduced affinity towards penicillin were those of low-molecular-weight (<50 kDa).

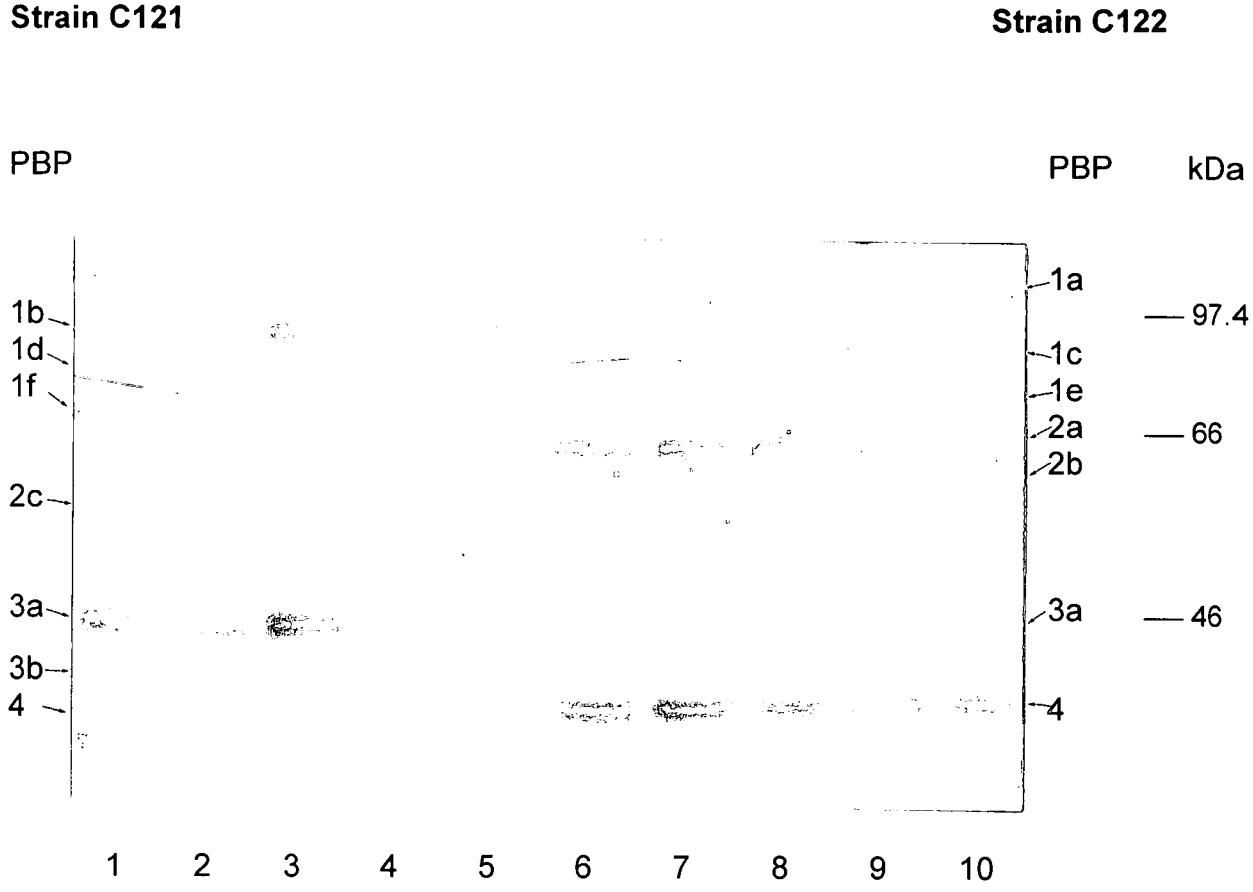


Figure 4.11a: PBP affinities of *Clostridium* spp. isolates. LANES: **1-5**, *C. tertium* strain C121 (penicillin MIC 1 µg/ml); **6-10**, *C. tertium* strain C122 (penicillin MIC ≤0.06 µg/ml). Strains were incubated with dilutions of [^{3}H]penicillin. Lanes: **1**, **6**: 5 µCi; **2**, **7**: 1 µCi; **3**, **8**: 0.5 µCi; **4**, **9**: 0.1 µCi; **5**, **10**: 0.05 µCi.

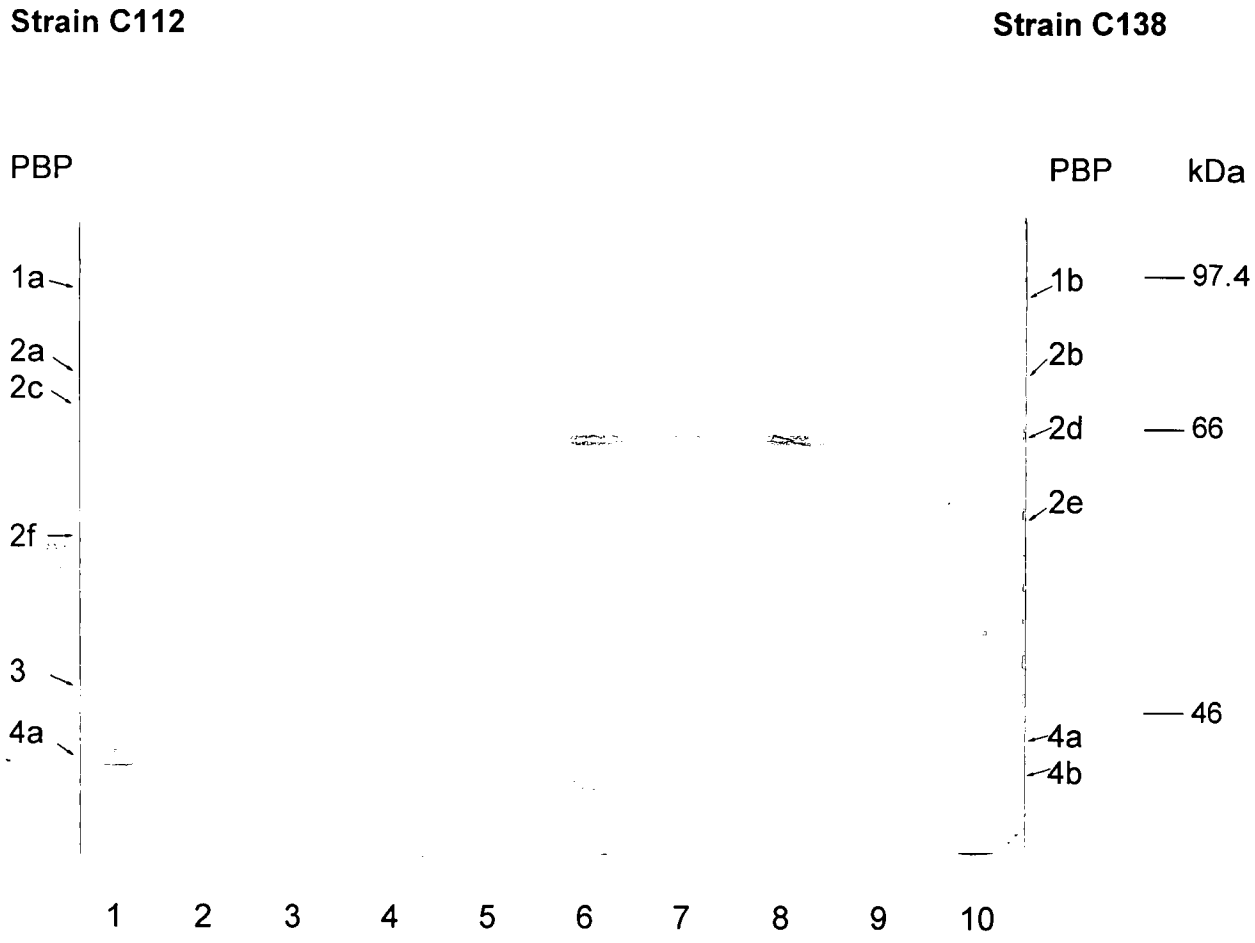


Figure 4.11b: PBP affinities of *Clostridium* spp. isolates. LANES: **1-5**, *C. sporogenes* strain C112 (penicillin MIC ≤0.06 µg/ml); **6-10**, *C. bifermentans* strain C138 (penicillin MIC 0.125 µg/ml). Strains were incubated with dilutions of [^{3}H]penicillin. Lanes: **1**, **6**: 5 µCi; **2**, **7**: 1 µCi; **3**, **8**: 0.5 µCi; **4**, **9**: 0.1 µCi; **5**, **10**: 0.05 µCi.

4.2.4 Piperacillin resistance in Veillonella spp.

Comparison of the PBP profiles of nine *Veillonella* spp. allowed the strains to be assigned to three groups: Group I (V2, V7); Group II (V4, V25, V1); Group III: (V8, V11, V14, V28) (Figure 4.12). The major PBP that exhibited the highest affinity for penicillin in Groups I and II was seen not to be the lowest-molecular-weight PBP. The low-molecular-weight PBPs are often associated with carboxypeptidase activity as found in *Streptococcus pneumoniae* and *Escherichia coli*, where inactivation of these PBPs has no affect on the killing action of ß-lactam antibiotics (Bryan & Godfrey, 1991; Spratt, 1994).

MIC determinations on *Veillonella* isolates revealed that 21/31 (68%) strains were selectively resistant to piperacillin (MICs >32 µg/ml) (Table 4.6). The MICs of the other ß-lactam agents tested were: penicillins ≤ 8 µg/ml, cephalosporins ≤ 16 µg/ml and carbapenems ≤ 4 µg/ml. All the isolates tested ß-lactamase negative (nitrocefin), excluding the production of a ß-lactamase directed against piperacillin.

The PBP (66 kDa) showing the greatest affinity for penicillin and ampicillin was seen to exhibit the lowest affinity for piperacillin (Figures 4.13 & 4.14). For many bacterial genera it is unusual that the affinities of different penicillins are focused on one PBP, yet for piperacillin, selective mutants had obviously resulted as MICs exceeded 128 µg/ml. This could be a possible explanation why resistance among Gram-negative anaerobic bacteria has been found against piperacillin, even in combination with tazobactam (García-Rodríguez, García-Sánchez & Muñoz-Bellido, 1995; Citron & Appelman, 1997; Betriu *et al.*, 1999; Labbé *et al.* 1999). In a previous study by Wren (1996) it was found that carboxypenicillins and ureidopenicillins were not active against all strains of *Veillonella*. Resistance to piperacillin/tazobactam in *Veillonella* spp. has also been reported by Mendes, Gordon & Mitchell (1997). The findings of the present study and those of other researchers certainly demonstrates that further investigations into piperacillin resistance development in *Veillonella* spp. are necessary.

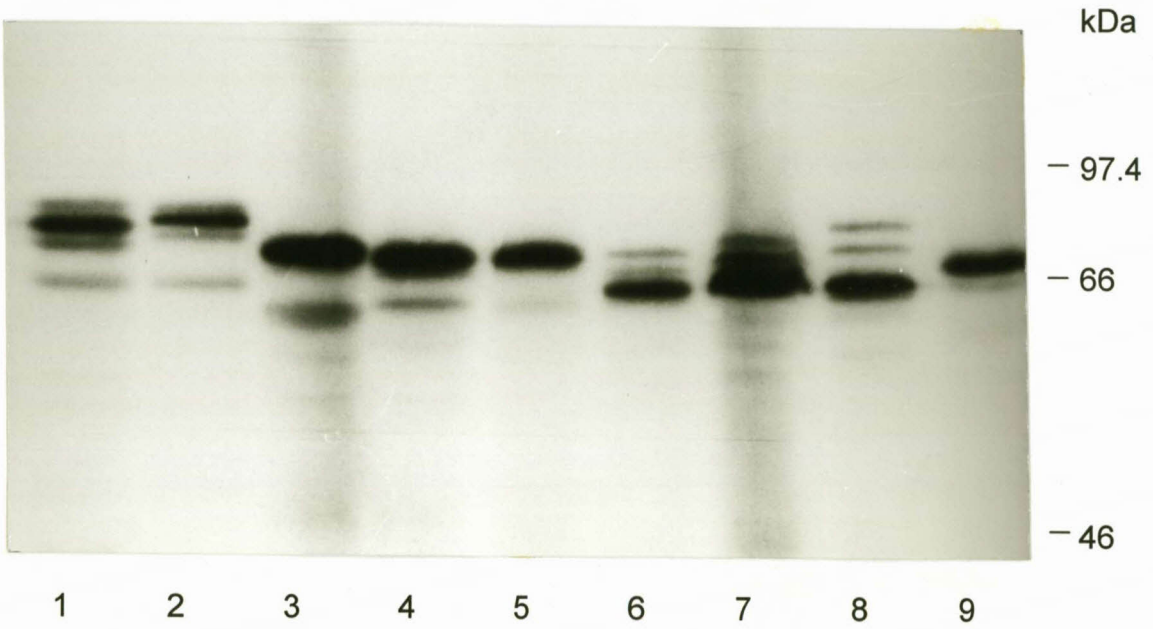


Figure 4.12: PBP profiles of *Veillonella* spp. MICs (µg/ml) of piperacillin and ampicillin respectively are given in brackets. Lanes: **1**, strain V2 (0.5, 0.125); **2**, strain V7 (0.25, ≤ 0.06); **3**, strain V4 (>128, 2); **4**, strain V25 (32, 4); **5**, strain V1, (>128, 4); **6**, strain V11 (16, 0.25); **7**, strain V14 (8, 0.125); **8**, strain V28 (>128, 0.5); **9**, strain V8 (8, 0.5).

Table 4.6: ß-Lactam/carbapenem MICs against 31 *Veillonella* strains (summarised from Chapter 3):

VEILLONELLA strains	MIC (µg/ml)							
	PENICILLINS			CEPHALOSPORINS			CARBAPENEMS	
	PIP	AMP	AMX	FOX	CPI	CPO	IMP	MER
V30	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.125	≤0.06
V7	0.25	≤0.06	≤0.06	0.25	≤0.06	≤0.06	≤0.06	≤0.06
V2	0.5	0.125	≤0.06	0.5	0.5	≤0.06	≤0.06	≤0.06
V14	8	0.125	0.125	0.25	2	1	0.125	≤0.06
V8	8	0.5	0.5	1	4	2	0.25	0.125
V11	16	0.25	0.25	0.5	2	2	0.125	0.125
V19	16	0.25	0.25	0.5	2	2	0.25	≤0.06
V21	16	0.25	0.25	0.5	2	2	0.125	0.125
V20	32	1	0.5	2	4	2	0.25	0.25
V25	32	4	2	8	8	4	0.5	1
V16	**64**	1	1	8	4	2	0.25	0.5
V31	**64**	1	1	2	16	4	0.25	1
V6	**128**	0.5	0.25	2	8	4	0.5	0.125
V3	**128**	1	0.5	8	8	4	1	0.5
V5	**128**	1	1	8	8	4	0.5	0.5
V29	**128**	2	1	4	8	2	0.125	1
V10	**128**	4	4	8	8	8	1	1
V28	**>128**	0.5	0.5	4	4	2	1	1
V12	**>128**	1	2	8	8	4	1	0.5
V18	**>128**	1	1	8	16	8	1	0.5
V26	**>128**	1	0.5	4	4	2	0.25	0.5
V4	**>128**	2	1	4	4	4	0.25	0.5
V32	**>128**	2	2	8	32	4	0.5	2
V1	**>128**	4	2	8	8	4	1	1
V13	**>128**	4	4	16	8	8	1	2
V17	**>128**	4	2	8	8	4	0.5	1
V22	**>128**	4	2	16	8	4	1	2
V24	**>128**	4	2	8	4	4	0.25	1
V27	**>128**	4	2	16	16	8	2	1
V9	**>128**	8	4	8	8	4	0.25	1
V15	**>128**	8	8	16	16	16	2	4

PIP = piperacillin, AMP = ampicillin, AMX = amoxicillin, FOX = cefoxitin, CPI = cefepime, CPO = cefpirome, IMP = imipenem, MER = meropenem.

Piperacillin resistance breakpoint ≥ 64 µg/ml

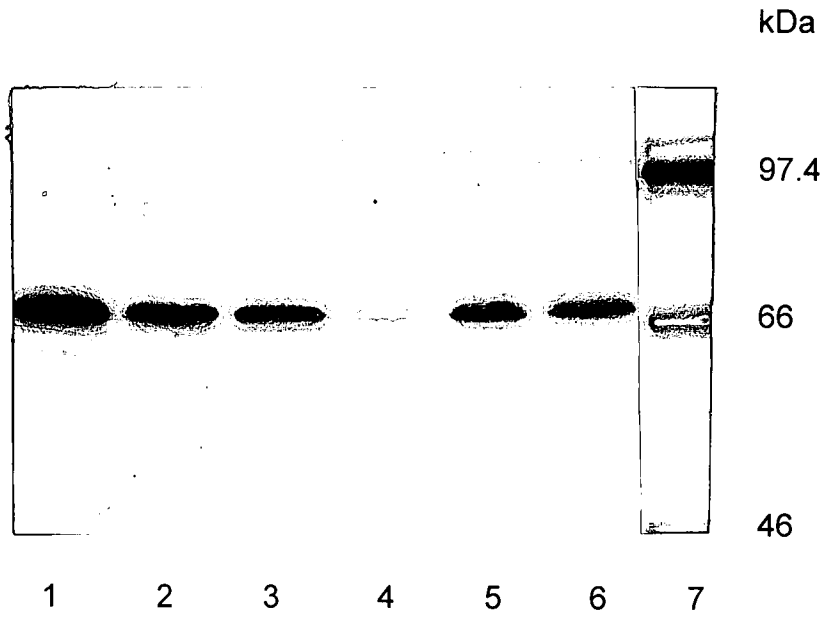


Figure 4.13: PBP penicillin affinity study on *Veillonella* strains V25 and V1. LANES: **1-4**, strain V25; **5-6**, strain V1. Whole cell lysates were incubated with dilutions of [^{3}H]penicillin: Lanes **1**, 5 µCi; **2**, 1 µCi; **3**, 0.5 µCi; **4**, 0.05 µCi; **5**, 10 µCi; **6**, 5 µCi. Lane **7**, MWM.

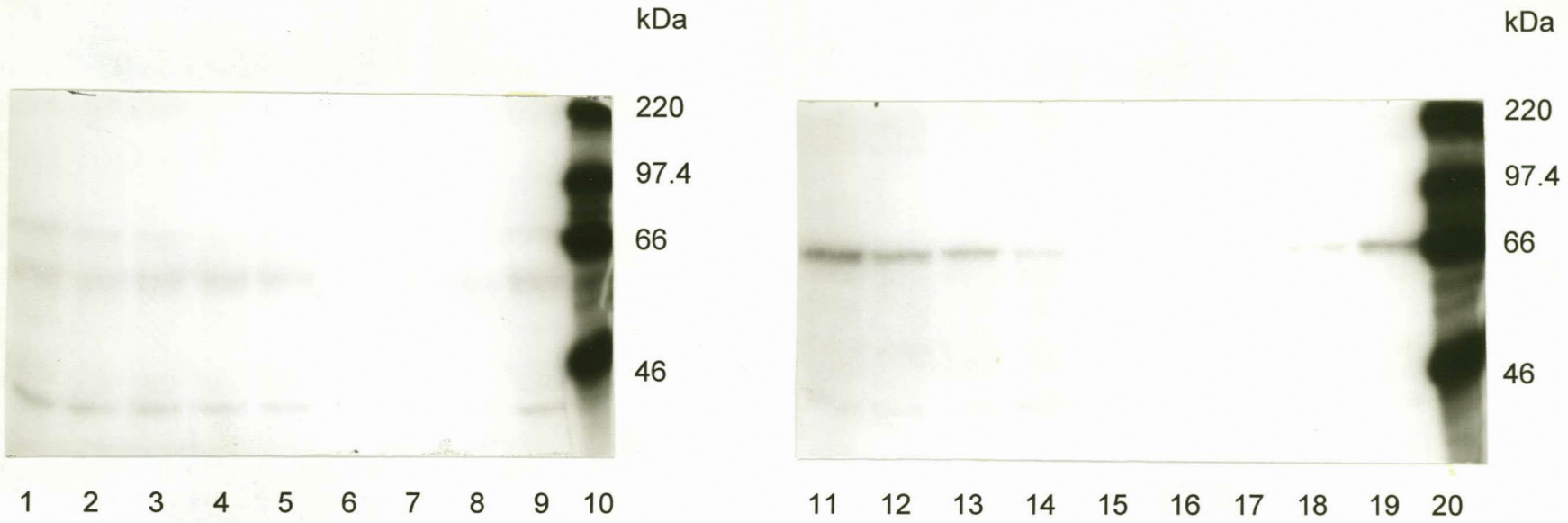


Figure 4.14: PBP penicillin affinity and piperacillin competition studies on membrane preparations of *Veillonella* strains V2 and V11. LANES: **1-9**: strain V2 (piperacillin MIC 0.5 µg/ml); lane **10**, MWM; LANES: **11-19**: strain V11 (piperacillin MIC 16 µg/ml); Lane **20**, MWM. Membrane preparations were incubated with dilutions of piperacillin and post labelled with 2 µCi [^{3}H]penicillin. LANES: **1-5**, **11-15**, preincubation with piperacillin; lane **1**, **11**: no piperacillin added; **2**, **12**: 0.01 µg/ml; **3**, **13**: 0.1 µg/ml; **4**, **14**: 0.5 µg/ml; **5**, **15**: 1 µg/ml. LANES: **6-9**, **16-19**: Membrane preparations incubated with dilutions of [^{3}H]penicillin. Lanes **6**, **16**: 0.01 µCi; **7**, **17**: 0.1 µCi; **8**, **18**: 0.5 µCi; **9**, **19**: 2 µCi.

PBP ampicillin/piperacillin competition studies were performed on eight *Veillonella* strains with piperacillin MICs ranging from 0.5 - > 128 µg/ml (Figures 4.14 and Figures 4.15 a, b, c & d). The PBP binding affinities of ampicillin and piperacillin and MICs of the eight *Veillonella* strains are summarised in Table 4.7. It was evident that ampicillin bound strongly (as with penicillin) to the high-molecular-weight PBP (66 kDa). In contrast, affinity of piperacillin for the 66 kDa PBP was considerably reduced in piperacillin resistant strains V1 (MIC >128 µg/ml), V4 (MIC >128 µg/ml), V18 (MIC > µg/ml), V28 (MIC >128 µg/ml) and V31 MIC 64 µg/ml) (Figures 4.15 a-c) when compared with the affinity of piperacillin in sensitive strains V11 (MIC 16 µg/ml) and V2 (MIC 0.5 µg/ml) (Figures 4.15 c-d).

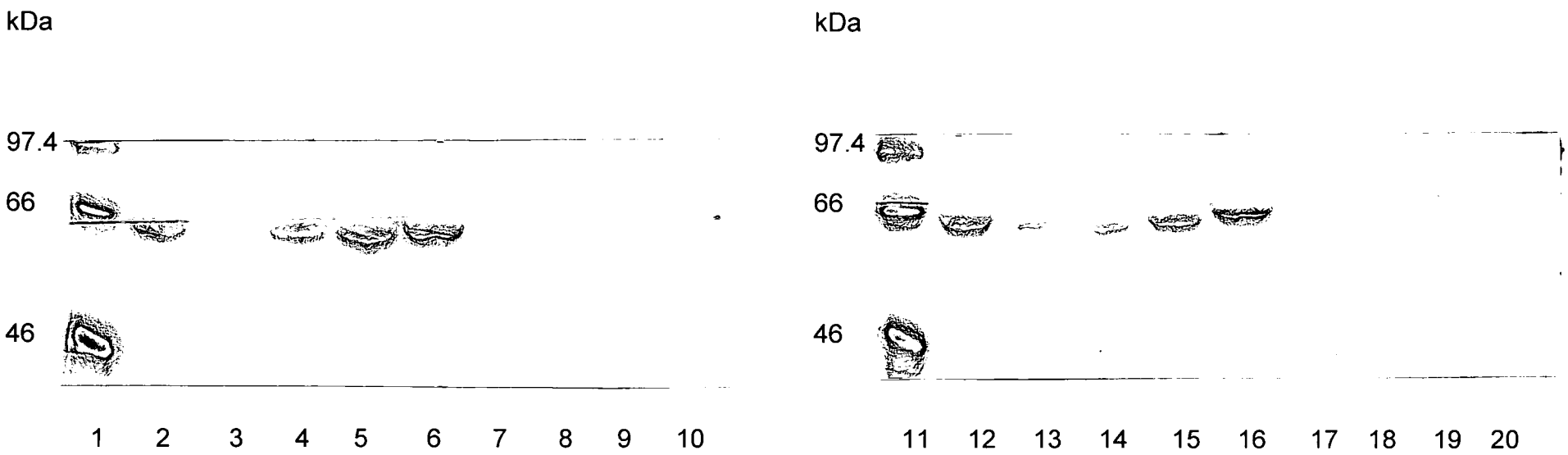


Figure 4.15a: Piperacillin and ampicillin competition studies on *Veillonella* strains V1 and V4. Lane **1**, MWM. LANES: **2-10**: strain V1 (piperacillin MIC >128 µg/ml, ampicillin MIC 4 µg/ml). Lane **11**, MWM. LANES: **12-20**: strain V4 (piperacillin MIC >128 µg/ml, ampicillin MIC 2 µg/ml). Whole cell lysates were incubated with dilutions of piperacillin or ampicillin and post labelled with 2 µCi [^{3}H]penicillin. Lanes **2**, **12**, no piperacillin or ampicillin added. LANES **3-6**, **13-16**: preincubation with piperacillin; lanes **3**, **13** 10 µg/ml; **4**, **14**, 5 µg/ml; **5**, **15**, 2 µg/ml; **6**, **16**, 1 µg/ml. LANES: **7-10**, **17-20**: preincubation with ampicillin; lanes **7**, **17** 10 µg/ml; **8**, **18**, 5 µg/ml; **9**, **19**, 2 µg/ml; **10**, **20**, 1 µg/ml.

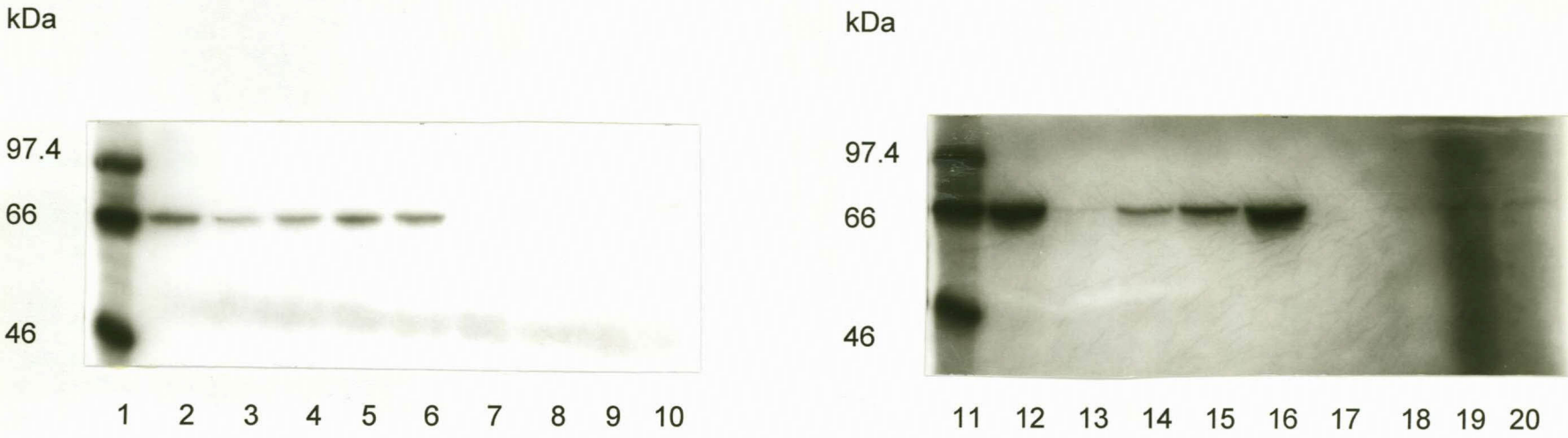


Figure 4.15b: Piperacillin and ampicillin competition studies on *Veillonella* strains V18 and V28. Lane **1**, MWM. LANES: **2-10**: strain V18 (piperacillin MIC >128 µg/ml, ampicillin MIC 1 µg/ml). Lane **11**, MWM. LANES: **12-20**: strain V28 (piperacillin MIC >128 µg/ml, ampicillin MIC 0.5 µg/ml). Whole cell lysates were incubated with dilutions of piperacillin or ampicillin and post labelled with 2 µCi [^{3}H]penicillin. Lanes **2**, **12**, no piperacillin or ampicillin added. LANES **3-6**, **13-16**: preincubation with piperacillin; lanes **3**, **13** 10 µg/ml; **4**, **14**, 5 µg/ml; **5**, **15**, 2 µg/ml; **6**, **16**, 1 µg/ml. LANES: **7-10**, **17-20**: preincubation with ampicillin; lanes **7**, **17** 10 µg/ml; **8**, **18**, 5 µg/ml; **9**, **19**, 2 µg/ml; **10**, **20**, 1 µg/ml.

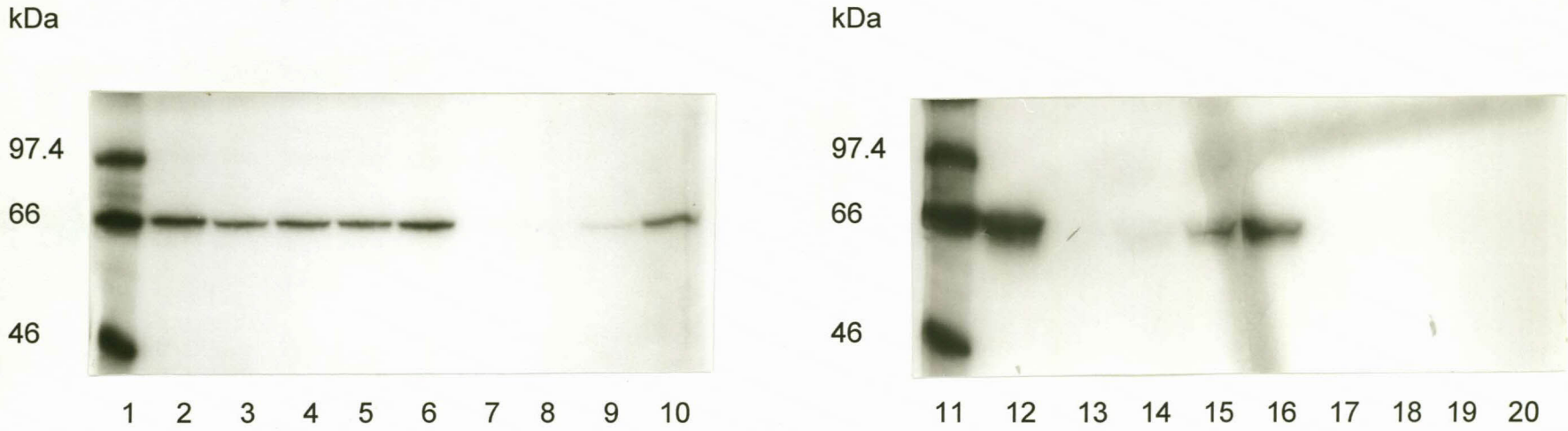


Figure 4.15c: Piperacillin and ampicillin competition studies on *Veillonella* strains V31 and V11. Lane **1**, MWM. LANES **2-10**: strain V31 (piperacillin MIC 64 µg/ml, ampicillin MIC 1 µg/ml). Lane **11**, MWM. Lanes **12-20**: strain V11 (piperacillin MIC 16 µg/ml, ampicillin MIC 0.25 µg/ml). Whole cell lysates were incubated with dilutions of piperacillin or ampicillin and post labelled with 2 µCi [^{3}H]penicillin. Lane **2**, **12**, no piperacillin or ampicillin added. LANES: **3-6**, **13-16**: preincubation with piperacillin; lanes **3**, **13** 10 µg/ml; **4**, **14**, 5 µg/ml; **5**, **15**, 2 µg/ml; **6**, **16**, 1 µg/ml. LANES: **7-10**, **17-20**: preincubation with ampicillin; lanes **7**, **17** 10 µg/ml; **8**, **18**, 5 µg/ml; **9**, **19**, 2 µg/ml; **10**, **20**, 1 µg/ml.

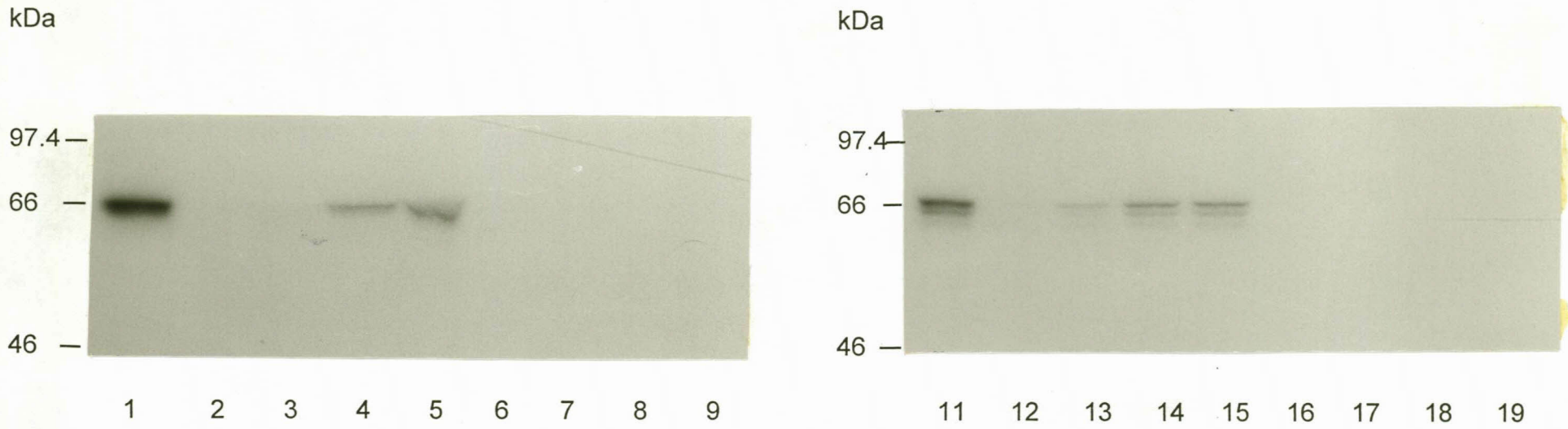


Figure 4.15d: Piperacillin and ampicillin competition studies on *Veillonella* strains V19 and V2. LANES: **1-9**: strain V19 (piperacillin MIC 16 µg/ml, ampicillin MIC 0.25 µg/ml). Lanes **10-18**: strain V2 (piperacillin MIC 0.5 µg/ml, ampicillin MIC 0.125 µg/ml). Whole cell lysates were incubated with dilutions of piperacillin or ampicillin and post labelled with 2 µCi [^{3}H]penicillin. Lane **1**, **10**, no piperacillin or ampicillin added. LANES: **2-5**, **11-14**: preincubation with piperacillin; lanes **2**, **11** 10 µg/ml; **3**, **12**, 5 µg/ml; **4**, **13**, 2 µg/ml; **5**, **14**, 1 µg/ml. LANES: **6-9**, **15-18**: preincubation with ampicillin; lanes **6**, **15** 10 µg/ml; **7**, **16**, 5 µg/ml; **8**, **17**, 2 µg/ml; **9**, **18**, 1 µg/ml.

Table 4.7: Interpretation of PBP competition studies performed on 8 *Veillonella* strains with piperacillin MICs ranging from 0.5 to >128 µg/ml.

Strain	MIC (µg/ml)		Extent of [^{3}H]penicillin binding	
	PIP	AMP	Prior exposure to PIP	Prior exposure to AMP
V2	0.5	0.125	++ 1-2 µg/ml, + 5-10 µg/ml	no binding at 1-10 µg/ml
V11	16	0.25	+++ 1-2 µg/ml, no binding at 5-10 µg/ml	no binding at 1-10 µg/ml
V19	16	0.25	+ 1-2 µg/ml, no binding at 5-10 µg/ml	no binding at 1-10 µg/ml
V31	64	1	+++ 1-10 µg/ml	+ 1-2 µg/ml, no binding at 5-10 µg/ml
V1	>128	4	+++ 1-10 µg/ml	+ 1-2 µg/ml, no binding at 5-10 µg/ml
V4	>128	2	+++ 1-10 µg/ml	+ 1-2 µg/ml, no binding at 5-10 µg/ml
V18	>128	1	+++ 1-10 µg/ml	+ 1-2 µg/ml, no binding at 5-10 µg/ml
V28	>128	0.5	+++ 1-2 µg/ml, ++ 5 µg/ml + 10 µg/ml	+ 1-2 µg/ml, no binding at 5-10 µg/ml

PIP = Piperacillin, AMP = Ampicillin

V2: susceptible to AMP and PIP

V11 and V19: susceptible to AMP, intermediate resistance to PIP.

V28: susceptible to AMP, resistant to PIP.

V1, V4, V18, V31: AMP MICs 1-4 µg/ml, PIP MICs 64->128 µg/ml.

CHAPTER 5

METRONIDAZOLE RESISTANCE

5.1 INTRODUCTION

Metronidazole is widely used for the treatment of anaerobic infections, often empirically, however, relatively little is known about its mode of action *in vivo.* Metronidazole is a nitroheterocyclic compound belonging to the 5-nitroimidazole group of antibiotics (Stratton, 1996). Although it has a wide spectrum of activity, including Gram-positive and Gram-negative bacteria, the efficacy of metronidazole is limited to micro-organisms with an anaerobic or at least microaerophilic metabolism, evidently indicating that it affects a biochemical reaction unique to anaerobiosis. The basis for the selective toxicity of metronidazole against anaerobes lies in the redox potential required for reduction of the nitro-group (Cederbrant, Kahlmeter & Ljungh, 1992).

Metronidazole resistance has been noted in strains of *B. fragilis* and *Peptostreptococcus* spp. (Wexler & Finegold, 1988; Pankuch, Jacobs & Appelbaum, 1993; Reysset, Haggoud & Sebald, 1993) as well as in *C. perfringens* (Edwards, 1993; Rasmussen, Bush & Tally, 1997), *C. clostridioforme* (Rolfe & Finegold, 1982) and *C. sordelli* (Spera, Kaplan & Allen, 1992). Reported resistance levels to metronidazole are thought to be grossly underestimated because of a general lack of reliable identification and susceptibility data. Metronidazole susceptibility determinations are hampered by multiple problems; the major problem causing inaccuracies pertains to mode of action, in that efficacy is dependant on strict anaerobiosis. Presumed incorrect resistance to metronidazole is abolished by "sufficient" anaerobiosis, indicating that resistance may be mediated through a decrease in the activation of anaerobic metabolic pathways under microaerophilic conditions (Smith & Edwards, 1995). The inhibitory effects of metronidazole and oxygen are competitive, it being suggested that oxygen is a more efficient electron

acceptor than metronidazole (Hamid *et al.*, 1997). Metronidazole is activated by accepting electrons from reduced ferredoxin as shown in Figure 5.1 (Edwards, 1993). Anaerobic organisms are thought to create a diversion from the normal fermentative pathway such that the activity of the pyruvate:ferredoxin oxidoreductase system is considerably decreased or abolished altogether, in this way causing resistance (Smith & Edwards, 1995). Antibiotic permeability studies on anaerobic bacteria have concentrated mainly on ß-lactam agents and the effect certain outer membrane proteins have on their uptake. Although reduced uptake has been a long proposed mechanism for metronidazole resistance development, no outer membrane protein has been identified that correlates with reduced permeability, neither has a metronidazole inactivating enzyme been identified in anaerobic bacteria.

From investigations on anaerobic bacteria two mechanisms of metronidazole resistance remain a) reduced pyruvate:ferredoxin oxidoreductase activity and b) reduced DNA interactions. Resistance genes have evaded conclusive identification (Müller, 1986; Edwards, 1993; Reysset, Haggoud & Sebald, 1993). Following conjugation experiments, three 5-nitroimidazole (*nim*) resistance genes have been sequenced and located on low-copy number mobilisable plasmids (Trinh & Reysset, 1996). These genes being *nimA* from *B. vulgatus*, *nimC* from *B. thetaiotaomicron* and *nimD* from *B. fragilis* (Roberts *et al.*, 1994; Trinh, Haggoud & Reysset, 1996; Trinh & Reysset, 1996). A fourth gene, *nimB*, has been mapped to the chromosome of a *B. fragilis* strain (Haggoud, Reysset & Sebald, 1992). The transfer of *nim* genes to susceptible recipients has been shown to increase metronidazole resistance and it is believed that the *nim* genes encode a 5-nitroimidazole reductase. The enzymatic reduction of 5-nitroimidazole antibiotics by *nim* gene products permits the cell to bypass toxic nitroradicals, thus avoiding DNA damage (Trinh & Reysset, 1998). A PCR-based method has been developed for the detection and screening of *nim* genes from *Bacteroides* spp. (Trinh & Reysset, 1996). Sequencing of the PCR products of the newly detected *nim* genes has begun and it is expected that the true diversity of these resistance genes among the *Bacteroides* species will be revealed (Trinh & Reysset, 1996).

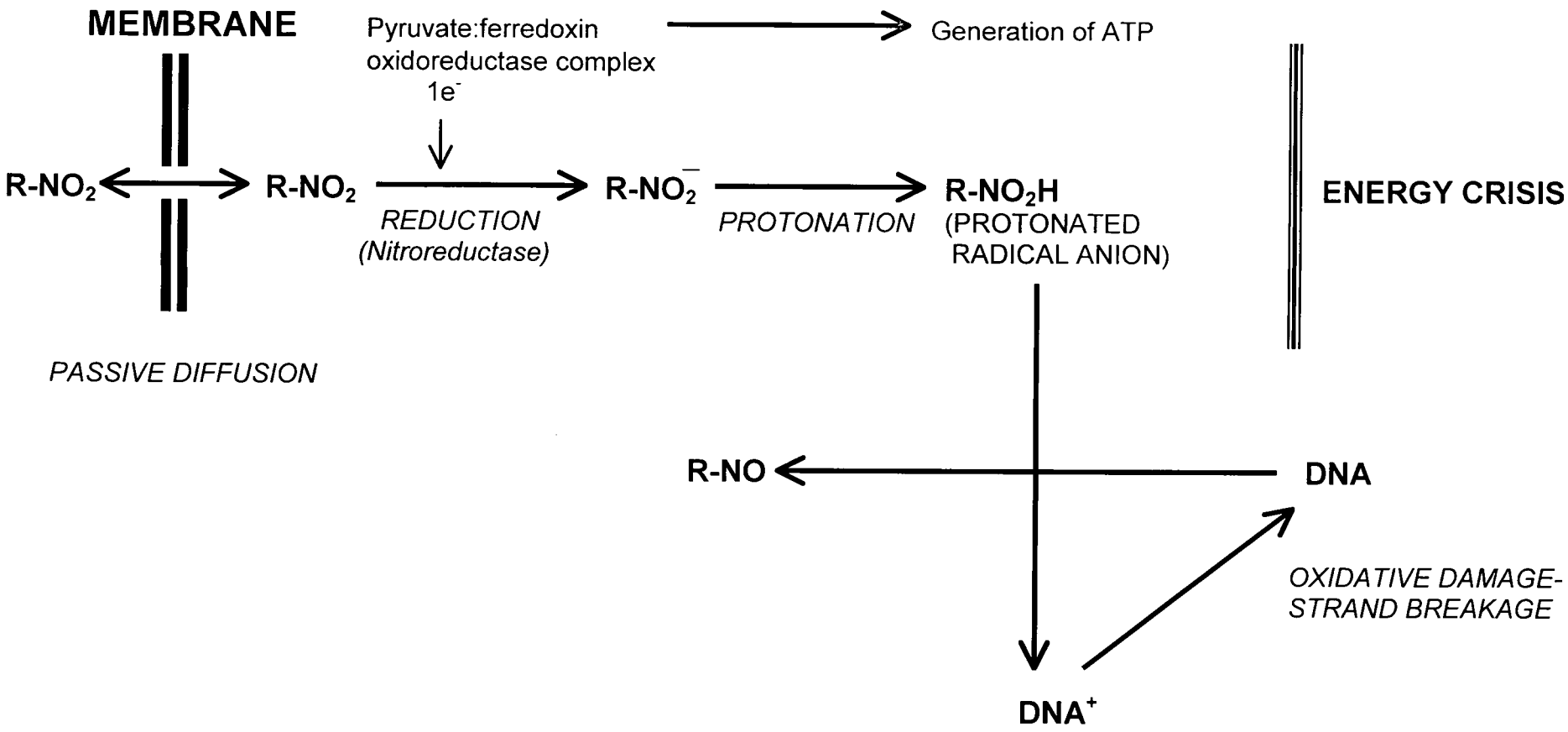


Figure 5.1: (From Figure 1.1) A simplified representation of the speculative pathway following proposed actions of metronidazole (Edwards, 1993).

Metronidazole is also used in combination therapy for infections caused by *Helicobacter pylori*, a major cause of peptic ulcer disease (Goodwin *et al.*, 1998). Resistance to metronidazole has consequently developed in *H. pylori* and investigations have revealed mutational inactivation of a *rdxA* gene that encodes an oxygen-insensitive NADPH nitroreductase, as a possible cause of resistance (Goodwin *et al.*, 1998; Jenks, Ferrero & Labigne, 1999). To date, because of the speculative and inconsistent nature of metronidazole resistance in both anaerobic bacteria (*nim* genes) and *H. pylori*, a microaerophilic pathogen (*rdxA* gene), the possibility of a similar mechanism of resistance (*rdxA* gene) developing or being acquired in anaerobes seemed to be of valid importance to investigate.

Accurate metronidazole susceptibility testing of anaerobic bacteria remains a challenge for clinical microbiology laboratories (Cormican, Erwin & Jones, 1996). Although resistance to metronidazole is not currently considered widespread, characterisation of proposed mechanisms of resistance are important in the elucidation of its mode of action and for the development of improved nitroimidazole analogs for clinical use (Dachs, Abratt & Woods, 1995).

The study was designed to optimise and correlate techniques that would assist in obtaining more reliable metronidazole resistance data and to investigate the role *nim/rdxA* genes and outer membrane proteins may have on resistance development.

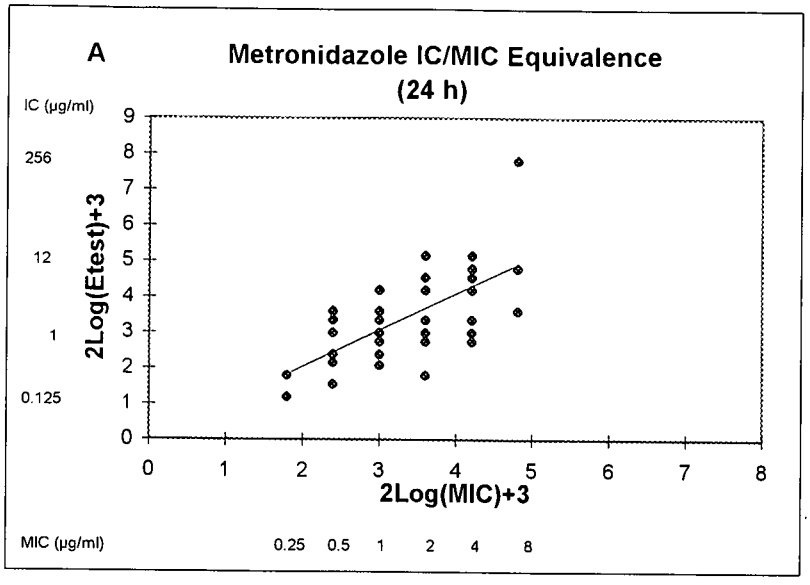
5.2 RESULTS AND DISCUSSION

5.2.1 Comparative metronidazole MIC and IC studies

Sixty-four strains comprising different species of *Peptostreptococcus* (18 strains), *Clostridium* (2 strains), *Propionibacterium* (8 strains), *Actinomyces* (17 strains), *Gemella* (1 strain), *Bacteroides* (11 strains) and *Prevotella* (7 strains) that exhibited metronidazole MICs ≥ 0.5 µg/ml were selected.

Regression analysis showed good correlation between agar dilution MIC and Etest inhibitory concentration (IC) values (Figure 5.2). A few deviations were noted with peptostreptococci as "resistant" colonies were seen within the inhibition ellipse of the Etest strip. However, such persisters were not recorded for MIC determinations, which could possibly be due to the inoculum prepared for MICs being lower than that of Etests. With the MIC cut off point of five colonies holding, MIC_{50} and MIC_{90} values of metronidazole for the 64 isolates were 1 µg/ml and 8 µg/ml respectively and 2 µg/ml and 8 µg/ml for the Etest. On comparing results obtained from the manufacturers' original lower range Etest strips (range 0.006 - 32 µg/ml) with the high range Etest strips (range 0.016 - 256 µg/ml) (Figure 5.3), the higher range was seen to be essential in order to accommodate some isolates with intermediate resistance (NCCLS intermediate breakpoint 16 µg/ml). Failure of the low range to accomodate these isolates and recommendations for high range at the time of the investigations were forwarded to AB BIODISK and so acknowledged (Claros & Rodloff, 1999). A facultative anaerobic *Propionibacterium* spp., as well as a *P. magnus* strain had MICs for metronidazole ≥128 µg/ml.

The metronidazole Etest compared favourably with the MICs providing a suitable, convenient alternative method for routine susceptibility testing of anaerobes. The Etest has equivalence with the MIC format in that a concentration is directly determined, when media, inocula and incubation conditions are standardised (Duerden, 1995).



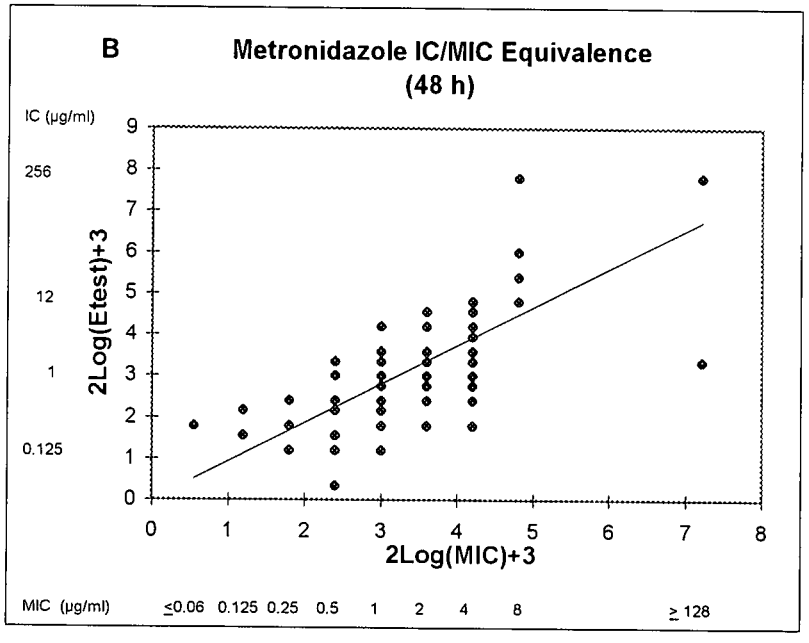


Figure 5.2: Regression analysis of Etest IC with MICs. **A**) Results recorded at 24 h, $r^2 = 0.47$; **B**) Results recorded at 48 h, $r^2 = 0.45$.

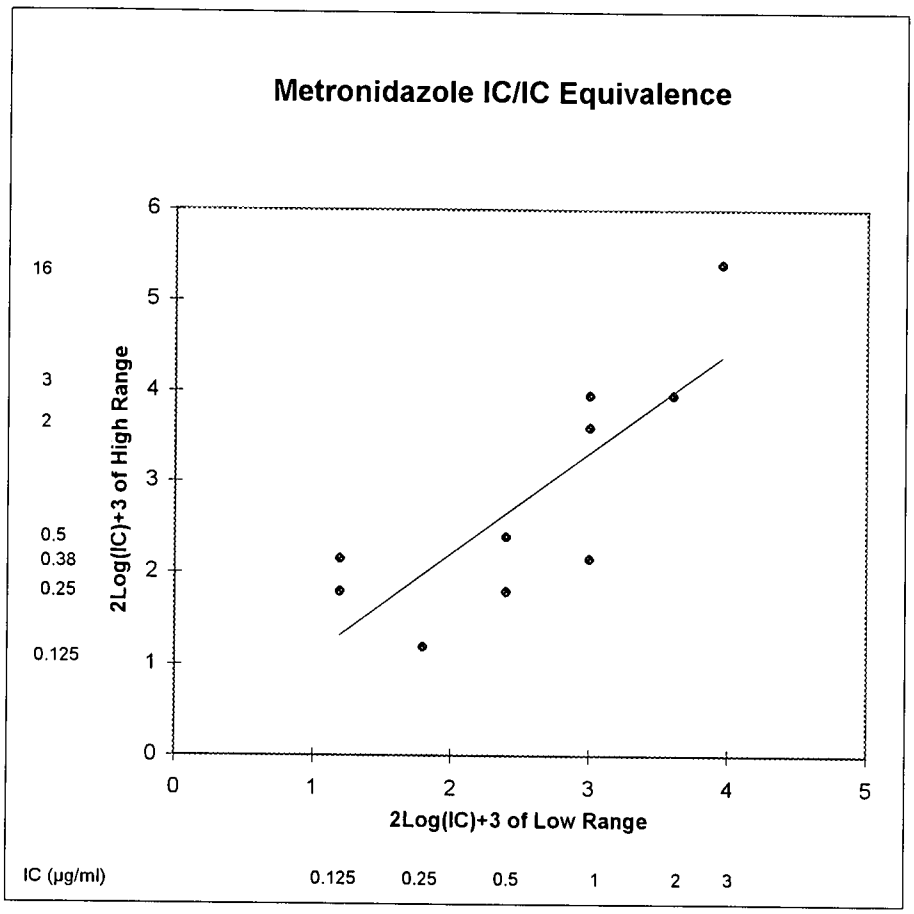


Figure 5.3: Comparison of Etest IC results obtained with the low range (0.006 - 32 µg/ml) and high range (0.016 - 256 µg/ml) metronidazole strips; $r^2 = 0.66$.

Most laboratories find the provision of diagnostic services for testing anaerobes very demanding as the agar dilution method is cumbersome and labour intensive (Citron, 1998). Disk susceptibility tests are regarded as unreliable for anaerobes other than those that are considered "fast growing" and are not sanctioned by the NCCLS (NCCLS, 1993; Duerden, 1995). The Etest is a simple agar diffusion method for susceptibility testing and holds promise for being accurate and flexible enough for use in most clinical laboratories (Sanchez & Jones, 1992; Rosenblatt & Gustafson, 1995). The Etest has recently become the candidate method for concurrent, accurate testing of anaerobes (Sanchez & Jones, 1992). As resistance to metronidazole appears sporadically in a number of different anaerobic bacterial species, the importance of the rapid Etest method for routine metronidazole susceptibility testing can not be overemphasised.

5.2.2 *Outer membrane protein analysis of Peptostreptococci and Veillonella strains*

A *P. prevotii* (Ps118) and a *Veillonella* spp. (V4) strain, both with metronidazole MICs of 4 µg/ml (parental strains), were selected for inducing resistance to metronidazole. The isolation of mutant strains exhibiting a two-fold increase in metronidazole MIC (8 µg/ml) from both parental strains resulted after exposure to increasing concentrations of metronidazole. None of these four isolates were shown to contain a *nim* gene (Table 5.1). A loss of seven outer membrane proteins (approximately 33, 34, 48, 51, 65, 68 and 71 kDa) and an additional two outer membrane proteins (32 and 133 kDa) were seen on comparing the parental strain of *Peptostreptococcus* with the mutant strain (Figure 5.4). A loss of six membrane proteins, three from the outer membrane preparation (38, 39 and 54 kDa) and three from the inner membrane preparation (42, 43 and 53 kDa), but an additional four membrane proteins, two from the outer membrane preparation (50 and 68 kDa) and two from the inner membrane preparation (41 and 68 kDa) were seen on comparing the parental and mutant *Veillonella* strains (Figure 5.4). The finding of multiple membrane protein alterations between parental and mutant strains that exhibited only a two-fold increase in MIC appears excessive and demonstrates complexities experienced in trying to pinpoint metronidazole resistance development.

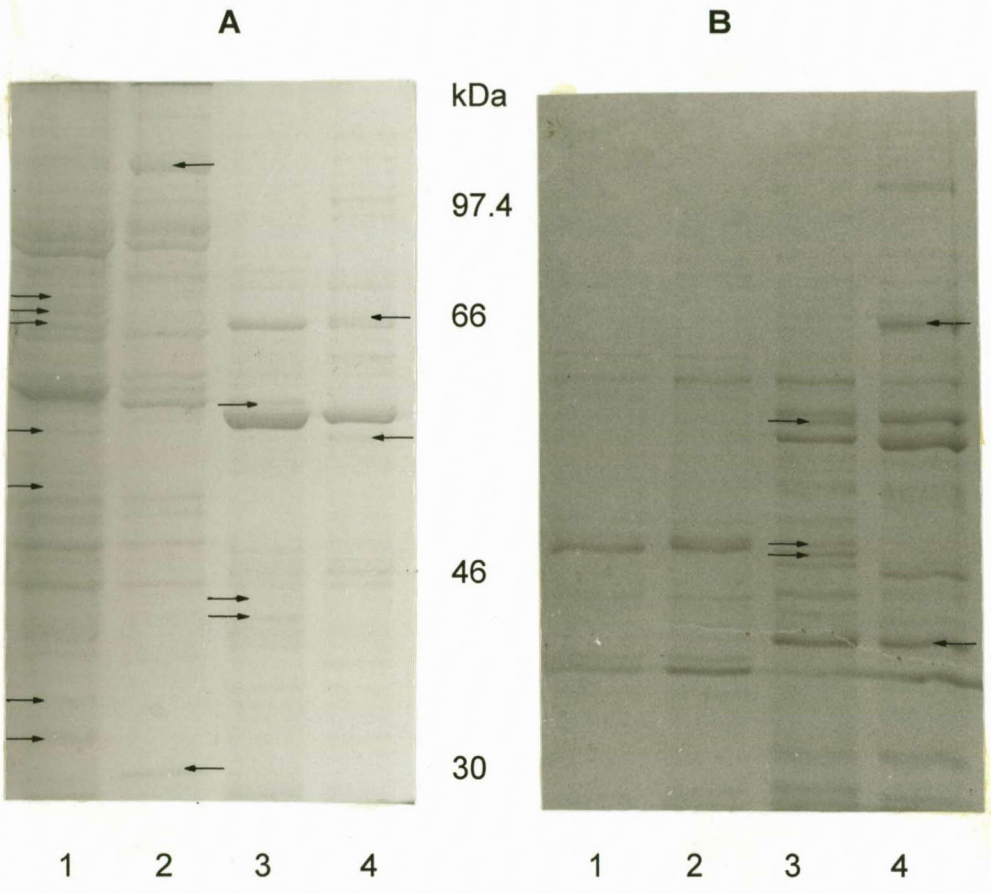


Figure 5.4: Membrane protein comparisons of parental and mutant strains of *P. prevotii* and *Veillonella* sp. **A**) Outer membrane proteins, **B**) Inner membrane proteins. Lanes: **1**, *P. prevotii* parent (Ps118, MIC 4 µg/ml); **2**, *P. prevotii* mutant (Ps118, MIC 8 µg/ml); **3**, *Veillonella* sp. parent (V4, MIC 4 µg/ml); **4**, *Veillonella* sp. mutant (V4, MIC 8 µg/ml).

5.2.3 Detection of rdxA genes

Sixteen strains exhibiting metronidazole MICs ≥ 1 µg/ml were examined for the presence of *rdxA* genes. Strains comprised six *B. fragilis*, one *B.* vulgatus, one *P. bivia*, one *C. bifermentans*, one *P. magnus,* five *Propionibacterium* spp. and one *A odontolyticus* strain. PCR products of approximate predicted *rdxA* gene size (937 bp) using Mtz6EF and MtzRBgl primers were found in five isolates; one *B. fragilis* B87, three *Propionibacterium* spp. Pr1, Pr45, Pr53 and one *A. odontolyticus* A10 strain (Figure 5.5a). Using a second *rdxA* gene primer pair *rdxA* 3 and *rdxA* 5, products of approximate predicted size (491 bp) were found in five isolates, two *B. fragilis* BF8, B50 and three *Propionibacterium* spp. strains, Pr1, Pr45, Pr53 (Figure 5.5b). However, on sequencing the above PCR products and comparing sequences with those published (Goodwin *et al.*, 1998), there were no similarities to indicate the presence of the *rdxA* gene in the isolates investigated.

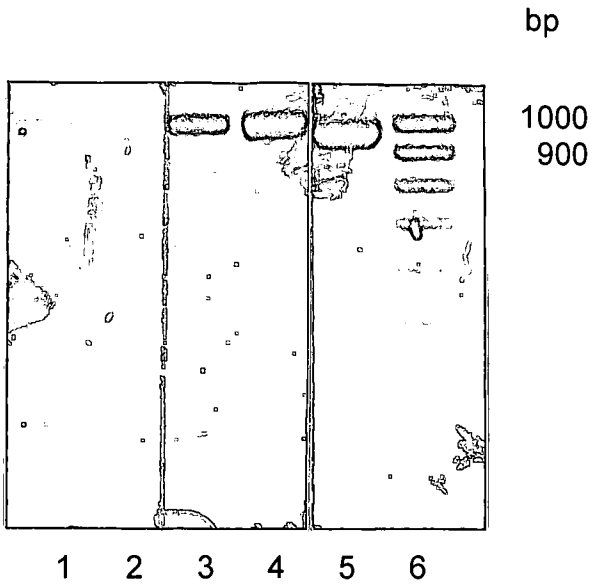


Figure 5.5a: PCR products (900 - 1000 bp) of five isolates considered as possibly harbouring a *rdxA* gene after amplification using *Mtz6EF* and *MtzRBgl* primers. Metronidazole MICs (µg/ml) are given in brackets. Lanes **1**, B85 *B. fragilis* (4); **2**, Pr53 *Propionibacterium* sp. (>128); **3**, Pr1 *Propionibacterium* sp. (>128); **4**, Pr45 *Propionibacterium* spp. (>128); **5**, A10, *A. odontolyticus* (>128); **6**, MWM.

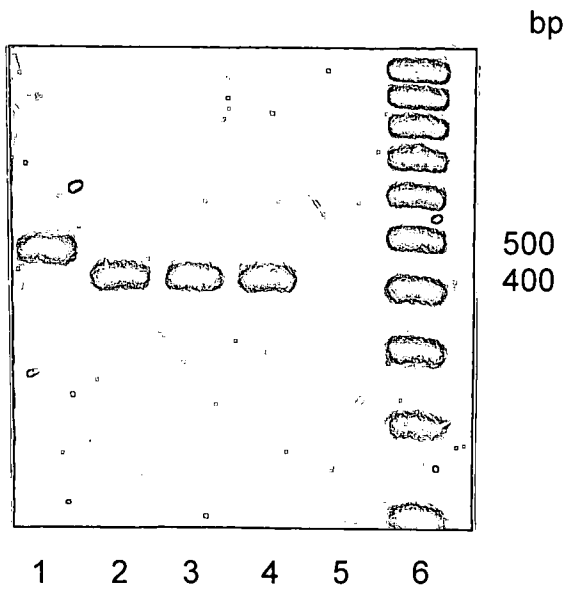


Figure 5.5b: PCR products (400 - 500 bp) of four isolates considered as possibly harbouring a *rdxA* gene after amplification using *rdxA* 3 and *rdxA* 5 primers. Metronidazole MICs (µg/ml) are given in brackets. Lanes **1**, B50 *B. fragilis* (0.5); **2**, Pr1 *Propionibacterium* sp. (>128); **3**, Pr45 *Propionibacterium* sp. (>128); **4**, Pr53 *Propionibacterium* sp. (>128); **5**, BF8, *B. fragilis* (*nim* gene control strain); **6**, MWM.

5.2.4 Detection and sequencing of nim genes

The current study expanded the spectrum of bacteria subjected to *nim* gene screening by including a wide variety of anaerobic/facultative anaerobic species. PCR products indicative of *nim* genes (458 bp) were produced in 22/64 strains at an annealing temperature of 52°C (Table 5.1). Amplified products of approximately 458 bp from seven strains were only demonstrated employing an annealing temperature of 52°C, due either to non-specific binding or non-complementary primer base pairing. Although amplified fragments of the predicted *nim* gene size were distributed across a number of diverse species, a high proportion of products with respect to number/genus was seen in propionibacteria (Table 5.1). Thirteen PCR products were obtained at 62°C and of sufficient quantity to enable purification and sequencing to be performed (Figure 5.6). An additional PCR product from a *P. magnus* strain, obtained at 52°C, was also sequenced. The sequence of nucleotides 31-410 was determined, the NIM-3 primer being situated at position 1-26 and NIM-5 primer at position 436-458 (Trinh & Reysset, 1996). *NimA* genes were identified in five *Propionibacterium* spp., one *A. odontolyticus*, one *C. bifermentans* and one *P. bivia* strain, while *nimB* genes were identified in five isolates of *B. fragilis* and one *P. magnus* strain (Table 5.1). *Nim* gene PCR products from three *Propionibacterium* spp. showed 100% nucleotide identity with the *nimA* gene, while those from three *B. fragilis* strains were 100% identical to the *nimB* gene. Minor nucleotide variations for the remaining strains are shown in Figure 5.7 and percent identity recorded in Table 5.1. The DNA sequences of *nimA* and *nimB* genes diverge by 30% and are presumed to represent two classes of genes that confer resistance via the same mechanism (Rasmussen, Bush & Tally, 1997).

The *nim* genes identified in the South African isolates exhibit a high degree of conformity in that sequence divergence was < 4% from their respective *nimA* or *nimB* gene sequences. The finding of a *nim* gene in a *P. magnus* strain is of major importance as *P. magnus* is by far the most common species and probably the most pathogenic of Gram-positive anaerobic cocci in human clinical specimens. Two studies and numerous case reports have documented *P. magnus* isolation in pure culture from a variety of anatomical sites (Murdoch, 1998).

Table 5.1: MICs of metronidazole and *nim* gene screening of the 64 anaerobic/facultative anaerobic bacterial species.

Strain (No. strains)	MIC (µg/ml)	Presumptive *nim* genes		*nim* gene (% identity)
		52°C	62°C	
Ps. anaerobius (2)	2-4	+	-	
Ps. anaerobius (7)	1-4	-		
Ps. magnus (1)	>128	+		*nimB* (99.5)
Ps. magnus (3)	0.5-128	-		
Ps. prevotii (2)	4	-		
Ps. micros (1)	4	-		
Ps. asaccharolyticus (2)	2-32	-		
C. innocuum (1)	1	-		
C. bifermentans (1)	1	+	+	*nimA* (99)
Pr. acnes (3)	>128	+	+	*nimA* (99.7; 100; 100)
Pr. acnes (2)	>128	+	weak	
Propionibacterium sp. (2)	>128	+	+	*nimA* (99; 100)
Propionibacterium sp. (1)	>128	+	-	
A. odontolyticus (1)	>128	+	+	*nimA* (98)
A. odontolyticus (2)	>128	+	-	
A. odontolyticus (14)	>128	-		
Gemella sp. (1)	0.5	-		
B. fragilis (4)	1-2	+	+	*nimB* (99.5; 99.5; 100; 100)
B. fragilis (1)	8	+	+	*nimB* (100)
B. fragilis (3)	1	-		
B. vulgatus (1)	0.5	-		
B. capillosus (1)	0.5	-		
Bacteroides sp. (1)	2	-		
P. bivia (1)	2	+	+	*nimA* (97)
P. bivia (1)	1	+	-	
P. bivia (1)	4	+	-	
P. intermedia (1)	2	-		
P. disiens (1)	1	-		
P. loescheii (1)	0.5	-		
Prevotella sp. (1)	1	-		

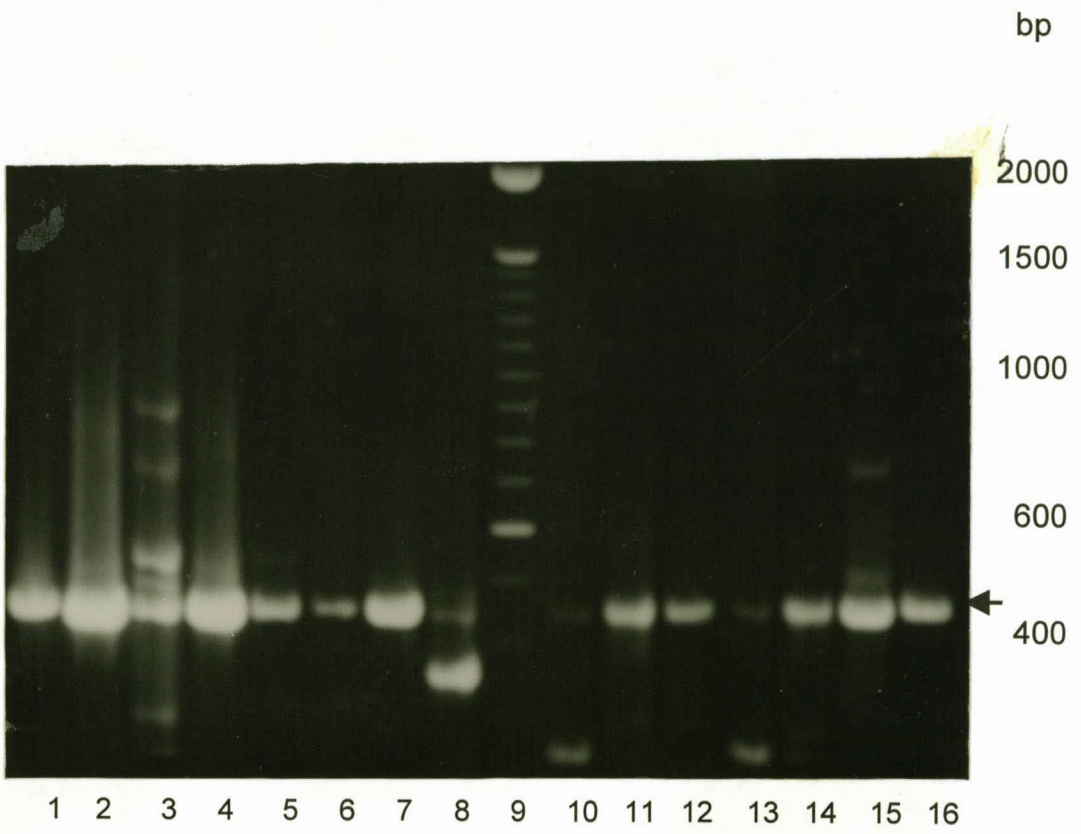


Figure 5.6: PCR products (approximately 458 bp) of 13 isolates obtained with an annealing temperature of 62°C. Lanes **1**, *B. fragilis* BF-8 (*nimB* control); **2**, *B. vulgatus* BV-17 (*nimA* control); **3**, *Propionibacterium* sp.; **4-5**, *Pr. acnes*; **6**, *Propionibacterium* sp.; **7**, *A. odontolyticus*; **8**, *B. fragilis*; **9**, DNA molecular weight marker; **10-11**, *B. fragilis*; **12**, *P. bivia*; **13-14** *B. fragilis*; **15**, *Pr. acnes* and **16**, *C. bifermentans*.

	Nucleotide																
	60	75	76	180	234	242	246	253	264	302	318	327	332	341	344	354	375	399
nimA	**A**	**C**	**G**	**G**	**G**	**G**	**A**	**G**	**A**	**G**	**G**	**G**	**C**	**T**	**G**	**G**	**G**	**G**
C. bifermentans (strain C69)	-	-	-	-	A	-	-	-	-	A	A	-	-	-	-	-	T	-
Pr. acnes (strain Pr5)	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-
Propionibacterium sp. (strain Pr53)	-	-	-	-	A	A	-	-	-	-	A	A	-	-	-	-	-	-
A. odontolyticus (strain A10)	-	G	-	T	A	A	-	T	-	A	A	-	-	-	-	-	-	-
P. bivia (strain P11)	C	-	A	-	-	A	C	-	C	-	-	A	T	G	T	C	T	A

	Nucleotide			
	27	306	365	373
nimB	**G**	**A**	**A**	**G**
B. fragilis (strain B97)	-	-	C	T
B. fragilis (strain B131)	-	C	C	-
P. magnus (strain Ps104)	A	-	-	-

Figure 5.7: Nucleotide diversity of *nimA* and *nimB* genes of the eight strains exhibiting alterations from those of the documented *nimA* and *nimB* gene sequences (Trinh & Reysset, 1996). Sites at which the same nucleotide is present are not shown.

Haggoud *et al.*, (1994) on investigating the hydrophobicity profiles of *nim* gene products have excluded a membrane location for the proteins and subsequent decreased penetration of metronidazole into the cells. Recently it has been proposed that the *nim* genes encode a 5-nitroimidazole reductase that converts 5-nitroimidazole to its non-toxic amino derivative, thus preventing the accumulation of toxic nitroradicals that would otherwise cause DNA damage (Carlier *et al.*, 1997; Trinh & Reysset, 1998). As facultative anaerobes are intrinsically resistant to metronidazole (MIC >128 µg/ml) it was not possible to relate the presence of *nim* genes in the propionibacteria to metronidazole MICs. No direct correlation was evident between the presence of *nim* genes and decreased susceptibility to metronidazole in the remaining anaerobic bacteria investigated (Table 5.1). As the presence of a *nim* gene did not always correspond to a dramatically increased metronidazole MIC it is possible that 1) *nim* genes may be prerequisites for the development of higher levels of resistance, or 2) silent *nim* genes are present (G. Reysset personal communication).

Plasmids were only demonstrated from two of the 14 anaerobic species shown to contain *nim* genes (Fig 5.8). Lysis was effective for all strains that underwent plasmid analysis, as residual chromosomal DNA was evident. The two isolates carrying plasmids were: 1) *B. fragilis* strain B131, containing two plasmids, 4.3 kb and < 2 kb, and 2) *C. bifermentans* isolate C69, a plasmid of 4.8 kb and a second plasmid between >16.2 - < 39 kb. Even though the four plasmids were of different sizes to those previously described as carrying *nim* genes (*nimA*, 7.7 kb plasmid; *nimC*, 10 kb plasmid, and *nimD*, 7.3 kb of Reysset, Haggoud & Sebald, 1992; Trinh & Reysset, 1996), possible plasmid location can not as yet be excluded in these two strains. The majority of South African isolates (12/14 strains), as with *Bacteroides* species isolated in France (21/33 strains) (Reysset, Haggoud & Sebald, 1993), were devoid of plasmids. A *nim* gene association with transposons or integrons has been suggested as homo- and hetero-specific transfer of chromosomal-borne *nim* genes has been performed *in vitro* (Trinh & Reysset, 1998).

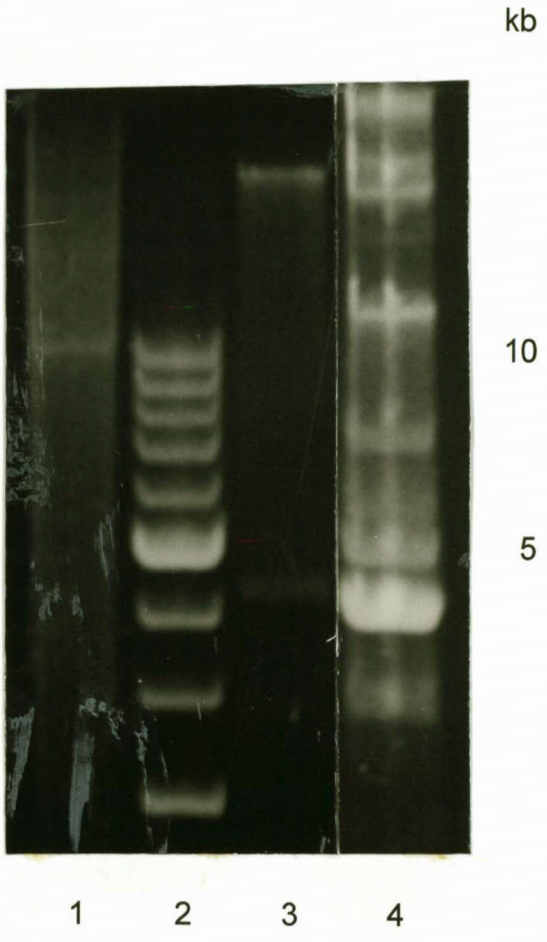


Figure 5.8: Plasmid profiles of *B. fragilis* (B131) and *C. bifermentans* (C69): Lanes: **1**, *C. bifermentans* (C69); **2**, Plasmid size marker, **3**, *B. fragilis* (B131); **4**, Control *Neisseria gonorrhoeae* strain plasmid 39 bp.

NimA and *NimB* genes have been localised both to the chromosome and various plasmids (7.7 - 10 kb) (Reysset, Haggoud & Sebald, 1993; Trinh & Reysset, 1996). Although there has been a report of a nitroimidazole resistance determinant carried on a 7.7 kb plasmid that was transferred by conjugation (Edwards, 1993), plasmids shown to harbour *nim* genes have only been shown to be transformable by electrotransformation (Reysset, Haggoud & Sebald, 1993). The expression of identified *nim* genes to date is due to the integration of an insertion sequence (IS) upstream of the resistance gene (Reysset *et al.*, 1992; Reysset, Haggoud & Sebald, 1993). Salyers & Shoemaker (1996) have suggested that since IS elements capable of activating resistance genes are widespread in *Bacteroides* species, further increases in promoter mutations can lead to increased expression. Clearly, reports of metronidazole-resistant anaerobes/facultative anaerobes should be taken seriously and active screening for such strains be instituted in diagnostic laboratories.

Although the full relevance of *nim* genes in the development of metronidazole resistance requires further investigation, the present study has shown their presence in a variety of anaerobes/facultative anaerobes. The origin of *nim* genes is unknown. However, the predominance of *nimA* genes in propionibacteria which are facultative anaerobes, may indicate a transferable *nimA* gene source in anaerobic environments.

GENERAL DISCUSSION

This is a summary of investigations into the prevalence of resistance development to antibiotics in current therapeutic regimens for anaerobic bacteria. The development of resistance is related to previously described resistance mechanisms and possible explanations for problematic treatment of anaerobic infections in South African hospitals are proposed.

MICs of 18 antimicrobial agents against 378 clinical isolates of anaerobic bacteria revealed several crucial areas of resistance development. Although reduced susceptibility to penicillin (MICs $\geq$ 1 µg/ml) was evident in 20 *Peptostreptococcus* strains and seven non-perfringens *Clostridium* species, resistance to penicillin appears to be declining, which could possibly be due to the retraction of treatment with penicillin as first choice for *Neisseria gonorrhoeae, Streptococcus pneumoniae* and nosocomial infections in South African hospitals. ß-Lactamases hydrolysing penicillins and cephalosporins were demonstrated in all *Bacteroides* and *Prevotella* isolates, but amoxicillin in combination with clavulanic acid (4 µg/ml) was effective against 30/31 of these isolates, with a *B. fragilis* strain maintaining intermediate resistance (8 µg/ml). Piperacillin exhibited good activity against the Gram-positive isolates with only eight strains of *Peptostreptococcus* spp. being resistant (MICs $\geq$ 64 µg/ml). However, 41% *Bacteroides* spp. were resistant to piperacillin and selective ß-lactam resistance to piperacillin observed in 68% *Veillonella* spp.

Of all the anaerobes investigated, 91% were susceptible to cefoxitin, but cefoxitin revealed better activity against Gram-positive than Gram-negative bacteria with only three *Clostridium* spp. resistant. Decreased activity to cefoxitin was noted for all *Bacteroides* and most *Fusobacterium* spp. (except *F. nucleatum*), susceptibility ranging from 57-68%, with *Prevotella, Porphyromonas* and *Veillonella* spp. tending to be more susceptible (82-100%). Except for ten non-perfringens *Clostridium* isolates, all Gram-positive anaerobes were susceptible to cefepime and cefpirome. This was not the case for the Gram-negative bacteria, in particular the *Bacteroides* spp. with susceptibility to cefepime < 50% and to cefpirome < 30%, and *Prevotella* spp. susceptibility to cefpirome 30-67% and to cefepime 55-78%. The majority of Gram-positive anaerobic strains investigated were susceptible to imipenem/ meropenem,

but among the Gram-negative anaerobes, high-level resistance to imipenem/ meropenem was seen for 13/37 (35%) *Fusobacterium* spp.

Three *C. perfringens* strains showed reduced metronidazole MICs (4-8 µg/ml), being two dilutions from the susceptibility breakpoint. Two *P. magnus* strains (MIC >128 µg/ml) and two *Prevotella* strains (MIC 32 µg/ml) were resistant to metronidazole. Of all the anaerobes investigated, 85% were susceptible to clindamycin, although only 57% *Bacteroides* spp. other than *B. fragilis* were susceptible. Dalfopristin/quinupristin exhibited excellent activity throughout the Gram-positive bacterial spectrum, except for one *Peptostreptococcus* sp. that showed reduced susceptibility (MIC 8 µg/ml). Poor activity was, however, seen against the Gram-negative bacteria, susceptibility ranging from 35% for *B. fragilis* group isolates to 39% for *P. loescheii* and only 3% of the *Veillonella* spp. susceptible. Trovafloxacin was effective against the majority of Gram-positive anaerobic bacteria with the exception of two *P. anaerobius* strains. Trovafloxacin demonstrated superior activity to ciprofloxacin against the Gram-negative isolates examined with only three *B. fragilis*, one *P. loescheii* and a *F. varium* strain showing MICs of trovafloxacin above the susceptibility breakpoint. Chloramphenicol was the most effective antibiotic tested with only two *Clostridium* spp. being resistant (MICs 16 µg/ml). MICs indicated overall susceptibility of Gram-positive anaerobic isolates to be higher than for the Gram-negative isolates.

Anaerobic susceptibility testing remains controversial, but should be considered under clinical circumstances in which decisions regarding the selection of antimicrobial agents are critical. Current knowledge of antibiotic resistance progression in anaerobic bacteria isolated in South Africa is limited, but the present study has emphasised the requirement of periodic susceptibility testing of anaerobic bacteria in conjunction with speciation as they can no longer be considered universally susceptible to antimicrobial agents. Of major clinical importance in the South African setting are anaerobic bacteria isolated from normally sterile sites, especially the brain, liver, lungs and blood. Forty trauma related injuries were noted with a variety of anaerobes identified from gunshot and stab wounds as well as bone and fracture-related infections. Current therapeutic regimens for central nervous system infections include chloramphenicol, all the strains isolated from brain abscesses being susceptible. For infections of unknown aetiology, empirical therapy

in Bloemfontein state hospitals would be clindamycin, cefoxitin or amoxicillin/ clavulanic acid. For treatment of mixed aerobe/anaerobe infections, the current trend is towards monotherapy. The results indicate antimicrobial agents such as the carbapenems (imipenem/meropenem) and quinolones as being of value for the treatment of polymicrobial infections. Despite reduced efficacy against some Gram-negative isolates (*Bacteroides* spp., *Prevotella loescheii*, *F. mortiferum*, *F. varium* and *Veillonella* spp.), dalf/quin proved to be excellent against the Gram-positive anaerobic bacteria and could be considered for therapy in mixed aerobe/anaerobe Gram-positive bacterial infections. In the present study, as in a previous study conducted in Bloemfontein, the non-perfringens clostridia and peptostreptococci were identified as requiring specific diagnostic attention regarding susceptibility testing. Additionally the current findings also recognise *Veillonella* and fusobacteria as being problem genera concerning resistance to ß-lactam/carbapenem antimicrobial agents.

The finding of high-level resistance to imipenem in 35% *Fusobacterium* spp. led to investigations into the production of carbapenemases/metallo-ß-lactamases. A bioassay indicated absence of enzymatic action against imipenem in the *Fusobacterium* isolates and none of the *Fusobacterium* isolates showed any indication of the presence of the *cfiA*, or *cphA* genes. *Bacteroides* spp. and *Prevotella loescheii* strains subjected to carbapenemase/metallo-ß-lactamase investigations did not reveal the full characteristics of metallo-ß-lactamase activities, but low levels of a carbapenem hydrolysing enzyme appeared to be present in three *B. fragilis* (B82, B97 and B121) and two *P. loescheii* strains (B71 & B54). PCR amplification revealed products of predicted size of the *cfiA* gene in two strains of *B. vulgatus* (B39 & B64), *a B. capillosus* (B38) and a *P. loescheii* (B71) strain, while products of predicted size of the *cphA* gene were seen in three *B. fragilis* strains (B82, B97 & B121), and a *P. loescheii* strain (B54). Sequencing could not confirm the presence of either gene in any of the isolates. No PCR products of predicted bla_{IMP} size were found in any of the *Bacteroides, Prevotella* or *Fusobacterium* strains investigated. Although screening for *cfiA, cphA* and bla_{IMP} genes in selected anaerobic bacteria failed to reveal definite results, early recognition of metallo-ß-lactamase-producing strains is imperative as susceptible clinical strains can convert to high-level ß-lactam/carbapenem resistance by simply increasing the level of expression of a "nearly" silent metallo-ß-lactamase gene (Rasmussen *et al.*, 1994). The *cfiA* gene status is considered to be a more reliable indicator of the emergence

of problematic strains than susceptibility testing and periodical screening, even in metallo-ß-lactamase negative strains, should be considered.

Investigations on penicillin-binding proteins as being implicated in resistance development to imipenem in fusobacteria were more indicative. Greatest penicillin-binding was seen for the low-molecular-weight PBPs (43 and 32 kDa) and the lowest-binding/affinity PBPs identified as PBPs 74, 66 and 62 kDa. Reduced imipenem PBP affinity was noted for the highest-molecular-weight PBP (74 kDa). Low affinity PBPs that appear to be involved in imipenem resistance are 74, 62 & 58 kDa. A resistant variant revealed the production of a PBP (±69 kDa) that was absent from the parental strain. Such an appearance of an extra PBP may be correlated with decreased susceptibility to ß-lactam antibiotics as has been reported by Tunér *et al.* (1996). The role of *Fusobacterium* spp. in infections of children has become increasingly important because of resistance development. Accurate identification of fusobacteria is of importance not only for taxonomic reasons but also for empiric treatment regimens. On comparing PBP profiles of 12 *Fusobacterium* strains seven major PBP groups could be identified and correlation with Rapid ID32A identification revealed the Rapid ID32A system as not being totally reliable in species identification of *Fusobacterium* spp.

PBP profiles were seen to distinguish species/subspecies of *Clostridium* isolates. PBP profiles of the *Clostridium* spp. other than *C. perfringens* were in no way similar to those of *C. perfringens. C. sordelli* and *C. tertium* identification by the Rapid ID32A system showed some correlation with PBP profiles, but not in most of the other *Clostridium* species identified, emphasising that commercial identification systems available for species identification of *Clostridium* spp. may not be as definitive as they should. PBP profiling may well be used as a control reference in the validity of identification results and may assist in the differentiation of *Clostridium* species. Varied results were found in PBP affinities for penicillin obtained from only four isolates, contributing to the problem of making reliable predictions for resistance development in the clostridia.

PBP profiles of *Veillonella* spp. assigned nine strains into three groups of species/ subspecies. The major *Veillonella* PBP that possessed the highest penicillin affinity was not a low-molecular-weight PBP (< 60 kDa) as is commonly observed in other

bacteria. Selective resistance to piperacillin was found in 21/31 *Veillonella* isolates, all of which were ß-lactamase negative, ruling out the production of a ß-lactamase directed against piperacillin. It is unusual that the affinities of different penicillins are focused on one PBP, yet the PBP (66 kDa) showing the greatest affinities for penicillin and ampicillin, exhibited the lowest affinity for piperacillin and may well be implicated in selective mutants with piperacillin MICs exceeding 128 µg/ml. Studies on resistance development to ß-lactam antibiotics in anaerobic bacteria have focused on the production of ß-lactamase enzymes, yet some interesting alternatives have emerged from this study.

Resistance to metronidazole appears sporadically in a number of different anaerobic bacterial species and the importance of a rapid and reliable method for routine metronidazole susceptibility testing is becoming imperative. In a regression analysis between agar dilution MIC and Etest inhibitory concentration (IC) values for metronidazole, the Etest compared favourably with the MICs providing a suitable, convenient alternate method for routine susceptibility testing of anaerobes. On comparing results obtained from the manufacturers' original lower range Etest strips (range 0.006 - 32 µg/ml) with the high range Etest strips (range 0.016 - 256 µg/ml) the higher range was seen to be essential to accommodate some isolates with intermediate resistance (16 µg/ml).

A comparison between membrane proteins of metronidazole susceptible parent and intermediate-resistant mutants of a *Veillonella* spp. and a *P. prevotii* strain revealed several alterations in the membrane protein profiles in both strains. These multiple membrane protein alterations between parental and mutant strains that exhibited only a two-fold increase in MIC appear excessive and demonstrate complexities experienced in trying to pinpoint metronidazole resistance development in anaerobic bacteria.

PCR amplification of 16 anaerobic strains with metronidazole MICs ≥ 1 µg/ml revealed products of predicted size of *rdxA* genes in seven strains, comprising three *Propionibacterium* spp., three *B. fragilis* strains and an *A. odontolyticus* strain. All three *Propionibacterium* strains were presumptive positive by amplification with two different sets of primers used. Sequencing of the PCR products and comparing

sequences with published data excluded the presence of the *rdxA* gene, emphasising the importance of sequence confirmation.

PCR products indicative of *nim* genes (458 bp) were produced in 22/64 strains at an annealing temperature of 52°C, and in 13 of the 22 strains at an annealing temperature of 62°C. Sequencing of the 13 PCR products from 62°C and a product obtained at 52°C, identified *nimA* genes in five *Propionibacterium* spp., one *A. odontolyticus*, one *C. bifermentans* and one *P. bivia* strain, while *nimB* genes were identified in five isolates of *B. fragilis* and one *P. magnus* strain. The *nim* genes identified in the South African isolates exhibit a high degree of conformity as sequence divergence was < 4%. Three of the *B. fragilis* strains harbouring a *nimB*, the control *B. fragilis* strain (BF8), as well as three of the *Propionibacterium* spp. harbouring a *nimA* gene, possibly produced the *rdxA* gene. No direct correlation was evident between the presence of *nim* genes and decreased susceptibility to metronidazole. As facultative anaerobes are intrinsically resistant to metronidazole (MIC >128 µg/ml), it was not possible to relate the presence of *nim* genes in the propionibacteria to metronidazole MICs. However, it is possible that *nim* genes may be prerequisites with which higher levels of resistance can be attained and the possibility of silent *nim* genes can not be excluded (G. Reysset personal communication).

The present study has shown the presence of *nim* genes in a variety of anaerobes/ facultative anaerobes and although the origin of *nim* genes is unknown, the predominance of *nimA* genes in propionibacteria which are facultative anaerobes, may indicate a transferable *nimA* gene source in anaerobic environments. Metronidazole-resistance genes may well be in an early stage of an evolutionary process and reports of metronidazole-resistance strains should be taken seriously. The study has confirmed that it is crucial to develop an efficient and effective metronidazole resistance diagnostic screening procedure as proposed by Trinh and Reysset (1996) for *nim* genes and to determine the dissemination potential of such resistance genes.

Effective treatment of anaerobic bacteria depends on the knowledge of the species involved and susceptibility data, as emerging antimicrobial resistance is ever present. In this study it was evident that resistance development among the anaerobic

bacteria in this country may be underestimated, emphasising the need for susceptibility monitoring and further in depth studies on the anaerobic bacteria to be prioritised in South Africa.

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APPENDIX

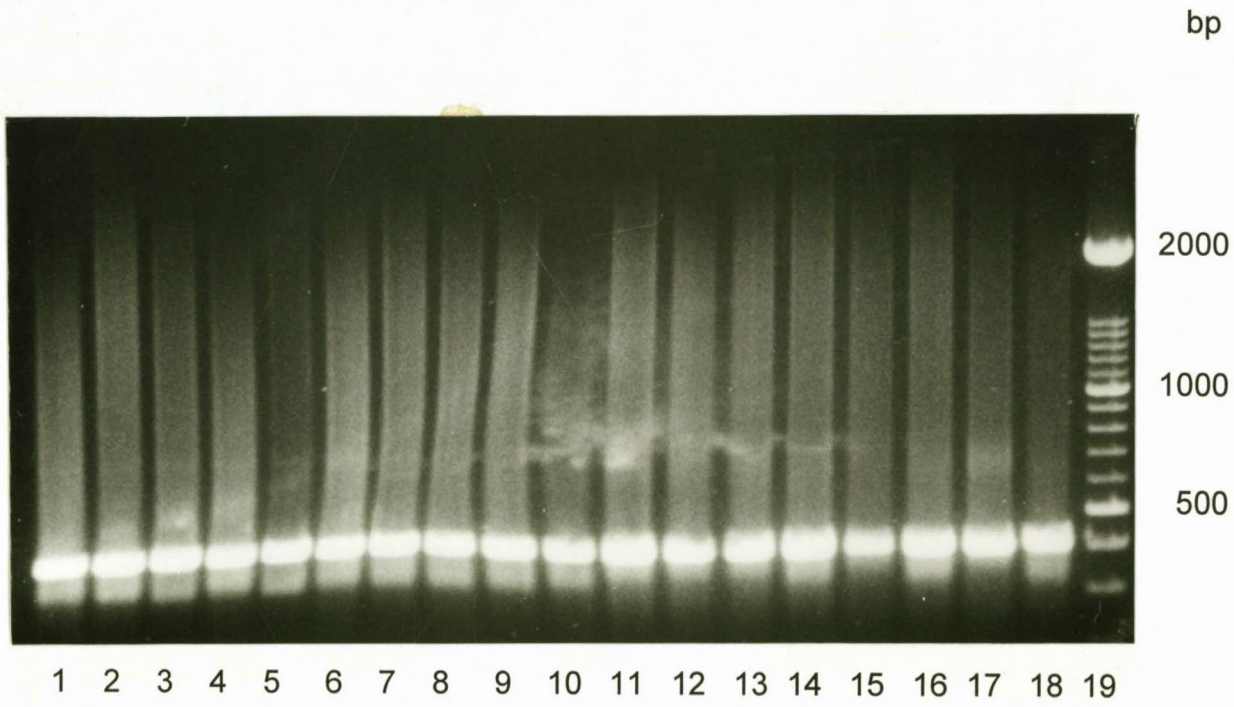

Figure A1: PCR products of nine *Bacteroides* spp. and nine *Fusobacterium* spp. strains, employing universal primers. Lanes, **1**, B38 *B. capillosus*; **2**, B53 *B. fragilis*; **3**, B64 *B. vulgatus*; **4**, B70 *B. vulgatus*; **5**, B82 *B. fragilis*; **6**, B90 *B. fragilis*; **7**, B97 *B. fragilis*; **8**, B110 *B. fragilis*; **9**, B121 *B. fragilis*; **10**, B51 *F. mortiferum*; **11**, F19 *F. mortiferum*; **12**, F23 *F. mortiferum*; **13**, F28 *F. mortiferum*; **14**, F35 *F. necrophorum* **15**, F39 *F. varium*; **16**, F44 *F. varium*; **17**, F47 *F. mortiferum*; **18**, F49 *F. mortiferum*; **19**, MWM.