ABSTRACT

BACTERIOLOGICAL PROFILE OF ANAEROBIC INFECTIONS AND THEIR ANTIBIOTIC SENSITIVITY PATTERN IN A TERTIARY CARE HOSPITAL, SOUTH INDIA

INTRODUCTION:

Anaerobic bacteria are the predominant indigenous microflora of humans and play an important role in infections , some of which are serious and carry a high mortality rate. These organisms are recovered only infrequently from cultures of infected materials because of common short coming in collection and transport procedures. The technical difficulties, time consuming processes along with the prohibitive cost involved in the elaborate "setup" required for its isolation and identification usually put a limitation to the routine search for the anaerobes in most laboratories. In addition, their evolving resistance to most of the commonly used antimicrobial agents has made the choice of empiric based therapy impossible nowadays. There is a need for a standardised simple , cost effective and reliable system, we plan to evaluate the usefulness of the rapid identification of anaerobic bacteria isolated by VITEK-2 system and MALDI TOF.

AIM OF THE STUDY: To determine the incidence of anaerobes among pyogenic infections.

OBJECTIVES:

1. To isolate the anaerobes isolated from clinical specimen by conventional anerobic culture methods .
2. To identify the isolated organism using VITEK-2 ID & MALDI-TOF.
3. To determine the antibiotic susceptibility pattern of anaerobes isolated.
4. To determine the prevalence of metronidazole resistance among anaerobes.

MATERIALS AND METHODS :

A total of 150 clinical samples from patients with pyogenic infections were processed using the anoxamat anerobe culturing system .Isolated and

conventionally identified anaerobes were compared with the automated VITEK-2 and MALDI TOF identification systems . All the isolated were subjected to antibiotic susceptibility testing .

RESULT AND ANALYSIS

About 28 (18.66%) anaerobes were isolated from intra-abdominal infections, periodontal infections . cellulitis, necrotizing fasciitis etc. Pure isolation of anaerobes and anaerobes mixed aerobes with were seen in (57.1%) and (42.8%) respectively. *Bacteroides fragilis* (32%) was the predominant isolate followed by *Prevotella disiens* (14%), *Clostridium clostridioforme* (7%).*E coli* was the commonest aerobe followed by *Klebsiella pneumoniae* , *Pseudomonas sp* and others When the VITEK-2 and MALDI-TOF automated identification systems were compared for anaerobes, identification upto Genus level was comparable in 75% and most with *B fragilis group* and *Prevotella sp* . Poor comparability was observed with *Clostridium species* All the anaerobes were sensitive to Metronidazole and Imipenem and were resistant to Ampicillin and Clindamycin.

CONCLUSION:

Isolation and identification of anaerobic infections should be routinely performed and newer automated methods should be standardized and their databases updated because they are simple, cost effective and rapid .Regular monitoring of drug resistance to commonly used anaerobic antimicrobial should be done to identify the trends in resistance.

INTRODUCTION:

Anaerobic bacteria are the predominant component of the bacterial flora of normal human skin and mucous membranes and are, therefore, a common cause of endogenous bacterial infections. Such infections may be serious and even life-threatening. They can involve all body systems and site.[1] but most often affect the abdominal and pelvic organs, the respiratory system, and the skin and soft tissues.

The diagnosis of anaerobic infections can be difficult, but it may be identified by the recognizing using certain clinical signs. Predisposing conditions and bacteriologic hints should alert the physician, who may apply diagnostic procedures to ascertain the nature of the pathogens and the extent of the infection. Almost all anaerobic infections originate from the patient's own microflora. Poor blood supply and tissue necrosis lower the oxidation-reduction potential and favor the growth of anaerobic bacteria. Any condition that lowers the blood supply to an affected area of the body can predispose to anaerobic infection. Therefore, malignancy, trauma, vascular disease, foreign bodies, surgery, colitis, edema, and shock may serve as predisposing factors.

Anaerobic infections can themselves provide clues to the presence of an underlying medical problem. The presence of putrid smell is the most specific clue to an anaerobic infection. The odor is caused by metabolic end-

products of the anaerobic organisms, which are mostly organic acids. However, the absence of a foul-smelling discharge does not exclude anaerobic infection, since not all anaerobic bacteria produce it. In deep-seated infections, these odors cannot always be appreciated.

There are different species of anerobes causing infections in human .*B fragilis* group is the most significant which constitute *B fragilis, B thetaiotamicron, B vulgatus, B distasonis, B ovatus* etc. *Porphyromonas* and *Prevotella* are encountered from oral cavity, head and neck, respiratory and urogenital infections. *Peptostreptococcus* , currently reclassified into different genera like *Shleiferella, Finegoldia, Micromonas* etc. are frequently encountered in blood culture , body fluids and various wound and abscess specimens .*Fusobacteria* are commonly involved in pleuropulmonary infections , brain abscess , chronic sinusitis , liver abscess and intraabdominal infections. *Clostridium sp* are associated with gas gangrene, food poisoning, necrotizing bowel disease , botulism, tetanus .Non sporing anaerobes *Actinomyces, Propionibacterium, Bifidobacterium* common normal flora but can produce few skin infections.

Because of the fastidious nature, anaerobes are difficult to isolate from infectious sites and are often overlooked. Isolation of these organisms when they cause infection is important, since failure to direct therapy against them

often leads to clinical failures. Isolating them requires appropriate methods of collection of the specimen in a way that avoids the contamination of the normal flora, transportation in a way that does not expose the organism to the deleterious effect of oxygen, and cultivation of specimens by specialized technicians by anerobiosis which include the use of anaerobic jars, bio-bags, PRAS (pre-reduced anerobic sterilization) method of hungate , anaerobic chamber or glove box . Traditional methods for the identification of anaerobic pathogens are not always available in clinical bacteriology laboratories and are often laborious and time-consuming .[2]Therefore, the need for a rapid and accurate method for the identification of anaerobic pathogens is highly desirable for appropriate treatment. Vitek-2 automated system and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are now being used in microbiology laboratories worldwide to identify aerobic and anaerobic bacteria and fungal species . [3] The Vitek 2 automated identification system, bioMérieux, Inc gives the advantage of identifying the anaerobes in few hours . The anaerobic and c*orynebacterium* identification card can identify 63 taxa of anaerobes and *corynebacterium.* MALDI-TOF MS gives the opportunity to obtain a reliable identification in a few minutes. MALDI-TOF MS can be used for the rapid identification of anaerobic bacteria e.g. *Prevotella sp., Fusobacterium*

sp., Clostridium sp., Bacteroides sp. and gram-positive anaerobic cocci (GPAC).

Antibiotics used against anaerobic bacteria are carbapenems, β lactam/β lactamase inhibitor, chloramphenicol and Metronidazole. The therapy of anaerobic infections are usually administered empirically because isolation of anaerobic bacteria are relatively slow and routine susceptibility testing of anaerobic isolates was not recommended previously. Susceptibility tests are usually indicated in conditions like recurrent bacteremia, infections of vascular grafts and prosthetic devices, endocarditis, brain abscess, osteomyelitis.

Treatment of anaerobic infections is complicated by the slow growth, their polymicrobial nature and resistance to antibiotics. An emerging increase in resistance to many antibiotic like Clindamycin, Metronidazole, quinolones, cefoxitin and piperacillin among the anaerobes have been observed .[4] Drugs which were effective like metronidazole, carbapenems, beta lactam and beta lactamase inhibitor combination are reported to be less effective recently leading to treatment failures. Recognition and control of antibiotic resistance among anaerobes is of increasing importance and warrants the need for routine susceptibility tests in anaerobes.[5,6]. Although

appropriately heightened attention has been given to resistant aerobic bacteria for several decades, awareness of similar developments among anaerobic bacteria is needed.

This study has been undertaken as anaerobic bacteria are often under appreciated as significant contributors to infectious disease processes. Most laboratories do not have the infra structure or the expertise in this area, so anaerobic pathogens often go unidentified. In addition, their evolving resistance to most of the commonly used antimicrobial agents has made the choice of empiric based therapy impossible nowadays. There is a need for a standardised simple, cost effective and reliable system, we plan to evaluate the usefulness of the rapid identification of anaerobic bacteria isolated by VITEK-2 system and MALDI TOF.

AIM OF THE STUDY:

To determine the prevalence of anaerobes among pyogenic infections.

OBJECTIVES:

1. To isolate the anaerobes isolated from clinical specimen by conventional anerobic culture methods .
2. To identify the isolated organism using VITEK-2 ID & MALDI-TOF.
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4. To determine the prevalence of metronidazole resistance among anaerobes.

REVIEW OF LITERATURE :

Anaerobes are bacteria that requires a reduced oxygen tension for growth and fail to grow on the surface of the solid media in 10% Co2 or in air (18% oxygen). Anaerobes are the most primitive bacteria and comprise the normal microbial flora of the gastrointestinal and urogenital tract of man and animals. They colonise the mucocutaneous surfaces of man and animals.

Historically, Hippocratus rendered an acceptable description of tetanus in fourth century BC, and Xenopan accurately described acute necrotizing ulcerative gingivitis in greek soldiers in fourth century AD. In the year 1690 Antonie Philip Van Leeuwenhoek, in his letter to the royal society reported that "animalcules" could exist in the absence of air. The animalicules of Leeuvenhoek were not only the first described microorganisms . They were also the first recognized anaerobes later .Spallanzani was next to take up the case of the anaerobes and his experimental approach was to dip a glass tube into the culture, seal one end and attach the other to a vaccum pump. He was surprised to find that they continued to live for five weeks.[7]

Anaerobiosis was rediscovered by Louis Pasteur almost 200 years after the preliminary description by Leevenhoek. In the year 1862,

Pasteur while observing the cover glass preparation of a culture containing a butyric fermenting bacillus microscopically , noticed that on the margin of the drop where there was contact with air, the organisms appeared to be non motile and the cells in the centre of the preparation were actively motile . This preliminary experimental step proved the inhibitory effect of atmospheric oxygen and established the concept of aerobic and anaerobic life though it was considered as “sour grapes”. He used the term”aerobies” and “anaerobies” for organisms that could live without atmospheric oxygen and he discovered the concept “anaerobiosis”. He discovered that butyric fermentation was caused by *Clostridium butyricumin* . Various nonsporing anaerobes were taken from the normal flora of mucous membranes surfaces of humans and animals and with naturally occurring diseases. [7]

Since Pasteur’s description of of anaerobes in 1860s, a waxing and waning of interest in these microbes were evident .However tetanus was identified in the earliest pages of medical history .In 1889, Kitasate first grew anaerobes including *Cl tetani* on solid media and obtained them in pure culture .Levy published the first report of a patient of a patient with an anaerobic infection, a post partum parametrial mass extending to produce a gas abscess in the upper thigh in1891.Anaerobic gram positive bacilli were recovered from the foul smelling pus along with *Streptococcus pyogenes*.

Veillon reported *Micrococcus* from foetid suppurative Bartholinitis in 1893.Veillon and zuber published a classic paper characterising a large number of various types of anaerobes cultured from 25 cases of gangrenous suppurative infections in 1897.[8]

The invention of Mc Intosh Fildes anaerobic jar , in the year 1916 , allowed plate cultures of anerobes which provided a means for the isolation and recognition of single colonies. At about the same time the Muriel Roberston described mixed cultures as the root cause of most of the anomalies cited in literature. With the discovery of a series of anaerobic bacteria, the etiology of various human diseases was known. By the end of the 19th century , most of the pathogenic clostridia and some of the non sporing anaerobes including *Peptostreptococci, Bacteroides* and *Fusobacterium* had been discovered. Economic importance of anaerobes were understood during the I world war . the necessity to synthesize rubber urged England to produce Butyl alcohol , 30,000 tons of acetone and large amounts of glycerol from anaerobes for the production of explosives. Thus, Anaerobes indirectly helped English to be victorious. Many gas gangrene cases were observed in battlefields.

During the next 60 yrs, Clostridia remained the main subject of interest , However Aaltemeierin in the United states emphasized the role of

non clostridial anaerobes in appendicitis and adnexal infections. [7] Many American workers like Mc Lennen, Gillespie and Guy, Gunn etc.were responsible for the better understanding of non- clostridial anaerobes including *B fragilis.* [7]

The clinical awareness and scientific recognition of anaerobic bacteriology had been extended to India during the 1980s. The first report on clostridial wound infection in India was published from the King Edward memorial Hospital and Seth GS Medical colleges, Bombay by Dhayagude and Purandare in 1949.This was also the first publication on anaerobes from our country .In 1977, a publication on anti-anaerobic agents for the management of anaerobic infections was described by the same group. [7]

NOMENCLATURE AND CLASSIFICATION OFANAEROBES:

Nomenclature of anaerobic bacteria keeps on changing and it is difficult to keep up with these changes. Formerly the characterization of anaerobic microorganism was based primarily on phenotypic characteristics and simple genetic analysis . They were classified as gram positive and gram negative on the basis of gram staining. They are also classified depend upon their spore formations, intolerance to oxygen and cellular morphology. [9] Biochemical reactions, serological techniques and DNA homology have increased the understanding of relatedness among anaerobes. The most

common gram positive and gram negative anaerobes isolated from human infections are clostridium species, *Actinomyces sp, Bacteroides sp, Prevotella sp ,Porphyromonas sp* and *Fusobacterium sp.*

Major taxonomic revisions were made in the classification of anaerobes in the recent past and numerous new genera and species have been introduced into the world of anaerobes and many members are renamed.[7]

1. *Anaerobiospirillum thomasii* – New species
2. *Sutterella wadsworthensis* – New genus & new species
3. *Atopobium minutum* - New genus & new species
4. *Actinobaculum schalii*- New genus & new species
5. *Holdmania filiformis* - New genus & new species
6. *Finegoldia* - New genus

CURRENT NOMENCLATURE	SYNONYMS
1. Tannerella forsynthensis	*Bacteroides forsythus*
2. Mituokella dentalis	*Prevotella dentalis*
3. Eggerthella lenta	*Eubacterium lentum*
4. Filifactor villosus	*Clostridium villosum*
5. Finegoldia magna	*Peptostreptococcus magnus*
6. Clostridium argentinense	*Clostridium botulinum G*

7. *Shleiferella asaccharolytica*	*Peptostreptococcus asaccharolyticus*
8. *Shleiferella indoloca*	*Peptostreptococcus indolicus*
9. *Shleiferella lacrimalis*	*Peptostreptococcus lacrimmalis*
10. *Micromonas micros*	*Peptostreptococcus micros.*

MEDICALLY IMPORTANT ANAEROBES[7]

I. Non sporing anaerobes

A. Cocci

a. Gram positive cocci – *Peptostreptococcus, Finegoldia, micromonas, Shleiferella, Coprococcus, Ruminococcus.*

b. Gram Negative Cocci - *Veillonella*

B. Bacilli

a. Gram positive bacilli – *Eubacterium, Lactobacillus, Bifidobacterium, Prpionibacterium, Actinomyces, Mobilincus*

b. Gram negative bacilli – *Bacteroides, Prevotella, Porphyromonas, Fusobacterium, Bilophilia, Sutterella, leptotrichia, Capnocytophaga, Selenomonas, Anaerobiospirillm*

C. Spirochaetes – *Treponema , Borrelia*

II. Sporing Anaerobes

Gram positive bacilli (Clostridium) – *Cl tetani, Cl perfringens, cl botulinum, Cl difficile*

The first description of anaerobic streptococci was Micrococcus foetidus isolated by Veillonin1893.Others are like *Anaerococcus, Coprococcus, Sacrina, Atopobium, Finegoldia, Micromonas and Peptostreptococcus*.[10] They are the part of the normal flora of the mouth, upper respiratory tract, gastrointestinal tract, female genital tract and skin. They are isolated from infections of the mouth, skin & soft tissue, upper respiratory tract infections, puerperal sepsis and brain abscess. They constitute about 25-30% of all anaerobic isolates.

They identified based on microscopic appearance on gram staining, standard biochemical tests such as nitrate reduction indole production, detection of volatile fatty acids gas liquid chromatography by gas liquid chromatography[GLC].Sodium polyanethol sulphonate[SPS] disk testing provides a method of presumptive identification of *Peptostreptococcus anaerobius*, which is the only sensitive Gram positive anaerobic cocci[GPAC].Gram positive anaerobic cocci are usually susceptible to wide

range of commonly used antimicrobial agents. But recent strains resistant to beta lactam agents and clindamycin has been reported and the resistance to beta lactam is mediated through modified penicillin binding proteins. [11]

The *Veillonellaceae* are a family of gram negative obligate anaerobic cocci that are divided into three genera as *Veillonella, Acidaminococcus, Megasphaera*. *Veillonella* occur as tiny diplococci in clusters, pairs or short chains and the colonies produce a brick red fluorescence with UV light. [12] *Veillonella* species have been encountered in patients with meningitis, osteomyelitis, abscess, aspiration pneumonia, burns ,bites, sinusitis.[13]

They are gram positive asporogenous rods, facultatively anaerobic or obligatively anaerobic ,motile or non motile, saccharolytic or assacharolytic and chemoorganotrophic. It includes *Actinomyces, Propionibacterium, Bifidobacterium, Eubacterium, Lactobacillus, Mobiluncus* etc.*Actinomyces* are gram positive branching filamentous rods.They are characterized as a chronic granulomatous lesion that become suppurative and that forms abscess and draining sinuses. [14] .*Actinomyces* are microaerophilic and are slow growers and produces molar tooth colony.

Bifidobacterium are rod like organisms with a characteristic Y or V shaped end. They inhabitat mainly the intestinal and vaginal mucosa of

humans and animals. They are rarely pathogenic and act as probiotics due to their potential beneficial roles in the intestinal tract.[15]

Eubacterium are pleomorphlic gram positive rods which comprise a substantial part of the normal flora of the mouth and large intestine. They are isolated from abscess, wounds,pleuro pulmonary infections and periodontitis.

Lactobacilli are pleomorphic rods or cocco-bacilli. They are the part of normal flora in the vagina, mouth & intestine. They are reported to cause meningitis, endocarditis, abscess, peritonitis. They are rarely pathogenic and might even be beneficial in the treatment of bacterial vaginosis and diarrhea.[14]

Mobiluncus is composed of gram variable to gram negative motile,curved ,non- spore forming rods with tapered ends. They occur as normal flora in the reproductive tract and rectum. They are isolated from bacterial vaginosis, Vulvovaginal abscess, salphingitis and septicemia.[14]

Propionibacterium are pleomorphic gram positive rods which occur as normal flora in skin and the moist epithelium of the conjunctiva, oral cavity and large intestine. As inhabitants of the skin, they are common contaminants of cultures of blood and body fluids. They have been identified

as causes of brain abscess, dental infection, pulmonary infections and peritonitis.[16]

Clostridium –Spore forming anaerobic gram positive bacilli are classified in the genus *clostridium* and they are found principally in the soil and some present as normal flora of the bowel .Pathogenic Clostridia may produce two types of infection: histotoxic infections such as gas gangrene and toxin induced disease in including the bowel and the nervous systems.

Clostridium tetani was an obligate anaerobic bacilli that is gram positive in fresh cultures, but may have variable staining in older cultures or in tissue sections .[17] The bacilli possess terminal endospores that give the sporing cells a drum-stick appearance. It is motile by peritrichous flagella. Growth may appear as a film because of the organisms vigorous motility ad swarming. It produces two important toxins such as tetanolysin and tetanospasmin and the latter is responsible for the various manifestation of tetanus-generalised, localized, cephalic and neonatal.

Clostridium perfringens is capsulated, non motile and has a box car appearance on gram stain of clinical material. On blood agar, the colonies are surrounded by double zone of hemolysis [target hemolysis], an inner zone of complete hemolysis due to alpha toxin. The production of lecithinase [alpha toxin] can be demonstrated with the Naglers reaction and *Clostridium*

perfringens are usually reverse CAMP test positive. The organism is relatively aerotolerant and shows stormy fermentation in milk.[18]

Clostridium botulinum is a large usually gram positive ,strictly anaerobic bacillus that forms a subterminal spore. *Clostridium botulinum* produces toxins –types A-G, which is responsible for the various clinical forms of botulism-infant botulism, food borne botulism and wound botulism.

Clostridium difficile is Large gram positive bacilli with subterminal spores .It is associated with antibiotics usage leading to necrotizing colitis.

Anaerobic gram negative bacilli are the most common anaerobes involved in infections. Some of the species are the most common antibiotic resistant types.

They are small non motile, gram negative bacilli or coccobacilli. They are non pigmented, bile tolerant, strongly saccharolytic organisms. Pleomorphism is common and large bizarre rods with round or oval swellings can be seen, mainly *Bacteroides fragilis* ,it fermentable carbohydrate is present in the media.

They grow well on neomycin blood agar incubated anaerobically at 37 ^{0} C to produce smooth, shiny, circular, convex, translucent and gray colonies at 24 hours. They are the important pathogens causing infections

particularly after accidental or surgical injury to the gastrointestinal tract or in association with pathological lesions of it. *Bacteroides fragilis* is the most common anaerobic organism and resistance to many antimicrobial agents. They are from most intra abdominal infections, diabetic foot infections, cerebral abscess, sinusitis, lung abscess, empyema, endocarditis, appendicitis ,other deep abscess, PID and bacteremia. Bacteroides bile esculin [BBE] is used as the selective media for *Bacteroides fragilis* group which produces black coloured colonies. A rapid screening test is available for *Bacteroides fragilis* group, which utilizes filter paper disks impregnated with 25 µg oxgall tested in conjuction with antibiotic identification disks [Kanamycin,Vancomycin,Colistin] resistance to Kanamycin and bile is taken as a presumptive identification *Bacteroides fragilis* group. [19]

Metronidazole is effective against Bacteroides species, that tend to be resistant to a wide range of antimicrobial agents. However, metronidazole resistant strains are beginning to emerge in india. [20] and resistance to metronidazole mediated through five nitroimidazole resistant genes nim-A to nim-E. [21] Resistance to beta lactam antibiotics in *Bacteroides fragilis* is mainly due to the presence of beta lactamase and a potent zinc dependent metalloprotease. *Bacteroides fragilis* is naturally resistant to some

of the β lactam agents includes monobactams and temocillin, because of poor affinity shared by its penicillin binding proteins [PBP] for these compounds.

Oliver and wherry [1921] described gram negative bacilli that produced black pigmented colonies grown on lysed blood agar. These pigmented strains were previously included under species *Bacteroides melaninogenicus*. This genus *Prevotella* comprises bile sensitive,sacchrarolytic ,some of which produce black brown colonies, pigment is enhanced on lysed blood agar.[22]They appear as small, pale staining cocco bacilli on gram stain.

Prevotella species are associated with infections of the oral cavity,head and neck ,lower respiratory tract and genital tract, pleuropulmonary infections. Species are *Prevotella melaninogenica, Prevotella bivia* and *Prevotella disiens*. *Prevotella melaninogenica* are found in infections with upper respiratory tract and *Prevotella bivia* and *Prevotella disiens* are associated with female genital tract infections. [23] .Many strains produce beta-lactamases and resistant to penicillin & cephalosporins.

Porphyromonas species are gram negative, non motile bacilli or coccobacilli.They are asacchrolytic,bile sensitive and produce black colonies due to over production of protoheme. *Porphyromonas* present as normal flora in the oral cavity and associated with periodontitis , root canal abscess,

axillary, breast infections, genital tract infections and perianal abscess. *Porphyromonas* spp are sensitive to Vancomycin by Kirby bauer method.

Kanamycin Vancomycin lysed blood agar and laked rabbit blood agar are the selective media for *Porphyromonas*. The pigmented *Prevotella* and *Porphyromonas s*pp may vary in the rapidity of pigment production depends on the composition of the base medium used in the agar. A period of 2 to 21 days may be required on laked rabbit blood agar to detect pigmentation ranges from buff to tan to black. The pigmented *Prevotella* and *Porphyromonas spp* fluoresce pink, orange or brick red under UV light. Fluorescence is best demonstrated in young cultures. In older cultures, especially on laked blood agar, the fluorescence is more or less masked depends on the intensity of pigment production..[24]

In 1923, Knorr proposed that pointed, fusiform, non-sporing gram negative bacilli found in the mouth called *Fusobacterium*. It act as a commensals in the upper respiratory tract, gastro intestinal tract and genitourinary tract. It is a gram negative bacilli, slender, spindle shape with tapered or pointed ends. It is most frequently isolated from infections of mouth, head and neck, pleuro- pulmonary infections, cerebral and liver abscess. They produce speckled, smooth or bread crumb like colonies and the colonies fluoresces chartreuse and produces greening of the agar.

Fusobacterium necrophroum is associated with Lemierres syndrome. It is a pleomorphic rod with rounded ends with variable bizarre shapes. It is the only lipase positive *Fusobacteria* .Josamycin-Vancomycin-Norfloxacin[JVN] media as the selective media for the isolation of fusobacteria. The addition of egg yolk to these medium will enhance the growth of *Fusobacterium necrophorum* due to the presence of lipase reaction. All *Fusobacterium spp* are sensitive to metronidazole, penicillin, Clindamycin, Tetracycline, Chlorampenicol. In contrast, Vancomycin and erythromycin showed limited activity and aminoglycosides are ineffective. More recently, increasing numbers of strains have been found that are resistant to some of these drugs. [24]

MECHANISMS:

Anaerobic bacteria will not grow in presence of oxygen and are killed by oxygen or toxic oxygen radicals. Anaerobic bacteria lack the enzyme catalase[peroxidase] and superoxide dismutase[SOD],which would allow toxic levels of H_2O_2 and superoxide dismutase[SOD] ions. Some of these enzymes are detected in certain species, especially in the more aerotolerant isolates.[25] Anaerobes grow at a low or negative oxidation reduction potential[Eh].The Ph and the oxidation reduction potential [Eh] were important in estabilishing conditions that favour the growth of anaerobes.

REQUIREMENTS OF GROWTH:

Generally anaerobes are fastidious organisms and require an enriched medium for growth. Growth factor requirement fulfilled by the addition of vitamin K ,hemin, yeast extract, blood[solid media],serum[liquid media],fermentable carbohydrates to the basal medium, and other additives such as arginine or cysteine required for some strains Solid media such as Neomycin blood agar, Columbia blood agar, Brucella based blood agar are preferable. Broth medium such as thioglycolate broth with supplemented carbohydrate, Cooked chopped meat medium containing carbohydrates are excellent broth medium. Selective medias are useful for isolation and presumptive identification of anaerobes from clinical samples, which often includes both facultative and anaerobic bacteria.

PATHOGENESIS:

Anaerobic infections are caused due to breakdown of a mucosal barrier and subsequent leakage of indigenous polymicrobial flora into previously sterile tissue or closed spaces..The predisposing factors are trauma, presence of foreign bodies, surgery, radiation therapy, malignancy, administration of steroids, immunosuppressive drugs and diabetes mellitus. [26]

VIRULENCE FACTORS:

A variety of virulence factors along with the synergistic bacteria play a role in the pathogenesis of anaerobes.

Capsule:

A polysaccharide capsule has been demonstrated to be an important virulence factor for strain of *Bacteroides fragilis*. Capsules also have been observed on other species such as *Prevotella, Porphyromonas* and other *Bacteroides* species. [27] Capsular material protects anaerobic bacteria from phagocytosis and killing by polymorphonuclear leukocytes. It is important in adhesion to mucosal epithelial cells and peritoneal mesoepithelium and also helps in haemagglutination due to the presence of capsular carbohydrate residue or in the bacterial surface, independent of fimbrial production.

Fimbriae (pili)

The role of Fimbriae is not clear but may be related to the adherence to mucosal surfaces.

Enzymes:

Various enzymes that may serves as as a virulence factors have been detected in various strains of anaerobic bacteria. It includes lecithinase, lipase, phopholipase, neuraminidase , N-acetyl glucosaminidase , α and β

glucosidase , collagenase, heparinase, chondrotin sulfatase and elastase. [28,29] these enzymes play a dual role in causing tissue damage and providing nutrients for infecting microorganisms. Varying amounts of superoxide dismutase and catalase production by *B fragilis* group provides the property of oxygen intolerance. But the level of production of these enzymes by a given strain and its virulence appears to have little relationship. The enzymes β – lactamases inactivates penicillin and is responsible for the resistance to β-lactam antibiotics. Another enzyme glutamic acid decarboxylase helps in the rapid detection by bringing about a colour change in the substrate and aid in the identification.

Metabolic products

Several metabolic products of anaerobes are toxic to mammalian cell .Short chain fatty acids (SCFA) are produced in large quantities as metabolic by products and accumulated in the infected site .SCFAs behave like leucotoxins and very useful for bacteroides sp. Sulphur compounds including hydrogen sulphide indole and amines are also the metabolic products responsible for tissue damage.[30]

Lipopolysaccharide:

Lipopolysaccharide has been demonstrated in strains of *Prevotella, Fusobacterium ,Bacteroides , Porphyromonas and Veillonella.* The lipopolysaccharides of *Bacteroides fragilis* appears to promote abscess formation in experimental animals. *Fusobacterium* has more potent biological lipopolysaccharide than *Bacteroides* species.[25] Until recently , it was thought that LPS from *Bacteroides, Porphyromonas, Prevotella* did not contain ketodeoxy octone (KDO) and hence were likely to cause classic manifestations of endotoxic damage. These species are now known to produce KDO that s detectable only after acid extraction.LPS of *Bacteroides fragilis* reduces the opsonic activity of the complement .

Metabolic synergy[27]

It is suggested 4 possible mechanisms by which one bacterial species may increase the pathogenicity of its bacterial sp may increase the pathogenicity of its bacterial partner in a mixed reaction. The effects on host defenses like –

1. Inhibits leucocyte function
2. Provision of essential nutrients
3. Improvement of local environment
4. Increased virulence of the organism

Since most of the anaerobic infections are polymicrobial , Symbiotic relationship exists between obligate facultative anaerobes. For example intraabdominal infections , metabolic interdependence between *B fragilis* and *E coli*. In addition *E coli* consumes oxygen and creates a reduced condition favorable to the growth of *B fragilis. B fragilis* can use the iron binding protein from e coli to overcome iron limiting condition, In turn *B fragilis* inhibits macrophage migration and impairs phagocytosis resulting in the protection of *E coli.* The presence of capsular material and depletion of serum opsonins by *B fragilis* contributes to this synergistic protection.

Toxins:

Toxins such as leukotoxin, endotoxin and hemolysin have been implicated as virulence factors particularly among gram negative bacilli Strains of *Bacteroides fragilis* producing enterotoxin with a variety of pathological effects on intestinal mucosal cells have been identified.[31].

In case of anaerobic spore bearers, the exotoxin acts as an important virulence factor. In *Clostridium tetani* produces two distinct toxins-hemolysin [tetanolysin] and a powerful neurotoxin [tetanospasmin]. Tetanolysin is a heat labile, oxygen labile hemolysin, antigenically related to the oxygen labile hemolysin produced by *Clostridium perfringens,, Clostridium novyii* and *Streptococcus pyogenes*. It is not relevant in the

pathogenesis of tetanus.Tetanospasmin is the toxin responsible for tetanus. It is oxygen stable but relatively heat labile. In *Clostridium perfringes* it produces four major toxins –alpha, beta, epsilon and iota which are responsible for the profound toxemia of gas gangrene. It is lethal, dermonecrotic and haemolytic.

Anaerobiosis

Anaerobiosis is the cultivation of anerobic bacteria . a number of methods have been described for achieving anaerobiosis. The Principle being –

1. Exclusion of oxygen
2. Displacement of oxygen with other gases
3. Absorption by chemical or biological means
4. Reduction of oxygen.

Anaerobic methods include the use of anaerobic jars, bio-bags, PRAS (Pre-reduced anaerobic sterilization) method of hungate , anaerobic chamber or glove box etc for the routine isolation and study of anaerobic bacteria from clinical material .

Oxygen may be displaced with gases like hydrogen, Nitrogen , Carbondioxide or helium. A popular but ineffective method is the candle jar. It provides a concentrate of carbondioxide which stimulates the

growth of most of the bacteria. In anaerobic jars , a mixture containing 90% hydrogen + 10% carbondioxide or 80% hydrogen + 10% carbondioxide + 10% nitrogen are used .

Absorption of oxygen – Alkaline pyragallol has the property of absorbing large amount of oxygen. First introduced by Buchner in 1888. Pyrogallic acid, added to a solution of NaoH in a large test tube inside an air tight jar provides anaerobiosis, but small amounts of carbon monoxide which is formed during the reaction, may be inhibitory to some of the bacteria.

The use of aerobic organism to absorb oxygen was originally described by fortner (1928). The organism such as coliform bacilli , *Pseudomonas aeruginosa* and *Bacillus subtilis* are suitable for this purpose. Anaerobiosis takes some time to develop, so that the method usually fails with exacting anaerobes.

The principles of achieving anaerobiosis in a single plate cultures was applied by kneteman (1957) by a plastic film technique in which an aerobic *Micrococcus* and the anaerobe were grown within the medium as pour plate culture . Oxygen from the air was prevented from diffusing into the medium by covering the surface of the agar with a film of oxygen impermeable plastic. Absorption of oxygen from small closed system has

been attempted by incubation along with germinating seeds or chopped vegetables . this method is slow and ineffective.

Reduction of Oxygen [7]: Reducing agents like 1% glucose, 0.15 ascorbic acid, 0.o5% cysteine, 0.15 thioglycollate (Shank,1963) have been attempted. An early prepared anaerobic medium was broth into which pieces of metallic iron flamed red hot were introduced and layered over with sterile liquid paraffin.

Broth containing fresh animal tissues such as rabbit kidney , spleen, tests or heart supports the growth of many anaerobes (Smith Noguchi medium) . Robertson's cooked meat medium is the most widely used fluid medium for the culture of anaerobes .It consists of fat free minced cooked meat in broth with a layer of sterile liquid paraffin over it . Unsaturated fatty acids and glutathione in RCM medium absorbs oxygen and this reaction is catalyzed by hematin present in meat particles . It permits the growth of even strict anaerobes and indicates their saccharolytic or proteolytic activities by the meat being turned red or black respectively. It is a very good medium for production of toxin by Clostridia as well as for the propagation of anaerobes as stock cultures.

Pre reduced Anaerobically sterilized roll tube: Hungate (1950) first developed this technique for the isolation of oxygen intolerant anaerobes from rumen fluid and sewage. In this system each culture tube is an individual anaerobic container . Water , mineral solutions ,resazurin and few glass beads are added to a flask and boiled until resazurin changes from pink to colourless. The PRAS medium can be inoculated using loop or Pasteur pipette . The advantages are it is not exposed to air and can be examined daily without interrupting anaerobic environment .

Anaerobic jars: [7] Anaerobic jars can be set up based on 2 principles

1. Evacuation and replacement technique.
    a. Mc Intosh Fildes jar
    b. BTL jar
2. Hydrogen – carbon dioxide generation technique
    a. Gas pak jar
    b. Dynamicro jar.

Mc Intosh Fildes jar: Consists of a glass or metal jar with a metal lid which can be clamped air tight with a screw.an outlet tube is connected to a vaccum pump to evacuate the air present in the jar . Gas inlet is connected to hydrogen supply. Lid consists of electrical terminals.

Leading from the terminals and suspended from the stout wires on the underside of the lid is a small grooved porcelain pool around which is wrapped by a layer of palladinised asbestos. Inoculated petri dishes are kept inside after evacuating jar by drawing a vaccum of 25 inches of mercury. A mixture of hydrogen , carbondioxide and nitrogen is filled in it . the Atmosphere in the jar is monitored by including an indicator to check anaerobiosis .BTL jar is modified Mc Intosh jar.

Dyna Micro Jar: This is based on the principle of producing Hydrogen and carbondioxide from sulphuric acid and Zinc or chromium salt (Marshall, 1960).

Gaspak Anaerobic system : Commercially available (Brewer & Allgeier,1966) as a disposable envelope containing chemicals which generate hydrogen and carbondioxide on the addition of water.after the incubated plates are kept in the jar , the gaspak envelope with water added , is placed inside the lid screwed tight. Hydrogen and carbondioxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment .The gas pack is simple and effective . It contains 3 tablets in

a plastic bag which includes Sodium bicarbonate, citric acid, Sodium borohydride.An alternative to gaspak is Vaidyalingam's method .*

Anoxomat : The Anoxomat is an automated method that employs the evacuation replacement technique to create an anaerobic environment in the jar. This fully automated system is similar to the technique already described but it also provides an internal quality assurance programme that executes additional tests when selected. These tests include leak checks,a test for sufficient catalyst activity and a test for adequate addition of replacement gas.

Glove box or anaerobic chamber: [7] Glove port and rubber gloves allow the operator to perform manipulations within the chamber. Media are placed within the airlock with the inner door remaining sealed. The Air is removed by vaccum pump connection and replaced with nitrogen. The inner door is opened and the media are placed within the main chamber containing atmosphere of hydrogen + Carbondioxide. A circulator circulates the gas atmosphere through the pellets of palladium catalyst causing any residual oxygen in the media to be used up by the reaction with hydrogen. After the media have become completely anaerobic, they can be inoculated and placed in an incubator located within the chamber.

Indicators of anaerobiosis [7]**:** Indicators are necessary in anaerobic work to ensure it is working well.

1. Resazurin has also been used as an Eh indicator in thioglycollate media.
2. Lucas semisolid indicator
3. The indicator of Brewer, Allgeier and Mc Laughlin – Used in anaerobic jars and gas pack.
4. Fildes and McIntosh indicator.
5. Bacteriological indicator- Using strict anaerobe such as *Cl tetani* or *B fragilis* and of a strict aerobe such as *Pseudomonas aeruginosa*.

LAB DIAGNOSIS OF ANAEROBIC INFECTIONS:

The most important considerations in the isolation of anaerobic bacteria from clinical specimen are -

1. Proper collection of the specimen in a way that avoids the contamination of the normal flora
2. Transport of the specimen without exposure to deleterious effect of oxygen .
3. Prompt cultivation of the specimen.

4. Use of freshly prepared media and maintaining anaerobic conditions.[16.Dowell VR,CDC lab manual 1987].

Specimen Collection : [23] Proper collection of clinical specimens is of primary importance in recovery of anaerobes. The specimens for anaerobic culture should be free of contamination with normal flora. The recommended collection methods for anaerobic culture are as follows-

1. Pulmonary specimen – Percutaneous, Transtracheal, Direct lung puncture for children, Plugged double or triple lumen catheter and bronchial brush with quantitative culture.
2. Pleural fluid – Thoracocentesis
3. Urinary tract – Percutaneous supra pubic bladder aspiration, Nephrostomy tube , suprapubic catheter
4. Abscess – Closed – Decontaminated and aspirated with needle / syringe.
5. Abscess – Open – Use of swab may yield secondary colonisers which have little clinical significance.
6. Female genital tract – Aspiration , Culdocentesis, Double lumen catheter
7. Sinus tract – Aspiration by syringe and small plastic catheter introduced as deep as possible through decontaminated skin surface.

8. Draining wound- specimens obtained from depth of wound or underlying bone lesion. Curetting and tissue biopsies.
9. Ulcers -Debrided and samples collected from the base or the progressive edge, where bacteria multiply

The specimens such as nasopharygeal swabs, throat swabs, gingival swabs, sputum, urine[voided/catheterized], cerival swab, vaginal swab which might be contaminated with normal flora are unsuitable for anaerobic culture.[17.Wadsworth Anaerobic 4thed.1985].

Transport of specimens: [7]

Anaerobic specimens are should be placed in anaerobic transporter immediately after collection, The transport system should be properly anaerobic with an oxygen indicator , non-nutritive with small sample dilution factor. Specimens should never be refrigerated , as oxygen diffuses better at lower temperature. However if the samples must be held for more than 3 hrs, refrigeration is advisable in an airtight container.

1. Aspirate : Can be transported in a syringe within 15-20 min of collection expelling air bubble and the needle embedded into a rubber stopper.

2. Anaerobic transport tube : Samples less than 2 ml are transported in a tube containing oxygen free gas , small volume of non-nutritive broth with an anaerobic indicator fluid and reducing agents .Eg: Reduced transport fluid (RTF). Anaerobes survive much longer in grossly purulent specimen greater than 2ml.
3. Swabs : Should be immediately placed into an anaerobic transport system sent to the lab . Ex : Cary Blair an anaerobic indicator.
4. Tissue and bone specimen: Transport anaerobic in a sterile container (within 15-20 mins of collection)
5. Biobag : If there is a delay, specimen should be sent in biobag containing indicator and gas generation system.

Specimen preparation and inoculation: Specimen processing should be ideally done in an anaerobic chamber. The steps involved includes

1. Vortex mixing
2. Homogenize piece of tissue / bone fragments with 1ml anaerobic medium
3. Swabs are extracted in broth and treated as liquid specimen
4. 1 drop / plate if purulent material
5. 2-3 drops / plate if non purulent material
6. 1 drop for staining

7. Rest of the sample in RCM / Thioglycollate

Direct microscopy:

The following methods are used to detect anaerobic bacteria by microscopic examination, Gram stain, Dark field or phase contrast microscopy Gram stained smear should always should be prepared from the specimens for anaerobic culture. Dark field microscopy and phase contrast mi microscopy may be helpful for the notation of spores[*Clostridium spp*). [22]

Culture: Always a non-selective and a selective medium is included for the isolation of anaerobes.

1. Brucella blood agar supplemented with 5% sheep blood, Vitamin K1 (1µg/ml) and hemin (5µg/ml) or brain heart infusion blood agar supplemented with yeast extract , hemin, vitamin K and cysteine – non selective media for all anaerobes.
2. Bacteroides bile esculin (BBE agar) for the selective isolation of *Bacteroides fragilis* and *Bilophila sp.*
3. Kanamycin Vancomycin laked blood agar (KVLB) foe pigmented *Porphyromonas* and *Prevotella* sp.

4. RCM or thioglycollate medium without indicator supplemented with vit K1, Hemin and reducing agent as a backup source of culture material in the case of failure of anaerobiosis / growth inhibition / small inoculum in the specimen .
5. If Clostridia are suspected , Egg yolk agar is to be included to check for the production of lipase and lecithinase.
6. Neomycin blood agar – Selective medium for all anaerobes
7. Rogosa S L broth – *Lactobacilli*
8. Propionibacterium selective agar – *Propionibacterium*
9. RLK & SA Medium

Inoculation and incubation:

1. Inoculated in on selective , a selective medium and RCM
2. The plates are streaked and a metronidazole disc (5µg) is placed for the presumptive identification of anaerobes.
3. Specimens collected from sites with anaerobes existing as normal flora, a semiquantitative isolation may be attempted by streaking in 4 quadrants.
4. Biological control is included everytime
5. The plates are incubated in anaerobic jars or anaerobic pouches.

6. Incubated for 48-72 ^{0} C .Negative culture plates should inoculated for a minimum period of 7 days for pigment producing porphyromonas , slow growing Actinomyces etc.
7. Liquid medium RCM/ thioglycollate may be observed for turbidity and subcultured on media like BA,NBA (Anaerobic) and proceed like primary cultures.

Presumptive identification of Anaerobes : Clue for presence of anaerobes in culture

1. A foul odor opening an anaerobic jar /pouch.
2. A zone of inhibition around the metronidazole disc.
3. Many colony type in the anaerobically incubated blood agar.
4. Black colonies on BBE
5. Red fluorescing or black pigmented colonies on KVLB /Laked blood agar.
6. Pitting colonies on NBA – *B ureolyticus*.
7. Target hemolysis (double zone of lysis) – *Cl perfringens*.
8. Bread crumb like speckled colonies with fusiform gram negative bacilli in smear – Fusobacterium.
9. Molar tooth shaped colony with Gram positive branching rod – *Actinomyces / Propionibacterium.*

10. Yellow ground glass colonies with glass colonies with horse stable odour on CCFA- *C difficile.*

After Gram staining each colony morphotype will be subcultured on Anaerobic and aerobic culture media to get pure growth of the isolate and also aerotolerance. Based on the morphology , gram staining and biochemical reactions the isolated anaerobe is identified.

SEROLOGY:

Various tests have been used for immune response in Bacteroides infection such as agglutination, complement fixation, indirect immunofluroescence, precipitation in agar gel, passive hemeagglutination and counterimmunoelectrophoresis. Counterimmunoelctrophoresis and precipitation in gel are user their expressed antigens. Indirect immunofluroscence and agglutination are used to detect antibodies to both thermolabile and thermostable antigens. Various antigens have been demonstrated for *Bacteroides melaninogenicus* and *Bacteroides fragilis.*[35]

RAPID TECHNIQUES IN THE DIAGNOSIS OF ANAEROBES:

Conventional techniques are laborious, time consuming and relatively expensive . Many technical factors like improper collection of specimen , delay in transport , exposure to oxygen are likely to reduce the frequency of isolation rate of anaerobes. Development of rapid and cost effective methods for the identification of anaerobes is the need of the hour.

Use of 4-Methyl Umbelliferone derivative:[36] Preformed enzyme glucosidase linked to a fluorescent substrate 4 methyl umbelliferone that has 1000 fold greater sensitivity than commercial test system s was reported due to the increased sensitivity .The incubation time is as less as 15 mins. The Whatman no-2 filter paper is soaked in substrate solution and a spot test was performed by smearing a heavy loopful of bacteria and incubating the filter paer strip at 37 0C for 15 min. The Filter strips were examined under a long wave length hand held lamp for characteristic light blue fluorescence of the hydrolytic product of enzymatic action on the glucoside derivatives, 4-methylumbelliferone. Based on the results, a flow scheme was developed for the presumptive identification of *B fragilis* group.

Use of Fibre optic light: Smokzynski et al (1993) [37]devised the use of fibre optic light in association with a movable stereoscope and gloveless

chamber, to enhance the ability to detect and manipulate bacterial colonies on plates used for primary culture of anaerobes, as early as 24hrs after inoculation. Decrease in time to identify the *B fragilis* group was more noticeable . It took only 4 days to identify *B fragilis* as against the 6 days , before installation of gloveless chamber / fibre optic light combination.

Detection of preformed enzymes [38]:Recent use of chromogenic or flurogenic substrates in detecting preformed enzymes of resting cells of bacteria has only recently been applied to the identification of anaerobic bacteria . Somer 1987.

Bacteriocin typing : A simple method to screen bacteriocin production and a typing scheme was developed for *B fragilis*. This is a valuable diagnostic and epidemiological tool.

Gas liquid Chromatography:[39] Anaerobes produce distinct short chain fatty acids (SCFA) such as acetic , propionic , succinic, butyric acid which can be identified by GLC. Although the anaerobic metabolism is not as efficient as that of facultative organisms, the intermediate products are left that can serve as markers of an organism's identity. Since the enzymes involved are genetically stable, the end products of its metabolism produce a fingerprint that is typical and useful for identification. This well advanced technique was initiated by James and Martin in 1952 and later

modified by Moore and colleagues and applied it in the taxonomy of anaerobes. The chromatograph includes a coiled, packed column in a small temperature controlled oven ,an inert carrier gas flow system , with a detector and a recorder. Upon introduction of the acids into the unit, the acid molecules are volatilized. The volatile molecules of each acid, cover a thermal conductivity detector wire in the chromatograph and cause in current flow that can be detected and recorded. Each acid has a different elution time and separation is easily accomplished. It can specifically identify pure culture of anaerobes and also mixed cultures. Direct analysis of clinical specimens employing GLC will enable the recognition of anaerobes within a few minutes of sample collection.

Vitek -2 Automated identification system : [2] The anaerobe and *Corynebacterium* (ANC) identification card has a database that includes 63 taxa of anaerobes and corynebacteria belonging to the genera *Actinomyces*, *Arcanobacterium*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Corynebacterium*, *Eggerthella*, *Eubacterium*, *Finegoldia*, *Fusobacterium*, *Lactobacillus*, *Microbacterium*, *Micromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Prevotella*, *Propionibacterium*, *Staphylococcus*, and *Veillonella*.

Electronic nose (e-nose):[40]An electronic nose is a device intended to detect odors . Over the last decade , “electronic sensing” or “e-sensing “ Technologies have undergone important developments from a technical and commercial point of view. The expression “ electronic sensing “ refers to the capability of reproducing human senses using sensor arrays and pattern recognition systems. The instrument recognize by comparing volatile compound fingerprint to those contained in its database. The stages of the recognition process are similar to human olfaction and are performed for identification, comparison, quantification and other applications, including data storage and retrieval.

High performance Liquid Chromatography(HPLC):[41] HPLC was evaluated for the rapid identification of *B fragilis* group by Radin et al ,1988. The different species of B fragilis group was inoculated into a defined chemical medium containing primarily carbohydrates and was incubated aerobically at 37 0c for 1 hr. after centrifugation , the pernatants are placed on ice to stop further enzymatic reaction. Specimens are injected into an Aminex HPX 87H column in order to determine carbohydrates and acid metabolic products. Peak areas of carbohydrates for each isolate are compared with those for uninoculated medium.

DNA Hybridization technique: DNA homology is considered as the gold standard for identification and characterization of microorganisms. DNA hybridization measures the amount of DNA sequences held in common between two organisms. Some of the commonly used hybridization formats in anaerobic bacteriology include – Checkerboard hybridization, reverse capture checkerboard hybrization , fluorescent in situ hybridization and DNA micro array. These techniques offer simultaneous and rapid detection of several bacterial species from a large number of samples in a single sitting.

The 16sRNA gene is the most useful phylogenetic marker to identify bacteria and to determine their evolutionary relationships. The introduction of 16SrRNA gene cloning and sequencing has a dramatic impact on the taxonomy of anaerobic bacteria and currently considered to be the gold standard for definitive identification of anaerobes, especially the yet not cultivable phylotypes.

In vitro MR spectroscopy: [42]Bacteria contain a number of macromolecules that could potentially contribute to their MR spectra. The principle of the technique is that protons in these molecules , when subjected to an intense magnetic field, have the possibility of orienting themselves with the field(lower energy state) or against the field (higher

energy state). Characteristic signals in the spectra are contributed to the cell wall constituents or metabolites of the microorganism. Since this varies in different organisms, the spectra obtained could contribute to the identification of the organism. Anaerobic metabolism is not as efficient as that of facultative organisms, hence, intermediate products are left that can serve as markers of an organism's identity.

Fourier transformed infrared (FTIR) spectroscopy:[43] There are reports on the use of vibrational spectroscopy for the study of bacteria. The main aim was to use the techniques of infrared or Raman spectroscopy for the rapid identification of microbial species. The major disadvantage of infrared spectroscopy is that the microbial suspension must be dried before spectra are collected because of the very strong absorption of the water.

Matrix assisted laser description ionization time of flight (MALDI-TOF):[3] This technology is about 20 years old. In MALDI-TOF , addition of an organic matrix to biomolecules like culture brings out rapid co-crystallization of the sample. The resultant product will be analyzed by exposure to a fixed , pulsed laser beam. The ions generated within nanoseconds, travel through a "flight" tube to detector. Differences in mass to ionic charge ratios result in separation of the ions. It seems highly

likely that in the future , it will provide solutions for bacterial identification even before in-vitro culture.

Polymerase chain reaction (PCR) : This is the most commonly used molecular method in clinical microbiology worldwide. This is mainly due to its simplicity , high sensitivity and specificity, flexibility and the ability to amplify the nucleic acid molecules of interest to millions of times in a matter of just a few hours. The commonly used variations are Multiplex PCR, Nested PCR, reverse transcriptase PCR, arbitrarily primed PCR and PCR restriction fragment length Polymorphism. The real time PCR has an paralleled specificity and sensitivity, is quantitative and can be easily adopted to automation, making it the most popular diagnostic methods in the world today.

Loop mediated Isothermal amplification:[44] This amplification method used for anaerobe detection has many advantages. Amplification takes place at a single temperature ($65\ ^{0}$C) . No need of thermocycler. This technique is economical since it does not need special equipment or costly reagent.

Microarray:[45] An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done on base pairing rules. Thousands of spotted samples known as probes (with known

identity) are immobilized on a solid support (a microscopic glass slides or silicon chips or nylon membrane). The spots can be DNA, cDNA or oligonucleotides.. These are used to determine complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. This technique was employed in the detection of Macrolide resistance genes of *B fragilis*, analysis of microbiota like *Fusobacterium* from gingival lesions in normal patients etc.

Fluorescence in situ Hybridisation (FISH): [46] This is a cytogenetic technique developed by biomedical researchers in the early 1980s, that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show high degree of sequence complementarily. This technique is finding application in the field of anaerobes.

Identification Of Anaerobic Isolates :There are three levels at which laboratories can operate with regard to anaerobic processing .[33]

LEVEL 1: Presumptive identification from primary plates, isolation and maintenance of an anaerobe in pure culture so that in important clinical

cases the isolate can be sent to a reference laboratory for complete identification and susceptibility testing.

LEVEL 2: Using simple tests to further group the anaerobes and speciate certain ones.

LEVEL3: Identify isolates using PRAS biochemicals, CDC thioglycollate biochemicals, biochemical systems [API and Minitek] ,rapid enzyme detection panels, gas liquid chromatography, toxin assays etc.

Antimicrobial treatment and resistance : Anaerobic infections usually involves appropriate antimicrobial therapy and or surgical management. The antibiotics used to treat anaerobic infections also have active against aerobic infections, as many of these infections are of mixed etiology. Anaerobes have uniform resistance to aminoglycosides as they lack electron transport systems need for the uptake of antibiotics.[47]

Bacteroides fragilis group, *Bacteroides fragilis* is generally the most susceptible, although more than 95% of all strains are resistant to penicillin and ampicillin.The carboxy and ureidopenicillins, ticarcillin and mezlocillin are more active than penicillin, but only less than 50% of isolates are susceptible. Piperacillin is the most active ureidopenicillins

against the Bacteroides fragilis group, although susceptibility has fallen from 90% to 70%.

The principle mechanism of resistance to penicillins is β –lactamase production.Thus, β lactum, β lactamase inhibitor combinations, such as amoxicillin-clavulanate,ampicillin-sulbactam,piperacillin-tazobactam, ticarcillin clavulanate are active against all strains of the *Bacteroides fragilis* group, with less than 2%resistance being cited in most of the reports.

Among the cephalosporins, cefoxitin has sensitive against *Bacteroides fragilis* group,with 80% to 90% of isolates being susceptible. A marked decrease in sensitive to clindamycin in *Bacteroides* species has been recognized worldwide. The clindamycin determinant is located on transferable plasmids and is linked to transferable tetracycline resistance.

Among fluoroquinolone drugs, trovafloxacin has active against most members of the *Bacteroides fragilis* group .Other fluoroquinolones are moxifloxacin and gatifloxacin have incomplete activity against most of the anaerobes

Among other agents, Metronidazole, chloramphenicol and carbapenems are active against *Bacteroides fragilis* group. The resistance to imipenem

is mediated by a zinc metallo enzyme that mediates resistance to β- lactam and β-lactamase inhibitor combination drugs.

Generally, *Prevotella* and *Porphyromonas* are more sensitive than the *Bacteroides fragilis group*. Half of the *Prevotella s*pecies are resistant to penicillin due to β-lactamase production.β lactamase production also present in *Fusobacterium* and *Porphyromonas* species.

Among the gram positive organisms,*Clostridium perfringens* has sensitive to most of the antianaerobic agents and fluoroquinolones. *Peptostreptococcus spe*cies are sensitive to all β lactams, β lactamase inhibitors, cephalosporins, some of the fluoroquinolones, carbapenems,Chloramphenicol and metronidazole. [48]

Antimicrobial agent	**Mechanism of action**
B-lactum	*B*-lactamase
Carbapenems	Imipenem hydrolyzing B lactamase, metallo E.coded for Cfia or ccrA Non metallo E+
Macrolides	Rrna methylases
Clindamycin	Transferable plasmide

Quinolone	GyrA &par C mutatiom ,R to DNA gyrase
Metronidazole	Nim gene on chromosome and on plasmids
Chloramphenicol	Production of chlorampenicol acetyl transferase

Antimicrobial susceptibility testing of anaerobes [49]

Indications:

A Specific infections like bacteremia, CNS infections, endocarditis, osteomyelitis, joint infection, prosthetic device infection, organism isolated from normally sterile site-infections not responsive to empiric therapy –infections which require long term therapy

B. To determine patterns of susceptibility in a particular hospital or geographic location (to be done at an interval of 4-6 months)

C. Evaluation of new agents.

Various methods for testing antibiotic susceptibility

1.Referance agar dilution test

2. Micro Broth dilution test

3.Macro Broth dilution test

4.E-test

5.Spiral Gradient and point systems

Many strains may be designated as resistance on one occasion and susceptible on another within the allowable error of technique. Recent clinical isolates should be used to determine current antibiograms. Antibiograms may be change in an institution over time. Adequate representation of all clinically relevant species should be ensured. Different species of the *B.fragillis* group should be reported separately .At least 10 of each species should be tested if inference to be made regarding susceptibility of a particular group of species.

Reference agar dilution testing method: The agar dilution method is the recommended method for all anaerobes and it is very useful when large numbers of strains are to be tested.

Macro broth dilution: It is convenient for small number of strains to be tested and useful for bacteria like clostridia which spreads on the surface of agar plates serial two fold dilution of antimicrobial stock

solutions are prepared in 2.5ml of brucella broth containing hemin (5ug/ml0,NaHCO3 (1 mg/ml) and Vitamin K1 (1ug/ml).Tubes should be prepared within 3 hours of use or else frozen at -70 ^{0} C.

The inoculum is a 1:200 dilution of a 0.5 McFarland in the supplemented Brucella broth. A final inoculum volume of 2.5ml is added to the broth containing the drug (the final inoculum will be ~3x 10 5 CFU/ml).Incubate the tubes in an anaerobic atmosphere at 37c for 48 h. Include an inoculated broth containing no antimicrobial agent as a growth control for each strain tested.

Micro both dilution

Plastic trays containing 8 or more rows of small flat bottomed U or V shaped wells are employed for the anti microbial agents economically and rapidly. Dilutions can be done manually or using automated dispensers.Trays are filled with 50µl each of serial dilutions of anti microbial agents sealed and frozen. The inoculum is prepared in the same manner as for macro dilution test.1.5ul of the inoculum is added to each well and the test are read after 24-48 hours of incubation

anaerobically.MIC is reported as the lowest conc of antimicrobial agent that doesn't permit any visible growth.

Broth disk elution test is a modification of broth tests to be used in where the selected concentrations of antimicrobial agents were incorporated into liquid media by means of elution from paper disks

E-Test:[50]

Uses a strip coated with a logarithmic agent, applied to an inoculated plate. After incubation, an ellipse of inhibition is formed. At the intersection of the ellipse with the strip, the MIC is read from the interpretive scale. Supplemented Brucella agar is used with an inoculum adjusted to Mc farland 0.5 standard and is applied with a sterile cotton swab. Antibiotic carriers are then applied pattern and incubated anaerobically for 48 hrs.

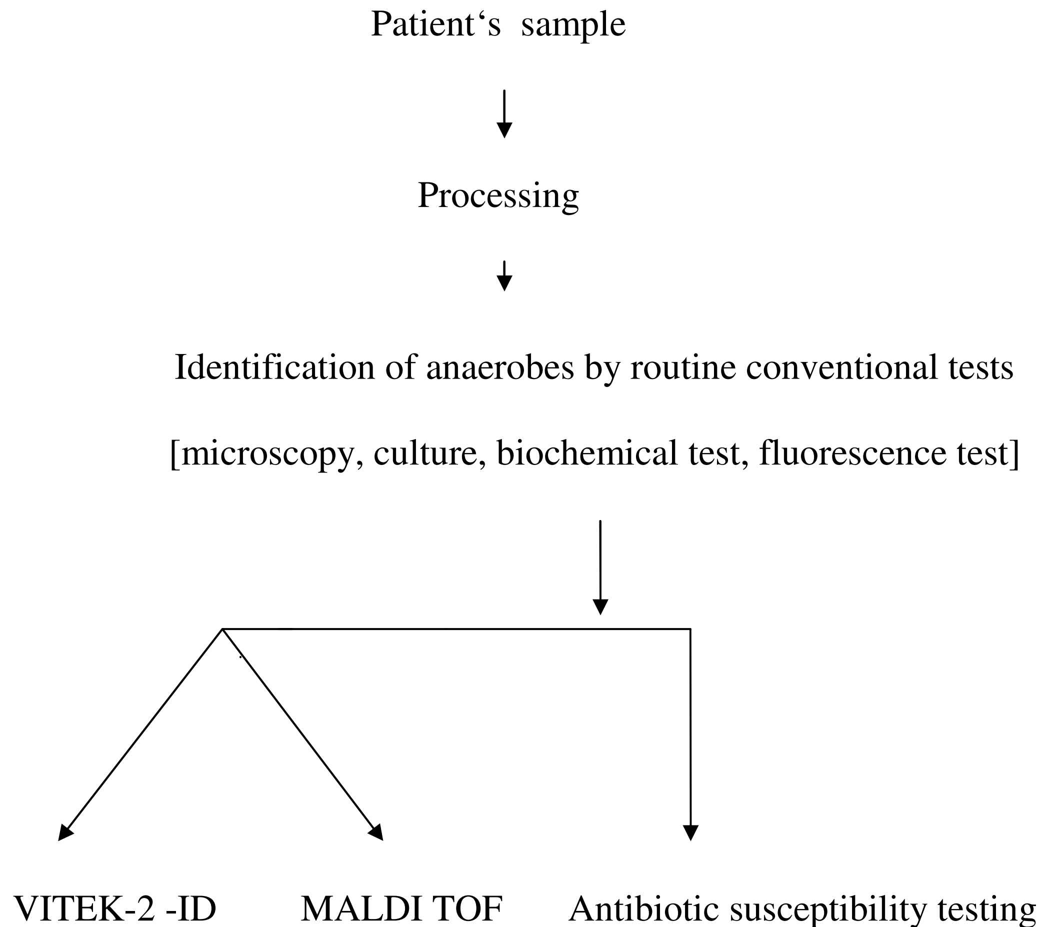
Spiral gradient End point system:[51]

The spiral streaker deposits the antimicrobial stock solution in a radially decreasing concentration gradient. The isolates are deposited on the plate using an automated inoculators in a radial pattern. MICs are determined by measuring the distance from the center of the plate

to the point where growth stops. A software program translates this number into the MIC.

MATERIALS AND METHODS : This study was study after obtaining the Institutional Human Ethics committee's approval.

Flow chart



Study Population: Samples received at the Diagnostic microbiology laboratory . PSG Hospital

Study Locale (geographic area) -In and around Coimbatore.

Sample Size: ~150 clinical samples

Sample Size Estimation: N=4pq/d^2 [p=40.;q=60;d=8]

Sampling Method: Consecutive Samples received at the diagnostic microbiology laboratory suspected with anaerobic infection

Inclusion Criteria: Aspirated fluid, Aspirated Pus, Tissue biopsy

Exclusion Criteria: Sputum , Stool , Gingival swab, Urine, Enotracheal aspirates.

METHODOLOGY

A total of 150 clinical samples from patients with pyogenic infections were collected and processed to detect anaerobic bacteria in the diagnostic microbiology laboratory , PSG Hospitals, Coimbatore. The aspirated pus samples were collected in a sterile syringe with the needle plunged with rubber cork to avoid exposure of air . Pus/ wound swab were taken only when aspiration was not possible . Tissue samples and biopsy sample were transported immediately to the lab in sterile saline. **Fig - 1** . In case of delay of transport, samples were kept in Robertson cooked meat medium to preserve the anaerobic organisms. Processing of all the samples were done as soon as possible to minimize oxygen exposure.

In the laboratory, gross inspection of the specimens were made to obtain information about the nature and quality of the specimen. Characteristics that were noted including purulence, blood, necrotic tissue, foul odour, and sulphur granules. Grossly purulent specimens were vortexed to ensure even distribution of microorganisms. Tissue samples were ground with 1 ml of liquid medium (Thioglycolate or chopped meat) to make a thick paste.

Gram stain of the samples were done .Gram stain will show the morphology and staining characteristic of the microorganisms and give presumptive information about the identification of anaerobic bacteria.

All the specimens were inoculated into Neomycin Blood agar (NBA) and thioglycolate broth. The thioglycolate broth was freshly heated in a boiling water bath for 10 minutes and rapidly cooled before inoculation. All the specimens were also inoculated into blood agar and Mc Conkey agar plates for aerobic culture. Metronidazole disk (5µg) was put in the beginning of the second streak line on the NBA plate for the presumptive identification of obligate anaerobe.

The NBA plates are incubated anaerobically in anaerobic jar using Anoxamat machine which produces anaerobiosis and kept at 37^0 C for 48 hours. Thioglycollate broth was kept at $37\ ^0$ C under aerobic conditions mainly to serve as a backup source of culture material in case of jar failure.

Method of processing using Anoxamat:[52Fig -2]

1. The inoculated plates were placed into a self-contained jar with programmed anaerobic gas filling system providing an atmosphere of 80-90% N2,5%H2, and 5-10%CO2; and an anaerobic indicator.

2. The jar was closed and incubated at 35-37°C .

3. The plates were incubated for 48 hours before opening the jar. This prevents exposure of tiny colonies to oxygen.

4. The catalyst , composed of palladium –coated alumina plates, was fresh and rejuvenated each time prior to use.

5. The plates are removed from the bag or jar to examine them and workup of the organisms are done as quickly as possible.

If growth was found on NBA plates, a single colony of each distinct morphotypes was subcultured into two blood agar plates. One of them was incubated aerobically for aerotolerance tests and the another was incubated anaerobically for purification. Each type of the colonies were subjected to Gram staining.

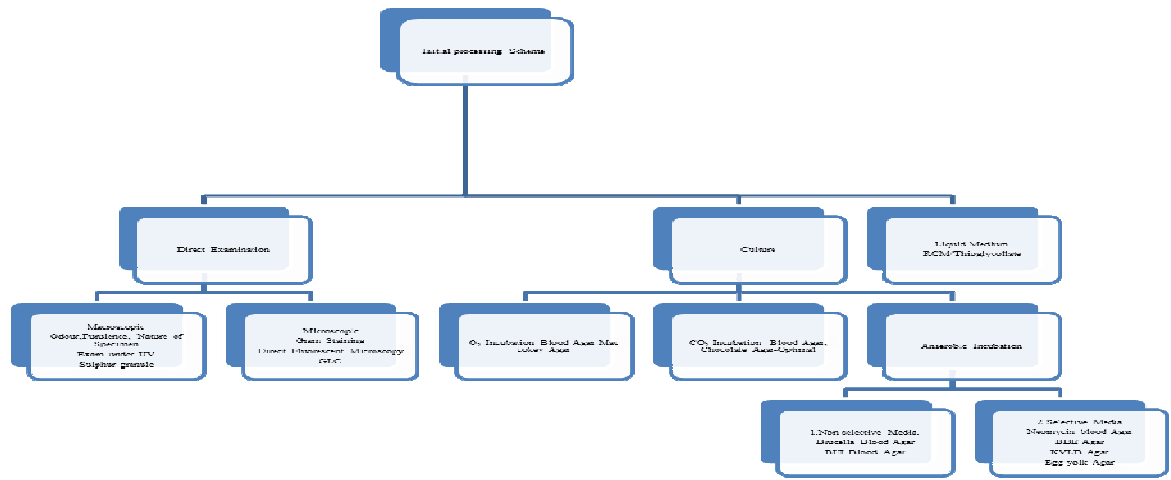
The anaerobic bacteria were differentiated from the facultative anaerobes by their inability to grow in the presence of oxygen and their susceptibility to metronidazole. [13]The Thioglycollate broth was inspected daily for turbidity

upto 1 week and when turbidity obtained, it was inoculated in Neomycin blood agar (anaerobically) and Blood agar, Mac Conkey agar(aerobically).

Aerobic bacterial colonies were identified by gram staining, colony morphology and appropriate biochemical reactions.[53]

Anaerobic bacterial colonies were identified by their colony morphology, gram staining, special potency antibiotic test susceptibility, and biochemical tests like spot indole test, nitrate reduction test, sodium polyanethol sulphonate (SPS) disk susceptibility, catalase test, lecithinase test, lipase test, 20% bile tolerance test, esculin hydrolysis test and sugar fermentation tests.[33]

Flow chart for identification of Anaerobes


Initial processing Scheme
Direct Examination
Culture
Liquid Medium
RCM/Thioglycollate
Macroscopic
Odour,Purulence, Nature of Specimen
Exam under UV
Sulphur granule
Microscopic
Gram Staining
Direct Fluorescent Microscopy
GLC
O_2 Incubation Blood Agar Mac cokey Agar
CO_2 Incubation Blood Agar, Chocolate Agar-Optimal
Anaerobic Incubation
1.Non-selective Media.
Brucella Blood Agar
BHI Blood Agar
2.Selective Media
Neomycin blood Agar
BBE Agar
KVLB Agar
Egg yolk Agar

Colony morphology

Description of each colony type such as pitting, swarming, hemolysis, pigment and greening the medium, etc. were noted.

.

Grams staining

The Gram stained smear of the each colony type was done and observed for the features such as pleomorphism, morphology of the bacteria , spore and its types.

Special potency antibiotic tests

A sterile swab was put into the inoculum and streaked evenly on the surface of blood agar plates For Gram negative bacteria, the following special potency discs were placed on the inoculated surface: 1.Colistin (10µg), 2.Kanamycin (1000µg) and 3.Vancomycin(5 µg). For Gram positive cocci, Sodium Polyanethol sulphonate [SPS] disc were placed near colistin disc for the identification of Peptostreptococcus anaerobius. The plates were kept in anaerobic environment at 37 ^{0}C for 48 hours. The organisms were identified based on their sensitivity table.

Table:1- Identification of anerobes by means of special potency antimicrobial agent dics [33]

ORGANISMS	Colistin (10µg)	Kanamycin (1000µg)	Vancomycin (5µg)
Gram positive	R	V	S*
Gram negative	V	V	R
Bacteroides fragilis Gp	R	R	R
Bacteroides ureolyticus Gp	S	S	R
Fusobacterium spp	R	S	R
Porphyromonas spp	R	R	S
Prevotella spp	V	R	R
Veillonella spp	S	S	R

S-susceptible; R-Resistant; V-Variable; *Rare strains *Lactobacillus spp* and *Clostridium spp*, may be resistant.

NAME OF THE ANTIBIOTICS	**ZONE SIZE**	**INTERPRETATION**

Colistin(10µg)	≥10 mm	Sensitive
Kanamycin(1000µg)	≥10 mm	Sensitive
Vancomycin(5µg)	≥10 mm	Sensitive
Sodium polyanethol sulphonate 5%	≥16 mm	Sensitive

Spot indole Test

A small piece of filter paper was placed on the surface of the glass slide and the paper was moistened with DMACA(ρ-dimethyl amino cinnamaldehyde) such that they are saturated but not dripping wet. Several colonies were removed from the agar medium that contains sufficient tryptophan (Brucella blood agar or Egg yolk agar). Development of blue or green colour on the filter paper around the inoculum within 30 seconds is considered positive and no colour change was considered negative .

Nitrate disk reduction test

The organism was inoculated on Brucella blood agar plate and a nitrate disk was placed on the heavy inoculated area. It was anaerobically incubated for 24 to 72 hours at 35 to 37 ^{0} C until heavy growth occurs around the disk. The disk are removed from the surface of the plate and placed in a clean petri dish or on a slide. One drop each of nitrate A and B is added . If no colour develops within 5 minutes, a pinch of zinc granules is added to the disk and allowed to stay for 5 minutes. Nitrate reduction is considered positive when there is development of red or pink colour after the reagents are added or no colour development after zinc is added. Nitrate reduction is negative if no colour occurs after the reagents are added and development of red colour after zinc is added.

Catalase Test

Growth from the neomycin blood agar was taken and added to a drop of 15%hydrogen peroxide on a slide and observed for effervescence of bubbles with in 20 to30 seconds. If catalase test has positive, immediate bubbling occurs. If negative , no bubbling occurs. Catalase test has use to detect *Clostridium species* and to speciate *Bacteroides fragilis* group.

Sodium polyanethol sulphonate (SPS)

A 5% sodium polyanethol sulphonate containing disc was placed on the heavy lawned growth of Brucella agar plate and kept anarobically for 48 to 72 hours at 35 to37 ^{0} C. If SPS susceptible, (zone of inhibition of $\geq$ 16 mm) helps in identifying *Peptostreptococcus anaerobius* (positive) from other anaerobic cocci which are resistant.

Esculin hydrolysis

Bacteroides bile esculin agar was used for identification of the *Bacteroides fragilis* group. Hydrolysis of esculin was observed by blackening of the media.

20% Bile test

Presence of growth in 20%bile plate indicates the presence of the *Bacteroides fragilis* group.

Lipase reaction

A portion of egg yolk medium is inoculated with the organism to be tested and kept it at 37 ^{0} C for 48 to 72 hours. Lipase reaction positive if oil on water appearance occurs.

Lecithinase reaction

A portion of egg yolk medium is inoculated with the organism to be tested and kept it at 37 ^{0} C for 48 to 72 hours and examined for the presence of white opacity surrounding the colony. Both lecithinase and lipase reactions are used to differentiate *Clostridium species*.

Urease test

Urea broth is inoculated with the organism to be tested and incubated under anaerobic condition for 1 hour. A bright pink colour indicates a positive reaction.

Fermentation of carbohydrates

A heavy suspension of anaerobes was inoculated in to thioglycolate broth contains 1% carbohydrates and incubated at 37 0 C for 48 hours in anaerobic jar. Bromothymol blue indicator was added , after incubated in anaerobic environment and before to interpret. The positive results are indicated by yellow colour appearance and negative results are by green colour. The following sugars are used: Glucose, Lactose, Sucrose, Maltose, Arabinose, Rhamnose and Trehalose.

1. Gram negative bacilli producing blackening on BBE agar and resistant resistant to all three antibiotics Vancomycin(5µg), Kanamycin(1000µg) and

Colistin(10µg, identified as *Bacteroides* were speciated into *Bacteroides fragilis* ,*Bacteroides stercoris* and *Bacteroides thetaiotaomicron* species were identified as follows: Fig-5a,b

B. FR	Gr ow	esc uli	In dol	cat ala	rha mn	ara bin
B.*fragilis*	+	+	-	+	–	–
B.*stercori s*	+	+	+	–	+	+
B.*thetaiot ao micron*	+	+	+	+	+	–

+ positive reaction; - negative reaction;

2.Gram negative bacilli or some coccobacillus forms.producing circular , convex colony about 1 to 2 mm in diameter producing brown -tan pigment to black pigment in 5 to7 days and resistant to Vancomycin(5µg), Kanamycin(1000 µg) and Variable sensitivity in Colistin (10µg).identified as *Prevotella* were speciated into *Prevoella disiens*, *Prevotella oralis*, *Prevotella bivia* and *Prevotella melaninogenica* as follows Fig -6a & b

Species	**Indole**	**Esulin**	**Gelatin**	**Lactose**	**Pigment**
P.intermedia	+	-	-	-	+

P.melaninogenica	-	-	-	+	+
P.disiens	-	-	+	-	-
P.bivia	-	-	+	+	-
P.oralis	-	+	-	+	-

+positive; - negative; NT-not tested

3. Gram positive bacilli with spores resembling *Clostridium* were speciated into *Clostridium septicum*, *Clostridum sporogens* and *Clostridium clostridioformis* as follows: Fig 7a & b

Species	**Indole**	**Glucose**	**Lactose**	**Sucrose**	**Lecithinase**	**Lipase**
C.*septicum*	-	+	+	-	-	-
C.*clostridioforme*	-	+	+	+	-	-
C.*sporogens*	-	+	-	-	-	+

+positive; -negative;

VITEK 2 SYSTEM :[2] Fig - 3

Bacterial colonies were suspended in 0.45% sodium chloride with a turbidity of 2.7-3.3 McFarland. Inoculums were then introduced into an ANC card in the VITEK 2 Compact automated identification system and incubated for

approximately 6 h. Through the three additional tests of Gram staining, cell morphology, and aerotolerance testing, the VITEK 2 system deduced interpretations for final identifications. In case of it is unidentified it was repeated again

MALDI – TOF[3].Fig -4

All the 28 anaerobic isolates were identification by MALDI-TOF (Vitek MS Brucker system) This was performed at Microbiological Laboratory, R S Puram coimbatore . Each isolate was directly smeared onto a disposable target slide and then covered by a small drop of matrix solution (VITEK MS-CHCA) and air dried. The loaded slide was then inserted into the VITEK MS system. The quality standard performed on each group was a spot of *E. coli* ATCC 8739. Microbial identification is achieved by obtaining a composite mass spectrum using MALDI-TOF technology and comparing the sample spectra to the reference spectra contained within the VITEK MS version 2.0 database

Antimicrobial sensitivity testing by reference agar dilution method (Wadsworth procedure) [49]

Agar dilution method is used to quantify the in vitro activity of an antimicrobial drugs against anaerobic isolates. The MICs were determined by the reference agar dilution method in accordance with CLSI guidelines . [49]

- The stock solution of the 4 antimicrobial drugs such as penicillin, clindamycin, Imipenem and metronidazole were made.
- For clindamycin the solvent and diluent used is water. For Ampicillin the Solvent and diluent used is 0.1 M Phosphate buffer,pH8. For Imipenem the solvent and diluents used is 0.01MPB, pH 7.2. For Metronidazole the solvent is DMSO and the diluents is water. Successive twofold dilutions of the antimicrobial drugs were made as per the dilution format given in CLSI interpretive break points.
- Brucella blood agar base supplemented with Vit K1 and hemin (needed for the test run) is prepared, autoclaved and kept refrigerated (upto 1 month)
- Each tube containing 17 ml of agar +1 ml of laked blood (added on the day of the test +2 ml diluted antimicrobial agent)
- If media is prepared in advance it is melted (or if prepare on the last day , it is autoclaved)and the medium is cool to 48^0C in a water bath and laked blood is added to 5% final volume.

- Preparation of the dilution of antimicrobial agents. Incorporate dilution (2ml of the drug solution) to a tube containing 18ml of molten agar with laked blood added to obtain 1:10 dil.

- Mixed thoroughly by gently inverting the tube 6 times and poured into a Petri dish.

- After the plates are solidified, place them in 35-37^{0}C incubator with top of the plates slightly for 30-45 min. to allow evaporation of excess moisture.

- Plates are refrigerated if the test is performed the next day (Except plates with imipenem).Plates are warmed to room temperature before the test run.

- The inoculum was prepared by picking up 2-4 colonies of the strain to be tested from a overnight broth culture and incubated into a tube of 5 ml supplemented (Vitamin k 1 and Hemin] Thioglycollate medium.

- After 4-6 hours of incubation at 37 ^{0} C, the culture broth was adjusted to the turbidity of McFarland 0.5.

- About 3 µl of the inoculum was applied on to the agar surface and allowed for drying. Drug free plates were inoculated as control.

- The control and the test plates were incubated anaerobically at 37 ^{0} C for 48 hours.

- The minimum inhibitory concentration [MIC] was taken as the lowest concentration of the drug at which no visible growth was seen after 48 hrs of incubation.fig-8

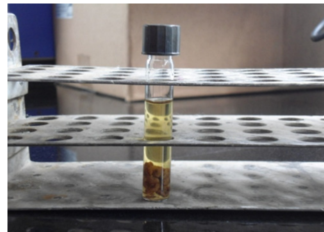
Table -2.Interpretative chart for MIC (Minimal inhibitory concentration) (µg/ml)[49]

No	Antibiotics	Sensitive	Intermediate	Resistant
1	Metronidazole	≤8	16	≥32
2	Imipenem	≤4	8	≥16
3	Clindamycin	≤2	4	≥8
4	Ampicillin	≤0.5	1	≥2

Fig -1: Clinical samples included in the study

Aspirated pus in a syringe

Tissue

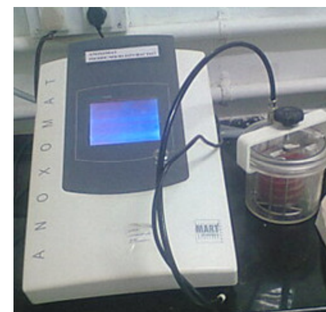


Robertson cooked meat

Fig – 2: Conventional anaerobic culture using Anoxamat system

Anaerobic jar

Anoxamat



Anoxamat-Jar

Fig -3- Vitek -2 automated identification system

Fig- 4: Maldi ToF – Identification system

Fig 5a - Identification of *Bacteroides sp*

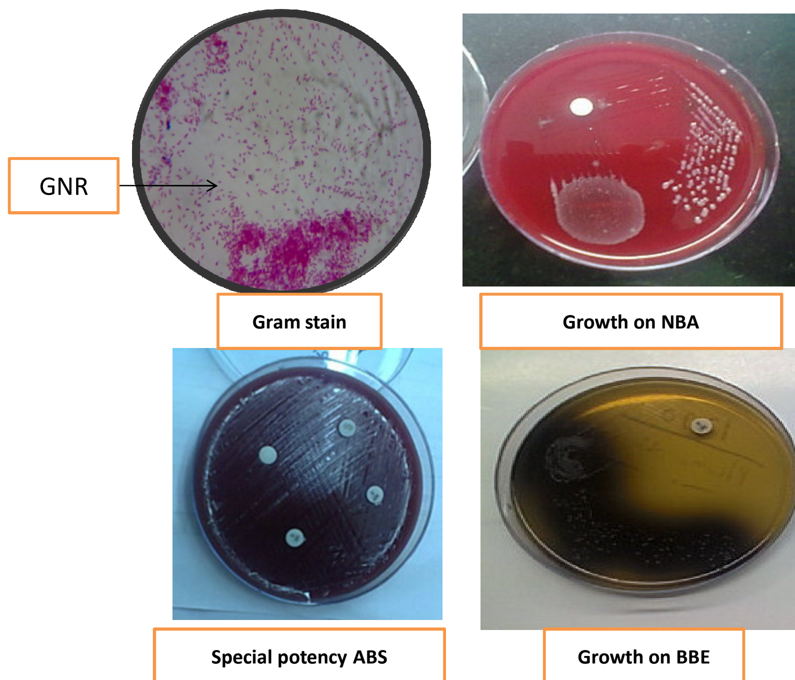


Fig – 5b: Biochemical tests to speciate *Bacteroides sp*

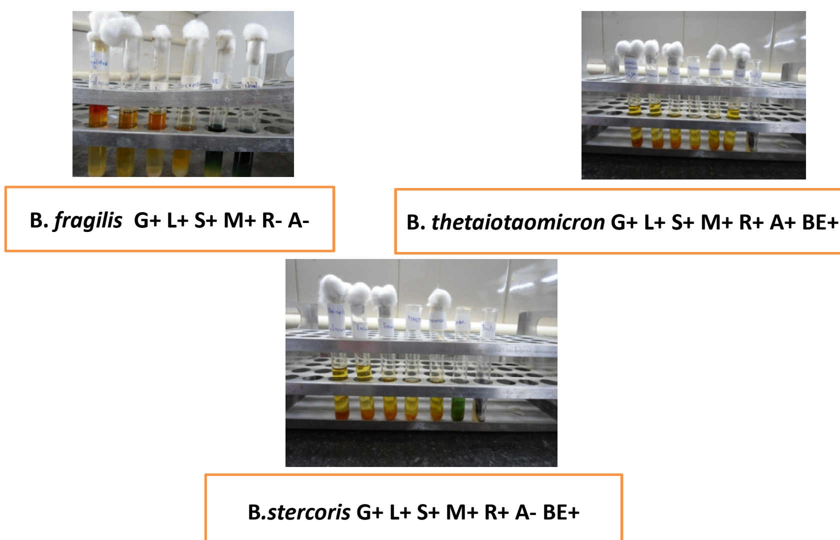


Fig - 6a : Identification of *Prevotella spp*

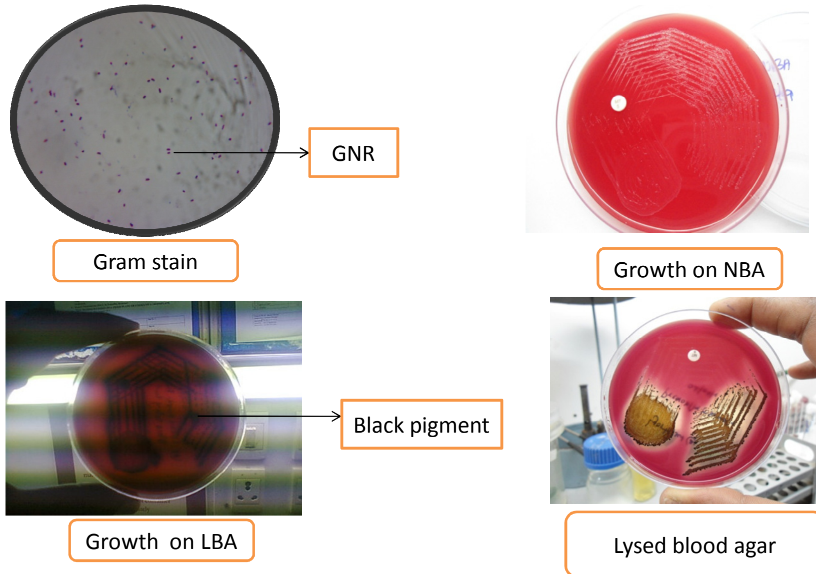


Fig -6b: Biochemical tests to speciate *Prevotella*

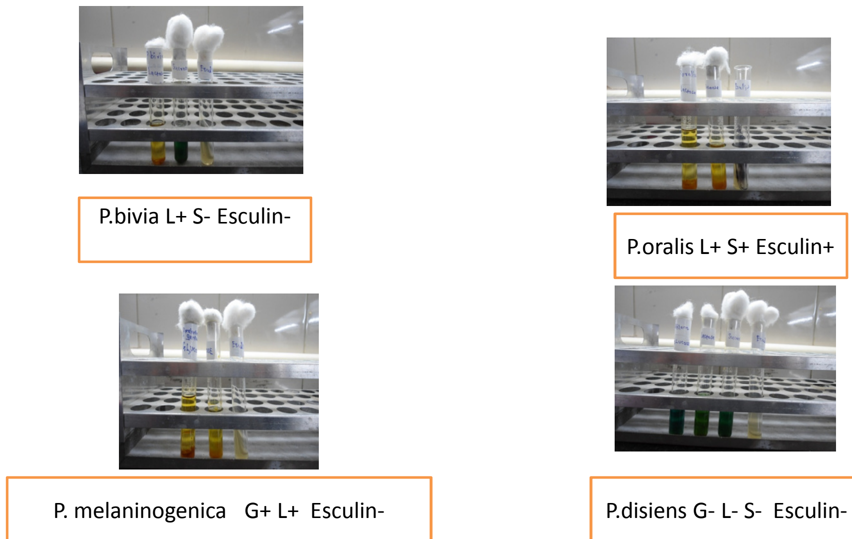


Fig 7a - Identification of *Clostridium spp*

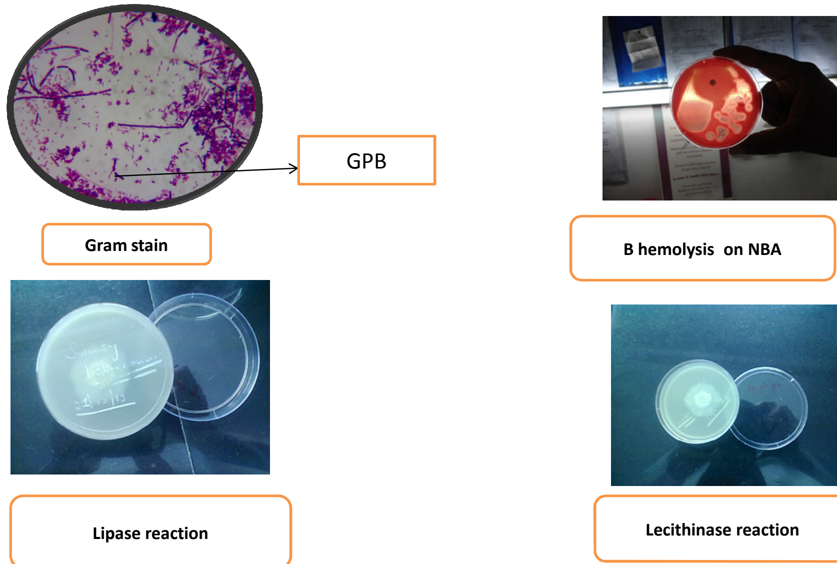


Fig -7b: Biochemicals to speciate *Clostridium spp*

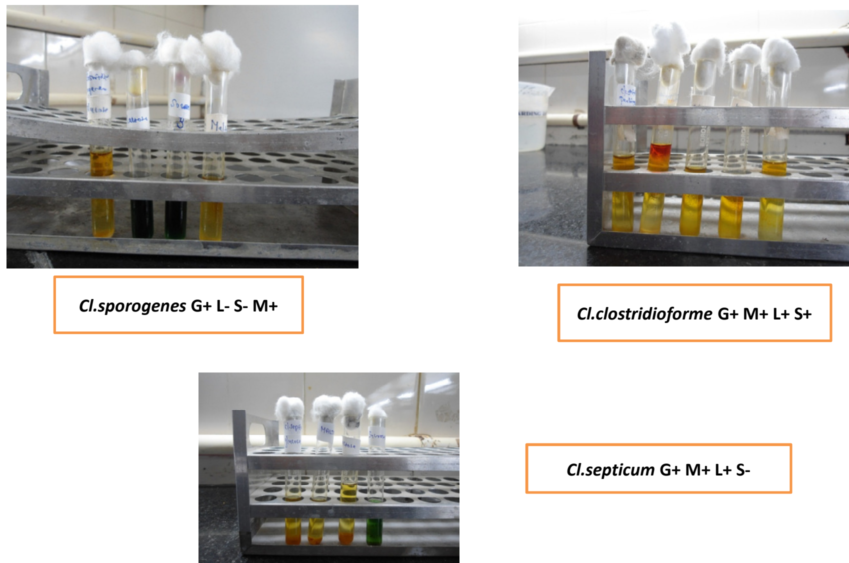
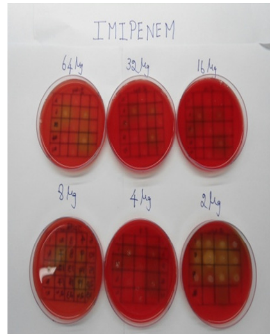
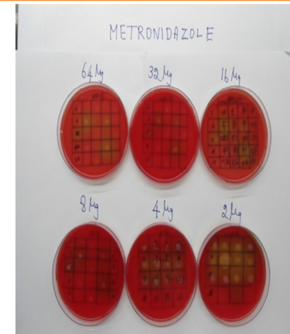


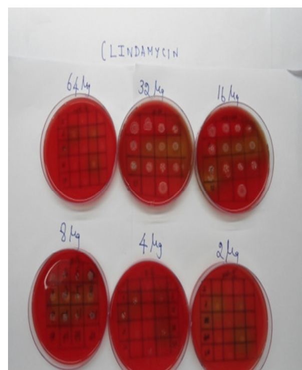
Fig -8:Antibiotic susceptibility



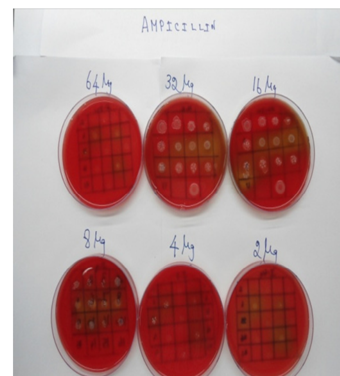
Imipenem



Metronidazole



Clindamycin



Ampicillin

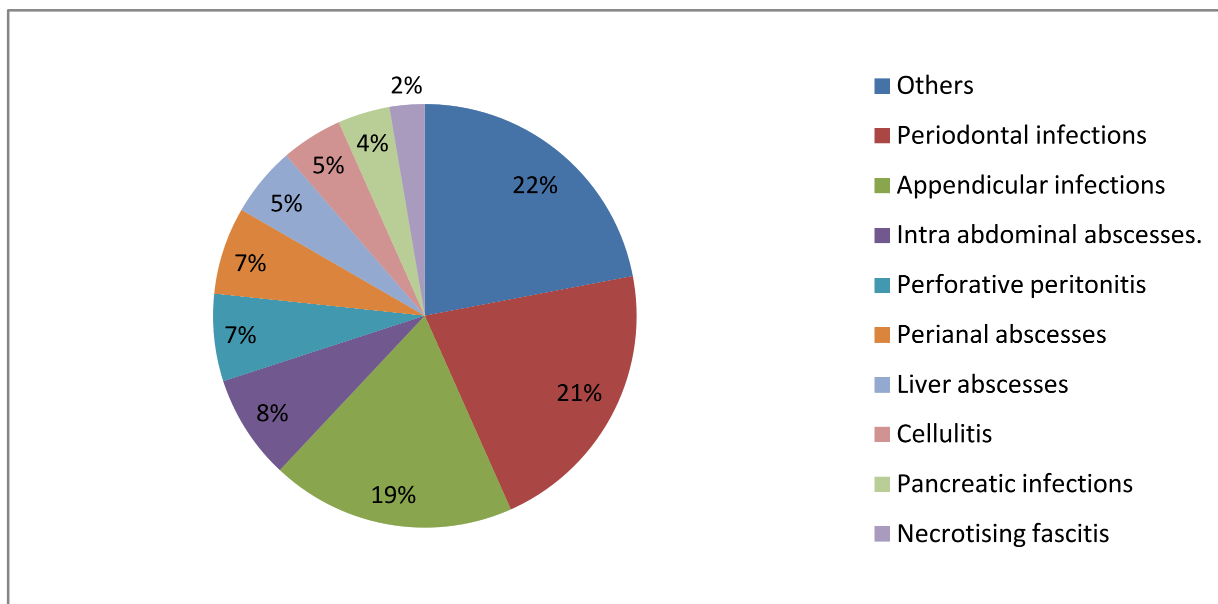
RESULTS & ANALYSIS:

A total of 150 pyogenic samples were collected from various patients attending clinical departments ,PSG hospitals, Coimbatore were included in the study . Periodontal abscess (21%), was the predominant sample followed by appendicular infections (19%), and other infections Table - 3, Fig – 8

Table–12 – Distribution of various clinical specimens included in the study

Sl no	Clinical diagnosis	No of samples
1	Periodontal infections	32
2	Appendicular infections	28
3	Intra abdominal abscesses	12
4.	Perforative peritonitis	10
5	Perianal abscess	10
6	Liver abscess	8
7	Cellulitis	7
8	Pancreatic infections	6
9	Necrotising fascitis	4
10	Others	33
	TOTAL	150

Figure –8 – Distribution of various clinical specimens included in the study



Out of 150 samples processed using the anoxamat anaerobic culture system , 28 anaerobic isolates (18.66%) were detected. Among the 28 anaerobic isolates, *Bacteroides* sp was the most predominant group followed by *Prevotella* and *Clostridium* species. Fig -9 Among the 28 of anaerobic isolates, Gram negative bacilli was the predominant bacilli, followed by Gram positive spore bearing bacilli. In Gram negative bacilli, *Bacteroides fragilis*(32%)(12 isolates) was the predominant followed by *Prevotella disiens*(14%)(4 isolates). In gram positive spore bearing bacilli, *Clostridium Clostridioforme* (11%)[3 isolates] the predominant bacilli, followed by *Clostridium sporogenes*(7%)[2 isolates].Fig-10

Fig-9: Distribution of various genus of anaerobes isolated

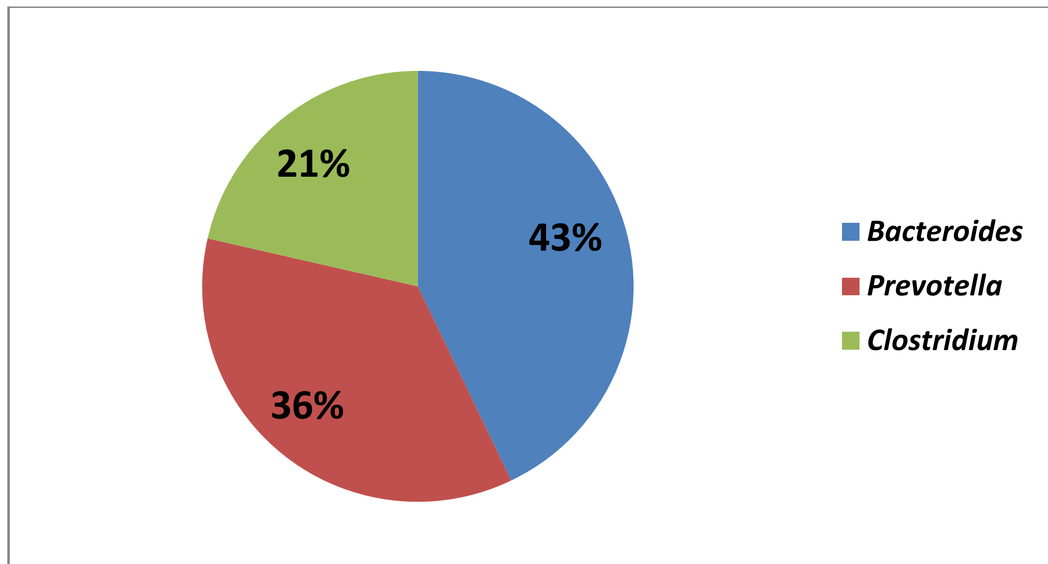
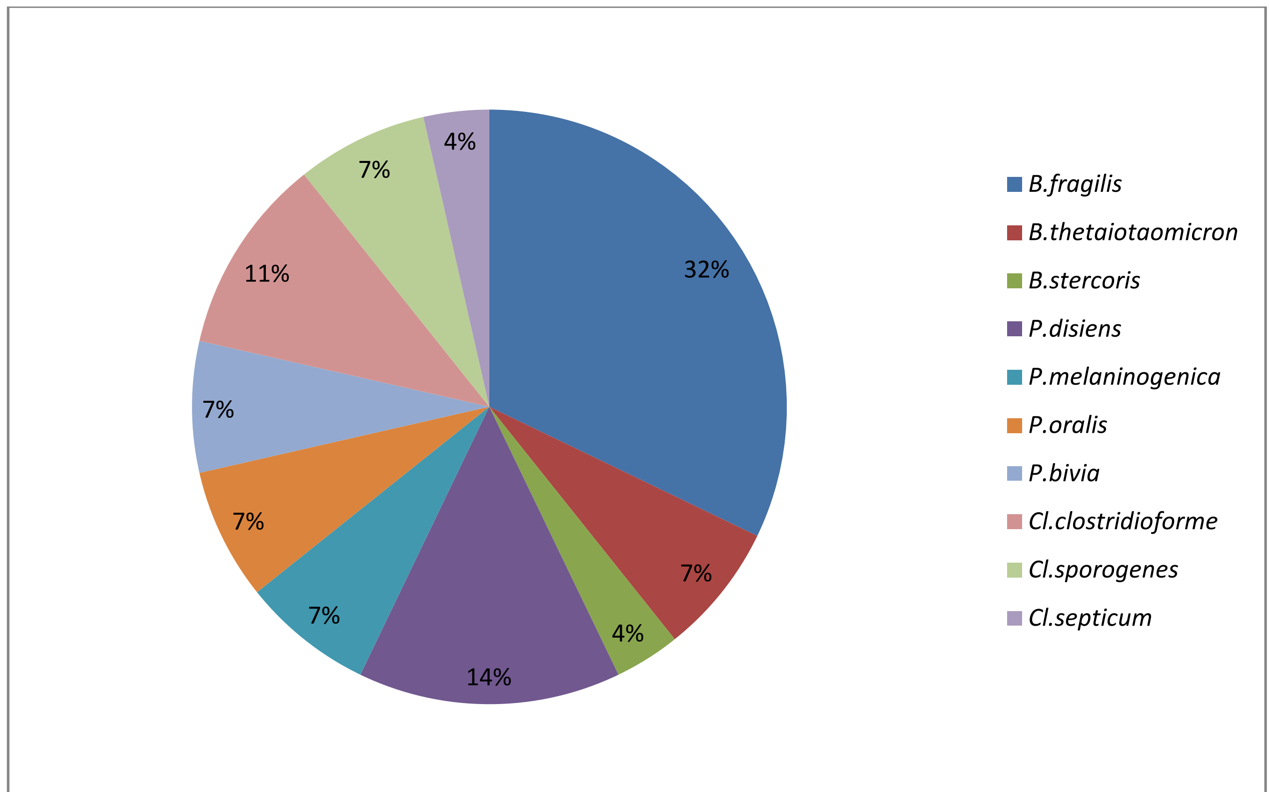
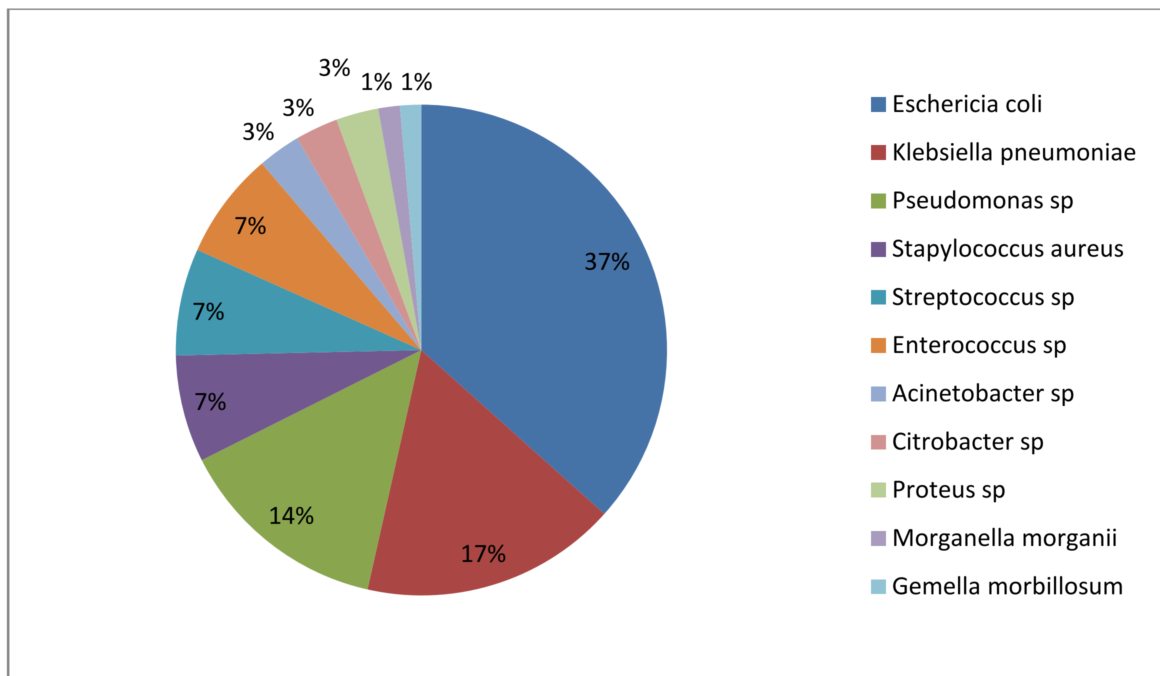


Fig- 10 : Distribution of various sp of anaerobes isolated



Out of 150 samples, 59 samples were pure aerobes,16 (57.1%) samples were pure anaerobes and 12(42.85%) samples were mixed with both aerobes and anaerobes. Fig -12

Fig- 21 : Distribution of various aerobes isolated



Among the aerobes, *Escherichia* coli (37%), was the predominant one followed by *Klebsiella pneumoniae (*17%) and *Pseudomonas* species(14%).All the *Pseudomonas* species were *Ps aeruginosa* except one *Ps fluorescence* isolated from peritoneal abscess .All *Staphylococcus aureus* isolates were methicillin sensitive.

Fig- 12 : Distribution of samples based on their isolation

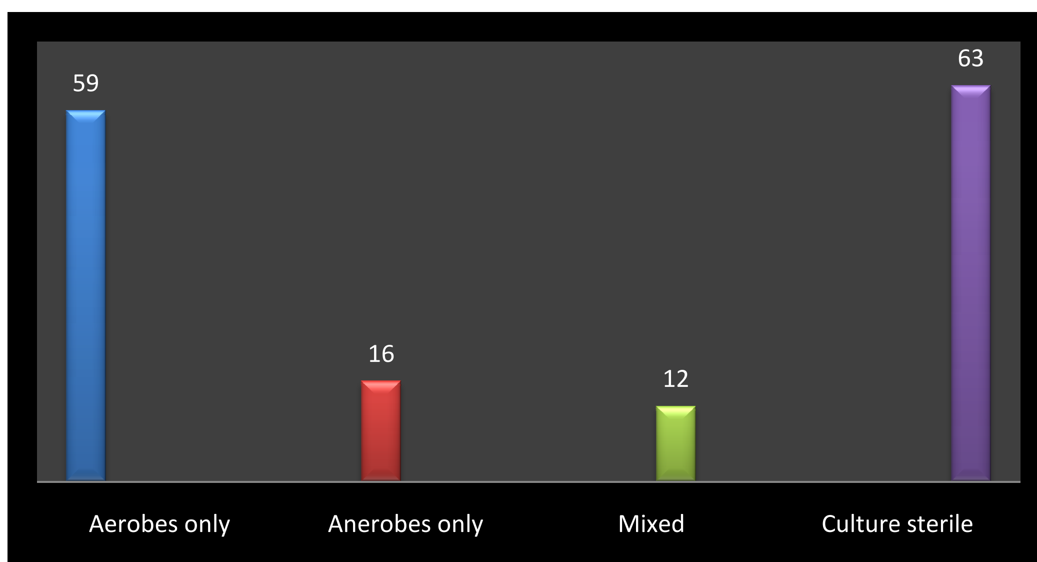


Table –4: Isolation of anaerobes among various infections

Sl no	Clinical diagnosis	Nos	Total anaerobes (%)	Anaerobes	Anaerobes mixed with aerobes	Bacteroides sp	Prevotella sp	Clostridium sp
1	Periodontal infections	**32**	**6 (18.75)**	2	4	-	4	2
2	Appendicular infections	**28**	**5 (17.85)**	3	2	3	1	1
3	Liver abscess	**8**	**-**	-	-	-	-	-
4	Perforative peritonitis	**10**	**2 (20)**	2	-	2	-	-
5	Intra abdominal abscess	**12**	**4 (33.3)**	4	-	2	-	2
6	Panreatitis	**6**	**-**	-	-	-	-	-
7	Perianal abscess	**10**	**4 (40)**	1	3	2	2	
8	Cellulitis	**7**	**3 (42.8)**		3	2	1	
9	Necrotising fascitis	**4**	**1 (20)**	1	-			1
10	Others	**33**	**3 (10)**	3	-	1	2	
	TOTAL	**150**	**28 (100)**	**16**	**12**	**12**	**10**	**6**

Table –5: Isolation of aerobes among various infections

Clinical diagnosis	**No of sample**	**Only aerobes**	**Aerobes mixed with anaerobes**	***E.coli***	***Klebsiella***	***Pseudomonas***	***Staphylococcus***	**OTHERS (*Streptococci, EnterococciAcinetobacter* &other spp)**
Periodontal infections	32	7	4	-	-	-	3	8
Appendicular infections	28	10	2	6	3	1		2
Liver abscess	8	4	-	1		1	-	2
Perforative peritonitis	10	6	-	2	1	2	-	1
Intra abdominal abscesses	12	8	-	4	2	2	-	-
pancreatitis	6	2	-	-	-	-	1	1
Perianal abscess	10	3	3	4	-	1	1	-
Cellulitis	7	2	3	1	1	1		2
Necrotising fascitis	4	3	-	1	1	-	-	1
Others	33	14	-	7	4	2	-	1
	150	**59**	**12**	**26**	**12**	**10**	**5**	**18**

Table –6: Isolation of various aerobic microorganisms with other anaerobes

Sl no	**Organisms**	**Total**	**Single aerobe**	**Mixed with another aerobe**	**Mixed with anaerobes**	**More than one aerobe with anaerobe**
1	*Escherichia*	26	16	8	1	1

	coli					
2	*Klebsiella pneumoniae*	12	7	4	1	-
3	*Pseudomonas aeruginosa*	10	7	1	1	1
4	*Staphylococcus aureus*	5	2	2	-	1
5	*Streptococci sp*	5	1	1	2	1
6	*Enterococci sp*	5	3	1	1	-
7	Others	8	2	4	2	

Table –7: Identification of the anerobes by various methods

Sl no	CONVENTIONAL	VITEK-2	MALDI-TOF (score value)
1	*Bacteroides fragilis*	*Bacteroides stercoris (v)*	*Bacteroides thetaiotaomicron (1.946)- PG*
2	*Bacteroides fragilis*	*Bacteroides stercoris (v)*	*Bacteroides fragilis(2.199)- PS*
3	*Bacteroides fragilis*	*Bacteroides stercoris(V)*	*Bacteroides fragilis(2.149)-PS*
4	*Bacteroides fragilis*	*Bacteroides fragilis(L)*	*Bacteroides fragilis(1.843)-PG*
5	*Bacteroides fragilis*	*Bacteroides fragilis(L)*	*Bacteroides fragilis(2.163)- PS*
6	*Bacteroides fragilis*	*Bacteroides fragilis(L)*	*Bacteroides fragilis(1.943)-PG*
7	*Bacteroides fragilis*	*Bacteroides fragilis(L)*	*Bacteroides fragilis(2.192)-PS*
8	*Bacteroides fragilis*	*Bacteroides thetaiotaomicron(V)*	*Bacteroides ovatus(1.861)-PG*
9	*Bacteroides fragilis*	*Bacteroides fragilis(L)*	*Bacteroides spp(1.523)-NR*
10	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron(V)*	*Bacteroides*

			thetaiotaomicron(1.834)-PG
11	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron(V)*	*Bacteroides thetaiotaomicron(1.97)-PG*
12	*Bacteroides stercoris*	*Bacteroides stercoris(V)*	*Bacteroides fragilis(2.199)-PS*
13	*Clostridium septicum*	*Clostridium sporogenes(L)*	*Clostridium septicum (1.993)-PG*
14	*Clostridium clostridioforme*	*Clostridium clostridioforme(L)*	*Streptococcus oralis(1.663)-NR*
15	*Clostridium clostridioforme*	*Clostridium clostridioforme(V)*	*Eubacterium limosum(1.448)-NR*
16	*Clostridium clostridioforme*	*Clostridium clostridioforme(V)*	*Actinomyces odontolyticus(1.966)-PG*
17	*Clostridium sporogenes*	*Clostridium sporogenes(L)*	*Anaerococcus murdochii(1.766)-PG*
18	*Clostridium sporogenes*	*Clostridium sporogenes(V)*	*Capnocytophaga ochracea(2.029)-PS*
19	*Prevotella disiens*	*Prevotella disiens (E)*	*Prevotella intermedia(1.428)-NS*
20	*Prevotella disiens*	*Prevotella disiens(E)*	*Bacteroides thetaiotaomicron(1.913)-PG*
21	*Prevotella disiens*	*Prevotella disiens(E)*	*Prevotella intermedia(1.183)-NR*
22	*Prevotella disiens*	*Prevotella disiens(E)*	*Prevotella intermedia(1.428) NR*
23	*Prevotella bivia*	*Prevotella bivia(L)*	*Prevotella intermedia(1.343) NR*
24	*Prevotella bivia*	*Prevotella bivia(E)*	*Prevotella intermedia(1.276) NR*
25	*Prevotella oralis*	*Prevotella oralis(V)*	*Prevotella intermedia(1.416) NR*
26	*Prevotella oralis*	*Prevotella oralis(E)*	*Bacteroides thetaiotaomicron(1.69) NR*
27	*Prevotella melaninogenica*	*Prevotella melaninogenica(V)*	*Prevotella intermedia(1.421) NR*
28	*Prevotella melaninogenica*	*Prevotella melaninogenica(L)*	*Prevotella intermedia(1.376) NR*

L-Low descrimination : V- very Good: E-Excellent /PG – Probable genus:PS-Probable species: NR-Not reliable

MALDI-TOF score values:

2.3 to 3 =highly probable species identification.

2 to 2.3 =secure genus identification, probable species identification.

1.7 to 2 = probable genus identification.

0 to 1.7 = not reliable identification.

Total number of Species similar by conventional , VITEK -2 and MALDI-TOF: 5 (17.85%) . Which included 4 *B fragilis* and 1 *B thetatiotamicron*. About 21 (75%) isolates were similar upto Genus level identification . Which included all the *Bacteroides spp*, 8 *Prevotella spp* which were identified as excellent by VITEK-2 system and one *Clostridium* . one isolate was totally dissimilar in species identification by MALDI- TOF, , conventional and Vitek-2. . Conventional (manual) identified an isolate as *Bacteroides fragilis* , VITEK -2 – *B stercoralis* and MALDI TOF – *B thetaiotamicron.* .Number of species similar by Conventional and VITEK-2. but dissimilar in MALDI TOF :16 (57.14%). Number of subspecies similar by both conventional and MALDI TOF, but dissimilar in VITEK were only 3(10.71%). None of the isolates were identified as Highly probable species by MALDI TOF. Excellent identification by VITEK -2 was given for 6 *Prevotella* isolates only Table – 7 .

Table – 8 : MIC (Minimal inhibitory concentration) (µg/ml) of 4 antibiotics

Sl.no	ISOLATES	Imipenem (µg/ml)	Metronidazole (µg/ml)	Clindamycin (µg/ml)	Ampicillin (µg/ml)
1	*Bacteroides fragilis*	4	8	8	8
2	*Bacteroides fragilis*	4	8	8	8
3	*Bacteroides fragilis*	4	4	8	4
4	*Bacteroides fragilis*	2	8	16	16
5	*Bacteroides fragilis*	4	8	>32	8
6	*Bacteroides fragilis*	4	8	8	8
7	*Bacteroides fragilis*	2	2	8	8

8	*Bacteroides fragilis*	4	8	8	4
9	*Bacteroides fragilis*	4	8	8	8
10	*Bacteroides thetaiotaomicron*	4	2	16	16
11	*Bacteroides thetaiotaomicron*	2	2	8	8
12	*Bacteroides stercoris*	4	4	16	>32
13	*Clostridium septicum*	4	8	8	8
14	*Clostridium clostridioforme*	4	8	8	8
15	*Clostridium clostridioforme*	2	4	16	4
16	*Clostridium clostridioforme*	4	8	>32	32
17	*Clostridium sporogenes*	4	8	8	8
18	*Clostridium sporogenes*	4	4	8	16
19	*Prevotella disiens*	2	8	8	8
20	*Prevotella disiens*	4	8	8	8
21	*Prevotella disiens*	2	8	16	4
22	*Prevotella disiens*	2	4	8	8
23	*Prevotella bivia*	2	8	8	8
24	*Prevotella bivia*	4	4	8	16
25	*Prevotella oralis*	4	4	8	>32
26	*Prevotella oralis*	2	4	16	8
27	*Prevotella melaninogenica*	2	8	8	4
28	*Prevotella melaninogenica*	4	8	8	8

All the anaerobic 28 (100%) isolates were sensitive to Metronidazole and Imipenem and were 100% resistant to Ampicillin and Clindamycin.Table-2 & 8.

DISCUSSION :

Anaerobic bacteria can cause various infections, and their incidence differ depending on the geographical location and the type of medical facility. We

investigated recent trends in anaerobe isolation and their susceptibility to commonly used empirical antibiotic to facilitate diagnosis and treatment of anaerobic infections.

The isolation of anaerobes from pyogenic infections in our study was 18.66% .Similar findings of 18.73% was reported by Anuradha de [54] and 22% isolation from intra abdominal infections by Neetu et al . [55] V lakshmi et al reported 15.3% isolation from brain abscess in their 24yrs retrospective data analysis. [56] Higher isolations of 74.6% from abscess from head and neck was reported by Kaniskha Guru et al[57] and 62.96% isolation of anaerobes from various samples at a cancer research centre was reported by Ravindra jadeja. [58.] A COMPACT TG anaerobic work station was used for the isolation of anerobes in their study . The anoxamat anaerobic culturing system used in our study improves the isolation of anaerobes and provides superior growth in terms of the colony size and density. [59] however, lesser rates of isolation may be attributed to the type, collection technique and transportation of the samples.

Obligate anaerobes alone were isolated in 57.1% and mixed with other facultative anaerobes / aerobes in 42.85%. Most studies showed higher isolation of mixed culture this is due to the synergistic effect between

facultative and obligatory anaerobic bacteria.* The ability of hydrogen production in the mixed culture was much better than that in the pure culture.[60]

Sydney Finegold described the most common 5 anaerobic organisms causing infections as *Bacteroides* , Pigmented and non pigmented *Prevotella*, *Fusobacterium nucleatum, Peptostreptococcus* and *Clostridium perfringens*[61] *Bacteroides(43),, Prevotella(26) and Clostridium(21)* were the common anaerobes isolated in our study .*Bacteroides fragilis* was the predominant followed by *Prevotella disiens*, *Clostridium clostridioforme* and others . *Bacteroides fragilis* as the commonest isolate was described by various researchers .[54,55,56,57] The type of anaerobe isolated depends on the samples included in the study. In our study *Bacteroides fragilis group* were isolated from appendicitis, perforative peritonitis , intra-abdominal infections, perianal abscess and cellulitis (Table – 4) . These organisms are normal GI commensals and predominate in intra-abdominal infections and all infections that originate from those flora. Some Enterotoxigenic *B fragilis* (ETBF) is also a potential cause of diarrhea in patients .[62] *E coli* was the commonest aerobe isolated from the pyogenic infections and mixed infections were commonest with *E coli* .

Prevotella sp and *Porphyromonas sp* are the most frequently isolated organisms from the periodontal infections . Four *Prevotella* species were isolated from periodontal infections of which three were *Prevotella disiens and a Prevotella bivia.* The most common anaerobe associated with adult periodontitis are the black pigmented *Pr. gingivalis* and *Pr. intermedia* .[62] *Prevotella disiens and prevotella bivia* are usually associated with gynaecological infection and not a common cause of periodontitis. *Cl clostridioforme and Cl sporogenes* were the other periodontal anerobic isolates in our study. The other common anaerobes reported from such infections are *Peptostreptococci* and *Fusobacterium*[63] but were not isolated. Two Pigmented *Prevotella melaninoogenica species* were isolated from cellutis and perianal abscess . Two *Prevotella oralis* were isolated from bartholin abscess and perianal abscess.

Clostridium septicum is associated with 1% of all clostridial infection and there is a strong association of this isolate in patients with adenocarcinoma . [64] .In our study, it was isolated from a patient with necrotizing fasciitis. In an appendicular infection *Clostridium clostridioformis*, was isolated, and from intraabominal infections *Clostridium clostridioformis* and *Clostridium sporogenes* were isolated. The 16sRNA sequencing studies had revealed that there are 3 species within the group

Clostridium clostridioforme: Clostridium bolteae, C. clostridioforme, and Clostridium hathewayi and they are associated with various infections.[65]

Trend analysis of drug resistance for various anaerobes was found to be changing and increasing.[66,67] Increasing drug resistance among anaerobes is a major problem among the *B.fragillis group* which is the commonest isolate and around 90% of them produce betalactamases .[68] The resistance of anaerobes has an impact on the selection of appropriate empirical therapy for infections caused by these pathogens. Anaerobic infections were treated most with clindamycin and metronidazole .[69] Metronidazole is the most commonly used drug for treating anaerobic infections in India. Metronidazole resistance of <5% - 63% has been reported to various anaerobic isolates .[70] However, all our isolates were found to be susceptible to metronidazole and Imipenem with MIC ≤8µg/ml and MIC ≤4µg/ml respectively . Around 17 of our isolates had a MIC of 8 for metronidazole. Carbapenem resistance is reported globally though in less percentages, About 1.5% to 5.4% for doripenem, 2 to 4.5% for ertapenem, 0.9 to 2.7% for imipenem, and 2 to 5.4% for meropenem . .[71]

One hundred percent resistance was observed with Clindamycin and Ampicillin tested with MIC ≥8/ µg ml and MIC ≥2µg/ml respectively and these drugs should not be used as a empirical monotherapy in critically ill

patients suspected to have anaerobic infections. Clindamycin resistance among *B fragilis* and *B non fragilis* isolates vary and higher resistance is observed among the non fragilis group . [72]. *erm*F gene is implicated in the resistance to clindamycin and also to the overexpression of efflux pumps *B. fragilis* (*bme*ABC1-16) resembling *Pseudomonas aeruginosa* MexAB-OprM . [72] The number of isolate in our study was small (28) but routine testing as per the CLSI should be routinely performed atleast using E test strips in all laboratories were anaerobes are isolated and identified.[68]

Anaerobic cultures are not routinely performed in most laboratories because it is labor intensive and technically challenging, Currently, automated and semi automated systems are available for identification. We compared the identification of anaerobic isolates by VITEK -2 system and MALDI TOF system .The identification of Genus level was comparable in 75% and most with *B fragilis* and *Prevotella* Poor comparability was observed with *Clostridium species* .However , molecular methods may be sought in the identification of these discrepant isolates.. Yang li et al in their comparative analysis described eight discrepant results among the 50 pairs of identifications produced by MALDI TOF and VITEK 2 ANC card the strains included in their study were confirmed by 16S rRNA gene sequencing.[3] In their study MALDI TOF produced superior accurate results

for *Clostridium difficile* identification over VITEK -2 and comparable results for *Bacteroides spp*.[3]

Though both systems can be used, the advantage of MALDI TOF over VITEK -2 is that it requires just a single colony and single attempt , whereas the turbidity needs to be matched with 2.7-3.3 Mc Farland opacity factor , use VITEK 2 ANC card and if the anaerobe is slow growing it needs 24hrs to grow and if the anaerobe is not identified in the first attempt it is repeated sometimes more than once, this would further increase the turnaround time and the cost of testing A cost assessment by Tan KE, Ellis BC, Lee R, et al [73] , showed that the MALDI TOF method provided on an average 1.45 days earlier and cost 56.9% less when compared with standard protocols, although the initial cost of the equipment was high .[3] The added advantage is , it is easy to perform , even inexperienced technician can perform this.

CONCLUSION:

Isolation and identification of anaerobic infections should be routinely performed and newer automated methods should be

standardized because they are cost effective and rapid .Regular monitoring of drug resistance to commonly used anaerobic antimicrobial should be done to identify the trends in resistance.

SUMMARY:

- A total of 150 pyogenic samples were processed using the Anoxamat culturing system .

- Samples that contributed highest (21%) were from periodontal infections.

- Out of 150 samples, 59(39.3%) yielded pure aerobes ,16 (57.1%) pure anaerobes and 12(42.85%) with both aerobes and anaerobes.

- Out of the 28(18.66%) anaerobes isolated.. Gram negative bacilli were the predominant followed by Gram positive spore bearing bacilli.

- *Bacteroides* fragilis group (43%) was the most commonest isolated followed by *Prevotella sp* (26%)and *Clostridium sp* (21%).

- *Bacteroides fragilis sp* (32%)was the predominant followed by *Bacteroides thetaiotamicron* and *Bacteroides stercoralis* . These were isolated from intraabdominal infections and cellulitis .

- Among the *Prevotella sp, Prevotella disiens*(14%) was the commonest followed by *P melaninogenica, P bivia and P oralis*. Non pigmented ones were isolated from periodontal infections. Two pigmented *P melaninogenica* were isolated from cellulitis and perianal abscess.

- In gram positive spore bearing bacilli, *Clostridium* c*lostridioforme* (11%) was the predominant bacilli, followed by *Clostridium sporogenes*(7%) and *Cl septicum* (4%).Cl septicum was isolated from necrotizing fasciitis , *Cl sporogenes* from periodental infections , intra abdominal infections and appendicitis

- Among the aerobes, *Escherichia coli*(37%) was the predominant followed by *Klebsiella pneumoniae*(17%) and *Pseudomonas* species(14%).

- When the VITEK-2 and MALDI-TOF automated identification systems were compared for Anaerobes, identification upto Genus level was comparable in 75% and most with *B fragilis group* and *Prevotella sp* . Poor comparability was observed with *Clostridium species*

- All the 28 (100%) anaerobic isolates were sensitive to Metronidazole and Imipenem and were 100% resistant to Ampicillin and Clindamycin.

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