

ANTIMICROBIAL SUSCEPTIBILITY OF ANAEROBIC ORGANISMS
ISOLATED FROM CLINICAL SPECIMENS AT CHARLOTTE MAXEKE
JOHANNESBURG ACADEMIC HOSPITAL

Sudeshni Naidoo

A research report submitted to the Faculty of Health Sciences, University of the
Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree
Of
Master of Science in Medicine

Johannesburg, 2009

TABLE OF CONTENTS

	Page
Table of contents	ii
Declaration	iii
Abstract	iv
Acknowledgements	v
Preface	vi
Abbreviations used in text	1
1.0 Literature review	3
1.1 Introduction	
1.2 Classification and characteristics of anaerobic organisms	
1.3 Epidemiology	
1.4 Pathogenesis	
1.5 Clinical manifestations	
1.6 Diagnosis of anaerobic organisms	
1.7 Mechanisms of resistance in anaerobic bacteria	
1.8 Management	
2.0 Rationale for study	67
2.1 Projected outcome	
3.0 Aims and Objective	68
3.1 Aim	
3.2 Objective	
4.0 Methods and Materials	69
4.1 Microscopy, culture and sensitivity	
4.2 Quality control	
4.3 Reading	
5.0 Results	80
6.0 Discussion	95
7.0 Conclusions	98
8.0 Literature cited	99

Declaration

I, Sudeshni Naidoo declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

The Ethics Committee University of Witwatersrand has approved this study.

None of the figures used in the text have been modified in any way from the stated references.

The anaerobe *Clostridium difficile* has not been included in this study as it has special requirements for isolation and culture that is not routinely performed at the Microbiology Laboratory at Charlotte Maxeke Johannesburg Academic Hospital. Upon request cytotoxin A and B detection is performed using a commercial kit.

.....

September 2009

Abstract

Anaerobic bacteria cause serious life-threatening infections such as endocarditis, sepsis, intra abdominal, pleuro-pulmonary and central nervous systems infections. Most infections are polymicrobial and involve aerobes and anaerobes. Empiric therapy is generally based on the expected pathogens and the particular type of infection. Even when specimens are cultured and anaerobes identified, not all laboratories perform susceptibility testing. The clinician often relies on published surveillance data when selecting treatment regimens. Antimicrobial susceptibility of anaerobic bacteria is becoming increasingly unpredictable. Resistance can vary significantly and patterns differ geographically, and even within units of the same hospital.

From June 2005 until February 2007, 180 consecutive anaerobes isolated from relevant, non-repetitive clinical specimens were tested routinely with the E test method for susceptibility to amoxicillin/clavulanate (XL), clindamycin (Cm), metronidazole (Mz), penicillin (Pg), ertapenem (Etp), cefoxitin (Fx), ceftriaxone (Tx), chloramphenicol (Cl), and piperacillin/tazobactam (Ptc). The results were read after 48hr incubation in anaerobic conditions. Specimen distribution was as follows: abdominal fluid (3), abscess (7), abdominal abscess (4), aspirates (3), blood cultures (27), bone (3), breast (3), drainage fluid (2), empyema (1), fluids (36), other (4), placental (1), pleural fluid (2), pus (41), tissues (34), umbilicus (1) and unknown sites (8).

Bacteroides fragilis was isolated from 81 (45%) clinically significant specimens, followed by *Clostridium perfringens* 23 (13%), *Peptostreptococcus anaerobius* 15 (8%) and *Prevotella melaninogenica* 15 (8%). *B. fragilis* demonstrated a 97.5% resistance to penicillin and 12.3% resistance to metronidazole. *C. perfringens* exhibited no resistance to penicillin and metronidazole while *P. anaerobius* had 40% resistance to penicillin and no resistance to metronidazole. *P. melaninogenica* was resistant to penicillin in 60% and 6.7% to metronidazole. Overall, chloramphenicol, piperacillin/tazobactam, ertapenem and amoxicillin/clavulanate demonstrated the highest activity to anaerobic isolates, 100%, 99.4%, 97.2% and 96.7%, respectively. Among the 180 tested anaerobes a total of 8.9% resistance has been observed with metronidazole and 81.7% sensitivity with clindamycin.

Periodic surveillance to monitor the susceptibility profile of the *B. fragilis* group and other anaerobic organisms is recommended to create empirical guidelines for appropriate use of antimicrobial agents.

Acknowledgements

This study was supported by research grant from Merck & Co Inc. I wish to thank Dr.Olga Perovic, Principal Pathologist of the Microbiology Laboratory, Charlotte Maxeke Johannesburg Academic Hospital for all her support, guidance and mentorship during my project. To all staff at the National Health Laboratory Services, Microbiology, Charlotte Maxeke Johannesburg Academic Hospital for the help in collection of the isolates, thank you.

Preface

Antimicrobial susceptibilities of anaerobes are generally thought to be more predictable than those of other bacteria. However, the increasing resistance of anaerobes to some agents has been observed, making their susceptibility patterns less predictable. Although isolation and identification are neither practical nor readily available for the individual patient, knowledge of the susceptibility of anaerobic antibiotics at individual hospitals is important in the selection of empirical antimicrobial therapy.

Variation in the susceptibility of anaerobes can be the result of differences in geographical distribution, site of infection, clonal population, as well as regional antimicrobial use. Various surveillance programs continue to monitor susceptibility to antimicrobial agents for common pathogens at local, regional, national, and global level. With increasing trends in antimicrobial resistance worldwide, it is essential to monitor emerging drug resistance at the local level to support clinical decisions, infection-control interventions, and antimicrobial resistance containment strategies.

Therefore, surveillance of antimicrobial susceptibility pattern is needed to detect extent of resistance and to guide clinicians to use antibiotics appropriately.

Abbreviations used in text

Abbreviation	Full Text
IL –17	Interleukin –17
TNF	Tumor necrosis factor
ICAM	Intracellular adhesion molecule
BFT	<i>Bacteroides fragilis</i> toxin
LPS	Lipopolysaccharide
CDC	Center for Disease Control
LT	Lethal toxin
HT	Hemorrhagic toxin
AAD	Antibiotic-associated diarrhea
ETBF	Enterotoxigenic strains of <i>B. fragilis</i>
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
SPS	Sodium polyanethol sulfonate
S	Susceptible
R	Resistant
MID	Mast identification
H ₂ O ₂	Hydrogen peroxide
PACA	p-dimethylaminocinnamaldehyde
LV	Lecitho-vitellin
KNO ₃	Potassium nitrate
RNA	Ribonucleic acid
Km	Kanamycin
Vm	Vancomycin
MIC	Minimum inhibitory concentration
CLSI	Clinical Laboratory Standards Institute
NCCLS	National Committee for Clinical Laboratory Standards
ICU	Intensive care unit
PBP	Penicillin-binding proteins
Nim	Nitromidazole resistance genes

gyrA	Gyrase
parC	Topoisomerase IV
Rna	Ribosomal Ribonucleic acid
kb	Kilo bases
µg	Micro grams
NBU	Non-replicating <i>Bacteroides</i> units
Mv	Mili volts
Eh	Redox potential
µm	Micrometer
FDA	Food and drug association
ml	Mili litre
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
Mw	Molecular weight
RCMM	Robertson's cooked meat medium

1.0 Literature review

1.1 Introduction

The broad classification of bacteria as anaerobic, aerobic or facultative anaerobic bacteria is based on the types of reactions they employ to generate energy for growth and other activities. Aerobic bacteria require molecular oxygen as a terminal electron acceptor in the energy building process and cannot grow in its absence. Anaerobes, as opposite, cannot grow in the presence of oxygen, which is toxic for them, and they depend on other substances as electron acceptors. Anaerobic metabolism is fermentative in which they reduce available organic compounds to various end products such as organic acids and alcohols. The facultative anaerobic organisms are the most versatile. They preferentially utilize oxygen as a terminal electron acceptor, but can utilize energy in the absence of oxygen by reducing other compounds such as, a molecule of glucose, which is completely catabolized to carbon dioxide and water in the presence of oxygen, that is only partially catabolized by a fermentative process in the absence of oxygen. The ability to utilize oxygen as a terminal electron acceptor provides organisms with an extremely efficient mechanism for generating energy. Understanding the general characteristics of anaerobiosis provides insight into how anaerobic bacteria can proliferate in damaged tissue and why special laboratory conditions are needed in processing clinical specimens that may contain them [53].

Anaerobic bacteria are part of normal flora in humans. Important oral anaerobes include *Fusobacterium nucleatum*, *Fusobacterium necrophorum*, *Prevotella oralis*, *Prevotella disiens*, *Prevotella oris*, *Prevotella buccae*, and *Bacteroides forsythus*. The microbiology of the mouth is complex, and many bacteria are dependant on other species for survival; for example, *P. melaninogenica* requires vitamin K produced by other species, and oral

spirochetes are dependent on metabolites produced by *Fusobacterium* species. Anaerobes are associated with the eruption of teeth and the gingival crevices as from childhood [64].

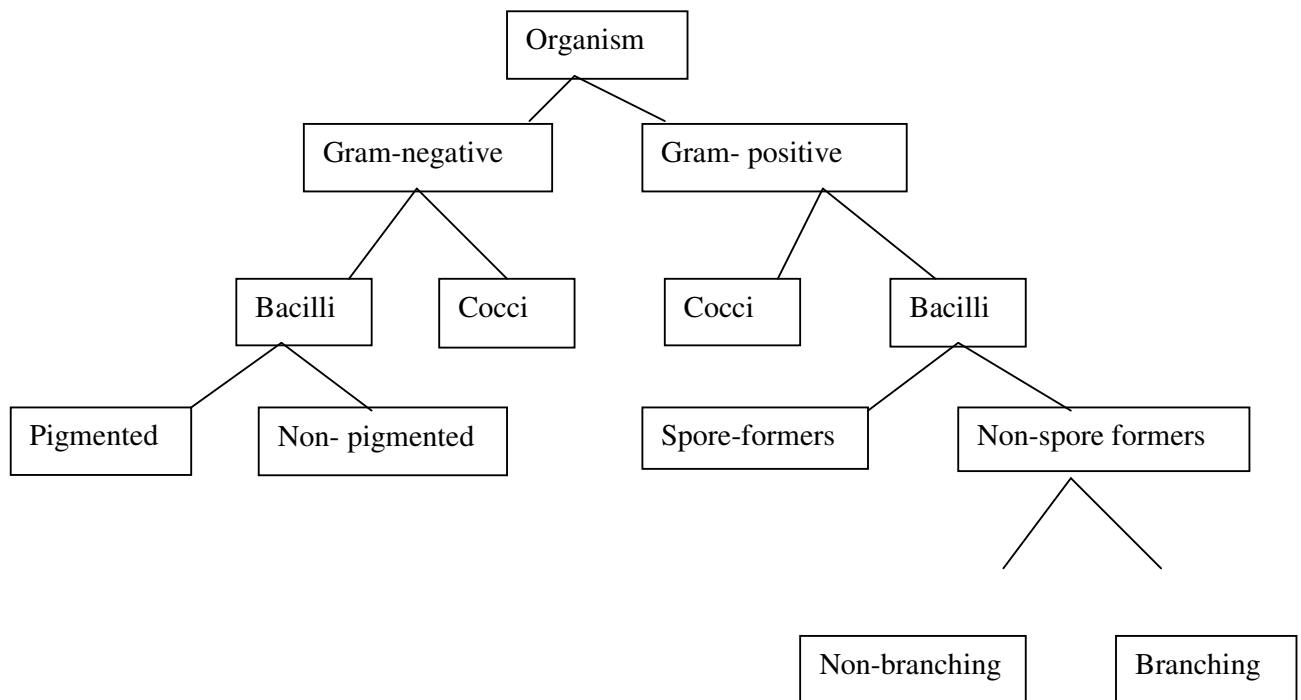
The anaerobic organisms as part of the gastrointestinal flora are responsible for the production of vitamin K demonstrating symbiotic and beneficial effect to the host, at the same time as the production of bile by these organisms is useful in fat absorption and cholesterol regulation [90]. But once these anaerobic organisms change sites and cause severe infections, they are associated with high morbidity and mortality and their outcome varies on the anaerobic species. Therefore, antibiotic therapy in conjunction with surgical intervention, including debridement of necrotic tissue or amputation of a limb, is strongly recommended, particularly in infections such as gas gangrene caused by clostridial species as well as in abscesses where antibiotics are less effective [102].

1.2 Classification and characteristics of anaerobic organisms

1.2.1 Classification of anaerobic organisms

Classification is based on the gram-stain morphology, the ability of spore formation and pigment production (Figure 1).

Figure 1: Flowchart classification of anaerobic bacteria



1.2.2 Characteristics of anaerobic organisms

1.2.2.1 Non-pigmenting gram-negative bacilli

- a. *Bacteroides fragilis*, the most common anaerobic organism causing infections in humans is a gram-negative bacillus with rounded ends 0.5 to 0.8 μm in diameter and 1.5 to 4.5 μm in length. Most strains are encapsulated. Vacuolization or irregular staining is common, particularly in broth media. Some pleomorphism also may be seen. By electron microscopy, the ultra structure of *B. fragilis* is similar to other gram-negative bacteria. The guanine-cytosine content is 42% [38].

B. fragilis has receptors for bacteriophage, which are species specific and active against most strains. Enzymes and toxins-bacteriocins are produced by strains of *B.*

fragilis and *B. thetaiotamicron*. Superoxide dismutase has been found in *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, and *B. ovatus*, with possibility to create tolerance to oxygen [39].

- b. Members of the genus *Fusobacterium* are spindle shaped, 5 to 10 µm long, often seen in pairs, have parallel sides and rounded ends. The guanine-plus-cytosine content ranges from 26 to 34%. Strains of *F. necrophorum* are elongated or filamentous, curved, and possess spherical enlargements and large, free, round bodies. *Fusobacterium nucleatum* is a virulent organism causing necrotizing oral or facial infections in severely debilitated or malnourished patients, mainly children. The lipopolysaccharide of *F. necrophorum* has a multilayered external coat structure. The endotoxin varies from strain to strain in its content of 2-ketodeoxyoctanate and sugars [38].

1.2.2.2 Pigmented gram-negative bacilli

Prevotella melaninogenica and *Porphyromonas asaccharolytica* are short coccoid gram-negative rods; they produce a distinctive pigment (brown to black), which is a heme derivative. In addition many strains of *P. melaninogenica* require vitamin K as well as heme [38].

1.2.2.3 Anaerobic gram-negative cocci

They are classified in the family *Veillonellaceae*. Three genera: *Veillonella*, *Acidaminococcus*, and *Megasphaera* had long been the members of the family, but in the year 2002 a fourth genus (*Anaeroglobus germinatus*) was described [102].

1.2.2.4 Anaerobic gram-positive cocci

Those of clinical significance are found in three gram-positive genera (*Peptostreptococcus*, *Gemella*, and *Streptococcus*). There are other genera of anaerobic cocci, but they are rarely isolated from clinical specimens. Anaerobic cocci may be proteolytic, saccharolytic or both. They produce a variety of short-chain volatile fatty acids (i.e., acetic, propionic, butyric, caproic, and lactic acids) from the fermentation of simple sugars and amino acids. Both *P. magnus* and *P. anaerobius* possess species-specific cell wall antigens, compared to other anaerobic cocci [104].

1.2.2.5 Anaerobic gram-positive spore forming bacilli

The genus *Clostridium* comprises this phylogenetically heterogeneous group of obligatory anaerobic, rod-shaped endospore-forming bacteria, mostly motile and peritrichously flagellated. The genus includes common free-living bacteria that belong to a group of important human pathogens, and can be subdivided physiologically into saccharolytic and/or proteolytic species [101].

- a. There have been 11 strains of *C. tetani* distinguished primarily on the basis of flagella antigens. They differ in their ability to produce tetanus toxin (tetanospasmin), but all strains produce a toxin that is identical in its immunological and pharmacological properties. Tetanospasmin is encoded on a plasmid that is present in all toxigenic strains. The bacterium synthesizes the tetanus toxin as a single 150kDa polypeptide chain (called the progenitor toxin), which is cleaved extracellularly by a bacterial protease into a 100kDa heavy chain (fragment B) and a 50kDa light chain (fragment A), which remain connected by a

disulfide bridge [98]. Mature organisms lose their flagella and develop a terminal spore. Growth of *C. tetanus* from wounds is not useful in diagnosis, as (1) anaerobic cultures may be negative; (2) a positive culture does not indicate whether the organism contains plasmid that produces toxin and (3) a positive culture may be present without disease in immunocompetent patients [13].

- b. Botulism is a life-threatening neuromuscular disease caused by antigenically distinct, heat-labile toxins of *C. botulinum*. Although seven toxin types (A, B, C, D, E, F and G) are produced by the different strains of *C. botulinum*, most cases of botulism in humans are caused by types A, B, E and F. Of these, type F is the least common. Types C and D are associated with botulism in birds and mammals, but not in humans. Type C can be subdivided into two types, C1 and C2. Although type G organisms have been isolated from autopsy samples of a few individuals who died suddenly, it is not clear whether type G organisms cause botulism in humans [102]. Botulinum toxin is synthesized as a single polypeptide chain with a molecular weight around 150kDa. In this form, the toxin has a relatively low potency. The toxin is nicked by a bacterial protease (or possibly by gastric proteases) to produce two chains: a light chain (the A fragment) with a molecular weight of 50kDa; and a heavy chain (the B fragment), with molecular weight 100kDa. The chains remain connected by a disulfide bond. The A fragment of the nicked toxin, on a molecular weight basis, becomes the most potent toxin found in nature [98].

- c. *Clostridium septicum* is a spindle-shaped, motile gram-positive rod. The organism produces alpha, beta, gamma and delta toxins. The organism is not strongly invasive, but has been associated with gas gangrene [105].
- d. *Clostridium sordellii* was first isolated in 1922 by the Argentinean microbiologist Alfredo Sordellii, who named organism as *Bacillus oedematis sporogenes* on the basis of morphology and the marked tissue edema [92]. In 1927, the organism was renamed *Bacillus sordellii* [49]. Two years later, it was shown to be identical to *Clostridium oedematoides* and the name *C. sordellii* was adopted [56]. Similarities in morphology and biochemical profile suggested that *C. sordellii* was simply a virulent strain of *Clostridium bifermentans*; however, urease production by *C. sordellii* clearly distinguished the two species [33]. In the late 1970's *C. sordellii* antitoxin was found to neutralize the cytotoxic effect of stool specimens collected from patients with *Clostridium difficile* associated pseudomembranous colitis. Colonies of *C. sordellii* appear translucent to opaque with small zones of β -hemolysis on sheep or rabbit blood agar [5].
- e. *C. difficile*, a gram-positive, spore-forming, toxin-producing bacillus, was first described in 1935 as a component of the intestinal flora in healthy newborn infants. The role of *C. difficile* in human disease was not appreciated until the 1970's, when it was identified as the causative agent of pseudomembranous colitis.

1.2.2.6 Anaerobic gram-positive non-spore-forming bacilli

Include species of *Actinomyces*, *Bifidobacterium*, *Arachnia propionica*, *Lactobacillus*, *Mobiluncus*, *Eubacterium* and *Propionibacterium* of which the former three produce branching characteristics [102]. *Actinomyces* and *P. propionicum* are irregular, nonspore-forming, gram-positive rods. *Actinomyces* contains 19 species, of which *A. israelii* and *A. gerencseriae* are the most common human pathogens [16]. Gram-stained smears prepared from lesions shows characteristic sulphur granules, which present granular microcolonies of the organism surrounded by purulent exudate [102]. The genera *Actinomyces* and *Propionibacterium* are defined by chemotaxonomic tests, e.g., cell wall composition, fermentation products, cellular fatty acids; DNA homology; and analysis of their ribosomal RNA (rRNA) [16].

1.3 Epidemiology

Anaerobic bacteria are prevalent among the bacterial populations of the human body, particularly on mucous membrane surfaces. In the mouth, anaerobes and aerobes are present in approximately equal numbers; in the lower intestine, anaerobes outnumber aerobes at least 1000 to 1; and on the skin, at least 10 to 1. The current use of more defined methods of collection, cultivation and identification of anaerobic bacteria will undoubtedly show them to be even more numerous than our present knowledge indicates [39].

Anaerobic bacteria are widespread in soil, lake and river sediments, the oceans, sewage, foods, and animals [102]. They are saprophytic in nature and are found in humans as part of normal flora [39]. Although a number of infections or intoxications due to anaerobic bacteria or their toxins arise from exogenous sources such as the soil, the greater part is of

endogenous origin arising frequently from the intestinal tract, the mouth, or the female genital tract. They colonize skin and mucous membranes.

a. Skin

The major groups of anaerobic bacteria that colonize the skin are *Propionibacterium* and the anaerobic gram- positive cocci. *Clostridium* species may also be found on the skin and represent transient contamination from the soil or faeces. Anaerobic cocci are found as commensal flora on all skin and mucosal surfaces and are, after the gram-negative bacilli, the second most common group of anaerobes encountered in human infection [66].

Approximately 10-15% of all isolates of anaerobic cocci come from pure culture infections, thus indicating that these organisms can be significant pathogens rather than innocuous commensals [104].

Propionibacterium acnes are frequent commensals in skin sebaceous glands, hair follicles, mouth, upper respiratory tract, and a frequent contaminant in laboratory cultures [43]. A role for *Propionibacterium acnes* in the pathogenesis of acne has been debated for decades, but never adequately proven [15].

Peptostreptococcus species and *Veillonella parvula* is universally present in the upper airways and normally inhabits the mouth, gastrointestinal tract, vagina, and skin in humans [66]. *Veillonella* and the anaerobic/ aerotolerant streptococci are the anaerobic cocci isolated most frequently from human bites [104].

b. Respiratory tract

The upper respiratory tract and mouth are inhabited by a variety of potentially pathogenic anaerobes in large numbers. Aspiration pneumonias and lung abscesses are frequently caused by non-spore forming anaerobes such as *Bacteroides melaninogenicus*,

Fusobacterium, anaerobic cocci and *Eubacterium*. Spirochetes also inhabit the mouth and are found in oral and pulmonary disease but their significance is unknown.

Actinomyces israelii, *A. gerencseriae*, *A. georgiae*, *A. naeslundii*, *A. odontolyticus*, *A. meyeri*, possibly *A. pyogenes* and *P. propionicus* are normal inhabitants of the human mouth and are found in saliva, on the tongue, in gingival crevice debris, and frequently in tonsils in the absence of clinical disease. Some data suggest that *A. israelii* may also be a common inhabitant of the female genital tract. The natural habitats are not known for the more recently described *Actinomyces* such as *A. bernardiae*, *A. neuui*, *A. radingae*, and *A. turicensis*. *Actinomyces bovis* is not found in humans and to date, the other species described from animals, with the exception of *A. pyogenes*, have not been found in humans. Actinomycosis occurs worldwide, but the disease appears more often in men than in women [16].

c. Gastrointestinal tract

The predominant bacteria in the intestinal flora are: *Bacteroides fragilis*, *Bifidobacterium*, anaerobic cocci and *Eubacterium* [39]. *Bacteroides* and *Prevotella* are abundant on all mucosal surfaces and reach their largest concentrations in the tonsillar crypts, the crypts of the tongue, dental plaque, and the gingival crevices in the oral cavity; the colon in the gastrointestinal tract and the vagina [64]. *Bacteroides fragilis* constitutes about 1-2% of the normal bacterial flora in humans [102].

d. Genitourinary tract

There are few studies of the normal flora of the genitourinary tract except for the vagina, where the normal flora is a predominantly anaerobic gram-positive non-sporulating bacillus. Anaerobic cocci are also commonly found. Although *Bifidobacterium* and

Lactobacilli are the most predominant bacteria present, most of the infections arising from the vagina are due to the anaerobic cocci or *Bacteroides* species [102].

e. Environment and human distribution

The genus *Clostridium* is widely distributed in nature, at present there are more than a 160 recognized species, but only a few species have been implicated in humans. In general, toxigenic or endogenous *Clostridium* species are recognized as causative agents of food poisoning, gas gangrene, tetanus, botulism, ulcerative colitis, septicemia, and postoperative infections in humans. In contrast, most environmental clostridia are considered seemingly harmless nonpathogenic bacteria [22].

The spores of *Clostridium tetani* are extremely stable in the environment, retaining the ability to germinate and cause disease indefinitely. They withstand exposure to ethanol, phenol, or formalin, but can be rendered noninfectious by iodine, glutaraldehyde, hydrogen peroxide, or autoclaving at 121°C [13].

The source of *C. sordelli* in obstetric/gynecological- associated infections is unknown, although two reports have documented prolonged vaginal carriage of *C. sordellii* in 0.5%-10% of healthy women, suggesting that some women may be natural *C. sordellii* carriers [72].

Improperly processed home-canned low-acid vegetables and contaminated meats are the usual cause of food-borne botulism. Foods associated with botulism include canned asparagus, green beans, garlic in oil, corn, soups, ripe olives, tuna fish, sausage, luncheon meats, fermented meats, salad dressings, and smoked fish. During the canning process, foods undergo a heating process and oxygen is removed, leaving the food in an anaerobic environment. Certain foods such as meat are able to bind oxygen to create an anaerobic environment. Home canning processes for low-acid foods present an extremely high risk

because processing times and temperatures are often inadequate and cans or jars may not be handled properly. Strains of *C. botulinum* can grow between 38°F and 115°F. This means that strains can grow at normal refrigeration and higher temperatures, making it difficult to prevent bacterial growth and toxin production. Infant botulism has been linked to the ingestion of *C. botulinum* spores in honey, corn syrup, and other foods. The *C. botulinum* spores germinate, colonize, and produce toxins in the intestinal tract of infants (intestinal toxemia botulism). Infants less than one year old lack the proteins in their intestines needed to destroy either the vegetative or spore form of *C. botulinum*, so these products are not recommended for infants [35].

C. difficile is ubiquitous in nature; the organism is more prevalent in the feces of some hospitalized adults who do not have diarrhea or colitis. It has been found in the feces of about 13-30% of hospitalized adults who were colonized but had no evidence of disease caused by *C. difficile* or antecedent antibiotic treatment [102].

1.4 Pathogenesis

Infections caused by anaerobes are usually a result of the breakdown of a mucosal barrier and subsequent leakage of indigenous polymicrobial flora into previously sterile closed spaces or tissue. In certain cases, such as aspiration pneumonia, anaerobic bacteria move into sterile sites [38]. Few anaerobic bacteria that survive in the infected site are those that have resisted changes in oxidation-reduction potential and host defense mechanisms [100]. The more aerotolerant anaerobes are more likely to survive after the normally protective mucosal barrier is broken and until conditions are satisfactory for their multiplication and invasion. Once anaerobes begin to multiply, they can maintain their own reduced environment by excreting end products of fermentative metabolism [38].

One of the major defenses of the body against infections by anaerobes is the normal Eh (+120 millivolts). Lowering the redox potential permits multiplication of anaerobes within tissues [39]. It is interesting to note that anaerobes inhabit areas of the body that are exposed to air: the skin, nose, mouth, and throat. It has been hypothesized that anaerobes can withstand oxygen at these sites, in part owing to the presence of aerobes and facultative organisms, which consume oxygen and reduce oxidation-reduction potential [100].

Under anaerobic conditions, the granulocyte phagocytic bactericidal system does not function effectively. Lowered oxidation – reduction potential also results from impaired blood supply, tissue necrosis and growth of facultative bacteria in a wound. Thus, vascular disease, epinephrine injection, cold, shock, edema, trauma, surgery, foreign bodies, malignancy, gas production by microorganisms, and aerobic infection may all predispose significantly to anaerobic infection [39].

1.4.1 Virulence factors

Virulence factors associated with anaerobic infections are:

- (1) Ability to evade host defenses,
- (2) Adherence to cell surfaces,
- (3) Production of toxins and /or enzymes and
- (4) Expression of surface antigens that contribute to pathogenesis [100].

MAJOR BACTERIAL VIRULENCE DETERMINANTS

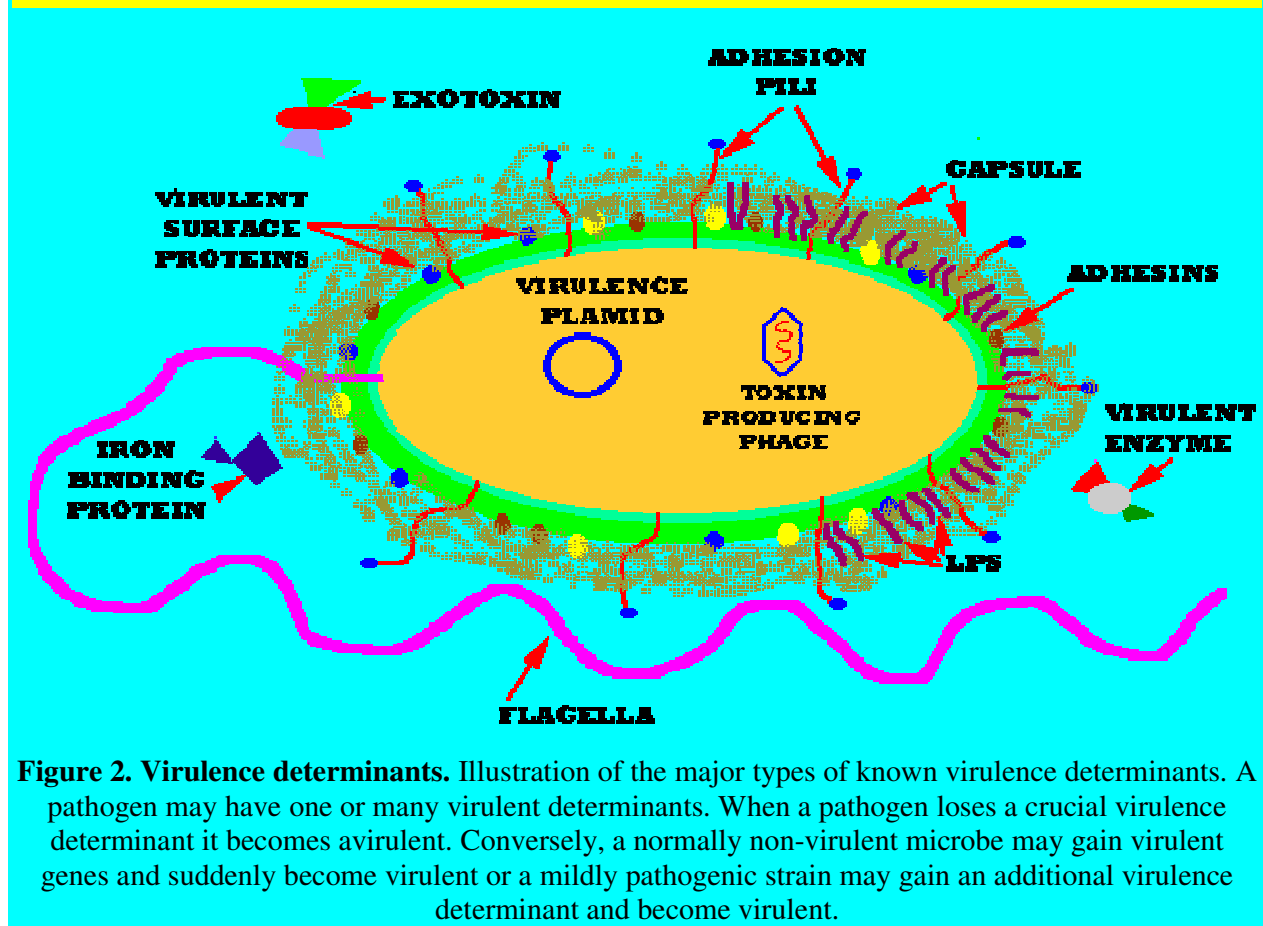


Figure 2. Virulence determinants. Illustration of the major types of known virulence determinants. A pathogen may have one or many virulent determinants. When a pathogen loses a crucial virulence determinant it becomes avirulent. Conversely, a normally non-virulent microbe may gain virulent genes and suddenly become virulent or a mildly pathogenic strain may gain an additional virulence determinant and become virulent.

[57]

1.4.1.1. Ability to evade host defences

Pathogens frequently cause disease by changing normal functions of the host cells. One way is to induce the host system to produce self-destructive chemicals that kill or inhibit its own cells. In doing this the body's own defence system is redirected towards its own destruction, leaving the invading pathogen to the nutritious remains. In fact many of the "diseases" caused by both viruses and bacteria are the result of the pathogen disrupting the normal processes of the host and escaping destruction or detection [57].

1.4.1.2. Adherence to cell surfaces

To avoid phagocytosis, many pathogens have evolved surface components that prevent the attachment and engulfment of macrophages and other host cellular immune responses. This may take the form of membrane bound proteins, slimy polysaccharide capsules, or "self" moieties scavenged by the microbe and bound to its cell surface [14]. Gram- positive bacteria are surrounded by a thick cell wall that has a low permeability to the surrounding environment, while gram- negative bacterial lipopolysaccharide (LPS) or "endotoxin" can protect against complement-mediated lysis. In addition, several antigens produced by both classes can inhibit adsorption [29].

Since many of the non-specific defences involve mechanically flushing away pathogens, a common virulence determinant of pathogens are cell components that stick the bacteria to the target cells. Like the attachment of viruses, these systems stick to each other. Two general attachment systems have been established:

- a) The pili are short protein rods or curled protein strands that have binding proteins on the ends that attach firmly to receptor molecules on the surface of other host cells.
- b) Capsules, on the other side are composed of sugar polymers (occasionally of protein polymers) [57].

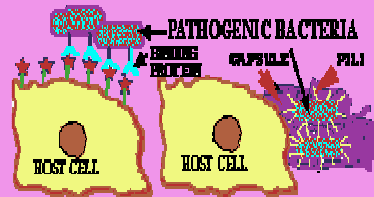


Figure 3. Attachment system that bind pathogens to their hosts. On the left binding proteins, usually pili, of the pathogen attach to the receptor molecules on the surface of the host cells. The pathogen may simply use this as an anchor by which it keeps from being flushed away by a flow of material or this may be the first step in destroying the target cell. On the right bacteria are shown embedded within a capsule that, along with pili, binds to each other and to the surface of a target cell. Again, the bacterial cells may use this as a way of not being washed away or it may be the preliminary step in a process that leads to the death of the target cell.

[57]

The first host barrier for many invading pathogens is usually a mucosal surface, such as the gut or respiratory tract. Since epithelial cell turnover is around 48 hours in these environments, the bacterium must attach and replicate sufficiently to avoid being swept away. Therefore, many have evolved motile or attachment elements like flagella and pili/fimbriae to cross the barrier and invade [86]. Simple attachment is mediated through a receptor on the host cell surface, and an adhesion on the bacterial one. Some may be species or even strain specific, while others exhibit tissue tropism or include the fimbrial protein subunit that binds D-mannose on host cell surfaces [71].

1.4.1.3 Production of toxins and or enzymes

Toxins are products of a pathogen that destroy one or more vital component of the host thus allowing the pathogen to survive and grow.

a) Secreted by viable pathogenic cells, bacterial protein exotoxins are amongst the most potent toxins known. Often encoded for by bacteriophage or plasmid, there are many classes and all are strongly antigenic but inherently unstable. Some act on host cell surfaces, while the majority (A/B toxins) bind to the target membrane with a receptor (B

subunit) and deliver a second moiety (A subunit) directly into the cytoplasm [4]. More specialized toxins involve "injection" of the protein into the host via a unique "type III" secretion system. The latter is only found in some gram- negative enteropathogens [55]. Toxins can also be grouped according to their biological activity in certain cells, such as leukotoxins and neurotoxins. These toxins are secreted from the cell or leak out of the cell after it dies. Generally they are soluble proteins and thus are carried throughout the body in the blood or lymph, doing damage at a distance from the infection site. Toxins tend to target specific cells in the body. Some are enzymes and others are proteins that bind to and inhibit crucial cellular activities that eventually lead to the death of cells [57].

b) Endotoxins produced by gram- negative bacteria have a general basic structure, but differ significantly in composition between species. Endotoxins are released in relatively small amounts as the cells grow, but in abundant amounts when the cells die. Different endotoxins differ in their degree of toxicity, but all are heat stable and can tolerate autoclaving [57]. Endotoxin or lipopolysaccharide (LPS) activates the host complement pathway, and is a potent inducer of inflammation. It is an outer membrane chemical moiety consisting of three sections: a toxic lipid (Lipid A) anchored in the outer membrane, an immunogenic polysaccharide core, and an antigenic O-linked series of oligosaccharides at the extracellular surface. It is considered a part of gram- negative bacterial pathology [34]. Endotoxins are toxic to most mammals, and can be lethal if encountered in too high a dose. Specifically, release of LPS into the host circulation promotes binding by a certain protein, dubbed "LPS-binding complex". This interacts with CD14 receptors on a variety of monocytes and macrophages, triggering inflammatory cytokine release, and activation of the complement and coagulation cascades [106]. They are often responsible for the cause of death of infections by gram-negative cells [57].

c) Enzymes affect physical barriers like tissue matrices and cell membranes. In this way, the bacterium can quickly spread through intercellular spaces [94]. There are several classes of invasive enzymes; while some dissolve strong tissues like the hyaluronic component of connective tissue other proteins create holes in cell membranes and cause cell lysis (lecithinases and phospholipases). Other obligate intracellular bacterial proteins have been called "invasins" and act exclusively on host actin filaments and induce engulfment of the microbe for colonization.

1.4.1.4 Expression of surface antigens that contribute to pathogenesis

Pathogens often evade a host's immune system by frequently changing its surface antigens. For example, HIV and malaria use this strategy to avoid destruction. In these cases a pathogen produces a few antigenic variants as its population increases. Meanwhile the host responds by making antibodies only against the major antigenic form. However, the variant(s) survives, thrives and increases in number, while continuing to harm the host. The host will then mount a second vigorous antibody attack against the new variant only to have another new variant escape. Eventually, the pathogen does so much damage that the weakened host is destroyed.

Furthermore a pathogen may hide from host defence cells. One way is to coat itself with a capsule which the white blood cells either doesn't recognize or for some other reason avoids ingestion thus allowing the capsule-covered pathogen to remain free and unhindered. In other cases the pathogen may enter and hide within host cells, thus escaping the ingestion of white blood cells and their antibodies. Some pathogens have even

developed ways of moving from one host cell to another without coming out of the cell so they avoid detection by the defending immune system [57].

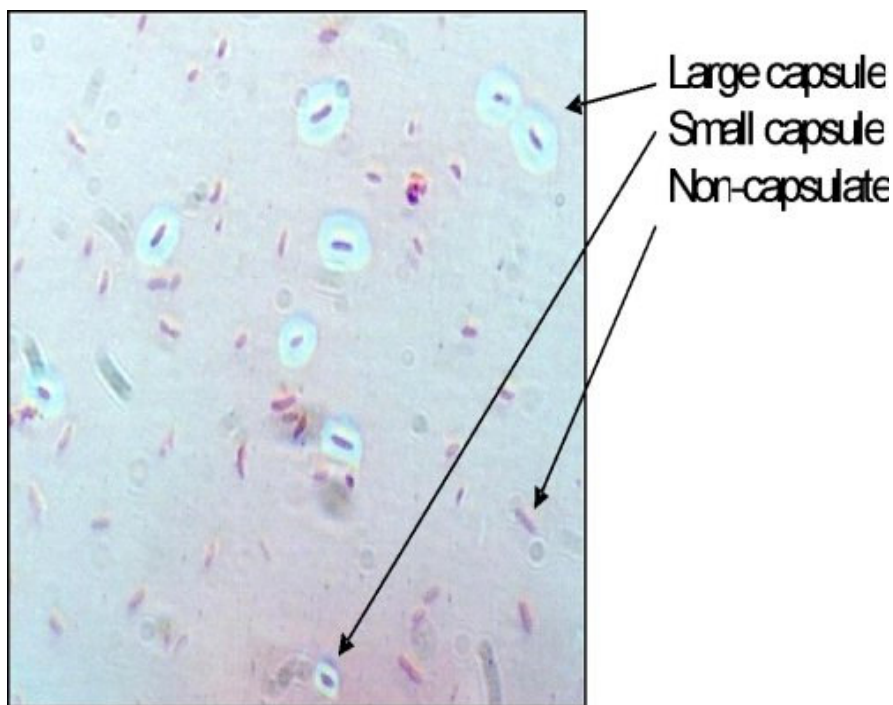
1.4.2 Pathogenicity of the gram-negative bacteria

Bacteroides, *Prevotella*, *Porphyromonas*, and *Fusobacterium* species may have the possibility to penetrate tissues and then to cause infection under certain circumstances such as surgical or other trauma or when tumors arise at the mucosal surface [38].

1. *Bacteroides* species produce many enzymes and toxins that contribute to pathogenicity. Enzymes such as neuraminidase, protease, glycoside hydrolases, and superoxide dismutases are all produced by *B. fragilis*. Also, this organism produces an entero-toxin with specific effects on host cells in vitro. This toxin, termed BFT (*Bacteroides fragilis* toxin), is a metalloprotease that is cytopathic for intestinal epithelial cells and induces fluid secretion and tissue damage in ligated intestinal loops [100]. Neuraminidase alters neuraminic acid-containing glycoproteins of human plasma; *Bacteroides* strains isolated from sterile sites have higher neuraminidase activity than do those isolated from stools, and strains of the *B. fragilis* group have greater activity of this type than do strains of other gram-negative anaerobic bacillary species. Hyaluronidase is produced by many strains of the *B. fragilis* group and pigmented gram-negative rods. DNase is also produced by *B. fragilis* and may be an important factor in infection. Many gram-negative anaerobic bacilli produce phosphatase. A heparinase produced by *B. fragilis* strains may contribute to intravascular clotting and hence increase the dosage of heparin needed for the treatment of septic thrombophlebitis in infections caused by this organism [38]. The septic thrombophlebitis commonly seen in anaerobic infections

may result from production of heparinase by anaerobic bacteria. This lesion may lead to metastatic abscesses and helps account for the difficulty in eradicating anaerobic infections [39]. The lipopolysaccharides of *B. fragilis*, *B. vulgatus*, and *F. mortiferum* activate the Hageman factor and thereby initiate the intrinsic pathway of coagulation. Fibrinolysin is produced by many *P. melaninogenica* group strains and by a few *B. fragilis* strains. Strains of *Bacteroides* and *P. gingivalis* degrade complement factors and immunoglobulins G and M [38]. The pathogenicity of *B. fragilis* is related to its carbohydrate capsule, outer membrane proteins, several enzymes and the enterotoxin called fragilysin [31]. Biologic and molecular studies of this virulence factor show that *B. fragilis* produce at least eight distinct capsular polysaccharides [62] they have been identified as major virulence factors and has a specific central role in the formation of abscesses [77].

Figure 4: Capsule of *B. fragilis*



[85]

These polymers activate host CD4 cells and promote the release of interleukin 17 (IL-17). In addition, the capsule induces the release of the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and IL-1 β from peritoneal macrophages. These cytokines potentiate the increase of cell adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) on mesothelial cell surfaces, which in turn leads to an increase in the binding of neutrophils to these cells and initiates abscess formation [100]. Pili (fimbriae) and lectin like adhesions may also be important in the adherence of *Bacteroides* cells to epithelial surfaces. Butyrate and succinate produced by *Bacteroides* show a cytotoxic effect [38].

Additional studies indicate that anaerobes can produce compounds such as succinic acid and short-chain fatty acids that inhibit the ability of phagocytes to clear facultative organisms [76]. Enterotoxigenic strains of *B. fragilis* (ETBF) produce a zinc-containing metalloprotease of 44.4kDa, which induces fluid accumulation in ligated intestinal loops and morphologic changes in HT-29 cells. ETBF has been found to cause diarrhea in animals and humans, especially children. The isolation of ETBF from 20.3% of diarrhea specimens from children in rural Bangladesh has been reported [26].

2. *Prevotella* species produce proteases- IgA proteases and metabolic products such as volatile fatty acids and amines. The degradation of IgA produced by mucosal surfaces allows *Prevotella* to evade this first line of host defence [100]. *Prevotella melaninogenica* is one of the few bacteria that produce collagenase. *Porphyromonas gingivalis* also produces collagenase and has trypsin-like activity. *Porphyromonas asaccharolytica* produces proteinases that render it capable of hydrolyzing gelatin, casein, coagulated protein, plasma protein, azacol, and collagen. A strain of *P.*

melaninogenica produces phospholipase A [38].

3. *P. gingivalis* possess a large number of virulence determinants, such as fimbriae and proteases [103]. It induces tissue destruction by the elevation of tumor necrosis factor (TNF) levels [65]. The bacterium has been shown to possess various bioactive components including a cytoplasmic membrane, peptidoglycan, outer-membrane proteins, LPS and fimbriae on its cell surfaces [75]. These components have been demonstrated to induce multiple cytokines in periodontal tissues [54]. The abilities of *P. gingivalis* to adhere to and invade host tissue, e.g. epithelial cells, endothelial cells, and gingival fibroblasts, as well as to express powerful lytic enzymes may also play a role in the destruction of the periodontal tissue [8]. Deshpande et al [30] indicated the existence of an association between *P. gingivalis* and cardiovascular disease because of the bacterium's ability to multiply in and activate endothelial cells. Lipopolysaccharide from *P. gingivalis* stimulates cytokine secretion in immune cells and initiates the inflammation associated with periodontitis [40].
4. *F. necrophorum* causes tissue necrosis in the oral cavity. Toxins such as leukotoxin, endotoxin, and hemolysin have been implicated as virulence factors. Both *F. nucleatum* and *F. necrophorum* produce a potent LPS (lipopolysaccharide) that is responsible for the release of numerous pro-inflammatory cytokines and other inflammatory mediators [100]. Certain *F. necrophorum* hemagglutinate the erythrocytes of humans. A bovine isolate of *F. necrophorum* demonstrates lysophospholipase activity. *Fusobacterium gonidiaformans* produces an appreciable inflammatory reaction when inoculated into the skin of rabbits; when injected intra-peritoneally into mice, it leads to

liver abscesses and occasionally to death. A specific toxin has not yet been isolated. *Fusobacterium necrophorum* may persist in Kupffer cells and impair macrophage functions [38].

5. The gram-negative anaerobe *Veillonella* is a common isolate in microbiological characterizations of human dental plaque and gingival crevices. The lipopolysaccharide (LPS) of this organism is highly endotoxic to mammalian tissue and has been implicated in the development of periodontal disease [99].

1.4.3 Pathogenicity of the gram-positive bacteria

Certain toxins account for the virulence of some anaerobic infections and cause serious intoxication without infection.

1. Tetanospasmin initially binds to peripheral nerve terminals. It is transported within the axon and across synaptic junctions until it reaches the central nervous system. There it becomes rapidly fixed to gangliosides at the presynaptic inhibitory motor nerve endings, and is taken up into the axon by endocytosis. The effect of the toxin is to block the release of inhibitory neurotransmitters (glycine and gamma-aminobutyric acid) across the synaptic cleft, which is required to inhibit nervous impulse. If nervous impulses cannot be checked by normal inhibitory mechanisms, it produces the generalized muscular spasms characteristic of tetanus. Tetanospasmin appears to act by selective cleavage of a protein component of synaptic vesicles, synaptobrevin II, and this prevents the release of neurotransmitters by the cells.

The receptor to which tetanospasmin binds has been reported as ganglioside GT and/or GD1b. Binding appears to depend on the number and position of sialic acid residues on the ganglioside. Isolated B fragments, but not A fragments, will bind to the ganglioside. The A fragment has toxic (enzymatic) activity after the B fragment secures its entry. Binding appears to be an irreversible event so that recovery depends on sprouting a new axon terminal [98].

2. The botulinum toxin is specific for peripheral nerve endings at the point where a motor neuron stimulates a muscle. The toxin binds to the neuron and prevents the release of acetylcholine across the synaptic cleft. The heavy chain of the toxin mediates binding to presynaptic receptors. Different toxin types seem to utilize slightly different receptors. The binding region of the toxin molecule is located near the carboxy terminus of the heavy chain. The amino terminus of the heavy chain is thought to form a channel through the membrane of the neuron allowing the light chain to enter. The toxin (A fragment) enters the cell by receptor-mediated endocytosis. Once inside a neuron, different toxin types probably differ in mechanisms by which they inhibit acetylcholine release. The affected cells fail to release a neurotransmitter, thus producing paralysis of the motor system. Once damaged, the synapse is rendered permanently useless. The recovery of function requires sprouting of a new presynaptic axon and the subsequent formation of a new synapse [98].

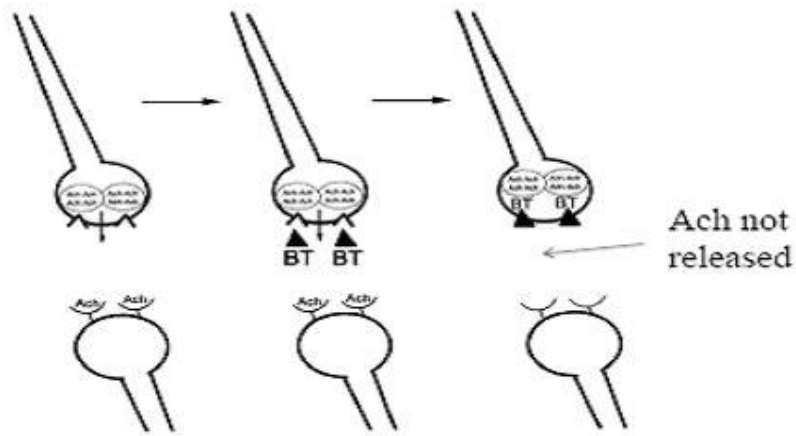


Figure 5: Action of botulinum toxin on a neuromuscular junction [89].

3. *Clostridium perfringens* produces the alpha-toxin, a potent lecithinase that causes haemolysis and necrosis. Other toxins include collagenase, hyaluronidase, deoxyribonuclease and proteinases [39]. Among the 12 toxins produced by *C. perfringens* are four major lethal toxins that are used to divide the species into five serologic types classified A to E. Additional virulence factors include enterotoxin, neuraminidase (sialidase), non- α - δ - θ -hemolysins, and the organism's vigorous metabolic activity [63]. As the clostridia multiply, various exotoxins (including hemolysins, collagenases, proteases, and lipases) are released into the surrounding tissue, causing local tissue necrosis, ischemia, gas formation, muscle involvement and systemic toxemia. As capillary permeability increases, the accumulation of fluid increases, and venous return is reduced, more tissue becomes involved, and clostridia release more toxins into the local tissue and the systemic circulation [105].
4. Virulence of *C. sordellii* is attributed to numerous exotoxins, although only 2, the lethal toxin (LT) and hemorrhagic toxins (HT) have been extensively studied.

Pathogenic strains of *C. sordellii* produce up to 7 identified exotoxins. Of these, lethal toxin and hemorrhagic toxin are regarded as the major virulence factors. Other exotoxins include an oxygen-labile hemolysin, neuraminidase, DNase, collagenase, and lysolecithinase. The roles of these toxins in pathogenesis have not been extensively investigated [92].

5. Actinomycosis results when bacteria resident in the mouth are introduced into the tissues. The mechanisms by which *Actinomyces* and *P. propionicum* produce disease are not clear. Pathogenesis may involve the ability of these organisms to suppress some of the immune functions of the host. Studies of oral disease have shown that *Actinomyces* are chemotactic, activate lymphocyte blastogenesis, and stimulate the release of lysosomal enzymes from polymorphonuclear leukocytes and macrophages. With the exception of *A. pyogenes* which produces a soluble toxin and a hemolysin that can be neutralized by antiserum, *Actinomyces* and *P. propionicus* do not produce exotoxins or significant amounts of other toxic substances [16]. Actinomycosis is almost always a mixed infection; a variety of other oral bacteria can be found in the lesion with *Actinomyces* or *P. propionicus*. The role of these associated bacteria is not clear. It has been shown that succinic acid produced by *Actinomyces* can promote the growth of some gram-negative anaerobes. In experimental infections, *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* enhance the virulence of *Actinomyces*. The mycelial masses of *Actinomyces* reduce the rate of penetration of antibiotics and may physically protect the associated bacteria. In addition, associated organisms that produce β -lactamases can complicate treatment. Therefore, the dense granules of *Actinomyces* and the presence of associated bacteria can enhance the virulence of

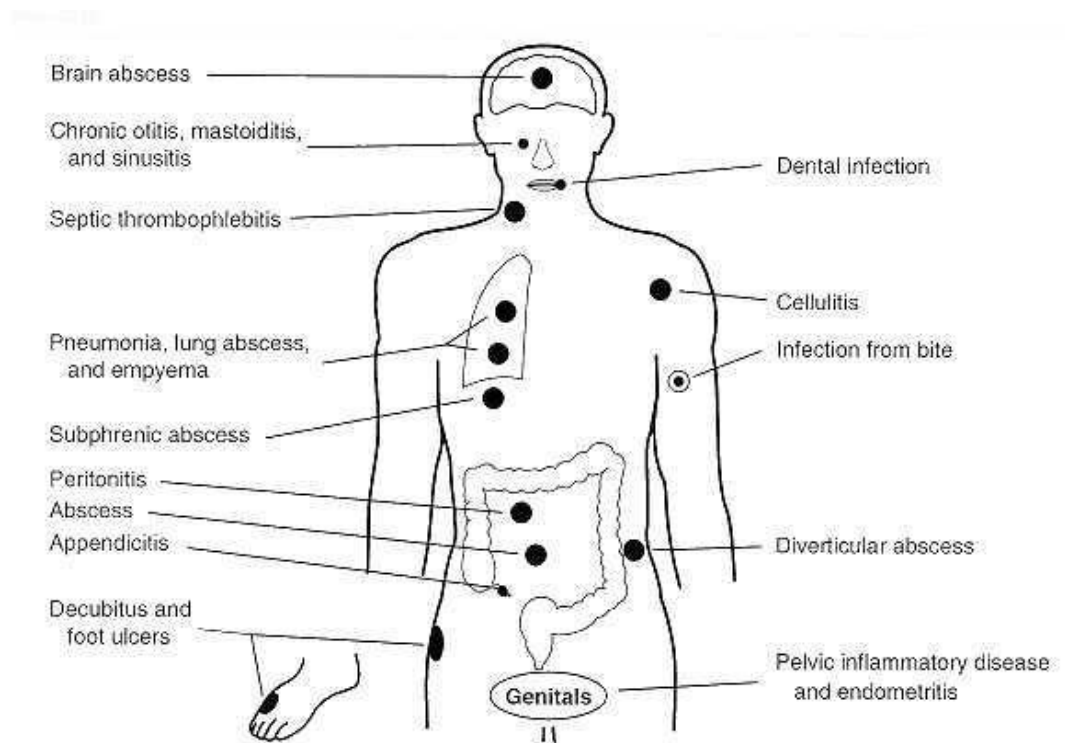
the infection and influence the mode of use of antibiotics, thereby adding to the difficulty of treating the disease [16].

1.5 Clinical manifestations

Anaerobic infections generally occur in patients who have impaired host defense mechanisms. Disruption of natural barriers such as the skin and mucous membranes, diabetes mellitus, connective tissue disorders, atherosclerotic disease, cancer (especially of the colon, uterus, and lung), irradiation damage, immunosuppressive treatment, and alcoholism predispose to anaerobic infections [39].

Anaerobic bacteria can cause infection practically anywhere in the body:

Figure 6: Types of anaerobic infections occurring in the human body.



[38]

- **Mouth, head, and neck:** Infections in the root canals, gums (gingivitis), jaw, tonsils, throat, sinuses, and ears.
- **Lung:** pneumonia, lung abscesses, infection of the lining of the lung (empyema), and dilated lung bronchi (bronchiectasis).
- **Intra abdominal:** abscess formation, peritonitis, and appendicitis.
- **Female genital tract:** pelvic abscesses, pelvic inflammatory disease, inflammation of the uterine lining (endometritis), and pelvic infections following abortion, childbirth, and surgery.
- **Skin and soft tissue:** diabetic skin ulcers, gangrene, destructive infection of the deep skin and tissues (necrotizing fascitis), and bite wound infections.
- **Central nervous system:** brain and spinal cord abscesses.
- **Bloodstream:** bacteraemia.

1.5.1 Clinical characteristics of infection caused by the anaerobic gram-negative bacilli

The clinical characteristics of infection with *Bacteroides*, *Prevotella* and *Fusobacterium* are similar, they include foul-smelling discharge, located in mucosal surfaces, cause tissue necrosis with gas production or discharges, associated with infections in cancer patients, infection related to the use of aminoglycosides or other agents with poor activity against anaerobes, septic thrombophlebitis and infection following human or animal bites [38].

B. fragilis one of the most common cause of anaerobic bacteremia, has been recognized as an organism with a potential mortality of 19% [80]. However, *B. ovatus* is occasionally isolated from the blood of compromised patients with poly-microbial bacteremia secondary to trauma or necrosis of the bowel [102].

The clinical presentation of *F. necrophorum* sepsis is distinctively characterized by sore throat and fever often accompanied by chills. A membranous tonsillitis with foul odour to the breath may be noted, and in the absence of effective therapy, bacteremia and widespread metastatic infections occurs [38].

Porphyromonas gingivalis, a black-pigmented rod, is considered to be associated with chronic periodontal disease [91]. *P. gingivalis* is a fastidious bacterium that can be detected more effectively by PCR (Polymerase chain reaction) than by culture and may need a longer incubation period than the 7 days applied in routine diagnostics [46]. Also this bacterium is considered to be the most pathogenic bacterium of all periodontal mixed anaerobic organisms that are part of normal flora [103]. It colonizes root canals in chronic apical periodontitis and is usually involved in periodontal diseases, which are characterized by alveolar bone loss [41].

1.5.2 Clinical characteristics of infection caused by the anaerobic gram- positive bacilli

1. Actinomycosis is a chronic disease characterized by the production of suppurative abscesses or granulomas that eventually develop draining sinuses. These lesions discharge pus containing the organisms. In long-standing cases, the organisms are found in firm, yellowish granules called sulphur granules. The disease is usually divided into three major clinical types:
 - Cervicofacial- infections involve the face, neck, jaw, or tongue and usually occur following an injury to the mouth or jaw or a dental manipulation such as extraction. The disease begins with pain and firm swelling along the jaw.
 - Thoracic- results from aspiration of pieces of infectious material from the teeth and may involve the chest wall, the lungs, or both. The symptoms are

similar to those of other chronic pulmonary diseases, and the disease is often difficult to diagnose. Thoracic disease may spread extensively to adjacent tissues or organs and often disseminates through the bloodstream, resulting in abscesses in distant sites such as the brain.

- Abdominal- often associated with abdominal surgery, accidental trauma, or acute perforative gastrointestinal disease. Persistent purulent drainage after surgery or abdominal masses resembling tumours may be the first sign of infection.

Primary infections may involve almost any organ. Secondary spread of the disease is by direct extension of an existing lesion without regard to anatomic barriers.

Actinomyces and *P. propionicus* cause a lacrimal canaliculitis with concretions in the canaliculi that are persistent. *A. naeslundii* and *A. odontolyticus* can also infect the eyes. In recent years, *Actinomyces* and *P. propionicus* have been isolated with increasing frequency from female pelvic infections associated with wearing an intrauterine contraceptive device [16].

2. *Propionibacterium propionicum* may resemble certain species of *Actinomyces* and it may be associated with human actinomycosis; cases involving neck abscess, a finger wound infection following a human bite, renal abscess, and lacrimal canaliculitis [102].
3. *Propionibacterium acnes*, an underestimated but significant cause of post - neurological infection, especially in the chronic sub-dural haematoma or cerebral tumor [43]. It is also known to be one of the most frequent causes of post-surgical endophthalmitis [88]. Severe infections due to *P. acnes* are rarely reported but it is

increasingly recognized as a cause of a number of infections, such as endocarditis, prosthetic joint infection, endophthalmitis, osteomyelitis, and central nervous system infections [20]. To the patients with an indwelling medical device such as a prosthetic heart valve, cerebrospinal fluid shunt or intra-ocular lens propionibacteria may be involved.

4. Eubacteria have been recognized as pathogens in infections of the female genital tract associated with intrauterine devices. They have been recovered from pleuro-pulmonary infections, (brain) abscesses, and osteomyelitis of the skull, peritonitis, wound infections, chronic periodontal infections, decubitus ulcers, and bites. Occasionally they have been reported as a cause of clinically significant bacteremia in patients with malignancies, gastrointestinal or obstetric disorders, and endocarditis.
5. Blood stream infections by bifidobacteria are diagnosed rarely, usually along with other organisms, in patients with complications associated with childbirth, gastrointestinal disorders, malignancies, or systemic lupus erythematosus. Involvement of bifidobacteria species has occasionally been reported in chronic otitis media, pleuro-pulmonary infections, cholesteatoma, peritonitis, abscesses in the head and neck, meningitis, and paronychia.
6. Lactobacteria have been involved in pleuro-pulmonary infections, (intra abdominal) abscesses, meningitis, conjunctivitis, dental caries, and endometritis. Several studies have indicated a protective effect of lactobacilli and bifidobacteria against potential pathogens in the gastrointestinal tract, linked to prevention of

colon cancer in several animals. Therefore, they have been in use as base of so-called probiotics, which are identified as bacteria that provide specific health benefits when consumed as a food component or supplement [67].

7. Clostridial wound infections may be divided into three categories namely gas gangrene/ clostridial myonecrosis, anaerobic cellulites and superficial contamination. Gas gangrene has a rapidly fatal outcome and requires urgent treatment [105]. Features of clostridial organisms are the production of gas at the infected site, activity of specific toxins and rarely massive intra-vascular hemolysis [63]. Initially tissue damages with impaired blood supply, the fever and edema, foul- smelling exudates and gas bubbles are products of anaerobic fermentation. Clostridial septicemia, although rare, may complicate in the late stages of the disease. Severe shock with massive hemolysis and renal failure is usually the ultimate cause of death. The incubation period, varies from 1 to 6 days, but it may be as long as 6 weeks. Average incubation times for the three most prevalent anaerobic organisms are for *C. perfringens* 10-48 hours, *C. septicum* 2-3 days and *C. novyi* 5-6 days, therefore the appropriate antimicrobial treatment is important to start as soon as possible to prevent development of gas gangrene. Clostridial cellulites, an infection of muscle tissue, with a more gradual onset than gas gangrene and appear without the systemic toxemia. Anaerobic cellulites may cause formation of gas bubbles, similar to gas gangrene. Some tissue necrosis does occur, caused by decreased blood supply and not invasion with the anaerobic organism. With appropriate treatment, anaerobic cellulites have a good prognosis [105].

8. Antibiotic-associated diarrhea (AAD) is a significant cause of morbidity and mortality, particularly in the hospitalized patients. People in good health usually do not get *C. difficile* disease. *C. difficile* produces two toxins: Toxin A is referred to as an enterotoxin because it causes fluid accumulation in the bowel. Toxin B is an extremely lethal (cytopathic) toxin [98]. Antimicrobial agents implicated in *C. difficile*-associated gastrointestinal illness have included aminoglycosides, penicillin's, cephalosporins, clindamycin, erythromycin, linomycin, trimethoprim-sulfamethoxazole, amphotericin B, rifampin and the fluoroquinolones [102]. *C. difficile* has been implicated in:
- a. Pseudomembranous colitis;
 - b. Diarrhea associated with methotrexate treatment and other anticancer chemotherapeutic agents;
 - c. Relapses of nonspecific inflammatory bowel disease (e.g., Crohn's disease; ulcerative colitis);
 - d. Obstruction or strangulation of the bowel and
 - e. A few cases of sudden infant death syndrome [102].
9. *C. tertium* is an aero-tolerant anaerobic organism that is usually considered nonpathogenic however; there are scattered reports of this organism causing bacteremia. Most cases have involved neutropenic patients, and the gastrointestinal tract appears to be the source of infection. It is possible that this organism causes many more cases of bacteremia than is currently appreciated as its aero-tolerant nature may result in its misidentification as a *Bacillus* species [105].

10. Clinical presentations of botulism are categorized by the CDC (Center for Disease Control) as:

- a. **Foodborne**- seen in adults from the ingestion of preformed toxin in contaminated food [102]. Clinical symptoms of botulism begin 18-36 hours after toxin ingestion with weakness, dizziness and dryness of the mouth. Nausea and vomiting may occur. Neurologic features soon develop, including blurred vision, inability to swallow, difficulty in speech, descending weakness of skeletal muscles and respiratory paralysis [98].
- b. **Wound** -results from production of botulinum toxin in an infected wound,
- c. **Infant** - seen most commonly with production of the neurotoxin within the infant gut [102]. The disease occurs in infants 5 - 20 weeks of age that have been exposed to solid foods, presumably the source of infection (spores). It is characterized by constipation and weak sucking ability and generalized weakness. The possible role of infant botulism in "sudden infant death syndrome-SIDS" has been suggested but remains unproven. *C. botulinum*, its toxin, or both have been found in the bowel contents of several infants who have died suddenly and unexpectedly [98].
- d. **Undetermined** (is identified in individuals who are older than 12 months, where no food or wound source of *C. botulinum* can be implicated [102].

Regardless of which antigenic type of *C. botulinum* (i.e. A, B, D, or E), is involved it may produce toxin which binds to synaptic vesicles of cholinergic nerves, preventing the release of acetylcholine at the peripheral nerve (including neuromuscular junctions) and consequently acute, flaccid, descending paralysis develops. The paralysis begins with bi-lateral impairment of cranial nerves resulting in paralysis of muscles of the face (including eyelids), head, and throat, then descends symmetrically to involve the muscles of the thorax, diaphragm, and extremities. Patients may die of respiratory

paralysis unless they have proper respiratory intensive care, including mechanical ventilation; death may also result from secondary pneumonia (caused by non-botulinum organisms) [13]. Confirmation of the clinical diagnosis of botulism is demonstrated by presence of botulinum toxin (mouse neutralization test) in serum or faeces, and/or *C. botulinum* in faeces. Isolation and identification of the organism is by conventional cultural biochemical procedures and the toxin neutralization test. Toxin has only rarely been detected in serum from an affected infant [102]. Toxin excretion may continue up to 1 month after the onset of illness, and stool cultures may remain positive for a similar period [13].

11. Tetanus is a severe disease caused by the toxin of *C. tetani* [105]. Most cases of tetanus result from small puncture wounds or lacerations, which become contaminated with *C. tetani* spores that germinate and produce toxin. The infection remains localized, often with only minimal inflammatory damage. The toxin is produced during cell growth, sporulation and lysis. It migrates along neural paths from a local wound to sites of action in the central nervous system [98]. The initial symptom is cramping and twitching of muscles around a wound. The patient usually has no fever but sweats profusely and begins to experience pain, especially in the area of the wound and around the neck and jaw muscles (trismus) [105]. The characteristic symptom of "lockjaw" involves spasms of the masseter muscle. Spasms of the pharyngeal muscles cause difficulty in swallowing [98]. Portions of the body may become extremely rigid, and opisthotonos (a spasm in which the head and heels are bent backward and the body bowed forward) is common [105]. In acute onset a very large number of patients die [13]. Death usually results from interference with mechanics of respiration [98].

Complications include fractures, bowel impaction, intramuscular hematoma, muscle ruptures, pulmonary, renal and cardiac problems [105].

Figure 7: Soldier dying of tetanus.



Sir Charles Bell's portrait of a soldier dying of tetanus. The characteristic rigidity of the body is referred to as opisthotonos and risus sardonicus [98].

12. Although most *Clostridium* species are not generally considered to be invasive, *C. clostridioforme* organisms have been isolated from bone, blood, liver abscesses, sub gingival areas, and foot (in diabetic patients) [107].

13. *Clostridium ramosum* is a member of the large bowel flora and is the second most common clostridium isolated from properly collected clinical specimens. It is common in intra abdominal infections following trauma and especially important clinically because of its resistance to penicillin G, clindamycin, and other antibiotics [102].

14. *Clostridium novyi* type A has been associated with an outbreak of serious illness and death amongst intravenous drug users. *Clostridium histolyticum* infections were also reported in this group of patients [101].
15. Many strains of *C. sordellii* are nonpathogenic; however, virulent strains cause lethal infections in several animal species, such as enteritis and enterotoxaemia in sheep and cattle [24].

1.5.3 Clinical characteristics of infection caused by the anaerobic cocci

Anaerobic cocci are not involved in specific disease process; rather, these infections may range in severity from mild skin abscesses, which disappear spontaneously after incision and drainage, to more serious and life-threatening infections such as brain abscess, bacteremia, necrotizing pneumonia, and septic abortion. Brain abscess, with a mortality rate of 40%, is one of the more serious infections involving anaerobic cocci that have been isolated in pure culture. Chronic otitis media or mastoiditis frequently, is the primary source of these organisms and may result as a direct extension of the infection into the brain. Pleuropulmonary infection, sinusitis, congenital heart defects, and bacterial endocarditis are other conditions predisposing individuals to brain abscess by blood-borne metastases.

Pleuropulmonary infections in which anaerobic cocci may be etiologic agents are lung abscesses, necrotizing pneumonia, aspiration pneumonitis, and empyema. The incidence of anaerobes in these infections is 50-90%; anaerobic cocci account for about 40% of the anaerobic isolates.

They are also involved in several skin and soft tissue infections that may be confused with clostridial myonecrosis (gas gangrene). These infections are identified as anaerobic streptococcal myonecrosis, progressive bacterial synergistic gangrene, necrotizing fasciitis, crepitant cellulitis, chronic burrowing ulcer, and synergistic necrotizing cellulitis. They are severe infections, and the mortality rates may be as high as 75%. These conditions may be characterized by purulent exudates, by varying degrees of tissue necrosis involving the skin, fascia, and/or underlying muscles, and sometimes by systemic toxicity and gas production. Anaerobic cocci often are isolated with other organisms in these infections. They are characteristically found with *Staphylococcus aureus* and *Streptococcus pyogenes* in progressive bacterial synergistic gangrene, and also are found with gram-negative aerobic or facultative bacilli or *Bacteroides* or both in synergistic non-clostridial myonecrosis and synergistic necrotizing cellulites. Diabetes mellitus and vascular insufficiency (often associated with trauma) are predisposing factors. Decubitis ulcers and postoperative wound infections are other soft-tissue infections from which anaerobic cocci have been isolated.

Anaerobic cocci have been recognized as significant pathogens in puerperal fever and septic abortion since the early 1900s. Other infections of the female genital tract in which anaerobic cocci have been implicated are pyometra, tubo-ovarian abscesses, postoperative wound infections following gynaecologic surgery, and pelvic inflammatory disease, often in association with gonococci.

Peridontal disease, peritonitis, intra abdominal abscesses of the liver, spleen, and pancreas are types of intra abdominal infections from which anaerobic cocci have been isolated as polymicrobial infections [104].

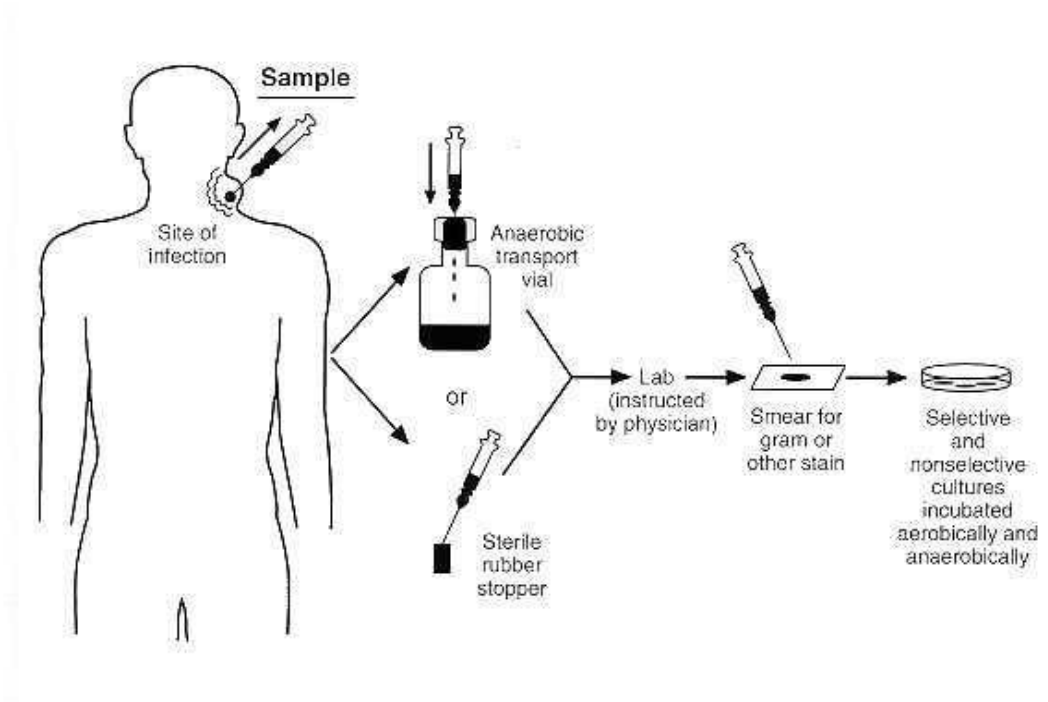
P. magnus, the most frequent isolated anaerobic coccus, is associated most often with chronic bone and joint infections and ankle ulcers. Pure cultures of this organism are not rare: they account for 15% of all *P. magnus* isolates. The presence of foreign bodies, such as prosthetic joints, seems to be particularly significant in *P. magnus* infection.

In one study, anaerobic cocci were isolated in 15 (6%) of 246 cases of mono-microbial bacteremia in cancer patients, indicating a relatively rare, but significant pathogenic potential for anaerobic cocci in this patient population [104]. Predisposing factors to *Peptostreptococcal* bacteremia include malignancy, recent gastrointestinal, obstetric, or gynaecologic surgery; ulceration of the extremities; dental extraction; and immunosuppression.

Peptostreptococcus species are among the most common organisms in anaerobic osteomyelitis and arthritis at all sites, including bites and cranial infections. When mixed with other aerobes and anaerobes, they may be involved in chronic wounds such as venous leg ulcers and severe soft tissue infections such as cellulitis, streptococcal myonecrosis, necrotizing fasciitis, and Fournier's gangrene. *P. anaerobius* is associated with infections of the female genital tract, intra abdominal, polymicrobial soft tissue and dental abscesses.

1.6 Diagnosis of anaerobic organisms

Figure 8: Procedure from collection to identification of anaerobes.



[47]

1.6.1 Collection of specimens

Specimens collected from sites such as body fluids other than urine, exudates from deep abscesses, fine-needle aspirates, and tissue biopsies should be cultured for anaerobic bacteria. However, since anaerobes normally inhabit the skin and mucous membranes as part of the normal indigenous flora, the specimens are virtually always unacceptable for anaerobic culture because the results cannot be interpreted. Gross examination of specimens may be valuable in assessing presence of anaerobes. A foul odor, purulent appearance of fluid specimens, and the presence of necrotic tissue and gas or sulphur granules are all valuable clues. A needle and syringe should be used whenever possible for collecting specimens for anaerobic culture. Swabs are not appropriate specimens for

isolation of anaerobic organisms. Once the specimens are collected, particular precautions should be taken to protect them from oxygen exposure and to deliver them to the laboratory promptly [102].

Unless the specimen is sent to the laboratory immediately, it should be placed in an anaerobic transport tube containing oxygen-free carbon dioxide or nitrogen. The specimen is injected through the rubber stopper in the transport tube and remains in the anaerobic environment of the tube until processed in the bacteriology laboratory.

Specimens from sterile sites, such as blood, spinal fluid, or pleural fluid, poses no problem provided the usual precautions are taken to decontaminate the skin.

Specimens should be transported to the laboratory and processed within 2 hours, but kept at room temperature if there are delays in processing. Commonly, specimens should not be stored for more than 24 hours, biopsy tissue may be maintained for up to 20-24 hours if stored at 25° C in an anaerobic transport system.

1.6.2 Selection and use of media for anaerobes

The primary isolation of obligate anaerobic bacteria from clinical specimens involves the use of nonselective, selective, and enrichment media. The choice of media will depend on the anatomic source of the specimen. The use of nutritionally adequate media supplemented with hemin and vitamin K₁ is of key importance. Because abscesses and wound infections frequently contain mixtures of obligate anaerobes, facultative anaerobes, and aerobes, species present in small numbers are likely to be missed if only nonselective media are used.

Most gram-positive bacteria encountered in clinical specimens are inhibited by a vancomycin containing media, whereas most gram-negative facultative anaerobes are inhibited by an aminoglycoside containing media.

Egg yolk-neomycin agar may aid in the isolation of *Clostridium* species from mixed microbial populations. The lipase and lecithinase reactions provide useful differential characteristics. The medium inhibits various facultatively anaerobic gram-negative bacteria but allows the growth of many gram-negative and positive anaerobes, including clostridia [7].

For susceptibility testing Brucella agar supplemented with 5% blood, 5ug/ml hemin and 1 ug/ml vitamin K₁ is used. This is an enriched, non-selective medium for the isolation and cultivation of a wide variety of obligatory anaerobic micro- organisms. It supports the growth of fastidious micro- organisms due to its content of peptones, dextrose and yeast extract. The sheep blood, hemin and vitamin K₁, provide essential nutrients for certain obligate anaerobes. The media should be reduced immediately prior to inoculation by placing under anaerobic conditions for 18-24 hours [42].

1.6.3 Use of anaerobic systems

Although several techniques are available for maintaining an oxygen-free environment during the processing of specimens for anaerobic culture, the anaerobic jar is the most common. It is a medium sized glass or plastic jar with a tightly fitting lid containing palladium-coated alumina particles, which serve as a catalyst. It can be set up by two methods. The easiest uses a commercially available hydrogen and carbon dioxide generator envelope (Gaspak) that is placed in the jar along with the culture plates. The generator is activated with water. Oxygen within the jar and the hydrogen that is generated are

converted to water in the presence of the catalyst, thus producing anaerobic conditions. Carbon dioxide, which is also generated, is required for growth by some anaerobes and stimulates the growth of others [53].

Anaerobic conditions should always be monitored when using either of the two jar techniques by including an oxidation-reduction indicator. Methylene blue strips available commercially for this purpose is blue when oxidized and clear when reduced. Thus, if anaerobic conditions are achieved, the methylene blue indicator solution will gradually turn colorless and will remain that way. If the solution turns blue after being colorless, this indicates that anaerobic conditions were not maintained and that the culture results may not be valid [102].

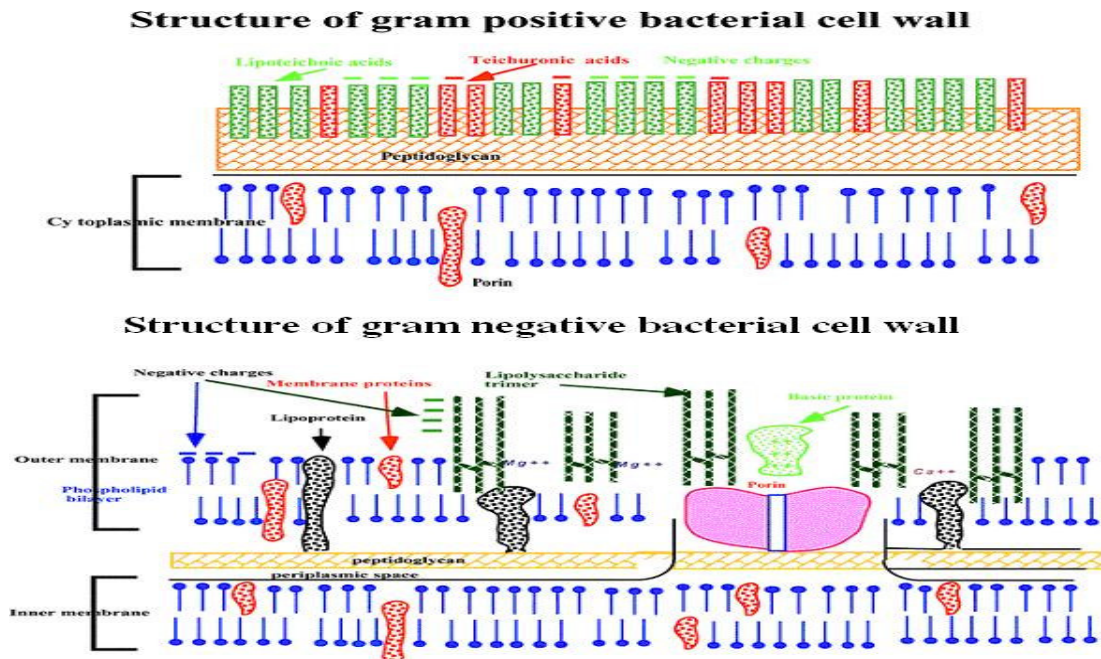
An alternative method for achieving anaerobiosis in the jar consists of evacuation and replacement. Air is evacuated from the sealed jar containing the culture plates and is replaced with an oxygen-free mixture of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide.

The anaerobic glove box isolator is an alternative. It is essentially a large clear-vinyl chamber, with attached gloves, containing a mixture of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. A lock at one end of the chamber is fitted with two hatches, one leading to the outside and the other to the inside of the chamber. Specimens are placed in the lock, the outside hatch is closed, and the air in the lock is evacuated and replaced with the gas mixture. The inside hatch is then opened to introduce the specimen into the chamber. Conventional bacteriologic procedures are employed to process the specimen in the oxygen-free atmosphere. Although these complex systems are needed to isolate anaerobic flora components, studies have shown that the anaerobic jar is adequate to recover clinically significant anaerobes. The extremely oxygen-sensitive bacteria of the micro flora apparently are not associated with infectious processes [53].

1.6.4 Laboratory identification

1. Gram stain is used to distinguish between gram- positive and gram- negative morphology of bacterial organisms. Crystal violet the primary stain, binds to the cell wall after treatment with iodine. Gram- positive organisms have the ability to retain the crystal violet after treatment with decolorizer due to the peptidoglycan structure of their cell wall and appear violet. Gram- negative bacteria loose the crystal violet primary stain when treated with decolorizer, because of the high lipid content of their cell wall. These decolorized bacteria then pick up the safranin counter-stain and appear red when observed under the microscope. Microscopy is a presumptive identification of anaerobic organisms [61].

Figure 9: Cell wall structure of gram- positive and negative bacteria



[84]

2. Sodium polyanethol sulfonate (SPS) discs- used for the differentiation of *Peptostreptococci*. Specimens are plated out onto 10% blood agar and a SPS disc placed on the initial inoculum and incubated anaerobically for 24 hours. The organism is SPS susceptible (S) if the zone of inhibition is $\geq 12\text{mm}$ and SPS resistant (R) if the zone of inhibition is $\leq 12\text{mm}$.
3. Mast identification (MID) 8 rings-for differentiation of anaerobes. The colistin, kanamycin, penicillin and vancomycin discs are utilized. A rifampicin disc is included for pigmenting gram-negative bacilli. Interpretation of the antibiotic discs: sensitive (S) if the zone of inhibition $\geq 10\text{mm}$ and resistant (R) if the zone of inhibition $\leq 10\text{mm}$.
4. Aesculin agar- is useful in differentiating several species of non-fermenting bacilli. Aesculin is a substituted glucoside that can be hydrolyzed by certain bacteria to yield glucose and aesculetin. When aesculin is hydrolyzed the medium turns black due to the reaction of aesculetin with the ferric ions in the medium. After touching the center of one well-isolated colony with a sterile inoculation loop, inoculate the organism onto the surface of the agar and incubate for 18 to 24hrs at 35°C anaerobically. Diffuse blackening of more than half of the inoculation within 24 to 48hrs indicates aesculin hydrolysis. On plates, black haloes should be observed around isolated colonies and any blackening is considered positive [61].
5. Catalase production. Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. A few drops of 3% hydrogen

peroxide are placed directly on a colony. Rapid effervescence indicates production of molecular oxygen and a positive test. Accurate results may be difficult to obtain if the test is performed on colonies growing on blood agar because of the presence of peroxidase in erythrocytes [61].

6. Rapid indole spot test – indole (benzyl pyrrole), is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan with the production of indole, pyruvic acid, and ammonia. Indole production is an important characteristic in the identification of many species of microorganisms. The indole test is based on the formation of a red complex when indole reacts with the aldehyde group of *p*-dimethylaminobenzaldehyde. This is the active chemical in Kovac reagent. A medium rich in tryptophan must be used. Rapid spot tests, using filter paper strips impregnated with *p*-dimethylaminocinnamaldehyde reagent, are useful in screening for bacteria that are prompt indole producers. A small portion of the colony to be tested is transferred from a nonselective medium, such as blood or chocolate agar, to a strip of filter paper that has been saturated with Kovac's reagent or *p*-dimethylaminocinnamaldehyde (PACA) solution. The immediate development of a red color with Kovac's reagent indicates the presence of indole and a positive test. PACA is more sensitive than Kovac's reagent, and a positive test reaction is indicated by the rapid development of a blue color [61].
7. Egg yolk agar. This medium is used for lecithinase, lipase and the Nagler reaction. Several clostridia produce phospholipases that give rise to a zone of opalescence extending beyond the colony or line of growth on human serum or egg-yolk media.

Proteolytic colonies produce zones of clearing on this media whilst opalescence restricted to the medium underlying a colony and associated with an overlying iridescent 'pearl layer' indicates lipase activity. The phospholipase reaction produced by *C. perfringens* is specifically neutralized by *C. perfringens* antitoxin. For the presumptive detection of *C. perfringens* in direct plate culture, prepare and dry a plate of good quality digest agar containing egg-yolk 5%. On one half of the plate spread 2-3 drops of *C. perfringens* antitoxin and allow drying. Then seed with the test organism or with the exudates under investigation, stroking from the antitoxin free to the antitoxin bearing half, and incubate anaerobically at 37°C for 24hrs. On the section containing no antitoxin, *C. perfringens* colonies show a surrounding zone of opacity, i.e. the Nagler reaction, whereas colonies of the organism on the remainder of the plate do not [27]. Lecitho-vitellin (LV) is the lipoprotein component of egg-yolk and can be obtained as a clear liquid by mixing egg-yolk with saline. When lecithinase-forming organisms are grown on a solid medium containing LV, the lecithinase diffuses into the agar and produces zones of opalescence around individual colonies. Lipolytic organisms also produce opalescence on LV agar and are often accompanied by a distinctive pearl layer or iridescent film [28].

8. β -lactamase testing- β -lactamases is heterogeneous bacterial enzymes that cleave the β -lactam ring of penicillins and cephalosporins to inactivate the antibiotic. β -lactamases are found in a wide variety of gram-positive and gram-negative bacterial species. The presence of β -lactamases may be detected quickly, providing an early clue that an isolate will not respond to the β -lactam antibiotics in question. The chromogenic cephalosporin test, employing nitrocefin, is the most sensitive.

Filter-paper disks impregnated with nitrocefin are commercially available. A loopfull of a colony is smeared on the disk and placed in a closed petri dish to prevent rapid desiccation. Organisms that contain β -lactamase will change the color of the disk from yellow to red. The reaction usually occurs within 30 seconds, but tests are read finally after 15 minutes [61].

9. Growth on bile agar. The bile test is based on the ability of certain bacteria to grow in the presence of bile salts. The medium contains 4% bile salts or 40% bile. With an inoculating wire or loop, two or three morphologically similar colonies are inoculated on the bile medium and incubated at 35°C for 24-48 hours in an ambient incubator. This is then observed for growth or no growth of the organism [61].
10. Motility nitrate media. Sterilize a solution of potassium nitrate (KNO_3) 2% in distilled water by autoclaving at 121°C for 20min. Use this to supplement PPY medium with KNO_3 200 $\mu\text{g/ml}$. To 5ml of the test culture in this PPY nitrate medium, add 0.25ml Reagent 1, then 0.25ml Reagent 2. A red color indicates the presence of nitrites. Reagent 1 = Addition of 100ml distilled water and 30ml glacial acetic acid to 0.5g sulphanilic acid and allow to dissolve. Reagent 2 = Dissolve 0.2g Cleves acid (5-amino-2 naphthalene sulphonic acid) in 120ml distilled water by warming in a water bath, then add 30ml glacial acetic acid [19].
11. Urease test. Microorganisms that possess the enzyme urease hydrolyze urea, releasing ammonia and producing a pink-red color change in the medium. For many of these species, a positive urease reaction is first detected by a pink-to-red color change in the slant portion of the agar. The slant initially turns red because of

the alkaline reaction, resulting from the splitting of small quantities of urea, and is augmented by the amines formed from the oxidative decarboxylation of the amino acids in the air-exposed portion of the medium. The surface of the agar slant is streaked with the test organism and incubated at 35°C for 18-24 hours. Organisms that hydrolyze urea rapidly may produce positive reactions within 1 or 2 hours; less active species may require 3 or more days. When there is no urea hydrolysis than the medium remains the original yellow color [61].

12. Rapid ID 32A Api panels. It is a system used for the identification of anaerobes.

This strip consists of 32 cupules, 29 of which are used as test cupules and contain dehydrated test substrates. After 4 hours of incubation in aerobic conditions, the reactions are read visually. Identification is obtained using the identification software [12]. The packaged kits test for preformed enzymes. Most of these systems use a battery of chromogenic substrates to rapidly test for various amino peptidases and glycosidases. Each system requires the preparation of a heavy cell suspension from the surface of a purity plate culture. All of the packaged systems provide numeric codes, computed databases and identification tables to aid in identification once an isolate has been characterized using the system [102].

13. Laboratory storage. Isolates can be stored in RCMM (Robertson's cooked meat medium). It is an excellent medium for the growth and maintenance of aerobic and anaerobic organisms. Cooked Meat Medium is prepared from heart tissue and has the ability to initiate growth of bacteria from very small inoculums and to maintain the viability of cultures over long periods of time. The products of growth do not rapidly destroy the inoculated organisms and therefore it is an excellent medium for

the storage of aerobic and anaerobic bacteria. The addition of glucose in the formulation allows rapid, heavy growth of anaerobic bacteria in a short time and leads to a more rapid identification of important anaerobes [83].

14. Laboratory sensitivity testing. Susceptibility testing of anaerobes is more challenging than aerobes, in part due to their fastidious growth and the need for specific diagnostic resources. Agar dilution is cumbersome to set up in a routine clinical laboratory for testing of patient isolates. Although the broth microdilution may be considered an alternative, up to 40% of clinical strains do not grow in broth [23]. Those that grow may do so poorly and thus resistance may potentially be underestimated. The CLSI and other expert groups do not recommend disc diffusion testing. The varying growth characteristics of different species and within species will directly influence zone sizes. E test uses a predefined and stable gradient covering 15 MIC (minimum inhibitory concentration) dilutions. It can be readily set up for daily testing and for resistance surveillance of all anaerobes to a wide range of antibiotics. It is the only commercial product cleared by the FDA (Food and Drug Association) for anaerobes. More than 100 scientific studies and publications have validated the reliability of E test for anaerobes. The CLSI anaerobe standard acknowledged that a system with a continuous concentration gradient, rather than two-fold dilution steps minimizes the uncertainty caused by MIC clustering around breakpoints [2]. The MIC is defined as the point on the epsilometer scale at which the curve of growth inhibition meets the test strip. Any intercept value that falls between two points, on the scale is rounded up to the next higher value [88]. However excessively wet plates prior to inoculation, insufficient

drying before applying strips and or unevenly streaked surfaces may give non-confluent growth, jagged edges and uneven MIC intersections [3].

1.7 Mechanisms of resistance among anaerobic bacteria

Administration of antibiotics, therapeutically or as prophylaxis, causes disturbances in the ecological balance between the host and the normal micro-flora. Disturbances in the micro-flora depend on the properties of the agents as well as of the absorption, route of elimination, and possible enzymatic inactivation and/or binding to fecal material of the agents. The clinically most common disturbances in the intestinal micro-flora are diarrhea and fungal infections that usually cease after the end of treatment. A well-balanced micro-flora prevents establishment of resistant microbial strains. By using antibiotics that do not disturb colonization resistance, the risk of emergence and spread of resistant strains between patients and dissemination of resistant determinants between microorganisms is reduced. Knowledge of the potential of different antibiotics to cause ecological disturbances in the normal micro-flora is of great importance, although individual variations of pharmacokinetics, composition, and susceptibility of the normal flora and degree of inactivation further determines the ecological outcome of antibacterial therapy [74].

Antibiotic resistance can be explained by two different mechanisms:

1. **Molecular:** including recombination or mutation, and
2. **Biochemical:** Bacteria that have acquired antibiotic resistance can overcome or avoid the effects of antibiotics through several basic mechanisms namely:
 - Production of β -lactamases,
 - Loss of outer membrane proteins,

- Alteration of target site and
- Expressing active efflux pumps

They can possess one or all of these mechanisms simultaneously [51].

1. Molecular

Bacterial resistance to antibiotics may develop as a result of a chance mutation or by exchange of DNA (deoxyribonucleic acid) with another bacterium. It is known that DNA transfer can occur between unrelated species. Plasmids are easily exchanged amongst same species, but this is not always the case. Genes that code for antibiotic resistance can be contained within these plasmids and transferred between bacteria [18]. Transfer of resistance genes has been described for anaerobes in the *B. fragilis* group and in *Prevotella*, *Clostridium*, and *Fusobacterium* species.

Bacterial conjugation appears to be the preferred method of transfer of antibiotic resistance genes. Resistance genes are harbored on transposons, plasmids, and chromosomal elements, many of which are mobile. Many of these elements are also small, carrying only the necessary genes for initiation of DNA transfer. Physical transfer of the DNA from cell to cell requires a mating bridge that appears to be encoded by much larger conjugative transposons, which are transferable.

Bacteroides encode an efficient conjugation system in which multiple, different transfer factors use mating-bridge pathways that are inducible. This phenomenon has significant implications for the spread of antibiotic resistance genes among organisms in the *Bacteroides* genus, as well as other gut commensals [50].

The most of β -lactamases produced by *Bacteroides* spp. are chromosomally mediated and produced constitutively. Some contain serine at their active site whereas others, known as

metallo-enzymes, require a Zn^{2+} at their active site for effective β -lactam hydrolysis. Although usually chromosomal, plasmid-mediated metallo- β -lactamases does occur. Conjugal transfer of clindamycin resistance has been shown to be plasmid-mediated. Many of these plasmids are self-transmissible and range in size from 14.6kb to approximately 82kb. Chromosomally encoded clindamycin resistance is linked to tetracycline resistance and the gene has been shown to lie within the tetracycline resistance transfer element. The frequency of transfer of resistance to tetracycline is however, very low unless an organism is exposed to tetracycline. The *tetQ* resistance gene is both inducible and transferable. Gene transfer occurs by conjugation mediated by the tetracycline resistance transfer element itself. Transfer is controlled by a two-component regulatory system. The two regulatory genes, *rteA* and *rteB*, are located in the *tetQ* operon downstream from the *tetQ* gene. Their expression is enhanced greatly by the presence of tetracycline, explaining the more efficient transfer of genetic material following tetracycline exposure. In addition to regulating the movement of the tetracycline resistance transfer element, *RteA* and *RteB* also are involved in the regulation of transfer of unlinked chromosomal elements called non-replicating *Bacteroides* units (NBU). Although most NBU do not result in any type of phenotypic expression, a ceftiofur-hydrolysing β -lactamase gene, *cfxA*, has been shown to reside on a NBU [59].

2. Biochemical:

2.1 Resistance against β -lactams

Some bacteria can synthesize enzymes that destroy the antibacterial agent or modify the agent to alter its entry or receptor binding [18].

Resistance to β -lactam antibiotics is mediated by 1 of 4 major resistance mechanisms:

- a. Inactivating enzymes (β -lactamases),
- b. Low-affinity, penicillin-binding proteins,
- c. Decreased permeability and
- d. Efflux pumps

- a. **Inactivating β -lactamases** is the most common and mediates the most diverse mechanism of resistance. Enzymes found among *Bacteroides* and *Prevotella* spp. are functional class 2e cephalosporinases. These enzymes are inhibited by the classical β -lactamase inhibitors: clavulanic acid, sulbactam and tazobactam [50]. Zinc metallo- β -lactamases is the most worrisome. These enzymes, encoded by the *ccrA* or *cfiA* genes, readily hydrolyze the carbapenems: imipenem, meropenem, ertapenem and other β -lactam agents. These β -lactamases are not inactivated by current β -lactamase inhibitors. It is important to note that as many as 4% of *Bacteroides* species actually carry the *ccrA* or *cfiA* genes, but the genes are not typically expressed to produce enzymes at a sufficiently high level to classify the strains as resistant (<0.8%) [50].
- b. **Low-affinity, penicillin-binding proteins.** Some bacteria develop antibiotic resistance by altering their cell-wall precursor targets, thereby preventing the antibiotic from disrupting the architecture of the cell wall [18]. Penicillin-binding proteins (PBP) such as PBP 1 complex and PBP 2, although essential to the activity of a β -lactam agent, have not been reported to be major mechanisms of resistance development among anaerobes [50]. Binding to PBP is the critical factor in determining whether a β -lactam antibiotic will effectively inhibit cell wall synthesis in a bacterium. Essential for bacterial growth, PBP function is the terminal stage of

cell wall synthesis. When a β -lactam effectively competes for the active site of an essential PBP, bacterial cell death results [59].

- c. **Decreased permeability.** In addition to enzyme production, permeability changes lead to β -lactam resistance among anaerobes. Pore-forming molecules associated with decreased permeability and β -lactam resistance has been described. Decreased permeability has also been associated with increased β -lactamase production, resulting in even higher levels of resistance to β -lactam antibiotics [59]. Pore-forming proteins of gram-negative anaerobic bacteria include identification and cloning of outer-membrane proteins from *Bacteroides*, *Porphyromonas* species, and *Fusobacteria*. The absence of ≥ 1 outer membrane protein has been found to be associated with resistance to ampicillin/sulbactam in some strains [50]. A decreased uptake or altered membrane permeability affect the ability of the drug to penetrate the bacterial cell wall.
- d. **Expressing active efflux pumps.** Increased elimination of the drug can occur through promotion of antibiotic efflux. Antibiotic efflux is an energy-dependent process that is related to the generation of an inner membrane protein by the resistant bacteria. Alterations that result in the increased expression of these pumps can increase the overall capacity of an organism to eliminate a drug, thus enabling the organism to resist higher concentrations of that drug [18].

1.7.1 Clindamycin resistance

Resistance to a wide variety of antibiotics may result from alteration of ribosomal binding sites. Failure of the antibiotic to bind to its target site(s) on the ribosome disrupts its ability to inhibit protein synthesis and cell growth. Alteration of ribosomal target sites is the principal mechanism of multiple-agent resistance among aerobic and anaerobic gram-positive bacteria. There could also be modification of the target enzyme so that it remains functional and insensitive to the antibacterial agent, while other bacteria develop resistance by over producing target enzymes [18].

Several genetic clindamycin- resistance determinants have been identified in the *B. fragilis* group of organisms (*ermF*, *ermG*, and *ermS*), *Clostridium perfringens* (*ermQ* and *ermP*), *C. difficile* (*ermZ*, *ermB*, and *ermBZ*), and *Porphyromonas*, *Prevotella*, *Peptostreptococcus*, and *Eubacterium* species (*ermF*). For both *B. fragilis* organisms and *C. difficile*, these determinants can be located on the chromosome, plasmids, or transposons and are transferable by conjugation. Resistance is mediated by a macrolide-lincosamide-streptogramin type 23S RNA (ribonucleic acid) methylase. However, not all clindamycin-resistant *bacteroides* poses *erm* genes, and alternative mechanisms for a minority of strains are likely [50]. Among *Bacteroides* spp., resistance is mediated by methylation of 23S rRNA at one of two adenine residues that prevents effective binding of clindamycin to the ribosomes, rendering them refractory to the drug's inhibitory properties. This mechanism may be either inducible or expressed constitutively [59].

1.7.2 Metronidazole resistance

Metronidazole is a nitroimidazole antibacterial agent that exhibits rapid bactericidal activity against some anaerobic organisms such as members of the *Bacteroides fragilis* group [95]. Metronidazole resistance is more common among gram-positive anaerobic

bacteria, including most isolates of *Propionibacterium acnes* and *Actinomyces* species, as well as some strains of lactobacilli and anaerobic streptococci.

The active antibacterial component of metronidazole is stable only under anaerobic conditions [50]. Resistance to metronidazole is attributed to a combination of reduced nitroreductase and decreased drug uptake by the organism. Both of these occur simultaneously [59].

Metronidazole activity is dependent on the reduction of the nitro group to form a reactive intermediate product [1]. Nitroimidazole resistance genes (*nim*) encode a nitroimidazole reductase, which reduces 4 or 5- nitroimidazole to 4 or 5- aminimidazole and prevents the formation of toxic nitrosoresidues necessary for the agent's activity. Six related chromosomal or plasmid-based *nim* genes (*nim* A-F) have now been reported in *Bacteroides* species. Insertion sequence elements, either identical or similar to those found in imipenem-resistant strains, are also found upstream of the *nim* genes, likely increasing their expression [50].

The entry of 5-nitroimidazoles into the cell depends on the rate of reduction of the nitro group. A decrease in the reducing environment within the bacterium will result in reduced nitroreductase activity and a concomitant reduction in drug uptake. Decreased pyruvate: ferredoxin oxireductase activity in combination with a compensatory increase in the lactate dehydrogenase activity results in a decrease in the reducing power of the bacterium [59]. Thus the in-vitro activity of metronidazole necessitates an immediate reduction of the drug at a low redox potential of at least Eh-450 mV to promote a continued uptake of the drug as the organism grows [1].

Two genes, designated *nimA* and *nimB*, capable of conferring moderate to high-level resistance have been described. DNA sequencing of these two genes shows approximately 73% similarity, and they presumably represent two unique genes that confer resistance by the same mechanism. These genes have been localized to the chromosome as well as to a variety of different non-self-transmissible plasmids. These plasmids can, however, be mobilized by other conjugal elements or acquired by transformation [59].

1.7.3 Quinolone resistance

Whether the typically low rate of susceptibility to most quinolones is due to poor drug penetration, low affinity for the target topoisomerases II and IV, or some other mechanism has not yet been clearly ascertained. Concomitant reduced cell permeability has, however, been described in clinical isolates of *B. fragilis* to cefoxitin and norfloxacin. Quinolones are generally bacteriostatic rather than bacteriocidal under anaerobic conditions [59].

In both aerobic and facultatively anaerobic bacteria, fluoroquinolones inhibit DNA gyrase and topoisomerase IV, both of which are essential for bacterial DNA replication.

Resistance in aerobes occurs by mutations in gyrase (*gyrA*) and topoisomerase IV (*parC*) genes and/or by increased expression of efflux pumps. Similar mechanisms have been discovered in anaerobic bacteria. In *B. fragilis*, both *gyrA* and *gyrB* were cloned from the bacteroides genome [50].

1.7.4 Tetracycline resistance

Protection or modification of the target site is the only documented mechanism of resistance to tetracycline in *Bacteroides* spp. The chromosomal *tetQ* gene encodes a protein that renders the ribosomal protein synthesis apparatus refractory to the inhibitory

effects of tetracyclines. DNA cross-hybridization studies indicate that a *tetQ* or *tetQ*-like gene is present in most tetracycline-resistant *Bacteroides* isolates. Identification of tetracycline-resistant isolates that do not contain *tetQ* DNA sequences indicates that another mechanism (e.g. efflux of tetracycline) or another class of ribosomal protection proteins also contributes to tetracycline resistance.

Tetracycline resistance genes have also been identified in *Costridium* spp. The *tetA* (P) and *tetB* (P) genes together form an operon encoding two unrelated proteins that result in tetracycline resistance mediated by two separate mechanisms. The *tet* gene product inactivates tetracycline by oxidation of the molecule, but is active only under aerobic conditions and has, therefore, not been shown to be operational in *Bacteroides* spp [59].

1.7.5 Chloramphenicol resistance

Two different classes of chloramphenicol resistance genes have been reported in *Bacteroides* spp. Both result in drug inactivation either through acetylation or by nitro-reduction at the p-nitro group of the benzene ring. The chloramphenicol acetyltransferase gene is transferable and plasmid-mediated [59].

1.7.6 Aminoglycoside resistance

It appears as if aminoglycoside resistance is the result of the drugs failure to reach their target sites. Aminoglycoside uptake is a two-step process involving both energy-independent and energy-dependent phases. Either oxygen or a nitrogen-dependent electron transport system has the capability to provide the energy necessary for the energy-driven phase of aminoglycoside uptake. Strict anaerobes lack these electron transport systems and are therefore impermeable to aminoglycosides [59].

1.8 Management

1.8.1 Surgical

In addition to antimicrobial therapy, surgical drainage of abscesses, excisions of necrotic tissue, relief of obstruction, and ligation or re-section of infected veins are important.

Percutaneous non-surgical drainage may be effective in certain patients. Lung abscess, which responds well to medical therapy, is the primary exception to the rule that abscesses require surgical drainage [38].

Treatment of gas gangrene includes radical surgical debridement coupled with high doses of antibiotics. Successful treatment of, less severe forms of clostridial wound infections includes local debridement and antibiotic therapy. The usefulness of gas gangrene antitoxin is a disputed matter. Some physicians maintain that the efficacy of this polyvalent antitoxin has been proved in the past, but better medical care now may have eliminated the need for its use. Others believe that because of insufficient data, antitoxin should be administered systemically as early as possible after diagnosis, and that the antitoxin should be injected locally into tissue that cannot be excised [105].

1.8.2 Prevention

There are two approaches in preventing anaerobic infections:

- i. Avoiding conditions that reduce the redox potential of the tissues and
- ii. Preventing the introduction of anaerobes of the normal flora to wounds, closed cavities, or other sites prone to infection.

Minimizing injury and devitalization of tissue during surgery protects against infection.

The use of closed methods of bowel resection, when feasible, decreases the likelihood of infection with members of the bowel flora. Appropriate therapy of established infections

such as chronic otitis media and sinusitis may prevent subsequent spread of infection such as intra-cranial abscess. Precautions to minimize aspiration are helpful in preventing anaerobic pulmonary infection [38].

Prevention of wound contamination is the single most important factor in controlling clostridial wound infections. In the past, immunization has been considered a possible preventive measure for gas gangrene; however, several factors have discouraged the use of active immunization, including difficulty in preparing a suitable antigenic toxoid, availability of prompt wound treatment, and accessibility of effective therapeutic agents [105].

Prophylactic antimicrobial therapy is effective in selected situations:

- ✓ Patients with acute leukemia on treatment with anti-tumor chemotherapy may require administration of an antimicrobial regimen designed to reduce the total body flora, including anaerobes.
- ✓ Anaerobic bacteremia following dental manipulation may be managed effectively by administration of an antibacterial agent 1 hour before manipulation.
- ✓ The effectiveness of prophylactic antimicrobial therapy before bowel surgery is now well established, as well as gynaecologic surgery [38].

A series of 4 primary immunizations against tetanus, followed by boosters q 10 yr, with the adsorbed (for primary immunization) or fluid (for boosters) toxoid is superior to giving antitoxin at the time of injury. Immunization in an unimmunized or inadequately immunized pregnant woman produces both active and passive immunity in the foetus and should be given at a gestational age of 5 to 6 months with a booster at 8 months [69].

Proper canning and adequate heating of home-canned food before serving are essential. Canned foods showing evidence of spoilage and swollen or leaking cans should be discarded. Infants < 12 months should not be fed honey, which may contain *C. botulinum* spores. Toxoids are available for active immunization of people working with *C. botulinum* or its toxins.

Although anaerobic infections can occur in any individual, good hygiene and general health management may help to prevent infections [69].

1.8.3 Antimicrobial administration

Since anaerobic infections are generally polymicrobial, where anaerobes are mixed with aerobic organisms, therapy should provide coverage of both types of pathogens. Treatment of anaerobic infections is also complicated by the slow growth of these organisms. The primary role of antimicrobials is to limit the local and systemic spread of infection [58]. Patients with nosocomially-acquired, intraabdominal infections are more likely to grow resistant pathogens. Inadequate empiric antimicrobial therapy is associated with treatment failure and death. Therefore, broader spectrum antimicrobial regimens are recommended for these patients [68].

Presently the drugs of choice for anaerobic gram- negative bacilli at Charlotte Maxeke Johannesburg Academic Hospital include amoxicillin- clavulanate, cefuroxime, metronidazole and alternate drugs include cefuroxime plus clindamycin or ertapenem. Lung abscess and necrotizing pneumonia may be caused by anaerobes of the oral cavity. In a comparative trial performed by Levison M.E et al., it was found that clindamycin to be clearly more effective than penicillin. A randomized study performed by Ferna'ndez-Sabe'

et al demonstrated that penicillin failures were associated with penicillin-resistant *Prevotella melaninogenica*.

Following these results most authorities recommend clindamycin as the drug of choice for the initial therapy of anaerobic lung infections. In the same study performed by Ferná'ndez-Sabé' all anaerobes and most facultative organisms isolated from respiratory samples have been susceptible to amoxicillin-clavulanate indicating that amoxicillin-clavulanate is a good therapy for patients with severe, community-acquired anaerobic lung infections, defined as lung abscess or necrotizing pneumonia. Amoxicillin-clavulanate provides coverage against the pathogens that are most likely to be involved in these infections, and it is effective in resolving the clinical signs and symptoms of the disease.

Both duration of fever and putrid sputum production were shorter in these patients treated with amoxicillin-clavulanate than in previously studied patients receiving clindamycin. An additional advantage, as in the case of clindamycin, is the possibility of using sequential therapy, beginning with intravenous administration for a few days and then switching to the oral route with the same antibiotic [37].

Clinicians should be aware that in vitro antimicrobial susceptibility tests have shown that some strains of anaerobic cocci are resistant to penicillin or to clindamycin. Metronidazole is typically active against most strains of anaerobic cocci [104]. Although Penicillin G is the drug of choice for treating infections involving anaerobic cocci, alternate choices would be a cephalosporin, clindamycin or chloramphenicol.

Penicillin is the drug of choice for all clostridial wound infections [105].

Trivalent antitoxin (A, B, E) is available for botulinism. Antitoxin does not inactivate toxin that is already bound at the neuromuscular junction; therefore, pre-existing neurologic

impairment cannot be reversed rapidly. However, antitoxin may slow or halt further progression. Antitoxin should be given as soon as possible after clinical diagnosis. Antitoxin is less likely to be of benefit if given > 72 hours after symptom onset [69].

Tetanus therapy requires maintaining adequate ventilation. Additional interventions include early and adequate use of human immune globulin to neutralize nonfixed toxin; prevention of further toxin production; sedation; control of muscle spasm, hypertonicity, fluid balance, and intercurrent infection; and continuous nursing care. If necessary, immune globulin or antitoxin can be injected directly into the wound, but this injection is not as important as proper wound care. Following injury, tetanus vaccination is given depending on wound type and vaccination history; tetanus immune globulin may also be indicated. Patients not previously vaccinated are given 2nd and 3rd dose of toxoid at monthly intervals [69].

Due to the mixed nature of the infection and the presence of granules successful treatment of actinomycosis requires long-term antibiotic therapy combined with surgical drainage of the lesions and excision of damaged tissue. *Actinomyces* spp. and *P. propionicus* are susceptible to penicillins, the cephalosporins, tetracycline, chloramphenicol, and a variety of other antibiotics. Penicillin is the drug of choice for infections with all species of *Actinomyces*; significant drug resistance is unknown [16].

Organisms to be considered for antimicrobial susceptibility testing because of their virulence, or because they are commonly resistant to certain antimicrobial agents include species of the *Bacteroides fragilis* group, species of the pigmented *Prevotella-Porphyrromonas* group, other *Prevotella*, *Fusobacterium mortiferum*, *F. varium*, and *F. necrophorum*, *Bilophila*, *Sutterrella*, and some *Clostridium* species [102].

2.0 Rationale for study

2.1 Projected Outcome

This study will provide information to the clinician about susceptibility to the antimicrobial agents of anaerobic bacteria from patients admitted at Charlotte Maxeke Johannesburg Academic Hospital. Antimicrobial resistance patterns have changed lately worldwide and this information will be used to create antimicrobial guidelines for empirical treatment of anaerobic infections in the local area.

The study will initiate and provide a foundation for a surveillance program for anaerobic organisms at the Microbiology Laboratory of the Charlotte Maxeke Johannesburg Academic Hospital, and finally, the data will be used to monitor the prevalence of antimicrobial resistance from a public health perspective.

3.0

3.1 Aim

This study aimed to determine the antimicrobial susceptibility patterns of anaerobic organisms isolated from clinical specimens and to analyze demographic data of patients admitted at Charlotte Maxeke Johannesburg Academic Hospital.

3.2 Objective

To initiate and organize this survey such that it can be performed on a regular periodic basis to update the data and identify changing trends in susceptibility and resistance.

4.0 Materials and methods

All anaerobes used for this study were routinely isolated from clinical specimens. After they were isolated and identified they were stored in RCMM until sensitivity was performed. The sensitivity testing was performed for purpose of this study in batches where each batch comprised of nine anaerobes including the reference strain controls.

4.1 Microscopy, identification and sensitivity

4.1.1 Microscopy

On all isolates gram-stain was performed.

Method:

- i. A thin smear of the organism was made and allowed to air dry after which it was fixed using methanol or heat.
- ii. The slide together with a control slide was stained. The control slide consisted of *E. coli* as the negative control and *S. aureus* as the positive control. First it was stained with crystal violet for approximately one minute and then washed with tap water.
- iii. Next it was stained with gram's iodine for approximately one minute and washed with tap water.
- iv. Thereafter it was decolorized with gram's decolorizer for only 10 seconds or less and washed with tap water.
- v. Lastly it was stained with safranin for approximately 30 seconds and washed with tap water.
- vi. The slides were allowed to air dry before examination under 100x oil immersion objective.

- vii. Ensuring that the control slides are correct, gram-positive organisms stained violet and gram-negative organisms stained pink in color.

4.1.2 Identification

All isolates were primarily cultured on basic 10% blood agar and amikacin agar plates.

For the identification of anaerobes, the Finegold System was implemented incorporating the following aspects:

4.1.2.1 Gram-positive cocci

Except for *Peptococcus niger* all former species of the genus *Peptococcus* were transferred to the genus *Peptostreptococcus*. The organisms were plated out on a 10% blood agar plate with a SPS disc and incubated in an anaerobic jar at 37°C for 24-48 hours. If the organism was SPS susceptible then it was reported as *Peptostreptococcus anaerobius* and if the organism was SPS resistant it was reported as *Peptostreptococcus* species.

4.1.2.2 Gram-positive bacilli

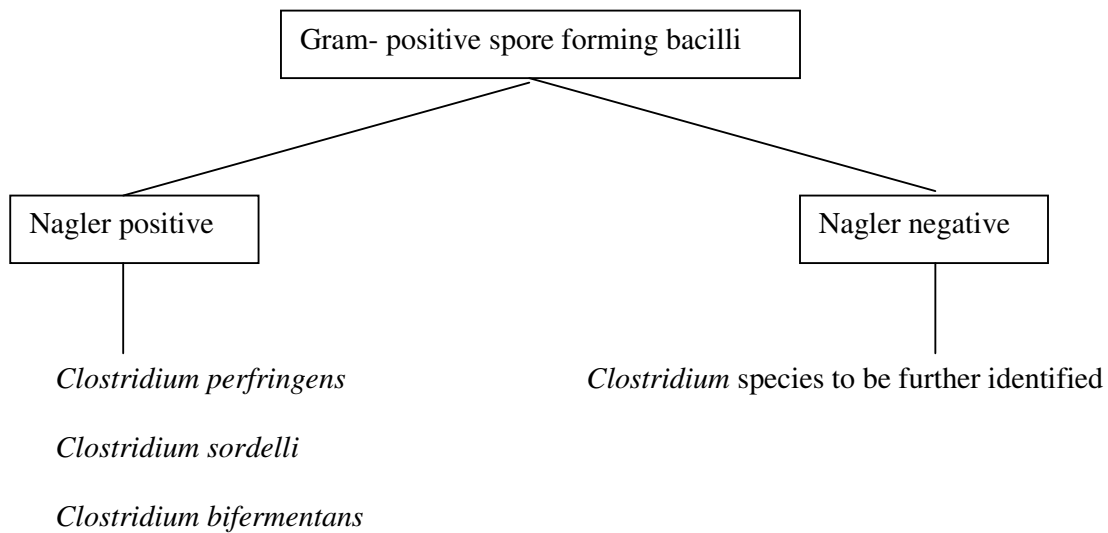
Anaerobic gram-positive bacilli of human clinical relevance are divided into two distinct groups, one genus of endospore formers (*Clostridium* species) and genera of non-spore-formers.

4.1.2.2.1 Gram-positive spore forming bacilli (Figure 10)

Certain clostridia for example: *C. tertium*, *C. inulinum*, *C. durum* and *C. carnis* are aero-tolerant and form colonies on chocolate agar incubated in 5% to 10% carbon dioxide (i.e. in micro-aerophilic conditions). The following criteria are used to determine whether an isolate is an aero-tolerant clostridia species or a facultatively anaerobic *Bacillus* species.

Aero-tolerant clostridia will rarely form spores when grown aerobically and are catalase negative, whereas *Bacillus* species will rarely form spores when grown anaerobically and are catalase positive. To demonstrate spores, gram-stained preparations are usually sufficient.

Figure 10: Identification of gram-positive spore forming bacilli



4.1.2.2.2 Gram-positive non-spore forming bacilli (Figure 11)

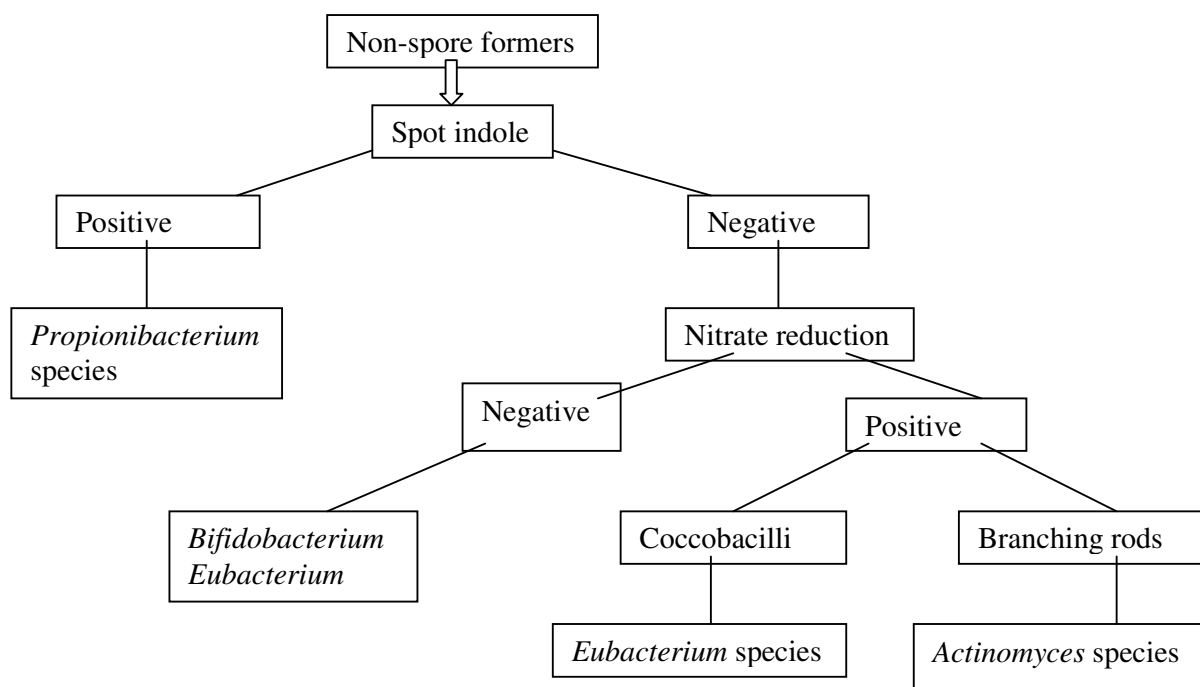
Included in this group are members of the following genera:

Branching bacteria: *Actinomyces* and *Bifidobacterium*

Non-branching bacteria: *Propionibacterium* and *Eubacterium*

Organisms were plated out on 10% blood agar for rapid indole. An aesculin plate was inoculated for aesculin hydrolysis and catalase and a nitrate medium was also inoculated. Incubation was 37°C for 24-48 hours in an anaerobic jar.

Figure 11: Identification of gram-positive non-spore forming bacilli



4.1.2.3 Gram-negative cocci

A motility nitrate medium was inoculated and incubated anaerobically in a jar at 37°C for 24 hours. A pink color indicates a positive reaction and was reported as *Veillonella parvula*. A negative result indicated by a yellow color was reported as an anaerobic gram-negative coccus.

4.1.2.4 Gram-negative bacilli

These species are identified on the basis of colonial and cellular morphology, pigment production, susceptibility to antibiotic discs and certain rapid biochemical characteristics:

1. *B. urealyticus*- presumptively identified by showing “pitting” growth.
2. *Prevotella* species are saccharolytic and produce a black pigment due to proto-heme production.
3. *Porphyromonas* species are asaccharolytic and also produce a black pigment.

A 10% blood agar plate was inoculated and a MAST MID8 ring placed in the center of the plate. An aesculin agar, basal agar and egg yoke for anaerobes was also inoculated. If the organism showed pitting growth a urea slope was added. All plates were incubated anaerobically in a jar for 24-48 hours at 37°C.

Table 1: Antibiotic discs used for identification of anaerobic gram-negative bacilli

Table 2: Identification tests used for the *Bacteroides* group

Table 3: Identification tests used for *Fusobacteria* and *Prevotella* species

Table 4: The cellular morphology characteristics of *Fusobacteria*

Table1. Antibiotic discs used for identification of anaerobic gram-negative bacilli.

Anaerobic organisms	Colistin (10µg)	Kanamycin (1000µg)	Vancomycin (5µg)	Penicillin (2 units)	Rifampicin (15µg)
<i>Bacteroides fragilis</i>	R	R	R		
<i>Bacteroides urealyticus</i>	S	S	R		
<i>Prevotella</i> species	S	R	R	R	S
<i>Prevotella intermedia</i>	S	R	R	S/R	S
<i>Porphyromonas</i> species	S	R	R	S	S
<i>Fusobacterium</i> species	S	S	R		

Table 2 Identification tests used for the *Bacteroides* group

	Aesculin hydrolysis	Indole spot test	Catalase	Bile agar	Urease
<i>Bacteroides fragilis</i>	Positive	Negative	Positive	Growth	
<i>Bacteroides thetaiotaomicron</i>	Positive	Positive	Positive	Growth	
<i>Bacteroides ovatus</i>	Positive	Positive	Negative	Growth	
<i>Bacteroides vulgatus</i>	Variable	Negative	Negative	Growth	
<i>Bacteroides urealyticus</i>	Negative	Negative	Negative	Inhibited	Positive

Table 3 Identification tests used for *Fusobacteria* and *Prevotella*

	Aesculin hydrolysis	Indole spot test	Catalase	Bile agar	Lipase
<i>Prevotella</i> species	Positive	Negative	Negative	Inhibited	
<i>Prevotella intermedia</i>	Negative	Positive	Negative	Inhibited	Positive
<i>Porphyromonas</i> species	Negative	Positive	Negative	Inhibited	
<i>Fusobacterium nucleatum</i>	Negative	Positive	Negative	Inhibited	
<i>Fusobacterium mortiferum</i>	Positive	Negative	Negative	Growth	
<i>Fusobacterium necrophorum</i>	Negative	Positive	Negative	Inhibited	Positive
<i>Fusobacterium varium</i>	Negative	Variable	Negative	Growth	

Table 4 Cellular morphology characteristics of the *Fusobacteria*

Species	Distinctive cellular morphology
<i>Fusobacterium nucleatum</i>	Slender with pointed ends
<i>Fusobacterium mortiferum</i>	Bizarre round bodies
<i>Fusobacterium necrophorum</i>	Large and pleomorphic
<i>Fusobacterium varium</i>	Pleomorphic

The following *Fusobacterium* species may produce hydrogen sulphide (This is seen by blackening of the colonies on aesculin agar): *Fusobacterium mortiferum*, *Fusobacterium necrophorum* and *Fusobacterium varium*.

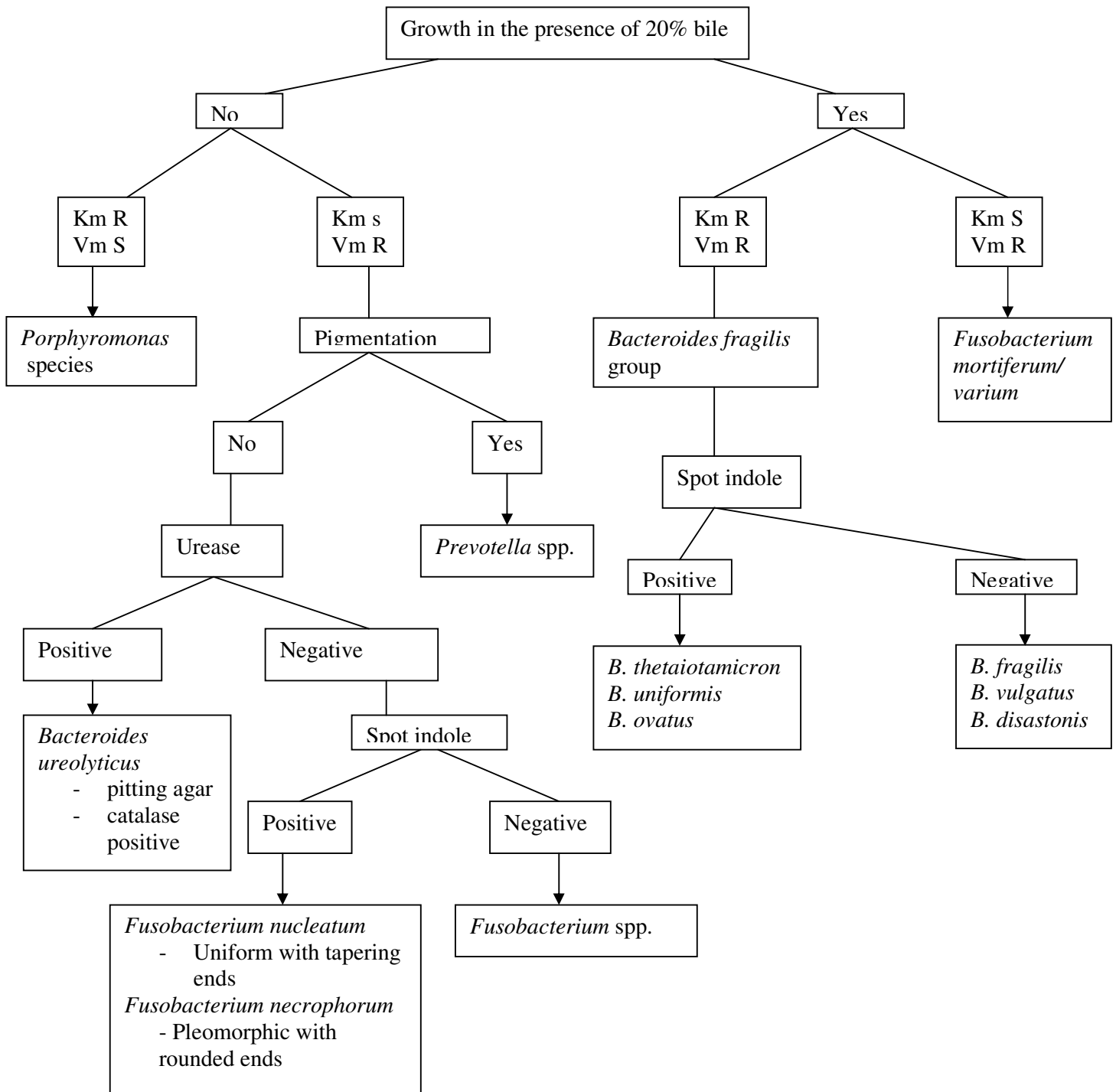
Fusobacterium necrophorum subspecies *necrophorum* is lipase positive whereas

Fusobacterium necrophorum subspecies *funduliforme* is lipase negative.

A new species, *Fusobacterium ulcerans* isolated from tropical ulcers, closely resembles *Fusobacterium mortiferum* but is aesculin negative.

Although *Bilophila wadsworthia* is similar to species of the *B. fragilis* group, and to certain *Fusobacterium* species that grow in the presence of 20% bile several of the phenotypic properties of *Bilophila wadsworthia* are different from those of the *B. fragilis* group and *Fusobacterium* species. *Bilophila wadsworthia* differs from the *B. fragilis* group by its failure to ferment carbohydrates, its production of urease, and its failure to produce a major amount of succinic acid. The production of strong catalase activity and its lack of butyric acid production are key characteristics that separate *B. wadsworthia* from *Fusobacterium* species [102].

Figure 12: Flowchart of the identification of gram-negative anaerobic bacilli.



Km = Kanamycin, Vm = Vancomycin, S = Sensitive, R = Resistant.

When anaerobes were not successfully identified using the Finegold system than they were tested using the rapid ID 32A Api panels.

4.1.3 Sensitivity

4.1.3.1 Materials used

- ❖ Mueller-Hinton broth for inoculum preparation.
- ❖ 1 McFarland standard
- ❖ E test strips, storage tubes, package insert, E test technical guides and CLSI guidelines. The following antimicrobial agents were tested (Table 5) and interpreted following CLSI guidelines (Table 6).
- ❖ Brucella agar plates with a depth of 4-5mm

Table 5: Antimicrobial agents tested

Antibiotic	Concentration of E test
Clindamycin	0.016-256µg/ml
Metronidazole	0.016-256µg/ml
Chloramphenicol	0.016-256µg/ml
Ertapenem	0.002-32µg/ml
Cefoxitin	0.016-256µg/ml
Piperacillin/tazobactam	0.016-256µg/ml
Benzympenicillin	0.002-32µg/ml or 0.016-256µg/ml
Amoxicillin/clavulanic acid	0.016-256µg/ml
Ceftriaxone	0.002-32µg/ml or 0.016-256µg/ml

Table 6 CLSI antimicrobial breakpoints for susceptibility testing for anaerobic organisms

Antibiotic	Abbreviation	Susceptible Break-point (µg/ml)	Intermediate Break-point (µg/ml)	Resistant Break-point (µg/ml)
Amox/clavulanic	Xl	≤ 4	8	≥ 16
Penicillin	Pg	≤ 0.5	1	≥ 2
Cefoxitin	Fx	≤ 16	32	≥ 64
Ceftriaxone	Tx	≤ 16	32	≥ 64
Chloramphenicol	Cl	≤ 8	16	≥ 32
Clindamycin	Cm	≤ 2	4	≥ 8
Ertapenem	Etp	≤ 4	8	≥ 16
Metronidazole	Mz	≤ 8	16	≥ 32
Pip/tazobactam	Ptc	≤ 32	64	≥ 128

4.1.3.2 Method

The E test storage tubes was removed from the freezer and allowed to reach room temperature for approximately 30 minutes.

1. Depending on the growth of the strain, individual viable colonies from a 24, 48 or 72 hour agar plate were suspended in Mueller-Hinton broth and mixed carefully minimizing aeration. The turbidity was adjusted to a 1 McFarland standard.
2. Ensuring that the agar surface was dry before swabbing, a sterile swab was seeded into the inoculum, excess fluid removed and the entire surface of the brucella plate swabbed evenly in three directions.
3. The surface of the plate was allowed to dry for 10 minutes.
4. The E test strips were applied to the surface of the agar with forceps and once applied the strip was not moved.

5. The plates were incubated immediately in an anaerobic jar for 24, 48 hours, or longer for slow growers.
6. The isolates were stored in Robertson's Cooked Meat Medium.

4.2 Quality control

Quality control was performed with organisms of known susceptibility. Separate plates of these organisms were tested with the same E test strips under the same conditions with each batch. Control strains that were used:

- *Bacteroides fragilis* ATCC 25285
- *Bacteroides thetaiotaomicron* ATCC 29741
- *Eubacterium lentum* ATCC 43055

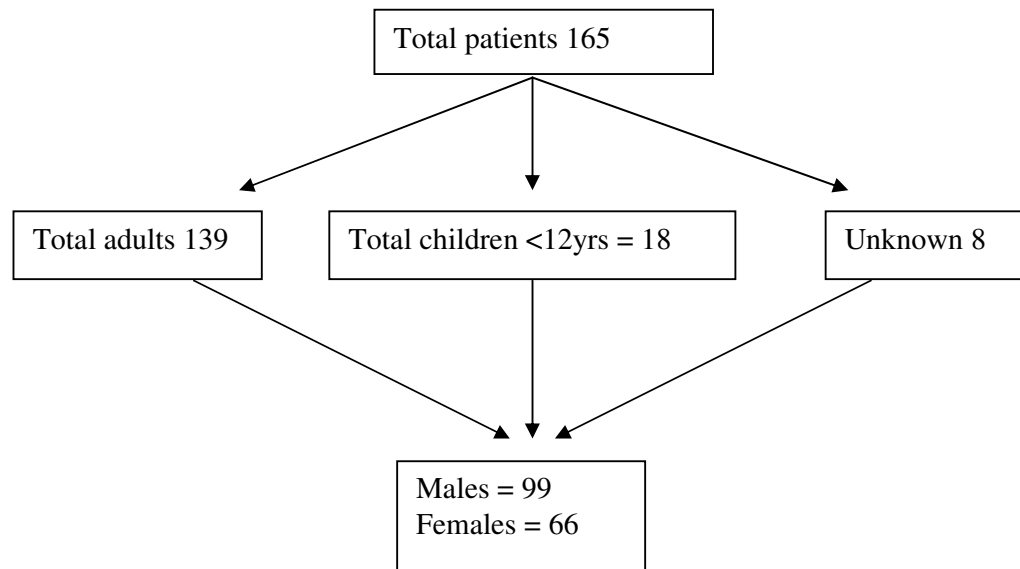
4.3 Reading

MIC interpretation was performed using CLSI guidelines. The MIC was defined as the lowest concentration of each antimicrobial that inhibited the visible growth including hazes and isolated colonies of the test isolate. The MIC was read after 24 to 48 hours, if sufficient growth was obtained and the ellipse was clearly visible. The clindamycin results were confirmed after 48 hours due to inducible resistance [1]. For the slow growers the MIC was read after 48-72 hours. When growth was inadequate or the inoculum too light, the test was repeated or eliminated.

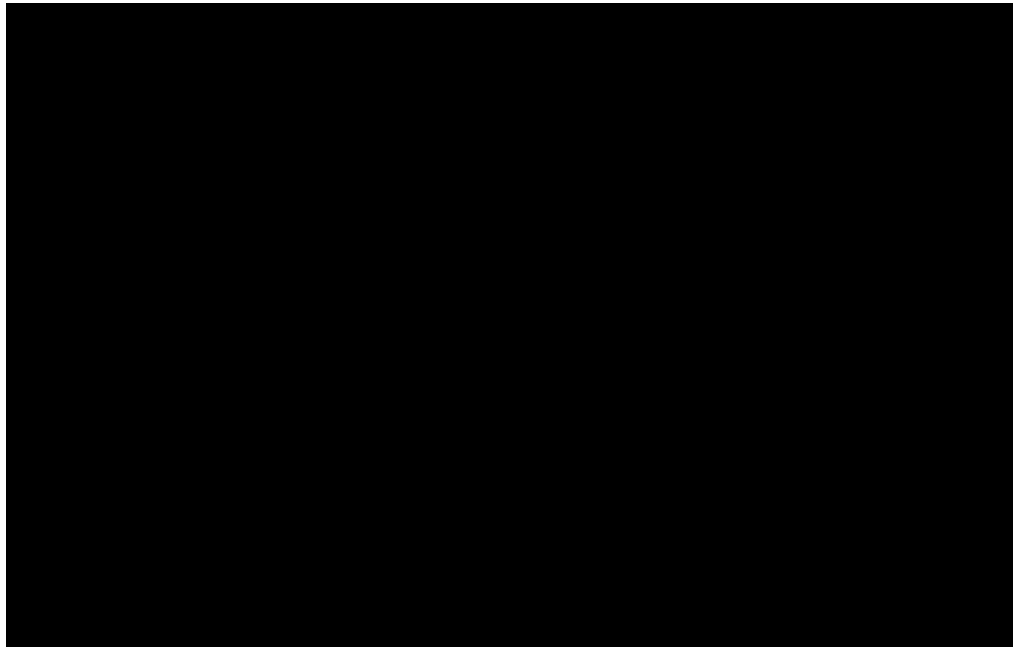
5.0 Results

A total number of 180 anaerobes from 165 different patients were tested. Multiple samples from a single patient that grew the same organism (even at different intervals or from different anatomical sites) were only done once hence all the isolates are non-repetitive. There were 14 patient samples that cultured more than one anaerobe. Patient demographics are seen in figure 13.

Figure 13. Patient demographics

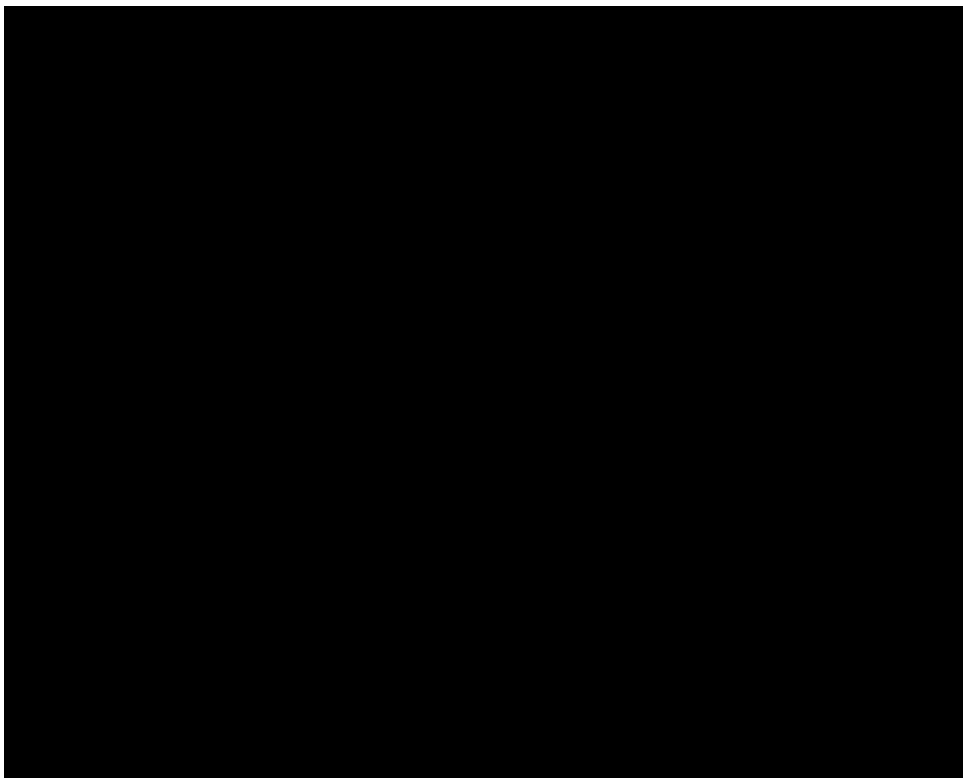


Total number of 26 patients provisionally diagnosed by the attending physician with sepsis is characterized in the figure 14.



Admission of the patients to various wards is presented in the figure 15.

Figure 15. Patient admissions at different wards in CMJAH.



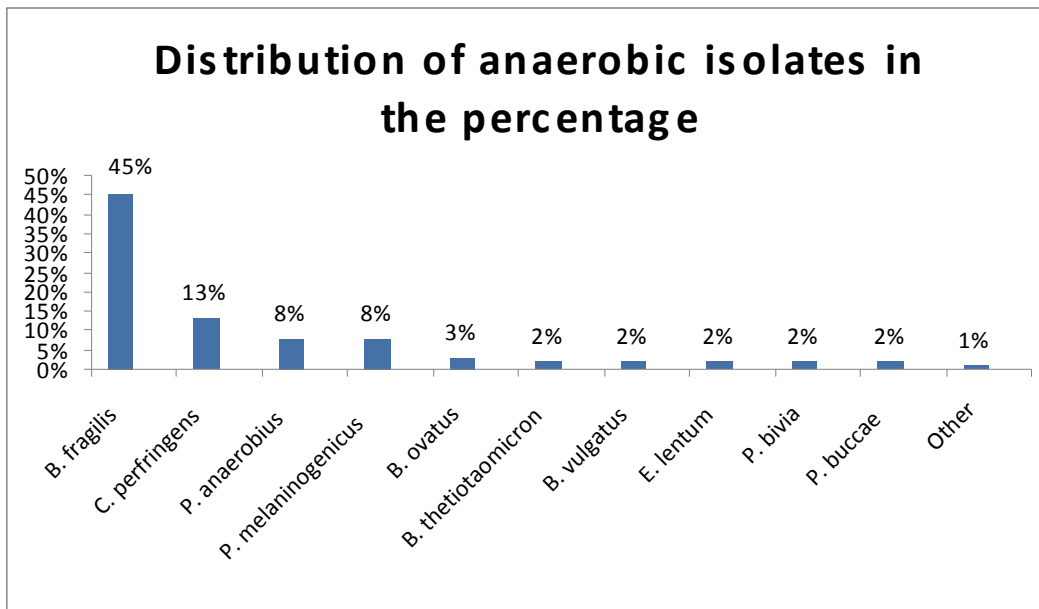
The type of specimens is listed in table 7.

Table 7. Type of specimens

Type	Total number
Abdominal fluid	3
Abscess	7
Abdominal abscess	4
Aspirate	3
Blood cultures	27
Bone	3
Breast	3
Drain fluid	2
Empyema	1
Fluids	36
Other	4
Placenta	1
Pleural fluid	2
Pus	41
Tissue	34
Umbilicus	1
Unknown	8

In view of isolation rates from all infection sources see figure 16.

Figure 16. Percentage of anaerobic species from 180 isolates



Routinely pus swabs are not cultured for anaerobes at the microbiology laboratory at CMJAH, unless they are transported in appropriate anaerobic transport media. None of the isolates in this study were isolated from a pus swab.

Analysis of the types of specimens from which the larger group of organisms originated is illustrated in figure 17 and 18.

Figure 17. Sources of *Bacteroides* group

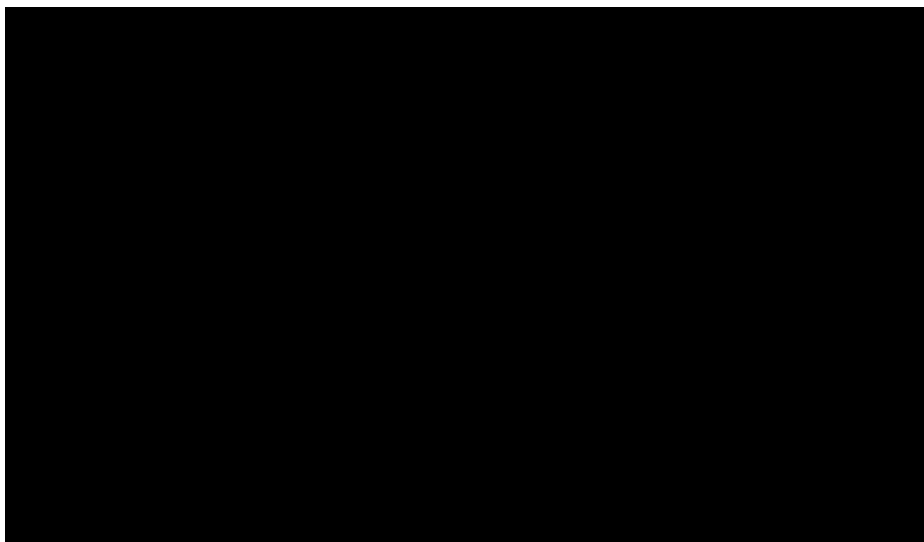
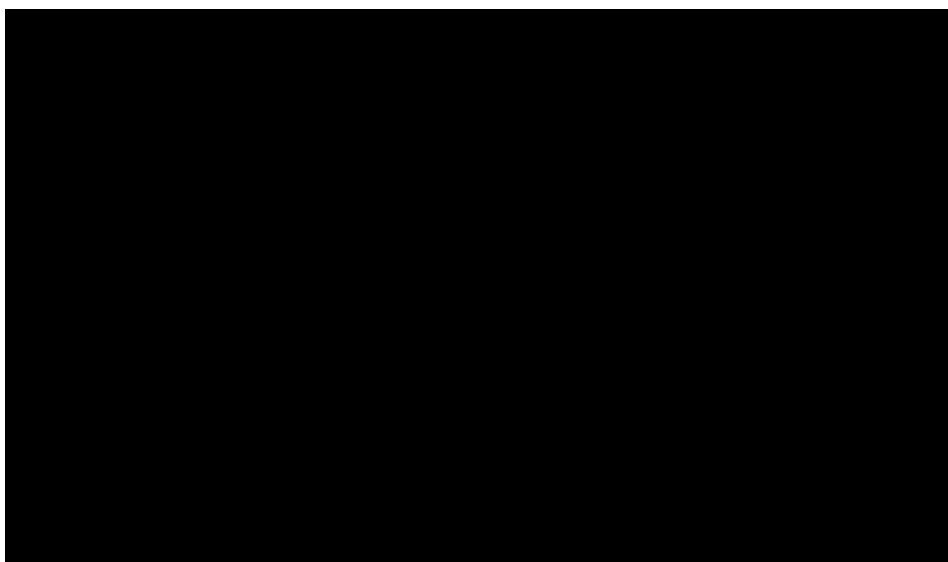


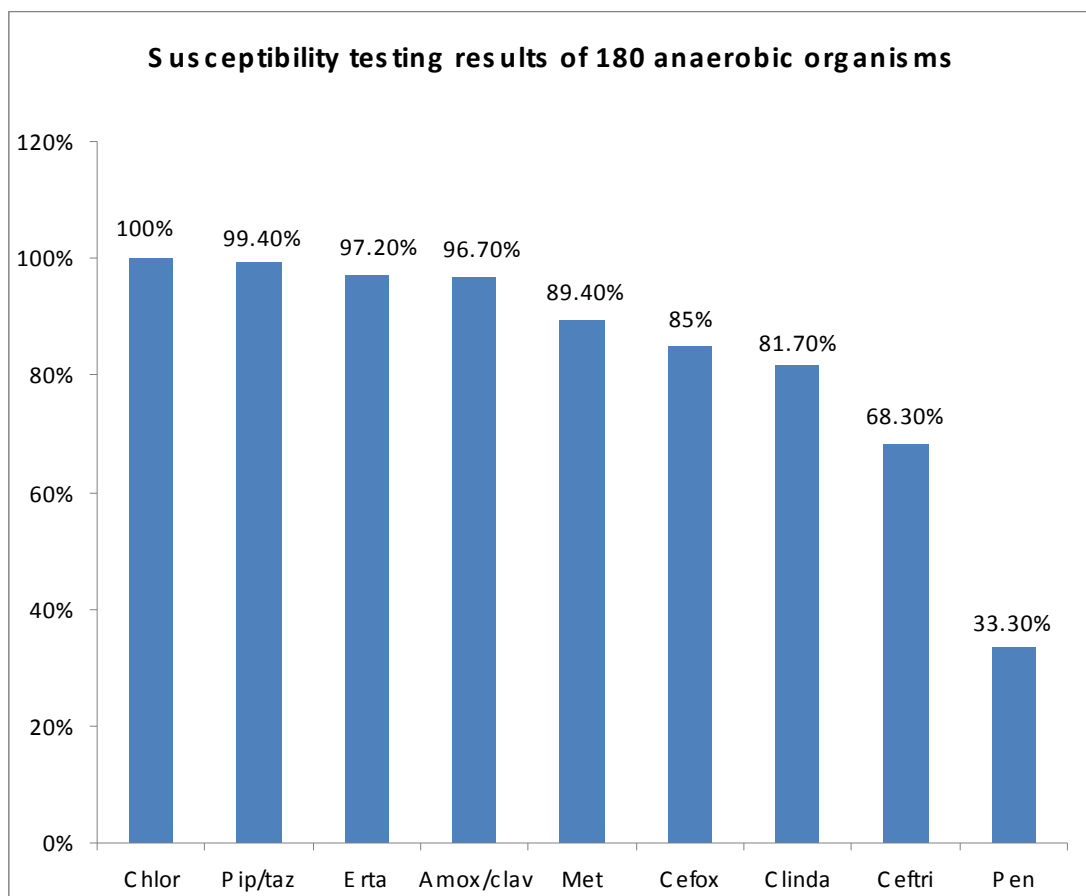
Figure 18. Sources for *Clostridium* group.



Susceptibility testing of nine antibiotics was performed on 180 isolates; penicillin demonstrated the highest resistance of 66.7%, followed with clindamycin 13.9%, metronidazole (8.9%), ceftriaxone (8.3%), ceftiofur (7.2%), amox/clavulanic acid (2.8%), ertapenem (1.1%), piperacillin/tazobactam (0.6%) and no resistance to chloramphenicol.

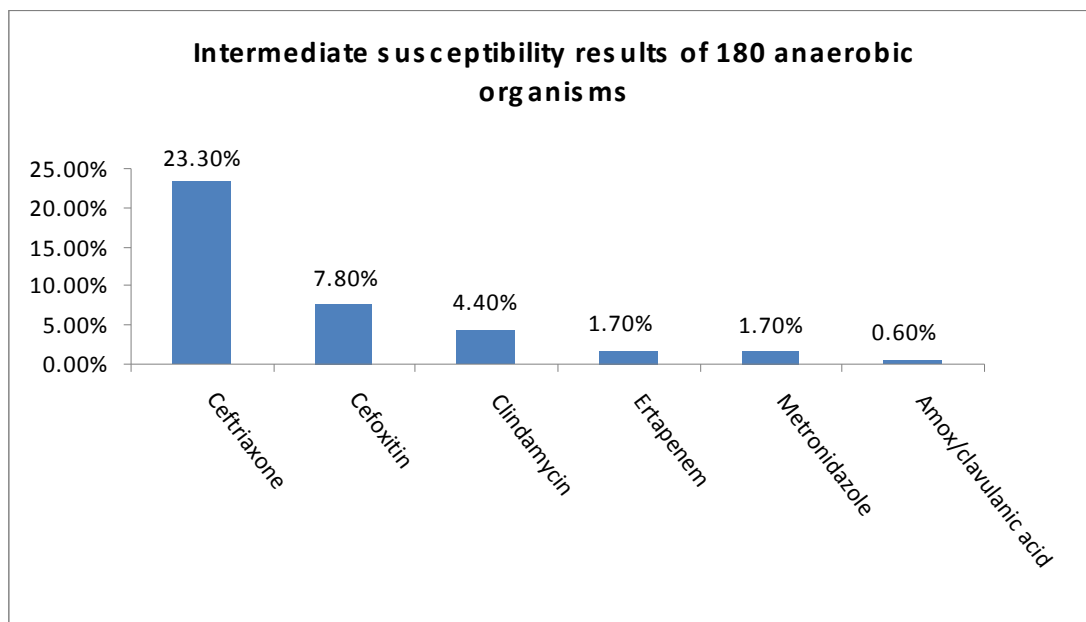
The study indicates the most active agents were: chloramphenicol with susceptibility of 100% followed with piperacillin/tazobactam (99.4%), ertapenem (97.2%), amoxicillin/clavulanic acid (96.7%), while metronidazole demonstrated 89.4% susceptibility, cefoxitin (85%), clindamycin (81.7%), and ceftriaxone (68.3%) and as expected penicillin exhibited poor susceptibility (33.3%).

Figure 19. Susceptibility testing results of 180 anaerobic organisms



However intermediate sensitivity was seen in ceftriaxone, cefoxitin, clindamycin, ertapenem, metronidazole and amoxicillin/clavulanic acid being 23.3%, 7.8%, 4.4%, 1.7%, 1.7% and 0.6% respectively to all the 180 tested anaerobes (Figure 20).

Figure 20. Intermediate susceptibility results of 180 anaerobic organisms



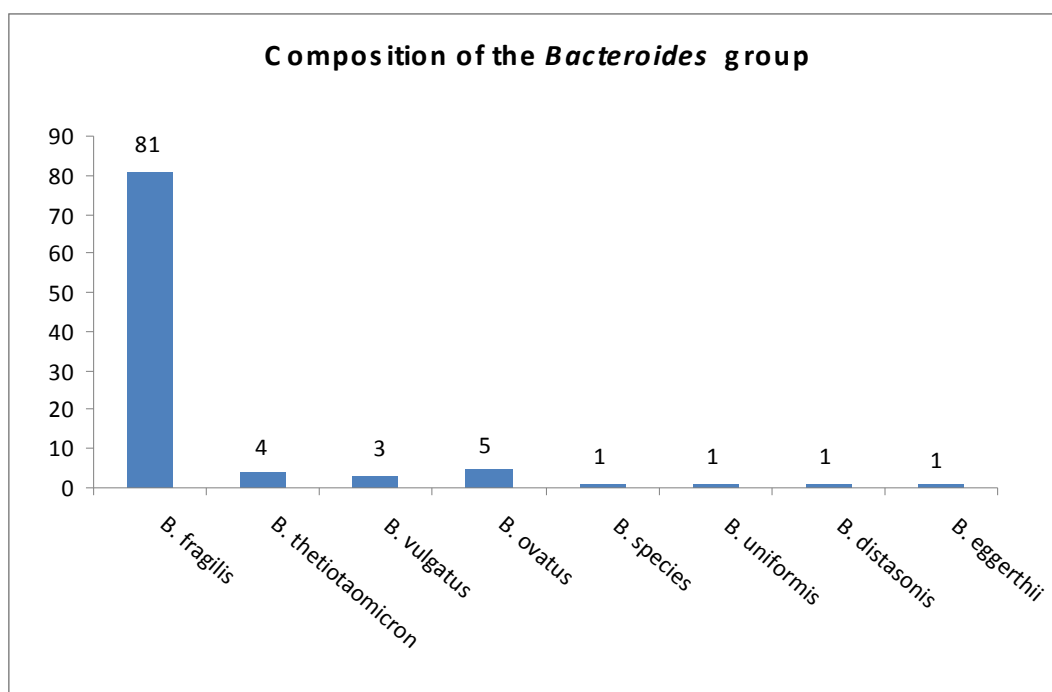
The MIC₅₀ and MIC₉₀ of all isolates to the nine antibiotics are illustrated in Table 8.

Table 8. The MIC₅₀ and MIC₉₀ of all isolates

Antibiotic	MIC ₅₀	MIC ₉₀	MIC Range
Amoxicillin/Clavulanic acid	0.19 µg/ml	1 µg/ml	0.016-96 µg/ml
Cefoxitin	3 µg/ml	32 µg/ml	0.016-256 µg/ml
Ceftriaxone	3 µg/ml	32 µg/ml	0.002-256 µg/ml
Chloramphenicol	1 µg/ml	2 µg/ml	0.016-8 µg/ml
Clindamycin	0.19 µg/ml	256 µg/ml	0.016-256 µg/ml
Ertapenem	0.125 µg/ml	1.5 µg/ml	0.002-32 µg/ml
Metronidazole	0.5 µg/ml	16 µg/ml	0.016-256 µg/ml
Penicillin G	12 µg/ml	32 µg/ml	0.002-256 µg/ml
Piperacillin/Tazobactam	0.19 µg/ml	4 µg/ml	0.016-256 µg/ml

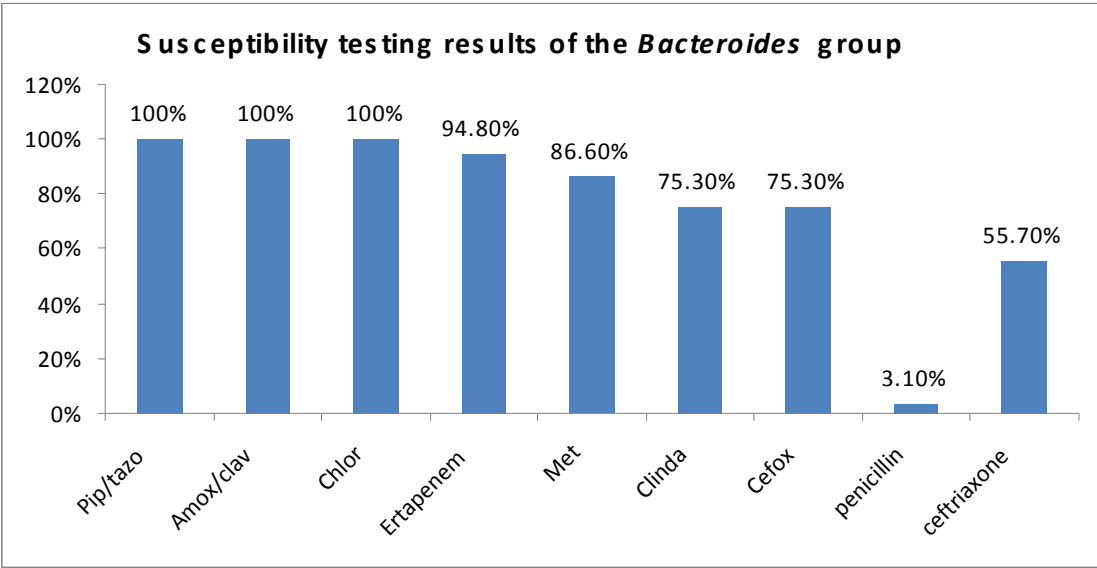
From 180 isolates tested, 97 organisms belong to the *Bacteroides* group: *Bacteroides fragilis* (81), *Bacteroides thetaiotaomicron* (4), *Bacteroides vulgatus* (3), *Bacteroides ovatus* (5), *Bacteroides species* (1), *Bacteroides uniformis* (1), *Bacteroides distasonis* (1), and *Bacteroides eggerthii* (1) figure 21.

Figure 21. Composition of the *Bacteroides* group



This group of anaerobic organisms presented with highest susceptibility (100%) to piperacillin/tazobactam, amoxicillin/clavulanate, chloramphenical, to ertapenem (94.8%), lesser to metronidazole (86.6%) and low down to both clindamycin and cefoxitin (75.3%) Figure 22.

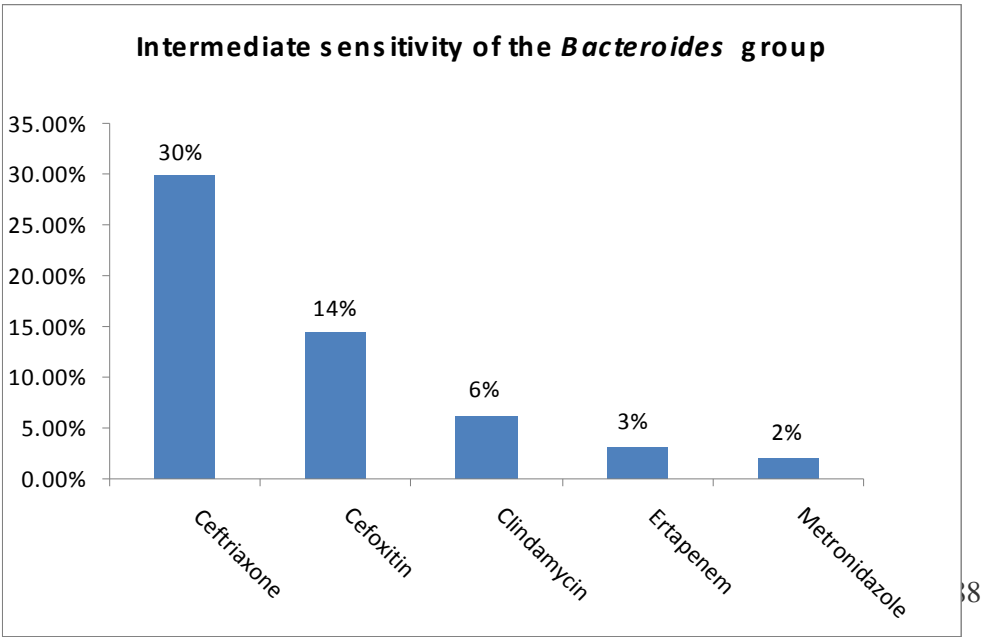
Figure 22. Susceptibility testing results of the *Bacteroides* group



Intermediate activity of the *Bacteroides* group has been seen in this study.

From the 97 isolates 29.9% were intermediately sensitive to ceftriaxone, 14.4% to ceftioxin, 6.2% to clindamycin, 3.1% to ertapenem and 2.1% to metronidazole, Figure 23.

Figure 23. Intermediate sensitivity of the *Bacteroides* group



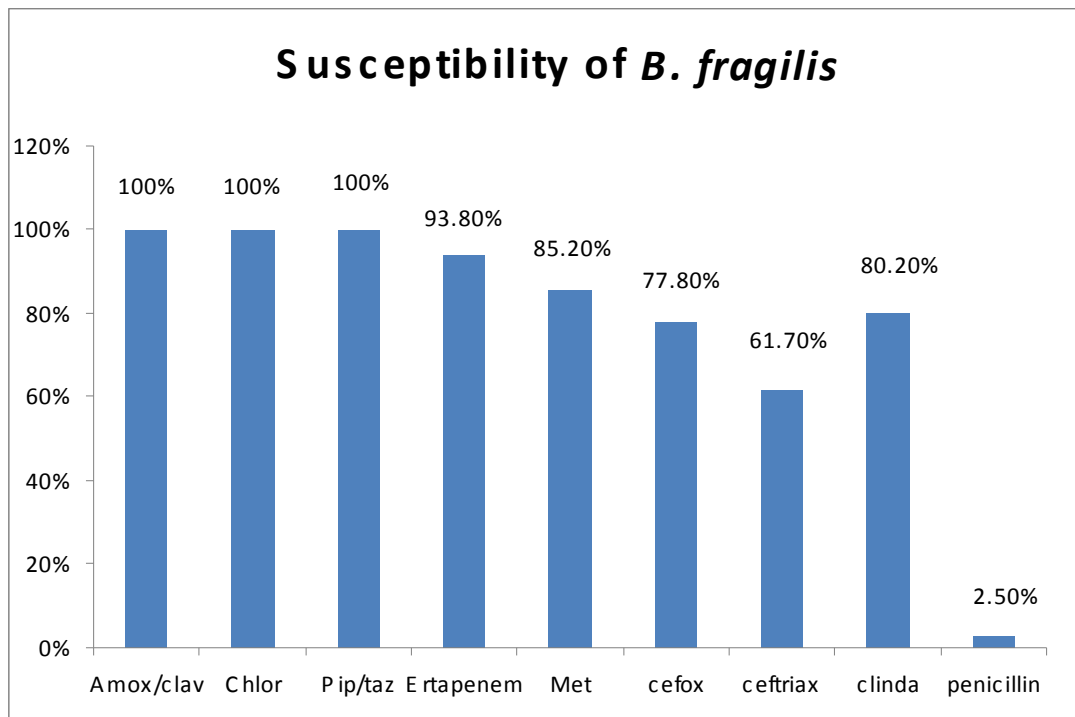
The MIC₅₀ and MIC₉₀ of the *Bacteroides* group are illustrated in Table 9.

Table 9. The MIC₅₀ and MIC₉₀ of the *Bacteroides* group

Antibiotic	MIC ₅₀	MIC ₉₀	MIC Range
Amoxicillin/Clavulanic acid	0.25 µg/ml	1.5 µg/ml	0.023-3 µg/ml
Cefoxitin	8 µg/ml	48 µg/ml	0.125-256 µg/ml
Ceftriaxone	8 µg/ml	256 µg/ml	0.008-256 µg/ml
Chloramphenicol	1 µg/ml	2 µg/ml	0.094-3 µg/ml
Clindamycin	0.5 µg/ml	256 µg/ml	0.016-256 µg/ml
Ertapenem	0.25 µg/ml	3 µg/ml	0.002-32 µg/ml
Metronidazole	0.5 µg/ml	128 µg/ml	0.023-256 µg/ml
Penicillin G	32 µg/ml	256 µg/ml	0.064-256 µg/ml
Piperacillin/Tazobactam	0.25 µg/ml	6 µg/ml	0.016-32 µg/ml

The most active antimicrobials to *Bacteroides fragilis* in this study have been observed with amoxicillin/clavulanic acid, chloramphenicol and piperacillin/tazobactam to which all the isolates were 100% sensitive. Excellent sensitivity was also seen in ertapenem and metronidazole being 93.8% and 85.2% respectively (Figure 24).

Figure 24. Susceptibility testing results of the 81 isolates of *Bacteroides fragilis*

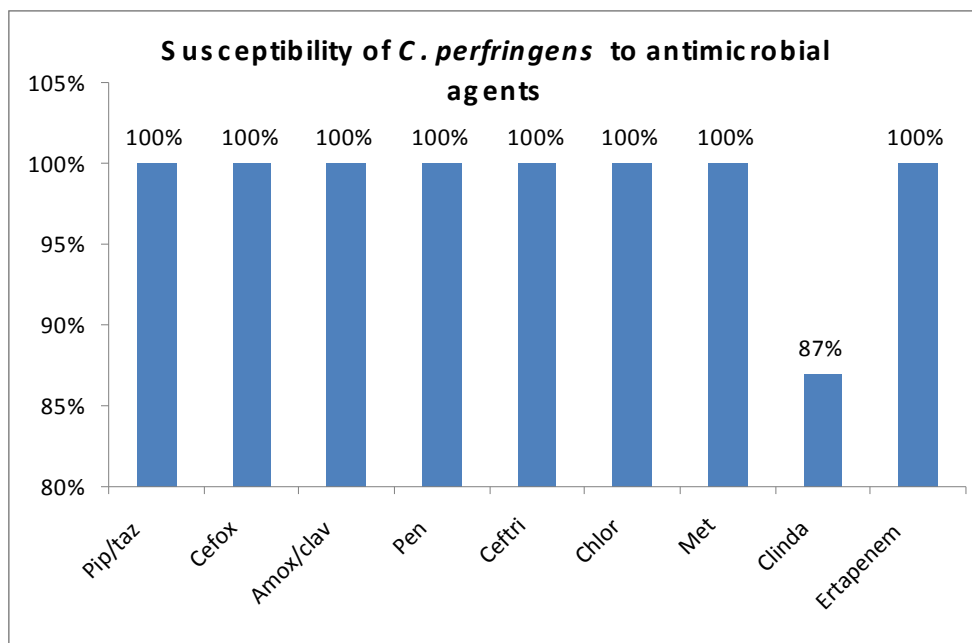


The highest antimicrobial resistance with this organism is seen with penicillin (97.5%), (*Bacteroides spp.* usually produce β -lactamases that confer resistance to penicillin), followed by clindamycin (14.8%), metronidazole and ceftriaxone both (12.3%), cefoxitin (8.6%) and ertapenem (2.5%).

A number of 23 *Clostridium perfringens* isolates was tested, which demonstrated excellent sensitivity to all the antimicrobials agents except lesser susceptibility to clindamycin (87%)

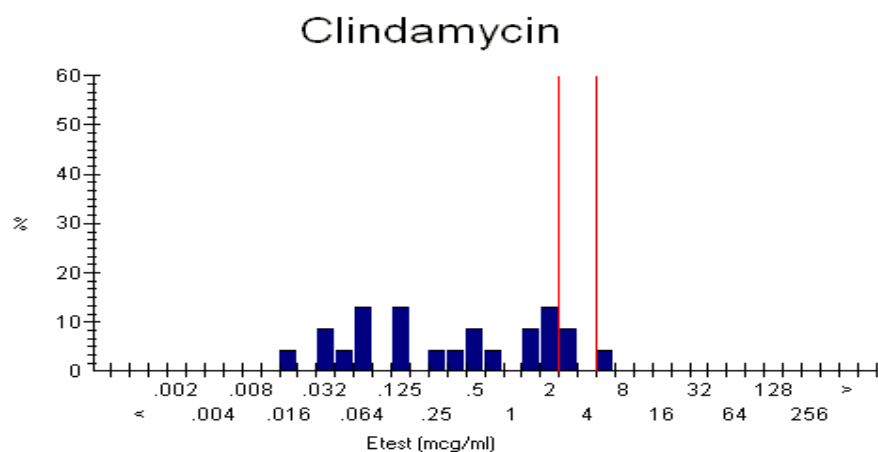
Figure 25

Figure 25. Susceptibility testing results of the 23 *Clostridium perfringens* isolates



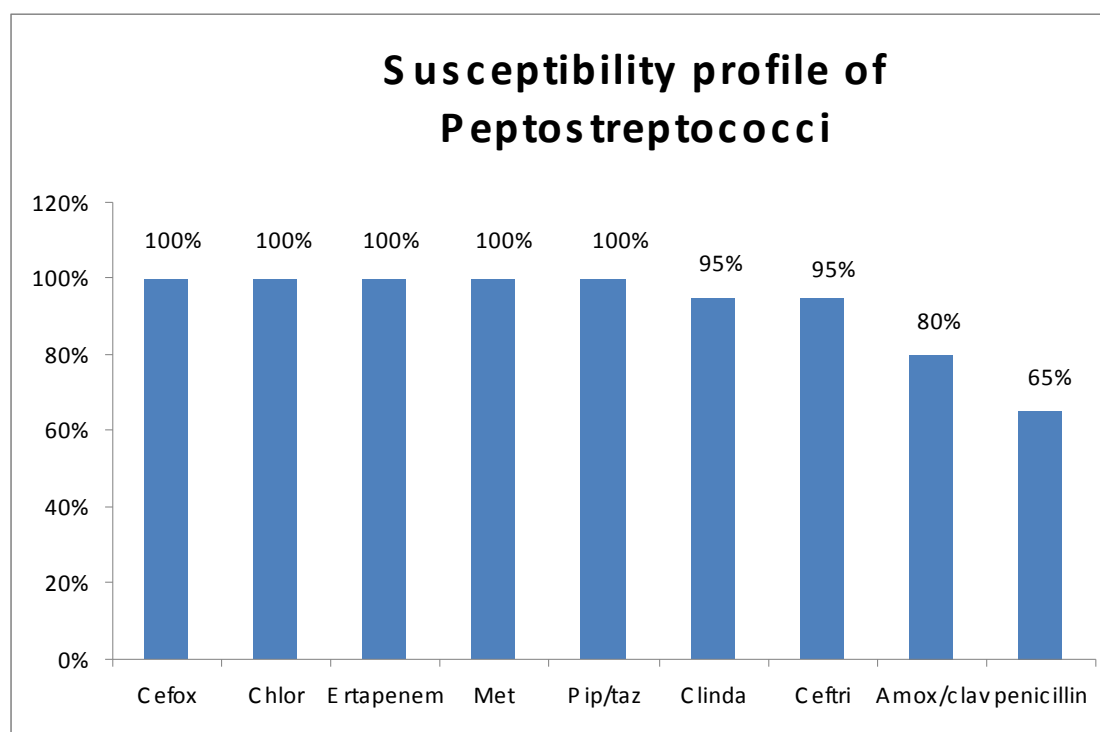
From the 23 isolates of *Clostridium perfringens*, 4.3% were resistant to clindamycin and 8.7% intermediately sensitive (Figure 26).

Figure 26. Distribution of sensitivity of *Clostridium perfringens* with clindamycin



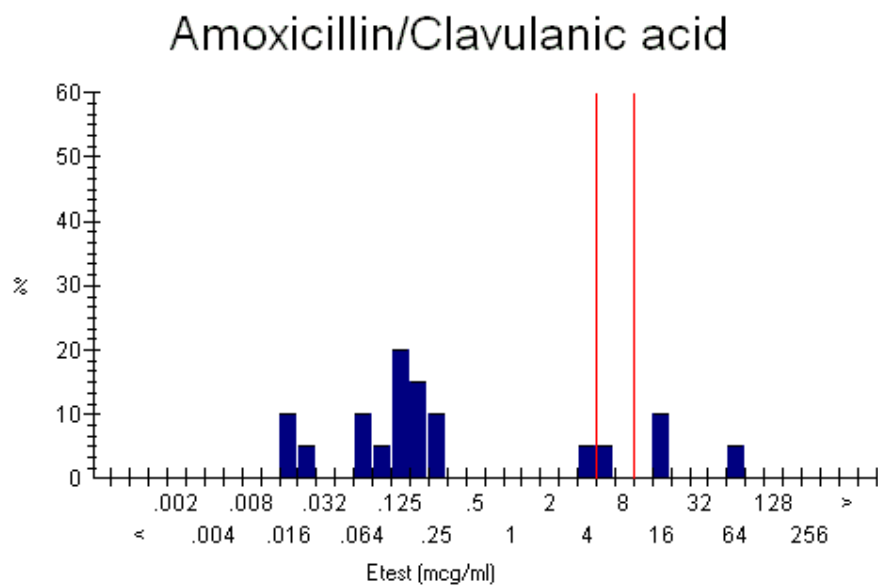
Overall 20 isolates of *Peptostreptococcus* species including: *Peptostreptococcus anaerobius* (15), *Peptostreptococcus* spp (2), *Peptostreptococcus prevotti* (1), *Peptostreptococcus asaccharolyticus* (1), and *Peptostreptococcus magnus* (1), demonstrated 100% sensitivity to cefoxitin, chloramphenicol, ertapenem, metronidazole and piperacillin/tazobactam, 95% to clindamycin and ceftriaxone and 80% to amoxicillin/clavulanic acid (Figure 27).

Figure 27. Susceptibility testing results of the *Peptostreptococcus* group



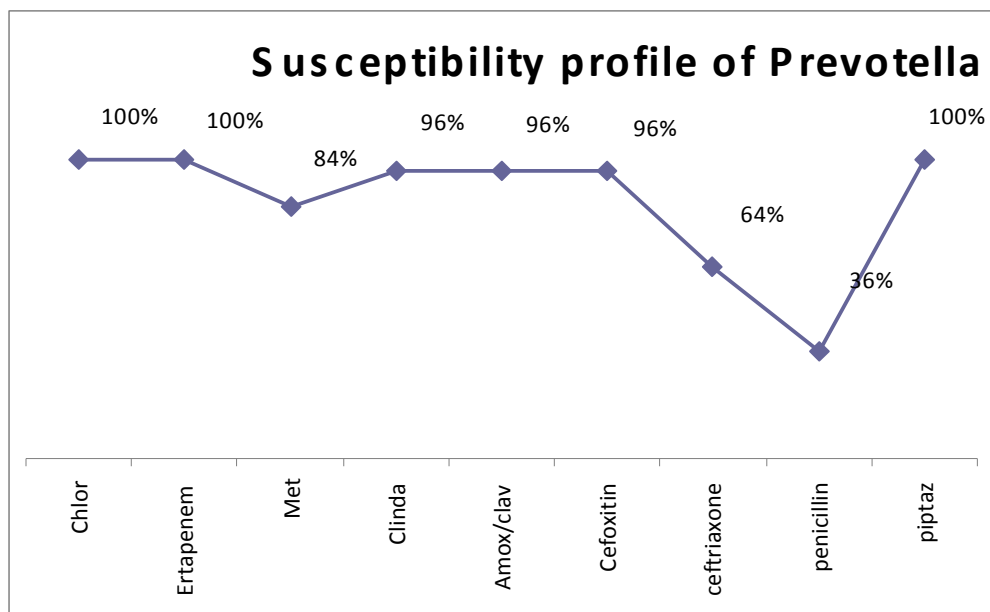
Intermediate sensitivity was observed with this species. 5% of the isolates were intermediately sensitive to amoxicillin/clavulanic acid and ceftriaxone (Figure 28).

Figure 28. Distribution of susceptibility of the *Peptostreptococcus* group to amoxicillin/clavulanic acid



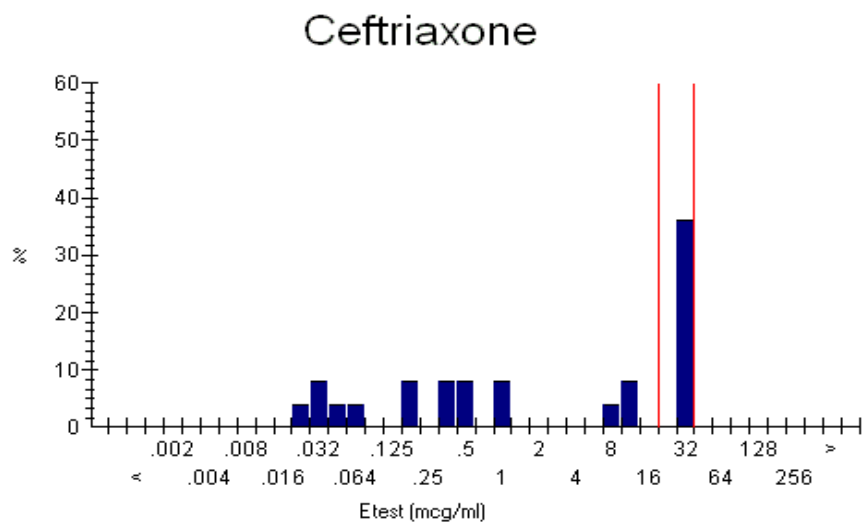
A total number of 25 strains of *Prevotella* species were identified as: *Prevotella melaninogenica* (15), *Prevotella buccae* (3), *Prevotella disiens* (1), *Prevotella bivia* (4), and *Prevotella oralis* (2). All isolates exhibit 100% susceptibility to chloramphenicol and ertapenem, 84% to metronidazole and 96% to clindamycin, amoxicillin/clavulanic acid and cefoxitin (Figure 29).

Figure 29. Susceptibility testing results of the *Prevotella* group.



Once more intermediate sensitivity was demonstrated in this group, 36% of the 25 strains of *Prevotella* species were intermediately susceptible to ceftriaxone and 4% to metronidazole (Figure 30).

Figure 30. Distribution of susceptibility of the *Prevotella* group with ceftriaxone



6.0 Discussion

Generally, chloramphenicol, piperacillin/tazobactam, ertapenem and amoxicillin/clavulanate demonstrated the highest activity to anaerobic isolates, such as 100%, 99.4%, 97.2% and 96.7%, respectively. Among the 180 tested anaerobes a total of 8.9% resistance has been observed to metronidazole and 18.3% to clindamycin.

From present study *Bacteroides fragilis* was isolated from 81 (45%) clinically significant specimens, followed by *Clostridium perfringens* 23 (13%), *Peptostreptococcus anaerobius* 15 (8%) and *Prevotella melaninigenicus* 15 (8%).

Susceptibility profiles of *Bacteroides fragilis* from our study to few antimicrobial agents were similar to study done in Brazil. The resistance rates reported in Brazil were for cefoxitin (12%), cefotaxime (15.1%), chloramphenicol (1%), clindamycin (18.2%) and tetracycline (75.7%). Sixteen isolates showed reduced susceptibility to metronidazole (MIC 2-4 µg/ml) [81] compared to the present study were resistance to cefoxitin was (8.6%) and intermediate susceptibility (13.6%), clindamycin (14.8%) with intermediate susceptibility of (4.9%), metronidazole (12.3%) with an intermediate susceptibility of (2.5%) and no resistance to chloramphenicol and amoxicillin/clavulanate. In this study MIC₅₀ was 0.5 µg/ml and MIC₉₀ was 128 µg/ml for metronidazole.

Bandoh et al and Betriu et al [10; 11] have reported clindamycin resistance rates as high as 33% for *B. fragilis*, 36% for *B. thetaiotamicron*, 49% for *B. distasonis*, and 46% for *B. caccae*. Also Oteo et al [78] reported an overall rate of resistance to clindamycin of 49% for the *B. fragilis* group. In this study, 18.7% of the *Bacteroides* group isolates were resistant to clindamycin; however 6% were intermediately susceptible.

C. perfringens exhibited no resistance to penicillin and metronidazole

The single isolate of *Clostridium fallax*, *Clostridium sordelli* and one isolate of *Clostridium septicum* were all sensitive to each of the tested antimicrobials. The other

isolate of *Clostridium septicum* showed high level of resistance to metronidazole with MIC 256 µg/ml. However the only isolate of *Clostridium paraputrificum* isolated and tested in this study was resistant to clindamycin and ceftiofur with MIC 256 µg/ml.

Koch et al [60] from Cape Town, South Africa demonstrated 4% of the *Clostridium perfringens* (26 isolates in total) resistant to benzylpenicillin and clindamycin, but sensitive to the other antibiotics (ceftiofur, metronidazole, chloramphenicol and co-amoxiclavulanic acid). Five *Clostridium spp.* were sensitive to all antibiotics tested. In comparison to current study, from the 23 strains of *C. perfringens* tested all were fully sensitive to penicillin but 4.3% were resistant to clindamycin and 8.7% were intermediately susceptible.

P. melaninogenicus was resistant to penicillin in 60% and 6.7% to metronidazole. From two isolates of *Veillonella parvula* tested, one was sensitive to ceftiofur, ceftriaxone, chloramphenicol, metronidazole, and ertapenem and intermediately sensitive to amoxicillin/clavulanic acid, while the other one was only resistant to penicillin and intermediately sensitive to ceftriaxone.

Three isolates of *Eubacterium lentum* were tested, two of which were sensitive to all the antimicrobials as the other was resistant to ceftiofur and clindamycin and intermediately sensitive to ceftriaxone.

Fusobacterium species and *Fusobacterium nucleatum* were sensitive to all the tested antimicrobial agents; *Fusobacterium varium* was highly resistant to ceftriaxone; and *Fusobacterium mortiferum* demonstrated resistance to penicillin, clindamycin and ceftriaxone.

The particular strain of lactobacillus that was tested was only resistant to metronidazole MIC 256 µg/ml.

In the present study, 97.2% of all isolated anaerobes were susceptible to ertapenem except 1.7% with intermediate sensitivity. Similarly Goldstein et al [45] demonstrated that ertapenem was uniformly active against 1001 isolates including all *Bacteroides fragilis* group species isolates, with the exception of 12 (20%) strains of 61 *Bilophila wadsworthia*, 3 strains of lactobacilli, and 1 isolate of *Acidaminococcus fermentans*.

A total of 370 clinical isolates of anaerobic bacteria from patients at a university hospital were investigated, in a prospective study, over a time period of 6 months by Pfister [82], with the exception of one strain of *Fusobacterium varium* all other anaerobic isolates were sensitive to ertapenem. In addition, one isolate of *B. fragilis* exhibited resistance to ertapenem (MIC 32µg/ml) while four other isolates demonstrated intermediate sensitivity. In the study performed by Koch [60], 20 strains of *Peptostreptococcus anaerobius* were tested and 10% were resistant to benzylpenicillin, cefoxitin and metronidazole; from others 17 *Peptostreptococcus* spp 12% showed resistance to benzylpenicillin and 6% to metronidazole and clindamycin whereas in the present study from the 20 strains of peptostreptococci 35% was resistant to penicillin and a 5% to clindamycin and no resistance to metronidazole.

Appelbaum and Chatterton [9] performed a similar study in Durban, South Africa, on 265 anaerobic bacteria from clinical isolates. Their results indicated low levels of resistance to antimicrobial agents that cover anaerobic organisms such as penicillin G, chloramphenicol, clindamycin, and metronidazole. In comparison to our results chloramphenicol remains 100% susceptible but clindamycin exhibited a susceptibility of 81.7%, metronidazole 89.4% and penicillin 33.3%, indicating increasing trends in resistance.

7.0 Conclusions

For first time the present study provides baseline data on antibiotic susceptibility patterns of anaerobic isolates from patients at Charlotte Maxeke Johannesburg Academic Hospital, and should be used as background information for future studies.

Our study demonstrates a change in the susceptibility patterns of anaerobic organisms and an increased resistance to metronidazole particularly to the *B. fragilis* group. Furthermore the high rates of intermediate susceptibility to other antimicrobial agents indicate need for periodic active surveillance to identify and record these emerging trends.

From a clinical perspective, our susceptibility results may guide clinicians to use antimicrobial agents such as amoxicillin-clavulanate or piperacillin-tazobactam and ertapenem for empirical treatment of mixed infections particularly those associated with anaerobic organisms.

8.0 Literature cited

1. AB Biodisk. 2000. MOOOOO24- M800, Customer Information Sheet CIS 003, Trouble shooting metronidazole. Available at: <[http:// www.abbiobisk.com](http://www.abbiobisk.com)> [Accessed 2007].
2. AB Biodisk. 1999. E test news. The importance of anaerobe susceptibility testing. EAL- A successful flying start. NO 25. Available at: <[http:// www.abbiobisk.com](http://www.abbiobisk.com)> [Accessed 2009].
3. <<http://www.abbiobisk.com>> [Accessed 2009].
4. Aktories, K. and Just, I. 2000. Bacterial and Protein Toxins. Handbook of Experimental Pharmacology. Vol 145.
5. Aldape, M.J., Bryant, A.E and Stevens D.L. 2006. *Clostridium sordellii* Infection: Epidemiology, Clinical Findings, and Current Perspectives on Diagnosis and Treatment. Clinical Infectious Diseases, Vol 43, pp 1436-46.
6. Aldridge, K.E., Ashcraft, D., Cambre, K., et al. 2001. Multi-center survey of the changing in-vitro antimicrobial susceptibilities of clinical isolates of *Bacteroides fragilis* group, *Prevotella*, *Fusobacterium*, *Porphyromonas*, and *Peptostreptococcus* species. Antimicrobial Agents and Chemotherapy, Vol 45, pp 1238-1243.
7. Allen. D Stephen., Siders. A Jean and Marler.M Linda. 1970. Isolation and Examination of Anaerobic Bacteria, Chapter 37 in Manual of Clinical Microbiology, 4th edition, Edwin H. Lennette., Albert Balows., William J. Hausler Jr et al.
8. Amornchat, C., Rassameemasmaung. S., W. Sriprajothikoon et al, 2003. Invasion of *Porphyromonas gingivalis* into human gingival fibroblasts in vitro. Journal of Periodontology, Vol 5, pp 98-105.

9. Appelbaum Peter C and Chatterton Shiela A. 1978. Susceptibility of Anaerobic Bacteria to ten Antimicrobial Agents. Antimicrobial Agents Chemotherapy, Vol 14, pp 371-376.
10. Bandoh, K., Veno, K., Watanabe, K et al.1993. Susceptibility patterns and resistance to imipenem in the *Bacteroides fragilis* group species in Japan: a 4-year study. Clinical Infectious Diseases, Vol 16 (Suppl 4), S382-S386.
11. Betriu, C., Gomez, M., Palau, M.L et al.1999. Activities of new antimicrobial agents (trovafloxacin, moxifloxacin, sanfetrinem, quinupristin-dalfopristin) against *Bacteroides fragilis* group: comparison with the activities of 14 other agents. Antimicrobial Agents and Chemotherapy, Vol 43, pp 2320-2322.
12. bioMerieux SA. Available at: <<http://www.biomerieux.com>> [Accessed 2005]
13. Bleck, Thomas P in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 2000. Chapter 242 and 243, 6th edition, Vol 2, Mandell Gerald L., Bennett John E., and Dolin Raphael, Elsevier Churchill Livingstone.
14. Bliss, J.M. and Silver, R.P. 1996. Coating the surface: a model for expression of capsular polysialic acid in Escherichia coli K1. Molecular Microbiology. Vol 21, pp 221-231.
15. Bojar, R.A and Holland, K.T. 2004. Acne and *Propionibacterium acnes*. Clinical Dermatology, Vol 22, pp 375-379.
16. Bowden George H.W. *Actinomyces*, *Propionibacterium propionicus* and *Streptomyces*. Available at: <<http://gsb.utmb.edu/microbook/ch034.htm.medmicro>> [Accessed 25.01.2009].
17. Boyanova, L., Kolarov, R., Gergova, G., et al. 2006. Anaerobic bacteria in 118 patients with deep-space head and neck infections from the University Hospital of

- Maxillo-facial Surgery, Sofia, Bulgaria. Journal of Medical Microbiology, Vol 55, pp1285-1289.
18. Braun, Phyllis C and Zoidis, John D. 2004. Update on Drug-Resistant Pathogens: Mechanisms of Resistance and Emerging Strains. RT for decision makers in respiratory care. Available at: <<http://www.rtmagazine.com>> [Accessed 03.02.2008].
 19. Brown.R., Collee.J.G., Poxton.I.R., et al.1989. *Bacteroides*, *Fusobacterium* and related organisms: anaerobic cocci: identification of anaerobes in: Mackie and McCartney Practical Medical Microbiology.13th edition. Vol 2 of Medical Microbiology, Collee, J.G., Duguid, J.P., Fraser, A.G et al.
 20. Bruggemann, H. 2005. Insights in the pathogenic potential of *Propionibacterium acnes* from its complete genome. Seminar in Cutaneous Medical Surgery, Vol 24, pp 67-72.
 21. Burrows, William. 1973. Textbook of Microbiology.12th edition.
 22. Carlier, J.P., K'ouas, G., Lozniewski, A., et al. 2004. Osteosynthesis-associated bone infection caused by a non-proteolytic, nontoxigenic *Clostridium botulinum*-like strain. Journal of Clinical Microbiology, Vol 42, pp 484-486.
 23. Citron D.M., Ostovon M.I., Karlsson A., et al.1991. Evaluation of the E test for susceptibility testing of anaerobic bacteria. Journal of Clinical Microbiology, Vol 29, pp 2197- 2203.
 24. Clark S. 2003. Sudden death in peri-parturient sheep associated with *Clostridium sordellii*. Veterinary Record, Vol 153, p340.
 25. Clinical Laboratory Standards Institute. Methods for Antimicrobial susceptibility testing of anaerobic bacteria, approved standards, 6th edition, M11-A6, Vol 24, No 2.

26. Cohen Stuart H., Shetab Razeq, Yajarayma J., et al. 2006. Prevalence of enterotoxigenic *Bacteroides fragilis* in hospital-acquired diarrhoea. Diagnostic Microbiology and Infectious Diseases, Vol 55, pp 251-254.
27. Collee.J.G., Brown.R., Poxton.I.R., et al.1989. Clostridia of wound infection in: Mackie and McCartney. Practical Medical Microbiology.13th edition. Vol 2 of Medical Microbiology. Collee, J.G., Duguid, J.P., Fraser, A.G et al.
28. Cowan S.T. 1974. In Cowan and Steel's Manual for the identification of Medical bacteria. 2nd edition. Cambridge University Press.
29. Cunningham, M.W. 2000. Pathogenesis of group A streptococcal infections. Clinical Microbiology Reviews, Vol 13, pp 470-511.
30. Deshpande, R.G., Khan, M and Genco, C.A., 1998-1999. Invasion strategies of the oral pathogen *Porphyromonas gingivalis*: implications for cardiovascular disease. Invasion Metastasis, Vol 18, pp 57-69.
31. Duerden.B.I., 1994. Virulence factors in anaerobes, Clinical Infectious Diseases, Vol 18 (Suppl.4), pp S253-9.
32. Edlund.C., 2003. In vitro activity of new antibiotics against anaerobic bacteria. Anaerobe, Vol 9, p 250.
33. Ellner P.D., Green S.S., 1963. Serological relationship between *Clostridium bifermentans* and *Clostridium sordellii* based upon soluble antigens. Journal of Bacteriology, Vol 86, p 605.
34. el-Samalouti, V.T., Hamann, L., Flad, H.D., et al. 2000. The biology of endotoxin. Methods in Molecular Biology, Vol 145, pp 287-309.
35. Extension Disaster Education Network. 2008. Available at:
<<http://www.extension.org>> [Accessed 02.01.2009].

36. Falagas M.E and Siakavellas E., 2000. *Bacteroides*, *Prevotella*, and *Porphyromonas species*: a review of antibiotic resistance and therapeutic options. International Journal of Antimicrobial Agents, Vol 15, pp 1-9.
37. Ferna'ndez-Sabe', N., Carratala', J., Dorca, J., et al. 2003. Efficacy and Safety of Sequential Amoxicillin-Clavulanate in the Treatment of Anaerobic Lung Infections. European Journal of Clinical Microbiology and Infectious Diseases. Vol 22, pp185-187.
38. Finegold Sydney M. Anaerobic gram-negative bacilli. Medmicro chapter 20. Medical Microbiology 4th edition edited by Samuel Baron. The University of Texas Medical Branch at Galveston. <<http://medmicrochapter20.htm>> [Accessed 13.07.2007].
39. Finegold Sydney M., Jon E.Rosenblatt., Vera L., et al. 1976. Scope monograph on anaerobic infections, 3rd edition.
40. Foster, N., Cheetham, J., Taylor, J.J., et al. 2005. VIP inhibits *Porphyromonas gingivalis* LPS-induced immune responses in human monocytes. Journal of Dental Research, Vol 84, pp 999-1004.
41. Fujise, O., Miura, M., Hamachi, T., et al. 2005. Involvement of *Porphyromonas gingivalis* fimA genotype in treatment outcome following non-surgical periodontal therapy. Journal of Periodontology, Vol 76, pp 1661-1666.
42. Gibbons Ronald J and MacDonald John B.1960. Journal of Bacteriology, Vol 80, p 164.
43. Godreuil, S., Marchandin, H., Lemoing, V., et al. 2003. Postoperative intracranial infections due to *Propionibacterium acnes*: report of ten cases. Anaerobe, Vol 9, pp 247-276.

44. Golan Y., McDermott L.A., Jacobus N.V., et al. 2003. Emergence of fluoroquinolone resistance among *Bacteroides species*. Journal of Antimicrobial Chemotherapy, Vol 52, pp 208-213.
45. Goldstein Ellie J.C., Citron Diane M., Merriam Vreni C., et al. 2000. Comparative In Vitro Activities of Ertapenem (MK-0826) against 1,001 Anaerobes Isolated from Human Intraabdominal Infections. Antimicrobial Agents and Chemotherapy, Vol 44, No 9, pp 2389-2394.
46. Gomes B.P., Jacinto, R.C., Pinheiro, E.T., et al. 2005. *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia* and *Prevotella nigrescens* in endodontic lesions detected by culture and by PCR. Oral Microbiology and Immunology, Vol 20, pp 211-215.
47. <<http://gsbs.utmb.edu/microbook/images/fig10-4.jpg>> [Accessed 2008].
48. Gynuity Health Projects. Frequently asked questions about fatal infection and mifepristone medical abortion. Available at:
<<http://www.gynuity.org/documents/FAQsCSordelliiinfectiontechnicalversionENGmay06.pdf>> [Accessed 01.02.2007].
49. Hall I.C., Scott J.P., 1927. *Bacillus sordellii*, a cause of malignant edema in man. Journal of Infectious Diseases, Vol 41, pp 329-35.
50. Hecht David W. 2004. Prevalence of Antibiotic Resistance in Anaerobic Bacteria: Worrisome Developments. Clinical Infectious Diseases, Vol 39, pp 92-7.
51. Hedberg. M. 2003. Multi-resistance in the *Bacteroides fragilis* group. Anaerobe, Vol 9, p 251.
52. Hedberg M and Nord C.E. 2003. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe. Clinical Microbiology and Infection, Vol 9, pp 475-488.

53. Hentges David J. Anaerobes: General Characteristics, Medmicro Chapter 17.
Medical Microbiology 4th edition edited by Samuel Baron. The University of Texas
Medical Branch at Galveston. Available at:
<<http://file:///F:/medmicrochapter17.htm>> [Accessed 13.07.2007].
54. Holt, S.C., Kesavalu, L., Walker, S., et al. 2000. Virulence factors of
Porphyromonas gingivalis. Journal of Periodontology, Vol 20, pp 168-238.
55. Hueck, C.J. 1998. Type III secretion systems in bacterial pathogens of plants and
animals. Microbiology and Molecular Biology Reviews, pp 329-433.
56. Humphreys F.B., Meleney F.L., 1928. The identity of *C. oedematoides* and *B.*
sordellii. Proceedings of the Society for Experimental Biology and Medicine, Vol
25, pp 611-4.
57. Hurlbert, R.E., 1999. Microbiology 101 Internet Text chapter XIII: How microbes
cause disease. Available at:
< [http //www.slic2.wsu.edu: 82/hulbert/micro101/pages/chap 3.html](http://www.slic2.wsu.edu:82/hulbert/micro101/pages/chap3.html)>
[Accessed 29.12.2008].
58. Itzhak Brook. 2007. Treatment of anaerobic infection. Expert Review of Anti-
infective Therapy, Vol 5, No 6, pp 991-1006.
59. Jenkins Stephen G. 2001. Infections due to anaerobic bacteria and the role of
antimicrobial susceptibility testing of anaerobes. Reviews in Medical
Microbiology, Vol 12, pp 1-12.
60. Koch Careena L., Derby Pamela and Abratt Valerie R., 1998. In-vitro antibiotic
susceptibility and molecular analysis of anaerobic bacteria in Cape Town, South
Africa. Journal of Antimicrobial Chemotherapy, Vol 42, pp 245-248.
61. Koneman Elmer W., Allen Stephen D., Janda William M et al.1997. Color Atlas
and Textbook of Diagnostic Microbiology 5th edition.

62. Krinos C.M., Coyne M.J., Weinacht K.G., et al. 2001. Extensive surface diversity of a commensal micro- organism by multiple DNA inversions. *Nature*, Vol 414, pp 555-558.
63. Lorber Bennett. 2000. Gas Gangrene and other *Clostridium*-Associated Diseases in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition. Mandell Gerald L., Bennett John E., and Dolin Raphael, Vol 2. Elsevier Churchill Livingstone.
64. Lorber Bennett. 2000. *Bacteroides*, *Prevotella*, *Porphyromonas*, and *Fusobacterium* Species (and Other Medically Important Anaerobic gram-negative bacilli) in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition. Mandell Gerald L., Bennett John E., and Dolin Raphael, Vol 2. Elsevier Churchill Livingstone.
65. Lin, Y.Y., Huang, J.H., Lai, Y.Y., et al. 2005. Tissue destruction induced by *Porphyromonas gingivalis* infection in a mouse chamber model is associated with tumour necrosis factor generation. *Infection and Immunity*, Vol 73, pp 7946-7952.
66. Mascini Ellen M and Verhoef Jan. 2000. Anaerobic Cocci in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition. Mandell Gerald L., Bennett John E., and Dolin Raphael, Vol 2. Elsevier Churchill Livingstone.
67. Mascini Ellen M and Verhoef Jan. 2000. Anaerobic gram-positive non-sporulating bacilli in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition. Mandell Gerald L., Bennett John E., and Dolin Raphael, Vol 2. Elsevier Churchill Livingstone.
68. Mazuski J.E., 2007. Antimicrobial treatment for intraabdominal infections. *Expert Opinion Pharmacotherapy*, Vol 8, No17, pp 2933-2945.

69. Merck manuals online medical library. The merck manual for healthcare professionals. Available at: < <http://www.merck.com> >
[Accessed, 30.12.2008].
70. Montravers, P., Gauzet, R., Muller, C et al. 1996. Emergence of antibiotic-resistant bacteria in cases of peritonitis after intraabdominal surgery affects the efficacy of empirical antimicrobial therapy. *Clinical Infectious Diseases*, Vol 23, pp 486-494.
71. Mouricout, M., 1997. Interactions between the enteric pathogen and the host. An assortment of bacterial lectins and a set of glycoconjugate receptors. *Advances in Experimental Medical Biology*, Vol 412, pp 109-123.
72. Miech R.P., 2005. Pathophysiology of mifepristone-induced septic shock due to *Clostridium sordellii*. *Annual Pharmacotherapy*, Vol 39, pp 1483-8.
73. NCCLS. National Committee for Clinical Laboratory Standards. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, M11-A5, Vol 21, No 2.
74. Nord, C.E., 2003. Impact of new antibiotics on the intestinal micro-flora. *Anaerobe*, Vol 9, p 249.
75. Offenbacher, S., 1996. Peri-dontal diseases: pathogenesis. *Annual Peridontology*, Vol 1, pp 821-878.
76. Onderdonk A.B., Bartlett J.G., Louie T., et al. 1976. Microbial synergy in experimental intraabdominal abscess. *Infection and Immunity*, Vol 13, pp 22-26.
77. Onderdonk A.B., Kasper D.L., Cisneros R.L., et al. 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and un-encapsulated strains. *Journal of Infectious Diseases*, Vol 136, pp 82-89.

78. Oteo J., Aracil B., Alo's, J.I., et al. 2000. High prevalence of resistance to clindamycin in *Bacteroides fragilis* group isolates. Journal of Antimicrobial Chemotherapy, Vol 45, pp 691-693.
79. Papaparaskevas J., Pantazatou A., Katsandri A., et al. 2005. Multi-centre survey of the in-vitro activity of seven antimicrobial agents, including ertapenem, against recently isolated gram-negative anaerobic bacteria in Greece. Clinical Microbiology and Infectious Diseases, Vol 11, pp 820-824.
80. Parkhill, J., 2003. The *Bacteroides fragilis* genome project. Anaerobe, Vol 9, pp 247-276.
81. Paula G.R., Falcao L.S., Antunes E.N.F., et al. 2004. Determinants of resistance in *Bacteroides fragilis* strains according to recent Brazilian profiles of antimicrobial susceptibility. International Journal of Antimicrobial Agents, Vol 24, pp 53-58.
82. Pfister W. 2004. In vitro activity of ertapenem in comparison with penicillin, piperacillin/tazobactam, clindamycin, and metronidazole against clinical isolates of anaerobic bacteria. European Society of Clinical Microbiology and Infectious Diseases. 14th European Congress of Clinical Microbiology and Infectious Diseases Prague/Czech Republic, Abstract No 902, pg 1887.
83. Robertson M. 1916. Journal of Pathology and Bacteriology, Vol 20, pp 327-349.
84. <www.rsc.org/ej/PP/2004/B311900A> [Accessed 2008].
85. <www.sanger.ac.uk/Projects/B_fragilis/photo.shtml> [Accessed 2009].
86. Sauer, F.G., Mulvey, M.A., Schilling, J.D., et al. 2000. Bacterial pili: molecular mechanisms of pathogenesis. Current Opinion in Microbiology, Vol 3, pp 65-72.
87. Sears.C.L., Myers.L.L., Lozenby.A., et al. 1995. Entero-toxigenic *Bacteroides fragilis*, Clinical Infectious Diseases, Vol 20 (Suppl.2), pp. S142-8.
88. Shames Robert., Satti Farhana., Vellozzi Ernestine M., et al. 2006. Suscetibility

- of *Propionibacterium acnes* Ophthalmic Isolates to Ertapenem, Meropenem and Cefepime. Journal of Clinical Microbiology. Vol 44, No 11, pp 4227-4228.
89. Shapiro, R.L. 1998. Annals of Internal Medicine, Vol 129, pp 221-228.
90. Shimada K., Bricknell K.S., and Finegold S.M., 1969. Deconjugation of bile acids by intestinal bacteria: Review of literature and additional studies. Journal of Infectious Diseases, Vol 119, pp 73-81.
91. Slots J., Bragd L., Wikstrom. M., et al.1986. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. Journal of Clinical Periodontology, Vol 13, pp 570-577.
92. Smith L.D.S., 1975. *Clostridium sordelli* in: The pathogenic anaerobic bacteria, 2nd edition. Springfield, IL: Charles C. Thomas Publishing.
93. Smith P.O., 2004. Decline in antimicrobial susceptibilities of anaerobic bacteria from the academic hospitals in Bloemfontein, South Africa, 1996-2003. European Society of Clinical Microbiology and Infectious Diseases.14th European Congress of Clinical Microbiology and Infectious Diseases Prague/Czech Republic, Abstract No 902, pg 789.
94. Song, L., Hobaugh, M.J., Shustak, C., et al.1996. Structure of Staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science, Vol 274, pp1859-1866.
95. Sprandel Kelly, A., Drusano George, L., Hecht David, W., et al. 2006. Population pharmacokinetic modeling and Monte Carlo simulation of varying doses of intravenous metronidazole. Diagnostic Microbiology and Infectious Diseases, Vol 55, pp 303-309.
96. Stein Gary E and Goldstein Ellie J.C. 2006. Fluoroquinolones and Anaerobes.

- Clinical Infectious Diseases, Vol 42, pp 1598-607.
97. Stelling J.M., O'Brien T.F., 1997. Surveillance of antimicrobial resistance: the WHONET program. Clinical Infectious Diseases, Vol 24: S157-S168.
98. Todor Kenneth. 2008. Todor's online Textbook of Bacteriology. Pathogenic Clostridia, including Botulism and Tetanus. Available at:
<www.textbookofbacteriology.net. > [Accessed 06.01.2009].
99. Tortorello Mary L and Delwiche Eugene A. 1984. Characterization of the lipopolysaccharide of *Veillonella parvula* ATN and growth conditions affecting its yield. Current Microbiology, Vol 2, No 2, pp 107-111.
100. Tzianabos Arthur O and Kasper Dennis L. 2005. Anaerobic Infections: General Concepts in: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. Mandell G.L., Bennett J.E., Dolin R., Vol 2, 6th edition, pp 2810-2816. Philadelphia: Elsevier Churchill Livingstone.
101. Van Mellaert, L., Barbe', S., and Jozef, Anne'. 2006. *Clostridium* spores as anti-tumor agents. Trends in Microbiology, Vol 14, No 4, pg 191.
102. Washington W Jr., Allen S., Janda W., et al. 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th edition, Lippincott Williams & Wilkins.
103. Welkerling H., Geißdorfer W., Aigner T., et al. 2006. Osteomyelitis of the Ulna Caused by *Porphyromonas gingivalis*. Journal of Clinical Microbiology, Vol 44, No 10, pp 3835-3837.
104. Wells Carol L and Wilkins Tracy D. Anaerobic Cocci. Medmicro chapter 19. Medical Microbiology 4th edition, edited by Samuel Baron. The University of Texas Medical Branch at Galveston. Available at:
< [http://file:///F:/Medmicro chapter19.htm](http://file:///F:/Medmicro%20chapter19.htm). > [Accessed 13.07.2007].

105. Wells Carol L and Wilkins Tracy D. *Clostridia: Spore-forming Anaerobic Bacilli*.
Medmicro chapter 18. Medical Microbiology 4th edition, edited by Samuel Baron.
The University of Texas Medical Branch at Galveston. Available at:
< [http://file:///F:/Medmicro chapter 18.htm](http://file:///F:/Medmicro%20chapter%2018.htm)> [Accessed 13.07.2007].
106. Wisconsin University Bacteriology lecture topics homepage, 2000. Available at:
<<http://www.bact.wisc.edu/Bact330/>> [Accessed 28.12.2008].
107. Yumi A. Warren., Kerin L. Tyrrell., Diane M.Citron., et al. 2006.
Clostridium aldenense sp.nov. and *Clostridium citroniae* sp.nov isolated from
Human Clinical Infections. Journal of Clinical Microbiology, Vol 44, No 7,
pp 2416-2422.