"CLINICO- BACTERIOLOGICAL STUDY OF ADULT PERIODONTITIS WITH SPECIAL REFERENCE TO DETECTION OF BETA-LACTAMASE PRODUCING ANAEROBIC BACTERIA"

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Under the Guidance of

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I hereby declare that this dissertation entitled **"CLINICO-BACTERIOLOGICAL STUDY OF ADULT PERIODONTITIS WITH SPECIAL REFERENCE TO DETECTION OF BETA-LACTAMASE PRODUCING ANAEROBIC BACTERIA"** is a bonafide and genuine research work carried out by me, under the guidance of **Dr. Jyoti. M. Nagmoti** Professor department of Microbiology, J. N. Medical College, Belgaum.

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LIST OF ABBREVIATION

Ac	Amoxyclav
Ak	Amikacin
Ce	Cefotaxime
Cf	Ciprofloxacin
DD	Double disc diffusion
E	Erythromycin
ESBL	Extended Spectrum Beta Lactamase
GNB	Gram negative bacilli
GNCB	Gram negative coccobacilli
GPC	Gram positive cocci
Ox	Oxacillin
PDD	Potentiated disc diffusion

ABSTRACT

Background and objective:

Periodontitis affects significant percentage of population. It is a major public health problem in India with a prevalence of 60-80%. Objectives of this study are to isolate and identify the predominant aerobic and anaerobic microflora, semiquantitative study of isolated microflora in pre-treatment and post-treatment groups, to test the aerobic and anaerobic isolates for the production of enzyme beta-lactamase in pre-treatment group and to study the antibiotic sensitivity pattern of aerobic isolates in pre-treatment group.

Material and methods:

The present study was conducted in the Department of Microbiology, JNMC, Belgaum. Material was collected from the subgingival pockets in patients with adult generalised chronic periodontitis attending the Periodontology, Outpatient Department at KLE's V. K. Institute of Dental Sciences, Belgaum over a period of one year from Jan 2010 to Dec 2010. The study comprised of 60 cases of clinically diagnosed new cases of adult chronic generalized periodontitis. Clinical samples were transported to the laboratory in fluid thioglycolate medium. Initially gram's stain and Fontana stains were done. Aerobic, anaerobic and microaerophilic culture were put up. For aerobic, 5% sheep blood agar, Macconkey agar and for anaerobic, blood agar with haemin and vitamin K, Kanamycin, Vancomycin laked blood agar (KVLB) and Bacteroides Bile Esculin agar (BBE) were used. The media were placed in McIntosh Fildes jar with Internal Gas Generating System and incubated at 37ºC for minimum of 3-5days. Anaerobic growth was identified using standard techniques. Antibiotic sensitivity testing of aerobic organisms were carried out by the Kirby Bauer's disk

diffusion technique. For microaerophilic, brain heart infusion agar and blood agar were placed in 5% CO_2 jar. Beta lactamase production was tested by Double disc diffusion test , Potentiated disc diffusion test and Nitrocefin disc method.

Results:

Sixty samples yielded 121 isolates of which 78.34% were polymicrobial, 11.66% were monomicrobial and oral commensals were grown in 10% cases. Out of 121 isolates 91.74% were anaerobic, 7.43% were aerobic and 0.83% were microaerophilic. *Fusobacterium spp* was the most common isolate among anaerobes. Out of 121 isolates 13.22% showed ESBL production and all of them were anaerobic GNB. None of the aerobic isolates were ESBL producers. Semiquantitative study was significant with P value of 0.001.

Conclusion:

This study has shown that anaerobic bacteria are important cause of chronic periodontitis, along with aerobes and microaerophilic organisms. Isolation of these anaerobic organisms and their antibiotic susceptibility should be determined to start appropriate antibiotic therapy as early as possible to overcome the morbidity and mortality associated with this disease and to prevent the development of resistant strains.

Key words: periodontitis, subgingival plaque, oral microbial flora, aerobe, anaerobe, β- lactamase production.

TABLE OF CONTENTS

SL.NO	SECTIONS	PAGE NO.
1	INTRODUCTION	1
2	OBJECTIVES	3
3	REVIEW OF LITERATURE	4
4	MATERIALS AND METHODS	49
5	RESULTS	70
6	DISCUSSION	83
7	CONCLUSION	91
8	SUMMARY	92
9	BIBLIOGRAPHY	94
10	ANNEXURES	
	Annexure I: Informed Consent	110
	Annexure II: Proforma	113
	Annexure III: Master Chart	121

LIST OF TABLES

Sl.No	TABLES	Pg. No.
1	Bacterial virulence factors	36
2	Bacterial Enzymes Capable of Degrading Host Tissues	38
3	Age distribution of cases	70
4	Sex distribution of cases	72
5	Distribution of isolates	73
6	Polymicrobial isolates	74
7	Distribution of type of isolates	75
8	Anaerobic GNB isolates	76
9	Anaerobic GPC isolates	78
10	Aerobic isolates	79
11	Results of ESBL test	80
12	Beta-lactamase producing anaerobic GNB	81
13	Antibiotic susceptibility pattern of aerobic isolates	81
14	Results of semiquantitative study	82
15	Comparison of sex distribution	84
16	Comparison of isolates in different studies	85
17	Comparison of spectrum of gram negative anaerobes in the literature	87

LIST OF FIGURES

Sl.No	FIGURE	Pg. No.
1	Anatomy of periodontal tissue	6
2	Diagrammatic representation of selected species in the microbial complexes identified in the subgingival microbiota	34
3	Age distribution of cases	71
4	Sex distribution of cases	72
5	Distribution of type of isolates	75
6	Anaerobic GNB isolates	77
7	Anaerobic GPC isolates	78

LIST OF PHOTOGRAPHS

Sl. No	PHOTOS	Pg. No.
1	Armamentorium for sample collection	63
2	Sample collection	63
3	Fluid thioglycolate medium	64
4	McIntosh Fildes jar	64
5	Modified Fontanna stained direct plaque sample smear showing spirochetes	64
6	Growth on primary blood agar plate	65
7	Growth of Prevotella intermedia on Brucella blood agar	65
8	Bacteroides fragilis showing resistance to Kanamycin, Vancomycin, and Colistin discs	66
9	Peptostreptreptococcus anaerobicus showing sensitivity to SPS disc on blood agar	66
10	Bacteroides urealyticus on BBE agar	67
11	Low power microscopic picture of A. actinomycetemcomitans showing star shaped colony	67
12	Gram stain showing Gram positive cocci in chains of Streptococcus mutans	68
13	Gram stain showing GNB of Bacteroides fragilis	68
14	Nitrocefin disc test showing positive reaction	69

INTRODUCTION

Multicellular creatures are supra-organisms consisting of a symbiosis between the host and one or more colonizing bacteria. With the realization of the enormity of the colonization of Homosapiens with bacteria has come the perception that most of the bacteria that we live with, from cradle to grave, cause no adverse events in our lives. We seem to have evolved to live in perfect harmony with these bacteria and by-and-large, our skin, gut and urogenital tracts show no sign that they are colonized by bacteria that could cause tissue pathology. However, there is one body site where this cooperatively between our prokaryotic and eukaryotic selves break down - the oral cavity. Unless daily care is taken to remove plaque bacteria, the mouth is subject to the incredibly common diseases, caries and periodontitis.[1]

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific microorganism or group of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both.[2] The prevalence of this disease is 13-57% worldwide.[3] In India it is the major public health problem with a prevalence of 65-80%.[4]

The search for the pathogens of periodontal diseases has been underway for more than 100 years and continues up today.[5] Chronic periodontitis in adult patients results from a complex interplay of mixed polymicrobial infection and host response. Periodontitis is associated with a widely diverse complex subgingival microbiobiota encompassing both Gram-positive and Gram- negative bacteria, facultative and anaerobic organisms and possibly yeasts.[6] Bacterial DNA sequencing study has suggested that nearly 19000 bacterial phylotypes exist in oral cavity.[7] At least nearly

500 bacterial strains have been recovered from the subgingival plaque. Most of these strains are thought to be commensals and a small number, potential pathogens.[6]

There are various methods for isolation of the oral bacteria mainly immunological, DNA probe assessment and bacterial enzyme detection but still culture remains economical and the gold standard for isolation of bacteria. Early isolation and initiation of antibiotic treatment is needed to arrest the disease process and to prevent the morbidity, mortality and antibiotic resistance.[8]

Although there are good number of studies are available from western world regarding the microbiodata of oral cavity both in normal and periodontitis condition, but data on anaerobic periodontal microflora in the Indian population is very scarce, hence this study was undertaken to know the nature of oral microbiodata in chronic periodontitis in this region of India.

AIMS AND OBJECTIVES OF THE STUDY

1. To isolate and identify the predominant aerobic and anaerobic microflora associated with adult generalized periodontitis.
2. Semiquantitative study of isolated microflora of adult generalized periodontitis in pre-treatment and post-treatment groups.
3. To test the aerobic and anaerobic isolates for the production of enzyme beta-lactamase in pre-treatment group.
4. To study the antibiotic sensitivity pattern of aerobic isolates in pre-treatment group.

REVIEW OF LITERATURE

I.HISTORY

Oral and periodontal microbiology occupies an important place in the history of microbiology since the first bacteria to be described by Antoni Van Leeewanchek in 1683 originated from the dental plaque. [9]

Hippocrates of Cos (460-377 BCE) was the father of modern medicine, the first to institute a systematic examination of the patient's pulse, temperature, respiration, excreta, sputum and pulse. He discussed the function and eruption of the teeth and the etiology of periodontal disease.[10]

Gingival and periodontal diseases in their various forms, have afflicted humans since the dawn of history and studies in paleopathology have indicated that destructive periodontal diseases as evidenced by bone loss affected early humans in such diverse culture as ancient Egypt as early pre-columbia America. Oral hygiene was practiced by the Sumerians of 300 BC and Assyrians. Peridontal disease was the most common of all diseases evidence in the embalmed bodies of the ancient Egyptians.

The medical works of ancient India devote a significant amount of space to oral and periodontal problems. The Susruta samhita contains numerous descriptions of severe periodontal diseases with loose teeth and purulent discharge from the gingiva.

The Italian physician, mathematician and philosophers Girolamo Cardano (1501-76) appears to have been the first to differentiate types of periodontal diseases.

Anton Van leeuwenhook (1632-1723) of Deltt Holland developed the microscope and used it to observe the microorganism, cellular structure, blood cells including tubular structure of dentin. Leeuwenhook described his findings in letter written originally in Dutch to Royal society of London, which translated hem into English and published them in philosophical transactions. Using material from his gingival tissue, Leeuwenhoek first described oral bacterial flora and his drawings offered a reasonably good presentation of oral spirochetes and bacilli. *"I did not clean my teeth for 3 days and then took the material that had lodged in small amounts on the gums above my teeth, I found a few living animalcules"*. He also described a great amount of bacteria in a man who had never cleaned his mouth.

The first individual to identify bacteria as the cause of periodontal diseases appears to have been the German dentist Adolph Wifzel (1847-1906) but the first true oral microbiologist was Willoughby D miller (1853-1907). He described the features of periodontal disease and considered the role of predisposing factors, irritational factors and bacteria in the etiology of pyorrhea alveolaris. [10,11]

J Leon Williams (1852-1932) recognized bacterial plaque.[10] G.V. Black (1836-1915) who in 1899 coined the term gelatinous microbic plaque. In the first third of 20th century, periodontitis flourished in central Europe, with two major centers of excellence: Vienna and Berlin.

The concept of systemic diseases originating in dental and oral infection had been mentioned in the Assyrian Clay tablets, by Hippocrates and by Girolamo Cardano and German Walter Humann Ryff in the 16th century. In 1818 Benamin Ruch and in 1828 Leonard Korcker recognized the role of oral sepsis in rheumatic and other

diseases. Later in the century W.D. Miller also mentioned oral infections as the cause of many diseases.[10]

II. ANATOMY OF PERIODONTAL TISSUE

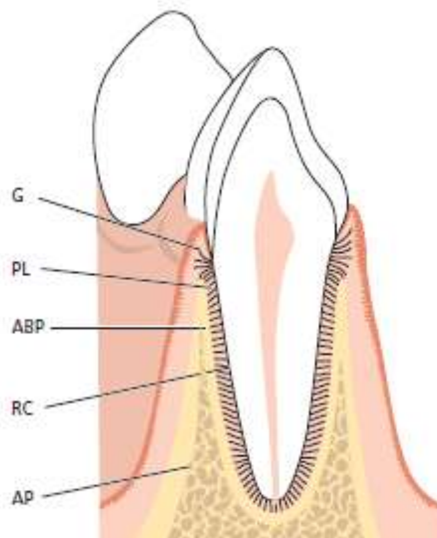
In periodontitis there is an apical extension of gingival inflammation to involve the tissues supporting the teeth including periodontal ligament and bone. The destruction of the fibre attachment results in a periodontal pocket.[12] Hence we need to know the anatomy of periodontal tissue.

The periodontium (peri= around, dontis=tooth) comprises the following tissues. 1) Gingiva

2) Peridontal ligament

3) Root cementum (RC)

4) The alveolar bone (AP).



G – Gingiva PL - Periodontal ligament

ABP – Alveolar bone process RC – Root cementum

Figure 1: Anatomy of periodontal tissue

Gingiva:

The oral mucosa is continuous with the skin of the lips and the mucosa of soft palate and pharynx. The oral mucosa consists of 1) masticatory mucosa which includes the gingival and covering of hard palate 2) specialized mucosa, which covers the dorsum of the tongue and 3) the remaining part is called lining mucosa.

The gingiva is that part of masticatory mucosa which covers the alveolar process and surrounds the cervical portion of the teeth. It consists of an epithelial layer and an underlying connective tissue layer called the lamina propria. The gingiva obtains its final shape and texture in conjuction with eruption of teeth.

In the coronal direction the coral pink gingiva terminates in the free gingival margin, which has a scalloped outline. In the apical direction the gingiva is continuous with the loose, darker red alveolar mucosa from which the gingiva is separated by a usually early recognizable border line called mucogingival junction or mucogingival line.

Two parts of the gingiva can be differentiated

1) The free gingiva (FG)
2) The attached gingiva (AG)

The free gingival margin is often rounded in such a way that a small invagination or sulcus is formed between the tooth and gingiva.

The free gingival margin is coral pink, has a dull surface and firm consistency. It comprises the gingival tissue at the vestibular and lingual/ palatal aspects of the teeth and the interdental gingival or the interdental papillae. On the vestibular and lingual side of the teeth , the free gingiva extends from the gingival margin in apical

direction to the free gingival groove which is positioned at a level corresponding to the level of cement-enamel junction. The attached gingiva is demarcated by the mucogingival junction in apical direction.

The attached gingiva extends in the apical direction to the mucogingival junction, where it becomes continuous with the alveolar mucosa. It is of firm texture, coral pink in colour and often shown small depressions on the surface. The depression named 'stippling' give the appearance of orange peel. It is firmly attached to the underlying alveolar bone and cementum by the connective tissue fibers and is therefore comparatively immobile in relation to the underlying tissue.

Periodontal ligament:

The periodontal ligament is the soft, richly vascular and cellular connective tissue which surrounds the roots of the teeth and joins the root cementum with the socket wall. In the coronal direction, the periodontal ligament is continuous with the lamina propria of the gingiva and is demarcated from the alveolar bone crest with the root.

Collagen fibers can be divided into following main groups

1. Alveolar crest fibers
2. Horizontal fibers
3. Oblique fibers
4. Apical fibers

Functions of the Periodontal Ligament

The functions of the periodontal ligament are physical,formative and remodeling, nutritional and sensory.

Physical Function- The physical functions of the periodontal ligament entail the following:

1. Provision of a soft tissue "casing" to protect the vessels and nerves from injury by mechanical forces
2. Transmission of occlusal forces to the bone
3. Attachment of the teeth to the bone
4. Maintenance of the gingival tissues in their proper relationship to the teeth
5. Resistance to the impact of occlusal forces (shock absorption)[13]

Root cementum:

The cementum is a specialized mineralized tissue covering the root surfaces and occasionally in small portion of the crown of teeth. It has many features in common with bone tissue. However , the cementum does not contain lymph, blood vessel or nerves. Does not undergo physiologic resorption or remodeling, but is characterized by continuous deposition throughout life. Like other mineralized tissues it contains collagen fibers embedded in an organic matrix.

Its mineral content, which is mainly hydroxyapetite, is about 65% by weight, a little more than that of the bone (i.e 60%). Cementum serves different functions. It attaches the periodontal ligament fibers to the root and contributes to the process of repair after damage to the root surface.

Alveolar bone:

Alveolar bone consists of 2 components, the alveolar bone process proper (ABP) and the alveolar process. Together with the root cementum and the periodontal membrane, the alveolar bone constitutes the attachment appartus of the teeth, the main

function of which is to distribute and resorb forces generated by, for example, mastication and other tooth contacts.

Blood supply of Periodontium:

The dental artery which is a branch of superior or inferior alveolar artery dismisses the intraseptal artery before it enters the tooth sockets. The terminal branches of the intraseptal artery penetrate the alveolar bone proper in canals at all levels of the sockets. The terminal branch of the intraseptal artery penetrates the alveolar bone proper in canals at all levels of the sockets. They anastomose in the periodontal ligament space together with blood vessels originating from the apical portion of the periodontal ligament and with other terminal branches from the intraseptal artery.

The gingiva recives its blood supply mainly through supraperiosteal blood vessels which are terminal branches of the subgingival artery, the mental artery, buccal artery, facial artery, greater palatine artery, infraorbital artery and posterior superior dental artery.

Lymphatic system of periodontium:

The lymph from the periodontal tissue drains to the lymph nodes of head and neck. The labial and lingual gingiva of the mandibular incisor region is drained to the submental lymph nodes. The palatal gingiva of the maxilla is drained to the deep cervical lymph nodes. The buccal gingiva of maxilla and the buccal and lingual gingiva in the mandibular premolar-molar region are drained to submandibular lymph nodes.

Nerve supply of periodontium:

Like other tissues in the body, the periodontium contains receptors which record pain, touch and pressure. Nerves recording pain, touch and pressure have their tropic center in the semilunar ganglion and are brought to the periodontium via the trigeminal nerve and its end branches. The gingiva on the labial aspect of the maxillary, incisor, canines and premolars is innervated by superior branches from the infraorbital nerve.

The buccal gingiva is innervated by branches from the posterior superior dental nerve. The palatal gingiva is innervated by greater palatine nerve, except for the area of incisior, which is innervated by long sphenopalatine nerve.The lingual gingiva in the mandible is innervated by subgingival nerve.The nerves enter the periodontal ligament through the perforaters (Volkamann's canals) in the socket wall. In the periodontal ligament, the nerves join larger bundles which take a course parallel to the long axis of the tooth.

III.PHYSIOLOGY:

Function of Periodontium-

The main function of the periodontium is to attach the tooth to the bone tissue of the tissue of jaws and to maintain the integrity of the surface of the masticatory mucosa of the oral cavity.

The peridonticum also called "the attachment apparatus or "the supporting tissue of the teeth" consists a developmental, biological and functional unit which undergoes certain changes with age and in addition, subjected to morphologic changes related to functional alteration.[14]

IV.ORAL MICROBIAL FLORA:

From the earliest days of the study of microbiology, the mouth has been identified as a habitat for large number of microorganisms. The seventeenth century microscopist, Anton van Leeuwanhook, provided initial description of the differing bacteria found within the oral cavity. Since then studies of the human bacterial flora in health and disease have progressed rapidly, keeping pace with technological advances in scientific investigation. The introduction of anaerobic culture techniques has greatly added the characterization of the normal oral flora.[15,16]

During birth and immediately thereafter, the neonate contacts the microbial inhabitants of birth canal and maternal environment. The initial microbial flora consists of lactobacilli, streptococci, staphylococci, enterococci, veillonellae, neisseriae and coliforms.

In early childhood, facultative species are dominant in the oral cavity. Then various obligate anaerobes are added. Major factors in establishing and maintaining microorganism in the oral cavity are bacteria–tissue interaction , interbacterial adherence and interbacterial metabolic interaction.[17,18]

Gram-positive cocci:

The predominant component of oral flora is streptococcal species, most commonly members of the alpha haemolytic viridans group. Currently 26 different group of viridans streptococci are recognized. They are divided into five major groups on the basis of phenotypic and molecular properties as follows- *S. mutans, S. salivarius, S. anginosus, S. sanguis, and S. mitis group*. Other gram positive cocci include anaerobic peptostreptococci species.

Within the mutans group two most common species isolated grom human oral cavity are *S. mutans and S. sobrinus*. The salivarius group has 3 main species in the human oral microflora. *S. salivarius, Str. vestibularis and str. infantarius. S. mitis* and *S oralis* are major components within the mitis group. *S. sanguinis, S. parasanguinis* and *S.gordonii* are the main constituents of sanguinis group. The anginosus group consists of three species *S. anginosis, S.milleri* and *S. intermedius*.[16]

Gram-positive bacilli:

Lactobacilli are found soon after birth but less in number as the neonate ages. Pleomorphic or filamentous gram positive rods, the so called "diphtheroids" are common inhabitants of the oral cavity. Other branching filamentous rods found within the oral cavity belong to Actinomyces species, including *Ac israelii, Ac. viscosus, Ac naeslundii* and *Ac.odontolyticus.*[16,19]

Gram- Negative cocci:

Anaerobic gram negative cocci are also found as part of the normal oral flora. Veillonella species such as *V. parvula, V. dispar and V. atypica* are found on the soft tissues of the mouth such as the tongue surface and in saliva and may constitute upto 5% of dental plaque biomass.[16,19]

Gram-negative bacilli:

Among the most numerous and varied components of the oral flora are gram negative rods. Anaerobic gram-negative rods constitute approximately 16% of the normal flora in the gingival crevices.

Commonly identified species include the black–pigmented rods previously classified within the bacteroides genus but now renamed as Prevotella or

Porphyromonas species. Bile sensitive saccharolytic bacteroids species now are classified within the genus Prevotella, bile sensitive asacchrolytic species are classified within the genus Porphyromonas.[20]

Apart from being the dominant microflora in the healthy gingival crevices, some seem to play a unique role in certain diseases states. For eg-*Prevotella intermedia* and *Porphyromonas gingivalis* are among the predominant species found in advanced periodontal diseases. Species such as *Bacteroides forsythus* are also identified.[21]

Numerous other gram-negative anaerobic or facultative rods within the oral cavity can contribute to different disease status. These include *Actinobacillus actinomycetemcomitans, Fusobacterium species* and *Eikenella corrodens.*[22]

Actinobacillus has been identified as an intraoral pathogen that contributes to localized juvenile periodontitis. Systemic dissemination by *E. corrodens* and *A.actinomycetemcomitans* are associated with the formation of cardiac valve vegetation and infective endocarditis.[22-24]

Spirochetes:

Spirochetes belong to the genus Treponema are found within the normal anaerobic flora of the oral cavity. A variety of morphotypes has been observed, but many remain uncultivable and are identified only by the molecular techniques. The most common species in the human oral flora include *Treponema denticola, Treponema orale* and *Tr vincentii*.

The anaerobic nature of the treponemes favours their isolation from subgingival tissues and within periodontal pockets. In disease states, treponemes

particularly *T. denticola* have been associated with periodontal destruction, where as others are linked to acute necrotizing gingivitis.[16,25]

V. PERIODONTITIS

Definition: It is an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both.[2]

Classification

AAP International Workshop for Classification of Periodontal Diseases, 1999 classify periodontitis into three major types based on clinical, radiographic, historical and laboratory characteristics.

1. Chronic periodontitis
2. Aggressive periodontitis
3. Periodontitis as a manifestation of systemic diseases

Chronic periodontitis:

Chronic periodontitis, formerly known as "adult periodontitis" or "chronic adult periodontitis," is themost prevalent form of periodontitis. It is generally considered to be a slowly progressing disease. Although chronic periodontitis is most frequently observed in adults, it can occur in children and adolescents in response to chronic plaque and calculus accumulation. This observation underlies the recent name change from "adult" periodontitis, which suggests that chronic,plaque-induced periodontitis is only observed in adults, to a more universal description of "chronic" periodontitis that can occur at any age.

Definition: Is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss.

Age: Commonly observed in adults increases in prevalence and severity with age

Sex: Both the sexes are commonly involved.

The following characteristics are common to patients with chroic periodontitis.

- Prevalent in adult but can occur in children
- Amount of destruction consistent with local factors
- Associated with a variable microbial pattern
- Subgingival calculus frequently forms
- Slow to moderate rate of progression with possible periods of rapid progression
- Possibly modified by or associated with the following
    - Systemic diseases such as DM and HIV infection
    - Local factors predisposing to periodontitis
    - Environmental factors such as cigarette smoking and emotional stress.

Classification: Chronic periodotitis may be further subclassified into localized and generalized forms and characterized as slight, moderate or severe based on the following specific features

Localized form: <30% of sites involved

Generalized form: >30% of sites involved

Slight: 1to2 mm of clinical attachment loss

Moderate: 3to 4 mm of clinical attachment loss

Severe: > 5 mm of clinical attachment loss. [26]

Aggressive Periodontitis:

Definition: Aggressive periodontitis comprises of group of rare often severe rapidly progressive forms of periodontitis often characterized by an early age of clinical manifestation and a distinctive tendency to aggregate in families.[27]

Age: Affects less than 30 years of age

Sex: Equally affects both the sexes

Aggressive periodontitis may be further classified into localized and generalized forms based on the common features described here and the following specific features.

Localized form

- Circumpubertal onset of disease
- Localized first molar or incisor disease with proximal attachment loss on at least two permanent teeth, one of which is a first molar.
- Robust serum antibody response to infecting agents

Generalized form

- Usually affecting persons under 30 years of age
- Generalized proximal attachment loss affecting at least three teeth other than first molars and incisors
- Pronounced episodic nature of periodontal destruction
- Poor serum antibody response to infecting agents

Periodontis as a manifestation of systemic diseases

Periodontitis may be observed as a manifestation of the following systemic diseases:

1. Hematologic disorders
    - Acquired neutropenia
    - Leukemias
    - Others
2. Genetic disorders
    - Familial and cyclic neutropenia
    - Down syndrome
    - Leukocyte adhesion
    - Papillon-Lefevre syndrome
    - Chediac-Higashi syndrome
    - Histiocytosis syndromes
    - Glycogen storage disease
    - Infentile genetic agranulocytosis
    - Cohen syndrome
    - Ehlers-Danlos syndrome(types IV and VIII AD)
    - Hypophospatasia
    - Others
3. Not otherwise specified [26]

Predisposing factors-

1) **Age**: Prevalence of periodontitis increases with the age. It is a age associated and not a age-related disease.

2) Prior History of Periodontitis

Although not a true risk factor for disease but rather disease predictor, a prior history of periodntitis puts patients at greater risk for developing further loss of attachment and bone, given a challenge from bacterial plaque accumulation.

3) Local Factors:

Plaque accumulation on tooth and gingival surfaces at the dentogingival junction is considered the primary initiating agent in the etiology of chronic periodontitis. Attachment and bone loss are associated with an increase in the proportion of gram-negative organisms in the subgingival plaque biofilm, with specific increases in organisms known to be exceptionally pathogenic and virulent. *Bacteroides gingivalis, Bacteroides forsythus* and *Treponema denticola*, otherwise known as the "red complex," are frequently associated with ongoing attachment and bone loss in chronic periodontitis.

Because plaque accumulation is the primary initiating agent in periodontal destruction, anything that facilitates plaque accumulation or prevents plaque removal by oral hygiene procedures can be detrimental to the patient. Plaque retentive factors are important in the development and progression of chronic periodontitis because they retain plaque microorganisms in close proximity to the periodontal tissues, providing an ecologic niche for plaque growth and maturation. Calculus is considered the most important plaque retentive factor because of its ability to retain and harbor

plaque bacteria on its rough surface. As a result, calculus removal is essential for the maintenance of a healthy periodontium. Other factors that are known to retain plaque or prevent its removal are subgingival and/or overhanging margins of restorations; carious lesions that extend subgingivally; furcations exposed by loss of attachment and bone; crowded and malaligned teeth; and root grooves and concavities [28]

4) Smoking:

Tobacco in any form increases risk for periodontitis. And also impair periodontal therapy.

5) Systemic Factors

a. Nutritional deficiency- Vitamin C, histiocytosis X

b. Blood Cells: Systemic haematological disorders have profound effects on the periodonticum by denying the oxygen supply, hemostasis and protection to the tissue of the Periodontium.[29]

c. Leukemia: Hemorrhagic gingival enlargement with or without necrosis is a common early manifestation of acute leukemia. Patients with chronic leukemia may experience similar but less severe periodontal changes. Chemotherapy or therapy associated with bone marrow transplantation may also adversely affect the gingiva. [30]

d. Diabetes mellitus:

Patients with undiagnosed or poorly-controlled Type 1(insulin dependent) diabetes mellitus or Type 2 (non-insulin dependent) diabetes mellitus may be particularly susceptible to periodontal diseases. Conversely, most well-

controlled diabetic patients can maintain periodontal health and will respond favorably to periodontal therapy.

6) **Stress**: By altering the function of the endocrine system and immune system causes periodontitis. Various authors observed that stress may be an immunoupressive.

7) **Drugs:**

Drugs can be a contributing etiologic factor in periodontal diseases. Drugs such as anticonvulsants, calciumchannel blocking agents, and cyclosporin may be associated with gingival enlargement. Oral contraceptives may be a contributing factor in alterations of gingival tissues. In addition, drugs can cause xerostomia, osteoporosis, lichenoid reactions, and other hypersensitivity reactions.

8) **Immune system disorders:**

Some forms of periodontal disease may be more severe in individuals affected with immune system disorders. Patients infected with human immunodeficiency virus (HIV), may have especially severe forms of periodontal disease. The incidence of necrotizing periodontal diseases may increase in the patient with acquired immunodeficiency syndrome (AIDS). Patients who have received organ transplants, are undergoing cancer treatment, or have certain autoimmune diseases may be taking immunosuppressing medications.[30]

8) Genetic Factors:

In addition to other factors, periodontal destruction is frequently seen among family members and across different generations within a family, suggesting the possibility of a genetic basis to the susceptibility to periodontal disease. Recent data

indicate that a genetic variation or polymorphism in the genes encoding interleukin 1-a and interleukin 1-ß is associated with an increased susceptibility to a more aggressive form of chronic periodontitis in subjects of Northern European origin.' In addition, smokers demonstrating the composite IL-1 genotype are at even greater risk for severe disease. A recent study suggested that patients with the IL-1 genotype increased the risk for tooth loss by 2.7 times; those who were heavy smokers and IL-1 genotype negative increased the risk for tooth loss by 2.9 times. The combined effect of the IL-1 genotype and smoking increased the risk of tooth loss by 7.7 times. [28]

VI. BACTERIOLOGY:

The search for the pathogens of periodontal diseases has been underway for more than 100 years and continues up today. The currently recognized key Gram negative periodontopathogens include: *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythus, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Capnocytophaga species, Campylobacter rectus.* Also the following bacteria could be isolated: *Eubacterium spp, Peptostreptococcus micros, Selenomonas spp* and *Spirochaetes*. [31-36]

The microorganisms could produce disease directly, by invasion on the tissues, or indirectly by bacterial enzymes and toxins. In order to be a periodontal pathogen, a microorganism must have the following:

- The organism must occur at higher numbers in disease-active sites than at disease-inactive sites
- Elimination of the organism should arrest disease progression

- The organism should possess virulence factors relevant to the disease process the organism should elicit a humoral or cellular immune response
- Animal pathogenicity testing should infer disease potential. [36]

Porphyromonas gingivalis:

It is a strictly anaerobic, Gram negative rod. It is a black-pigmented microorganism which produces a black pigment. Many virulence mechanisms have been identified. It has a carbohydrate capsule on its outer surface which prevents opsonization by complement and inhibits phagocytosis and killing by neutrophils. The lipopolysaccharide which is produced is not very strong, but it could inhibit chemotaxis and killing by leucocytes. This organism possesses several putative virulence factors (including proteases which degrade immunoglobulin, complement, collagen fibres, hyaluronic acid; adhesins, endotoxins and cytotoxins) that can directly affect the periodontium or elicit host functions that result in the gingival tissue and bone damage typical for periodontal disease [37]. Porphyromonas expresses three major virulence factors-fimbriae, gingipains and lipopolysaccharides.

Its importance as a periodontal pathogen is also highlighted by the research efforts aimed at developing a vaccine aimed at immunization against this bacterial species and thus preventing a chronic periodontal disease. [38-40]

Aggregatibacter actinomycetemcomitans:

This species has recently been renamed *Aggregatibacter actinomycetemcomitans* from its former name of *Actinobacillus actinomycetemcomitans* (Norskov-Lauritsen & Kilian 2006).

A. actinomycetemcomitans is a small, non-motile, Gram-negative, saccharolytic, capnophilic, round ended rod that forms small, convex colonies with a "star-shaped" center when grown on blood agar plates. [41] Several virulence factors are reported: the leukotoxin is the most important, cytolethal distending toxin, immunosuppression factors, inhibition of PMNS functions etc. Leukotoxin, is an RTX (Repeats in Toxin) toxin and shares sequence similarity with the α-hemolysin from *Escherichia coli*, the cytolysin from Pasteurella haemolytica and the leukotoxin from *Actinobacillus pleuropneumoniae* [42,43]. Leukotoxin from *A.actinomycetemcomitans* could kill human and non-human primate polimorphopnuclear leukocytes and peripheral blood monocytes. [44,45] So, the innate immune response could be attacked directly. Its endotoxin has the potential to modulate host responses and contribute to tissue destruction. The ability of the lipopolysaccharide to stimulate macrophages to release interleukin IL-1, IL-1β, and tumor necrosis factor (TNF) is of big importance. These cytokines, among other their activities, are capable of stimulating bone resorption .[46]

Prevotella intermedia:

P.intermedia is the black-pigmented Bacteroides. The levels of this Gram-negative, short, round-ended anaerobic rod have been shown to be particularly elevated in acute necrotizing ulcerative gingivitis, certain forms of periodontitis and progessing sites in chronic periodontitis. [41] This species resists phagocytosis, probably by virtue of its capsule. It is an important periodontal pathogen, in association with *P.gingivalis and A.actinomycetemcomitans*. [6]

Fusobacterium nucleatum:

F. nucleatum is a Gram-negative, anaerobic, spindle shaped rod that has been recognized as part of the subgingival microbiota for over 100 years. This species was the most common isolate found in cultural studies of subgingival plaque samples, comprising approximately 7–10% of total isolates from different clinical conditions. *F. nucleatum* was prevalent in subjects with periodontitis and periodontal abscesses and was reduced after successful periodontal therapy.

Invasion of this species into human gingival epithelial cells in vitro was accompanied by an increased secretion of IL-8 from the epithelial cells. The species can induce apoptotic cell death in mononuclear and polymorphonuclear cells induces epithelial cells to produce collagenase 3, and produces a 65 kDa serine protease. In addition, F. nucleatum induces cytokine, elastase, and oxygen radical release from leukocytes. Perhaps the most important role of F. nucleatum in the subgingival ecosystem is its function as a "bridging" species, facilitating coaggregation among species.[41]

Tannerella forsythus

Formerly known as Bacteroides forsythus - is a non-pigmented saccharolytic anaerobic gram-negative rod. *T. forsythus* possesses several virulence factors including the production of a trypsin-like protease and lipopolysaccharide but recently, its ability to penetrate into host cells or induce apoptosis. [47-50]

Micromonas micros:

Micromonas micros formerly known as *Peptostreptococcus micros* is an anaerobic, Gram positive small asachrolytic coccus which is associated with

periodontal disease as well as several other polymicrobial infections in other systemic diseases. [51-54] The prevalence of *Micromonas micros* in advanced adult periodontitis was reported as 58% to 63%, with *M.micros* representing 12% to 15% of the cultured viable counts.[54] In periodontal patients, the prevalence of *M.micros* was higher in those with active disease, which supports an etiological role of *M.micros* in progressive attachment loss.[53] This finding was supported in another study where *M.micros, Wolinella recta, and Fusbacterium nucleatum* were the only species detected in one or more samples from patients with active disease sites [.55] *M.micros* was also positively associated (18% of the time) with recurrent or refractory periodontal disease and implant failure . All of these present a strong association of *M.micros* in periodontal disease and periimplantitis.[6]

Eikenella Corrondens:

E. corrodens is a Gram-negative, capnophilic, asaccharolytic, regular, small rod with blunt ends. It has been recognized as a pathogen in other forms of disease, particularly osteomyelitis, infections of the central nervous system and root canal infections . This species was found more frequently in sites of periodontal destruction as compared with healthy sites in some but not all studies. In addition, *E. corrodens* was found more frequently and in higher levels in actively breaking down periodontal sites and in sites of subjects who responded poorly to periodontal therapy. [41]

Spirochetes:

Spirochetes are Gram-negative, anaerobic, helicalshaped, highly motile microorganisms that are common in many periodontal pockets. Two important species – *Treponema denticola* and *Treponema vincentii*. They could be implicated in periodontal disease. Both of them produce a lipopolysaccharide and unusual

metabolic end products, like indole, hydrogen sulphide, ammonia, which are potentially toxic to host cells. Spirochetes were observed in a higher proportion of patients with periodontal disease than in periodontally healthy patients. [56, 57]

Treponema.denticola is frequently isolated from severely diseased sites in patients with a periodontal disease. Many studies have attempted to elucidate the role of *Treponema.denticola* in periodontitis. *Trep.denticola* has been shown to attach to human gingival fibroblasts, basement membrane proteins, as well as other substrates by specific attachment mechanisms, the binding of the spirochete to human gingival fibroblasts resulted in cytotoxicity and cell death due to enzymes and other proteins [.6]

Takeuchi et al. [58] (2001) found that *Treponema socransky, T.denticola and P.gingivalis* were frequently detected in patients with a periodontal disease by PCR technique in plaque and saliva samples. They also found that the presence of *T.socranskii* was associated with periodontitis, as well as that *T.socranskii* was more frequently detected in plaque samples from aggressive or chronic periodontitis patients than from healthy subjects. *T.socranskii* were detected more frequently at sites where a severe periodontal tissue destruction was observed.

VII. PATHOGENESIS

The following requisites are indispensable for microbes to invade subgingival sites: (i) the ability to attach to the tissue surface; (ii) the ability to multiply; (iii) the ability to compete against other microbial species and; (iv) the ability to defend against host responses. The innate bacterial characteristics dictate the first three features. Where the host genotype becomes important is mainly in relation to (iv), where the huge machinery of inflammation and immunity comes into play in terms of recognizing bacteria and attempting to kill and remove them. [1]

The initial lesion in the development of periodontitis is the inflammation of the gingiva in response to a bacterial challenge. Changes involved in the transition from the normal gingival sulcus to the pathologic periodontal pocket are associated with different proportions of bacterial cells in dental plaque.[59]

DENTAL PLAQUE: A HOST ASSOCIATED BIOFILM

Periodontal health can be considered to be a state of balance when the bacterial population coexists with the host and no irreparable damage occurs to either the bacteria or the host tissues. Disruption of this balance causes alterations in both the host and biofilm bacteria and results ultimately in the destruction of the connective tissues of the periodontium.

Macroscopic Structure and Composition of Dental Plaque

Dental plaque can be defined as the soft deposits that form the biofilm adhering to the tooth surface or other hard surfaces in the oral cavity, including removable and fixed restorations.

'Dental plaque is broadly classified as supragingival or subgingival based on its position on the tooth surface. Dental plaque is composed primarily of microorganisms. Cultivation studies, in which bacteria are isolated and characterized in the laboratory, indicate that more than 500 distinct microbial species are found in dental plaque. 6s Recent studies have focused on molecular approaches to bacterial identification, which rely on analysis of ribosomal DNA sequences rather than cultivation. Nonbacterial microorganisms that are found in plaque include Mycoplasma species, yeasts, protozoa, and viruses.[12] The microorganisms exist within an intercellular matrix that also contains a few host cells such as epithelial cells, macrophages, and leukocytes. The intercellular matrix, estimated to account for

20% to 30% of the plaque mass, consists of organic and inorganic materials derived from saliva, gingival crevicular fluid and bacterial products. Organic constituents of the matrix include polysaccharides, proteins, glycoproteins and lipid material. Glycoproteins from saliva are an important component of the pellicle that initially coats a clean tooth surface, but they also become incorporated into the developing plaque biofilm. Polysaccharides produced by bacteria, of which dextran is the predominant form, contribute to the organic portion of the matrix. Albumin, probably originating from crevicular fluid, has been identified as a component of the plaque matrix. The lipid material consists of debris from the membranes of disrupted bacterial and host cells and possibly food debris.

The inorganic component of plaque is predominately calcium and phosphorus, with trace amounts of other minerals such as sodium, potassium, and fluoride. The source of inorganic constituents of supragingival plaque is primarily saliva; as the mineral content increases, the plaque mass becomes calcified to form calculus. Calculus is frequently found in areas of the dentition adjacent to salivary ducts (e.g., the lingual surface of the mandibular anteriors and the buccal surface of the maxillary first molars), reflecting the high concentration of minerals available from saliva in those regions.

Formation of Dental Plaque

Dental plaque may be readily visualized on teeth after 1 to 2 days with no oral hygiene measures. Plaque is white, grayish, or yellow and has a globular appearance. The process of plaque formation can be divided into three phases: formation of the pellicle coating on the tooth surface, initial colonization by bacteria, and secondary colonization and plaque maturation.

Formation of the Dental Pellicle:

Initially there is formation of the acquired pellicle which is nothing but the saliva coated over the teeth. Acquired pellicle contains glycoprotein, phosphoprotein, histidine rich protein and other molecule that can act as the receptor for adhesion for the bacteria. The mechanism involved in the enamel pellicle formation includes electrostatic, van-der Walls and hydrophobic force.

Initial Colonization of the Tooth Surface :

Within a few hours, bacteria are found on the dental pellicle. The initial bacteria colonizing the pellicle-coated tooth surface are predominantly gram-positive facultative microorganisms such as *Actinomyces viscosus and Streptococcus sanguis.* These initial colonizers adhere to the pellicle through specific molecules, termed adhesins, on the bacterial surface that interact with receptors in the dental pellicle. For example, cells of *A. viscosus* possess fibrous protein structures called fimbriae that extend from the bacterial cell surface. Protein adhesins on these fimbriae specifically bind to proline-rich proteins that are found in dental pellicle, resulting in the attachment of the bacterial cell to the pellicle-coated tooth surface. The plaque mass then matures through the growth of attached species, as well as the colonization and growth of additional species. In this ecologic succession of the biofilm, there is a transition from the early aerobic environment characterized by gram positive facultative species to a highly oxygen-deprived environment in which gram-negative anaerobic microorganisms predominate.

Secondary Colonization and Plaque Maturation:

Secondary colonizers are the microorganisms that do not initially colonize clean tooth surfaces, including *Prevotella intermedia, Prevotella loescheii,*

Capnocytophaga spp., Fusobacterium nucleatum, and Porphyromonas gingivalis. These microorganisms adhere to cells of bacteria already in the plaque mass. Extensive laboratory studies have documented the ability of different species and genera of plaque microorganisms to adhere to one another, a process known as coaggregation. This process occurs primarily through the highly specific stereochemical interaction of protein and carbohydrate molecules located on the bacterial cell surfaces",in addition to the less specific interactions resulting from hydrophobic, electrostatic and van der Waals forces.

The significance of coaggregation in oral colonization has been documented in studies of biofilm formation in vitro' as well as in animal model studies. Well characterized interactions of secondary colonizers with early colonizers include the coaggregation of *F. nucleatum* with *S. sanguis, P. loescheii* with *A viscosus*, and *Capnocytophaga.ochracea* with *A. viscosus*. Most studies of coaggregation have focused on interactions among different gram-positive species and between gram-positive and gram-negative species. In the latter stages of plaque formation, coaggregation between different gram-negative species is likely to predominate. Examples of these types of interactions are the coaggregation of *F. nucleatum with P. gingivalis or Treponema denticola.*[60]

In a study by Ganier et al.[61] first time demonstrated the co aggregation between *P. gingivalis and T. denticola*. They also observed that the coaggretation is bimodal and is inhibited by the heating of both the partner and also by L-arginine. The coaggration of *P.gingivalis* with *Fusobacterium nucleatum and actinomyces viscosus* are also observed in the literature. The coaggretation phenomenon described is

responsible for the adherence of the bacteria to the epithelial surface and each other to cause periodontitis.

Microscopic Structure and Physiologic Properties of Dental Plaque-

Morphologic and microbiologic studies of subgingival plaque reveal distinctions between the tooth-associated and tissue-associated regions of subgingival plaque . The tooth-associated (attached) plaque is characterized by gram-positive rods and cocci, including *Streptococcus mitis, S. sanguis, A. viscosus, Actinomyces naeslundii,* and *Eubacterium spp.* The apical border of the plaque mass is separated from the junctional epithelium by a layer o f host leukocytes, and the bacteria o f this apical tooth-associated region show an increased concentration of gram-negative rods. Studies of tissue-associated plaque indicate a predominance of species such as *S. oralis, S. intermedius, P. micros, P. gingivalis, P. intermedia, Bacteroides forsythus,* and *F. nucleatum. Bacteria found* in tissue-associated plaque *(e.g., P. gingivalis)* also have been found in host tissues .

Thus the physical proximity of these bacteria to the host tissues in the plaque mass may be important in the process of tissue invasion. The transition from gram-positive to gram-negative microorganisms observed in the structural development of dental plaque is paralleled by a physiologic transition in the developing plaque. The early colonizers (e.g., *streptococci* and *Actinomyces species*) use oxygen and lower the reduction-oxidation potential of the environment,which then favors the growth of anaerobic species. Gram-positive species use sugars as an energy source and saliva as a carbon source. The bacteria that predominate in mature plaque are anaerobic and asaccharolytic and use amino acids and small peptides as energy sources . [60]

Significance of the Biofilm Environment

Studies of oral microbial communities as biofilms in vitro further highlight the significance of structural and physiologic interactions between bacterial species in plaque. For example, the presence of *F. nucleatum* in experimental mixed biofilm communities is critical to the survival of high numbers of the anaerobic species *P. nigrescens* and *P. ingivalis*. The ability of *F. nucleatum* to coaggregate with both the facultative and anaerobic species may facilitate the survival of the anaerobes. In addition, *F. nucleatum* can reduce the reduction-oxidation potential of its environment so that it may function to provide a protective econiche for other anaerobic species. In a study by Socransky et al. involving more than 13,000 plaque samples of 40 subgingival microorganisms using a DNA-hybridization methodology was used to define "complexes" of periodontal microorganisms. The composition of the different complexes is based on the frequency with which microorganisms are recovered together. Interestingly, the early colonizers are either independent of defined complexes *(A. naeslundii, A. viscosus)* or members of the yellow *(Streptococcus spp.)* or purpcomplexes *(A. odontolyticus)* .The microorganisms primarily considered secondary colonizers fell into the green, orange or red complexes. The green complex includes *E. corrodens, Actinobacillus actinomycetemcomitans serotype a, and Capnocytophaga spp.* The orange complex includes Fusobacterium, Prevotella, and Campylobacter spp. The green and orange complexes include species recognized as pathogens in periodontal and nonperiodontal infections. The red complex consists of *P. gingivalis, B. forsythus, and T. denticola*. The red complex is of particular interest because it is associated with bleeding on probing, which is an important clinical parameter of destructive periodontal diseases. [96] The existence of complexes of

species in plaque is another reflection of bacterial interdependency in the biofilm environment.[62]

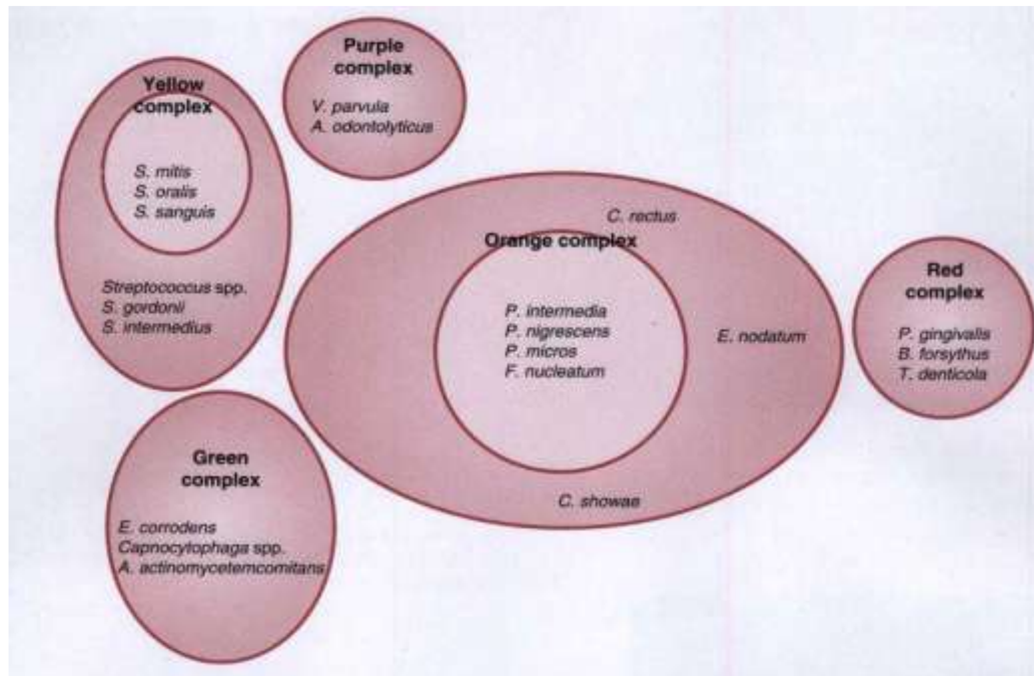


Figure 2: Diagrammatic representation of selected species in the microbial complexes identified in the subgingival microbiota. Each defined grouping is identified by a color. (Adapted from Socransky et al: Microbial complexes in subgingival plaque. J Clin Periodontol 1998; 25:134.).[62]

Virulence factors

To function as a pathogen, a bacterium must colonize the appropriate host tissue site and then cause destruction of the host tissues.. Thus virulence properties can be broadly categorized into two groups: factors that enable a bacterial species to colonize and invade host tissues, and factors that enable a bacterial species to directly or indirectly cause host tissue damage.

Bacterial Adherence in the Periodontal Environment - The gingival sulcus and periodontal pocket are bathed in gingival crevicular fluid, which flows outward from the base of the pocket. Bacterial species that colonize this region must attach to

available surfaces to avoid displacement by the fluid flow. Therefore adherence represents a virulence factor for periodontal pathogens. The surfaces available for attachment include the tooth or root, tissues and preexisting plaque mass. Numerous interactions between periodontal bacteria and these surfaces have been characterized and in some cases the molecules responsible for mediating these highly specific interactions have been determined. Bacteria that initially colonize the periodontal environment most likely attach to the pellicle or saliva coated tooth surface. A relevant example is the adherence of *Actinomyces viscosus* through fimbriae on the bacterial surface to proline-rich proteins found on saliva coated tooth surfaces.

Bacterial attachment to preexisting plaque is studied by examining the adherence between different bacterial strains (coaggregation). One of the best-characterized interactions is the adherence of *A. viscosus* through surface fimbriae to a polysaccharide receptor on cells of *Streptococcus sanguis*. These types of interactions are thought to be of primary importance in the colonization of the periodontal environment. In addition, the adherence of bacteria to host tissues is likely to play a role in colonization and may be a critical step in the process of bacterial invasion. Thus the ability of *P. gingivalis* to attach to other bacteria, epithelial cells and the connective tissue components fibrinogen and fibronectin are all likely to be important in the virulence of this microorganism.

Host Tissue Invasion-Bacteria may enter host tissues through ulcerations in the epithelium of the gingival sulcus or pocket and have been observed in intercellular spaces of the gingival tissues. Another means of tissue invasion may involve the direct penetration of bacteria into host epithelial or connective tissue cells. Laboratory investigations have demonstrated the ability of *A. actinomycetemcomitans, P*

gingivalis, P nucleatum and Treponema denticola to directly invade host tissue cells.[63]

Table No. 1 Bacterial Virulence factors[63]

HostDefense Mechanism	Bacterial Species	Bacterial Property	Biologic Effect
Specific antibody	*P. gingivalis* *P intermedia* *P. melaninogenica* *Capnocytophaga sp.*	IgA and IgG degrading proteases	Degradation of specific antibody
Polymorphonuclear Leukocytes	*A.actinomycetemcomitans* *F. nucleatum* *P. gingivalis* *P. gingivalis* *T. denticola*	Leukotoxin heat-sensitive surface protein Capsule Superoxide production	Inhibition of PNM function Apoptosis (programmed cell death) of PMN Inhibition of phagocytosis Decreased bacterial Killing
Lymphocytes	*A.actinomycetemcomitans* *A.actinomycetemcomitans* *F. nucleatum* *T. forsythus* *P. intermedia* *T. denticola* *A.actinomycetemcomitans*	Leukotoxin Cytolethal distending toxin heat-sensitive surface protein Cytotoxin Suppression	Killing of mature B and T cells; nonlethal suppression of activity impairment of function by arresting of lymphocyte cell cycle Apoptosis of mononuclear cells Apoptosis of lymphocytes decreased response to antigens and mitogens
Release o f IL-8	*P. gingivalis*	Inhibition of IL-8 production by epithelial cells	Impairment of PMN response to bacteria

Microbial Mechanisms of Host Tissue Damage

Research on virulence factors has focused on the properties of bacteria related to the destruction of host tissues. These bacterial properties can be broadly categorized as those resulting directly in degradation of host tissues and those causing the release of biologic mediators from host tissue cells that lead to host tissue destruction. Some bacterial products inhibit the growth or alter the metabolism of host tissue cells; these include a number of metabolic by-products such as ammonia; volatile sulfur compounds; and fatty acids, peptides, and indole.

An important class of molecules in tissue destruction is the variety of enzymes produced by periodontal microorganisms . These enzymes appear to be capable of degrading essentially all host tissue and intercellular matrix molecules . In particular, a wide range of proteolytic enzymes have been identified from P. gingivalis , including a trypsin-like enzyme and those that degrade collagen, fibronectin, and mmunoglobulins. Bacterial enzymes may facilitate tissue destruction and invasion of bacteria into host tissues. However, the exact role of bacterially derived proteases in the disease process has not been determined because similar enzymes (e.g., collagenases) in the periodontal environment originate from host tissue cells. Indeed, one mechanism by which bacteria may indirectly cause tissue damage is by induction of host tissue proteinases such as elastase and matrix metalloproteinases. The host immune system involves a complex network of interactions among cells and regulatory molecules. Bacterial products may perturb the system, resulting in tissue destruction. Well-characterized interactions involve the release of interleukin-1 (IL-1), tumor necrosis factor (TNF), and prostaglandins from monocytes, macrophages and PMNs exposed to bacterial endotoxin (lipopolysaccharide). These host-derived

mediators have the potential to stimulate bone resorption and activate or inhibit other host immune cells.

A trypsinlike enzyme is present in *T denticola, P gingivalis,* and *T forsythia* and is absent for at least 60 other subgingival plaque organisms. The ability of subgingival plaque to hydrolyze BANA was associated with elevated levels and proportions of spirochetes and with probing depths greater than 6 mm. subsequently, BANA hydrolysis was shown to be related to the *T denticola* and *P gingivalis* content of the plaque and to the clinical diagnosis of health or disease.[64,65]

Table No. 2: Bacterial Enzymes Capable of Degrading Host Tissues [64,65]

Bacterial Enzyme	Species
Collagenase	*P. gingivalis* *A. actinomycetemcomitans*
Trypsin-like enzyme	*P. gingivalis* *A. actinomycetemcomitans* *T. denticola*
Keratinase	*P. gingivalis* *T. denticola*
Arylsulfatase	*C. rectus*
Neuraminidase	*P. gingivalis* *B. forsythus* *P. melaninogenica*
Fibronectin-degrading enzyme	*P. gingivalis* *P. intermedia*
Phospholipase A	*P. intermedia* *P. melaninogenica*

Adapted from Socransky SS, Haffajee AD: Microbial mechanisms in the pathogenesis of destructive periodontal diseases: A critical assessment. Periodontal Res 1991; 26:195; and Loesche WI: Bacterial mediators in periodontal disease. Clin Infect Dis 1993; 16(suppl 4):5203.

VIII. CLINICAL FEATURES

The clinical features of chronic periodontitis include symptoms such as

1. Color, texture and volume alterations of the marginal gingiva
2. Bleeding on probing from the gingival pocket area
3. Reduced resistance of the soft marginal tissues to probing (increased pocket depth or periodontal pocketing)
4. Loss of probing attachment level
5. Recession of the gingival margin
6. Loss of alveolar bone
7. root furcation exposure
8. Increased tooth mobility
9. Drifting and eventually exfoliation of teeth. [29]

Characteristic clinical findings in patients with chronic periodontitis include supragingival and subgingival plaque accumulation that is frequently associated with calculus formation, gingival inflammation, pocket formation, loss of periodontal attachment and loss of alveolar bone.

Chronic periodontitis can be clinically diagnosed by the detection of chronic inflammatory changes in the marginal gingiva, presence of periodontal pockets, and

loss of clinical attachment. It is diagnosed radiographically by evidence of bone loss. These findings may be similar to those seen in aggressive disease. A differential diagnosis is based on the age of the patient, rate of disease progression over time, familial nature of aggressive disease and relative absence of local factors in aggressive disease compared with the presence of abundant plaque and calculus in chronic periodontitis.[28]

IX. TREATMENT

Phases of Periodontal Therapy

Preliminary phase

Treatment of emergencies:

- Dental or periapical
- Periodontal
- Other

Extraction of hopeless teeth and provisional replacement if needed (may be postponed to a more convenient time)

Etiotropic phase (Phase I therapy)

Plaque control and patient education

- Diet control (in patients with rampant caries)
- Scaling and root planning
- Correction of restorative and prosthetic irritational factors

- Excavation of caries and restoration (temporary or final, depending on whether a definitive prognosis for the tooth has been arrived at and on the location of caries)
- Antimicrobial therapy (local or systemic)
- Occlusal therapy
- Minor orthodontic movement
- Provisional splinting and prosthesis

Surgical phase (Phase II therapy)

Restorative phase (Phase III therapy)

Maintenance phase (Phase IV therapy)

Local Therapy

The cause of periodontitis and gingivitis is bacterial plaque accumulation on the tooth surface in close proximity to the gingival tissue. The accumulation of plaque can be favored by a variety of local factors, such as calculus, overhanging margins of restorations and food impaction. The removal of plaque and of all the factors that favor its accumulation is therefore the primary consideration in local therapy.

Systemic Therapy

Systemic therapy may be employed as an adjunct to local measures and for specific purposes, such as the control of systemic complications from acute infections, chemotherapy to prevent harmful effects of posttreatment bacteremia, supportive nutritional therapy and the control of systemic diseases that aggravate the patient's periodontal condition. [66]

X. PERIODONTAL DISEASE AS A RISK FOR SYSTEMIC DISEASE:

Periodontitis if untreated thought to act as a risk factor for systemic diseases like atherosclerosis, cardiovascular and cerebrovascular diseases, pregnancy complications diabetes mellitus and others. [67]

Atherosclerosis and other cardiovascular diseases-

A number of pathogenic species involved in the periodontal infections display tissue invasion properties. Circulating levels of several cytokines (IL-1 beta, IL-2, IL-6 and IL-8) induced during the course of several infection, but also locally in the periodontal tissue in conjuction with periodontitis have been identified as biomarkers of cardiovascular disease. Interestingly, these pro-inflammatory cytokines have also been detected within atheromatous lesions. In line with the observation that chronic infection may contribute to a pro-coagulant state, elevated von Willenbrand factor antigen, a measure of endothelial cell damage, has been demonstrated in individuals with multiple dental infections. [68] Herzberg et al. [69] observed that during periodontitis, dental plaque microorganisms may disseminate through the blood to infect the vascular endothelium and contribute to the occurrence of atherosclerosis and risk of myocardial ischemia and infarction.

A number of studies have examined the presence of oral bacteria in atheromatic plaque lesions. Chiu et al. [70] investigated the relationship between the presence of multiple infectious agents in human carotid endarterectomy specimens and pathoanatomic features of the corresponding carotid plaques and reported positive immunostainings for *P.gingivalis* and *Streptococcus sanguis* in several carotid plaque specimens.

In an another study by Haraszthy et al.[71] polymerase chain reaction, reported that 30% of the carotid endarterectomy specimens examined were positive for *T. forsythia,* 26% for *P. gingivalis,* 18% for *A.actinomycetemcomitans* and 14% positive for *Pr. intermedia*.

Several studies have investigated the association between periodontitis and subclinical atheroscorosis, commonly measured by means of carotid artery intima media thickness (IMT) assessments. Increased IMT has been documented to be directly associated with increased risk of myocardial infarction and stroke. [72]

Among the group of studies focusing on the potential association of periodontitis and stroke, an early case-control study by Syrjanen et al. [73] compared the level of dental diseases in 40 patients who had suffered a cerebrovascular accident with 40 randomly selected community controls, matched for gender and age and reported that severe chronic dental infection was associated with cerebral infarction in males under 50 years of age.

Mattila et al [74] conducted two separate case-control studies that totaled 100 patients with acute myocardial infarction and 102 controls selected from the community at random. Patients in the first series were 40 consecutive men aged 50 years or less admitted because of acute myocardial infarction and the controls were matched for age, sex and neighborhood from the community and invited for examination when the patient was admitted. The second series consisted of 60 consecutive cases, either men aged 60 years or less or women aged 65 years or less. The dental examination was performed while patients were in the hospital or shortly thereafter. The dental index, which was used in all of Mattila's publications, was the sum of scores for the number of carious lesions, missing teeth, probing depth

measures (including pus in the pocket), the number of periapical lesions and the presence or absence of pericoronitis. These index scores showed that patients had worse dental health than controls in both studies. Logistic regression analysis indicated that the association between poor dental health and coronary heart disease persisted after controlling for age, total cholesterol, high-density lipoprotein, triglycerides, C peptide, hypertension, presence of diabetes, and smoking.

Rheumatoid arthritis:

The association of periodontal disease and rheumatoid arthuritis as been well established. In a study by Mercado et al. [75] did a study to determine whether there is a relationship between disease experience of rheumatoid arthritis and periodontal disease. In patients referred for periodontal treatment, the prevalence of self-reported rheumatoid arthritis was 3.95% which is significantly higher than that seen in patients not referred for periodontal treatment (0.66%) and also that reported in the general population (1%). Of those referred patients with rheumatoid arthritis, 62.5% had advanced forms of periodontal disease.

Pregnancy complications:

Preterm infants are born prior to completion of 37 weeks of gestation. Despite the established role of genitor-urinary tract infections in the pathobiology of preterm birth,women with preterm labour do not invariably present with positive amniotic fluid cultures, leading to the hypothesis that preterm birth may be indirectly mediated through distant infection resulting in translocation of bacteria , bacterial vesicles or LPS in the systemic circulation. The possibility that periodontal infections may constitute such maternal infections that adversely influence birth outcome was raised for the first time in the late 1980s

Transient bacteremias occur commonly in subject with inflamed gingiva and may conceivably reach the placental tissue, providing the inflammatory impetus for labour induction.[76]

Offenbacher et al. [77] stated that periodontal diseases are gram-negative anaerobic infections that can occur in women of child bearing age (18-34 yrs). In the present investigation they sought to determine whether the prevalence of maternal periodontal infection could be associated with preterm low birth weight (PLBW), controlling for known risk factors and potential covariates. A control study of 124 pregnant or postpartum mothers was performed. Preterm low birth weight mothers cases were defined as a mother with a birth of less than 2,500 g and one or more of the following such as gestational age <37 weeks, preterm labor (PTL), premature rupture of membranes (PROM). Controls were normal birth weight infants (NBW). Assessments included a broad range of known obstetric risk factors, such as tobacco use, drug use, and alcohol consumption, level of prenatal care, parity, genitourinary infections, and nutrition. Each subject received a periodontal examination to determine clinical attachment level. Preterm low birth weight cases and primiparous preterm low birth weight cases n=93 had significantly worsened periodontal disease than the respective NBW controls. Multivariate logistic regression models, controlling for the risk factors and covariates demonstrated that periodontal disease is a statistically significant risk factor for preterm low birth weight with adjusted odds ratio of 7.9 and 7.5 for all preterm low birth weight cases and primiparous preterm low birth weight cases, respectively. This data indicates that periodontal diseases represent a previously unrecognized and clinically significant risk factor for preterm low birth weight as a consequence of either preterm low birth weight or preterm premature rupture of membrane.

Diabetes mellitus:

The role of diabetes as a risk factor for periodontitis has been observed, however limited data seems to suggest that an inverse relationship may also be present. In line with the concept that infection may contribute to impaired metabolic control of diabetes. In a study by Taylor et al. on 39 diabetic subjects over a median follow up period of 6 years showed significantly higher prevalence of proteinuria and cardiovascular complications in the severe periodontitis group. [78] In a study by Ainamo et al (1990) [79]reported the rapid periodontal destruction in adult humans with poorly controlled diabetes mellitus in 2 patients out of a pool of 12 middle-aged or elderly diabetic patients demonstrating rapid periodontal break-down. The common feature of all 12 patients was that they were either unaware of or unable to control their diabetic condition at the time of the active, stage of their periodontal disease. The bone loss progressed in spite of specialist periodontal care and the patients again responded to treatment only after their elevated blood glucose levels had been brought back to normal. Thus, the rapid periodontal breakdown was not found to be associated with the diabetic condition per se, but rather with the hyperglycemia.The observations suggested that there may be an inter-relationship between rapid periodontal breakdown and elevated blood glucose levels.

Periodontitis as a risk for respiratory infections-

Scannapieco, Wang et al. [80]conducted preliminary studies in their laboratory and found that oral bacteria may modulate the adhesion of respiratory pathogens to epithelial cell lines. In addition, oral bacterial products or cytokines in oral or pharyngeal aspirates may stimulate cytokine production from respiratory epithelial

cells, resulting in recruitment of inflammatory cells. The resulting inflamed epithelium is more susceptible to respiratory infection.

XI. ANTIMICROBIAL RESISTANCE BY ORAL PATHOGENS:

Broad spectrum beta-lactams are generally considered to be the primary choice of antibiotics in emergency cases, although the increasing frequency of beta-lactamase producing oral bacteria may result in prescription failure. [81]

Amoxicillin is a broad spectrum antibiotic that is active against both obligate anaerobic flora and facultative anaerobic flora. Its use causes the selection of natural or acquired resistants (beta-lactamase-producing stains). Beta-lactamase-producing stains may be present in the oral cavity from a very early age. [82]

Extended-spectrum beta-lactamases (ESBL) are enzymes that mediate resistance to extended spectrum cephalosporins such as cefotaxime, ceftriaxone and ceftazidime and monobactum aztreonam. One mechanism of bacterial resistance to β-lactam antibiotics is enzymatic inactivation of the antimicrobial agent. Production of β-lactamase is mediated via either chromosomes or plasmids. Beta-lactamase production may be constitutive or inducible. Induction after β-lactum exposure can result in significant increase in enzyme production. One of the first report on plasmid-mediated resistance to a β-lactum antibiotic was in 1974 and since than an increase in β-lactam resistance has been noted in gram-negative bacteria such as *Pseudomonas aeruginosa, Enterobactor cloacae, Serratia species* and *Haemophilus influenza* have been reported. The clinical consequence of resistance can be therapeutic failure and relapse.[83]

Walker et al. [84], testing whole subgingival plaque samples found β-lactamase activity in 36% of all ≥ 6mm periodontal pockets in patients with adult periodontitis. They also concluded that the subgingival concentration of enzymes can be sufficiently high to degrade subgingival penicillin levels. In a study of Kinder et al.[85] β- lactamase have been detected in dark-pigmented Prevotella species, in Haemophilis and Capnocytophage species in patients with adult periodontitis. Appelbaum et al. [86] reported that the occurrence of β- lactamase positive strains of Fusobacterium nucleatum from mainly non- oral sites can be 21%. Inhibitors of β-lactamase such as clavulanic acid can be combined with for instance amoxicillin resulting in β- lactamase resistant drug. This combination has shown beneficial clinical effects in the treatment of active periodontitis and refectory adult periodontitis.

Amoxicillin, in combination with metronidazole has been used successfully in the treatment of severe Actinobacillus actinomycetemcomitans- associated periodontitis. Others have advocated the use of amoxicillin + clavulanic acid in combination with metranodazole. More information on the occurrence of penicillin – degrading subgingival bacteria in adult periodontitis is needed for proper selection of potential antimicrobial therapies.[83]

MATERIAL AND METHODS

The present study was conducted at the Department of Microbiology, JNMC, Belgaum.

Source of data: Material (Plaque) was collected from the subgingival pockets in patients with adult generalised chronic periodontitis attending the Periodontology, Outpatient Department at KLE's V. K. Institute of Dental Sciences, Belgaum over a period of one year from Jan 2010 to Dec 2010. The study comprised of 60 cases of clinically diagnosed new cases of adult chronic generalized periodontitis

Detailed clinical history regarding age, sex, chief complaints, past history and treatment history was obtained from each patient. Oral examination was done to record periodontal pocket depth, plaque index and gingival index. Plaque samples collected from the subgingival pockets were studied in the department of Microbiology, J.N. Medical College Belgaum.

Sample size-

Allowing 10% absolute error and taking 80% prevalence, the sample size is calculated by using formula

$$n=4pq/d^2$$
$$= 4x80x20 / (10)^2 = 6400/100$$
$$=64\sim60$$

Sample size n = 60

n = Sample size

p = Prevalence

q = 100-p

d = 10% of error

Method of Collection of data

Inclusion Criteria:-

Clinically diagnosed new cases of adult generalized periodontitis of both sexes attending Periodontology outpatient department.

Exclusion Criteria:-

- Patients with history of systemic conditions such as diabetes mellitus, nutritional deficiencies.
- Pregnant women.
- History of antibiotic usage in the last 3 months.
- History of undergoing any other dental procedure in the last 3 months.

Plaque sample collection:

After isolating the teeth with cotton rolls, the tooth surfaces will be dried with sterile gauze to avoid contamination by saliva. Subgingival plaque sample was collected using sterile periodontal Gracy curette and placed in sterile test tubes containing fluid thioglycolate medium.

Laboratory methods-

Sample in the fluid thioglycolate medium is transferred to 1 ml of trypticase soya broth and vortexing is done for 1 min.

Microscopic examination:-

Direct smear was made by using a loop full of sample . The smear was stained by Gram's method [Hucker's modification of Gram's stain was followed where in counterstain safranin was used and kept for 5 mins (details given below)][87] for an

immediate presumptive diagnosis of the number and type of microorganisms present in the sample. Morphology of the organism and other observations in the gram stained smear were recorded.

Gram stain Procedure: [Hucker's modification]

1) A thin smear of the material was made on a clean glass slide and allowed to air dry.
2) The material was fixed by passing the slide three to four times through the flame of a Bunsen burner so that the material does not wash off during staining procedure.
3) Slide was placed on staining rack and the smear overlayed with crystal violet solution.
4) After 1 minute the slide was washed thoroughly with distilled water.
5) Then the smear was overlayed with Gram's iodine solution for 1 minute and washed again with water.
6) The smear was held between the thumb and fore finger and the surface flooded with a few drops of acetone-alcohol decolorizer, until no color washes off.
7) The smear was washed with running water and placed on staining rack. The surface was overlayed with safranin counter stain for 5 minutes and washed with running water.
8) The smear was placed in a upright position in a staining rack, allowing excess water to drain off and smear to try.

9) The stained smear was examined under 100 X (oil) immersion objective of the lens.

Modified Fontana staining procedure:[88]

1) A thin smear of the material was made on a clean glass slide and allowed to air dry.
2) The slides then were placed vertically in 100 ml capacity beaker containing reagent A and allowed for two minutes.
3) They are removed by forceps, blotted with tissue paper and dipped in a beaker containing absolute alcohol or methanol or rectified spirit for three minutes.
4) The undersurface of the slides were cleaned with paper and the smear was air dried.
5) The slides were then dipped in a 100 ml beaker containing reagent B pre-heated to 75^0 C in hot water bath and allowed to react for a minute.
6) The slides were rinsed in distilled water and air dried.
7) The slides were dipped in a beaker containing reagent C pre-heated to 75^0c and allowed to react for a minute.
8) The slides were rinsed with distilled water, the under surface cleaned, air dried and examined under bright field microscope.

Reagent A-Fixative

Glacial acetic acid-1ml

Formalin(40% formaldehyde)-2 ml

Distilled water- 100 ml

Reagent B-Mordant

Phenol - 1 gm

Tannic acid – 5 gm

Distilled water-100 ml

Reagent C- Silver stain

Silver nitrate solution – 60 ml & 40 ml

Silver nitrate solution 60 ml + few drops of ammonia solution
brown precipitate appears

Add 0.5 – 1 ml of ammonia till all precipitate dissolves

From 40 ml alliquot, transfer solution to above solution till precipitate reappears

Bacteriological methods

Aerobic culture methods-

With a standard loop, a loopful of sample is taken from vertexed solution and inoculated onto,

1) 5% sheep blood agar
2) Macconkey agar plate
3) Brain heart infusion agar

Lawn culture was done and the plates were incubated at 37^0C for 24 hrs aerobically (Mac Conkey agar and blood agar) and under 5% CO_2 (blood agar and brain heart infusion agar). All the isolates were identified and characterized biochemically by standard procedures as described by Mackie & Mc Cartney practical medical microbiology.

Antibiotic sensitivity testing

The antimicrobial susceptibility testing was done for aerobic and microaerophilic isolates by disc diffusion method as described by Kirby and Bauer, on Mueller Hinton agar.

Different antibiotics and concentration of discs used were as follows:

	Antibiotics	Concentration per disc
1)	Amikacin(Ak)	30 mcg
2)	Ciprofloxacin(Cf)	5 mcg
3)	Amoxicillin / clavulanic acid (Ac)	30 mcg
4)	Cefotaxime(Ce)	30 mcg
5)	Erythromycin (E)	15 mcg
6)	Oxacillin (Ox)	1 mcg

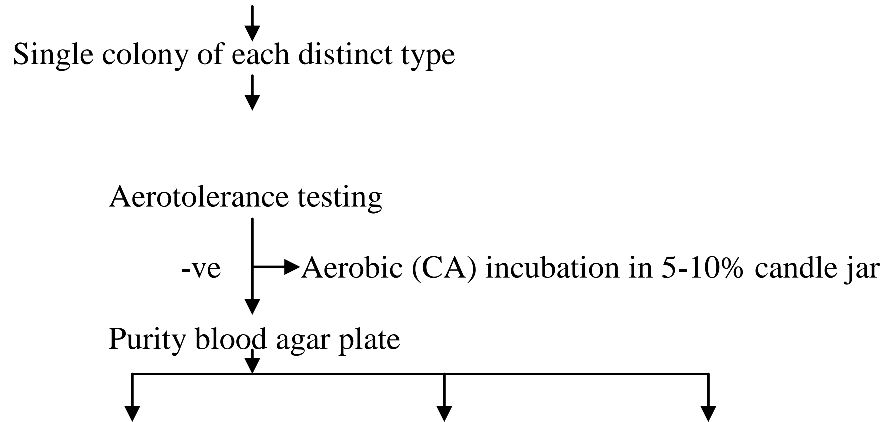
For antimicrobial sensitivity testing a single colony was inoculated in peptone water and incubated at 37^0C for 4 to 6 hrs and turbidity adjusted to Mac Farland's 0.5. Muellor Hinton agar plate was inoculated with culture by means of cotton swab and antibiotic discs were applied and incubated over night at 37^0C. zone of inhibition was measured. Interpretation was made according to the Kirby Bauer chart.87

Control stain used was Staphylococcus aureus ATCC 25923.

Culture for anaerobic organisms [89]

Each sample was inoculated onto

1) Blood agar supplemented with Haemin and Vit K.



Add antibiotic	**Perform**	**Record**
Identification discs	1. Gram stain	1. Colony Morphology
- Sodium polyanethol Sulphonate (SPS) disc	2. Spot indole test	2. Pigment
	3. Catalase test	3. Haemolysis
- Nitrate disc		4. Fluorescence
- Susceptibility to Kanamycin (1mg) Vancomycin (5µg), Colistin (10µg)		5. Pitting

(1) Blood agar was supplemented with Haemin (5mcg/ml) and Vit K (10mcg/ml). Blood agar plates used for anaerobic isolation were prepared with Brucella agar base.

(2) Kanamycin(75mcg/ml) Vancomycin(7.5mcg/ml) laked blood agar (KVLB). KVLB agar inhibits the growth of most facultative bacteria and allows for earlier pigmentation of *Prevotella melaninogenicus*. Most *Bacteroides spp*. grow well on it, while *Fusobacterium spp*. and most gram positive anaerobes are inhibited.

Preparation:

First Brucella agar base was prepared to which 75µg/ml Kanamycin base was added and autoclaved. Vancomycin 7.5µg/ml and laked blood (5%) was aseptically added after autoclaving. Laked blood was prepared by freezing whole blood overnight and then thawing.

(3) Bacteroids bile esculin agar (BBE) containing Gentamycin 100µg/ml, 20% bile, 0.1% esculin, 0.05% ferric ammonium citrate (HI MEDIA) was used.

BBE agar is useful for rapid detection and isolation of members of the *B. fragilis* group. Members of the *B. fragilis* group grow well, producing dark colonies with brown to black halos.[89]

The method used for obtaining anaerobiosis in the jar was "internal gas generating system" described by Lakshminarayana and Vaidhyalingam. [90]

Catalyst:

Palladium pallets reactivated every time before use by drying at 150^0 C-160^0 C for 1-2 hrs.

Indicator:

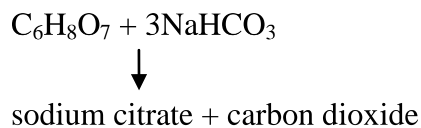
Methylene blue preparedby mixing equal volumes of,

a. 6% glucose containing 1mg/ml thymol as preservative
b. Sodium hydroxide solution prepared by diluting 6ml of 0.1 N NaOH to 100ml of distilled water.
c. Methylene blue prepared by adding 3ml of 0.5% w/v solution of methylene blue to 97ml of distilled water. Mixture is placed in anaerobic jar after it is made colourless by heating in a boiling water bath.

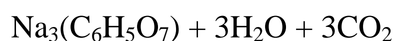
Principle

In this system the hydrogen and CO_2 gas mixture required for creating anaerobiosis is obtained from the following reactions.

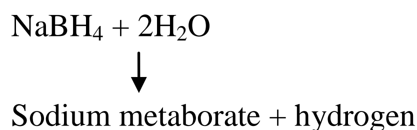
(1) Citric acid + sodium bicarbonate



sodium citrate + carbon dioxide



(2) Sodium borohydride + hydrogen



Sodium metaborate + hydrogen

Operation of the gas generator

a) 1g sodium borohydride was taken in 30 ml test tube

b) 1g sodium bicarbonate and 1g citric acid were taken in the 5ml test tube, which was placed inside the 30 ml test tube.

c) The stem of 20 ml funnel was plugged lightly with cotton to control the flow of water. The funnel was placed in 30 ml test tube in such a way that the stem of funnel dips into 5 ml test tube. Entire unit was kept inside the jar with the indicator. 20 ml of distilled water was poured in the funnel just before closing the lid of the jar.

The water poured into the funnel drips into the 5 ml test tube liberating CO_2. CO_2 being heavier stays within displacing the air. Once the 5 ml test tube is filled with water, it overflows into the 30 ml test tube liberating hydrogen, which being lighter gas, rushes out with CO_2. The palladium catalytically reduces the oxygen present within the jar to form water. Catalyst is exothermic, so warming of the lid of the jar can be felt. [90]

After 72 hours of incubation at 37^0C anaerobic jar was opened. The plates were examined for the presence of colonies. When the colonies appeared on the anaerobic plates each predominant distinct colony was subcultured to purity blood agar plate (BAP). From a pure culture on a BAP, following was recorded,

- Colony morphology, including size of colony, shape, color, internal appearance (such as speckling), general appearance (eg: mucoid transparent, opaque) and colony count
- Pigment

- Haemolysis
- Fluorescence
- Pitting

Single colony of each distinct type was plated on to blood agar plates with antibiotic identification discs.

- Sodium polyanethol sulphonate (SPS) disc for rapid presumptive identification of Peptostreptococcus anaerobius.
- The 3 antibiotic discs Kanamycin 1 mg, Colistin 10µg and Vancomycin 5µg were placed on the first quadrant of the purity BAP, which aid in preliminary grouping of anaerobes and serve to verify the Gram's stain.
- A nitrate disc was placed on the 2nd quadrant for subsequent determination of nitrate reduction.

Chocolate agar plate was inoculated for incubation in candle jar at 37^0C to test for aerotolerance.

If there was no growth on plates after 72 hours of anaerobic incubation, the plates were reincubated for an additional period of 48 hours and for a maximum period of 1 week.

The following tests were done from the purity plate.

Catalase test : Growth was removed from blood agar plate to a drop of 15 % hydrogen peroxide on a glass slide and observed for evolution of bubbles.

Spot Indole test : A loopful of growth from a pure culture on a blood agar plate was removed and this growth was smeared on filter paper that has been saturated with 1 %

paradimethylaminocinnamaldehyde in 10 % (V/V) concentrated hydrochloric acid. A positive reaction was indicated by the rapid development of blue colour around the growth. Negative reaction gave no color change or a pinkish color.

Nitrate test : This test was done using nitrate discs. The disc was removed from surface of plate and placed in a clean petridish. One drop each of reagents A and B were added. Development of pink to red color indicated nitrate had been reduced to nitrite. If no colour developed in few minutes, a small amount of zinc dust was added and waited for 5 minutes. Development of red colour indicated that nitrate was not reduced. If no colour developed it was taken as positive test. [89]

Nitrate reagents

Solution A

Sulfanilic acid 0.5g

Glacial acetic acid 30.0ml

Distilled water 120.0ml

Solution B

1,6-Cleve's acid 0.2g(5-amino-2-naphthalenesulfonic acid)

Glacial acetic acid 30.0ml

Distilled water 120.0ml

Tests for beta-lactamase production:

Three tests were done to know the production of beta-lactamase by both aerobic and anaerobic organisms. These tests were

1) Double disc diffusion test
2) Potentiated disc diffusion test
3) Nitrocefin disc method

For anaerobic isolates Brucella agar supplemented with hemin(5 μg/ml), vitamin K(1μg/ml) and lysed horse blood(5%) was used. For aerobic isolates Muller-Hinton agar (MHA) was used.

For double disc diffusion test and potentiated disc diffusion test A standardized inoculum may be prepared by either growing the microorganisms to the turbidity equivalent to a 0.5 McFarland standard or suspending colonies directly to achieve the same density as above.

Double disc diffusion test-

First using the detection test described by JRLIER et al synergy was determined between a disc of amoxicillin-clavulunate (20mg/10mg) and a 30 mg disc of third generation of cephalosporin test antibiotic placed at a distance of 20mm from center to center on Brucella blood agar which was inoculated with the test isolate and incubated in anaerobic condition at 37 degree centigrade for 48 hours.

Clear extention of the zone of inhibition 20mm of cephalosporin towards the amoxiclav disc was interpreted as positive for ESBL production.[91-94]

Potentiated disc diffusion test :

A disc of ceftazidime (30mg) alone and ceftazidine+clavalanic acid (30mg or 10mg) were placed at a distance of 25 mm center to center on BBA plate inoculated with the test isolates and incubated anaerobically at 37 degree centigrade for 48 hours.

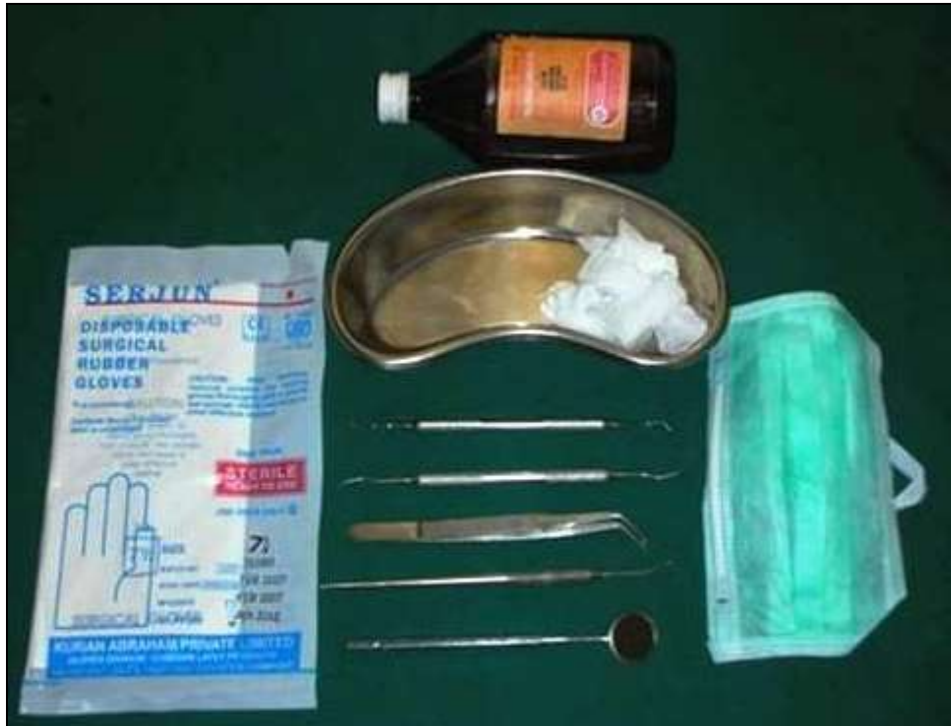
An increase in the inhibition zone diameter of > 5mm for a combination disc versus ceftazidime disc alone confirmed ESBL production. [83,95]

NITROCEFIN DISC METHOD:

Nitrocefin disc were inoculated with a small portion of growth from blood agar plates and observed for a change in color from yellow to red. Disks which did not show color change within 10min at room temperature were additionally incubated for 60 min at 37 degree centigrade

A test was considered positive when yellow colour turned to pink/ red. [83]

PHOTOGRAPHS

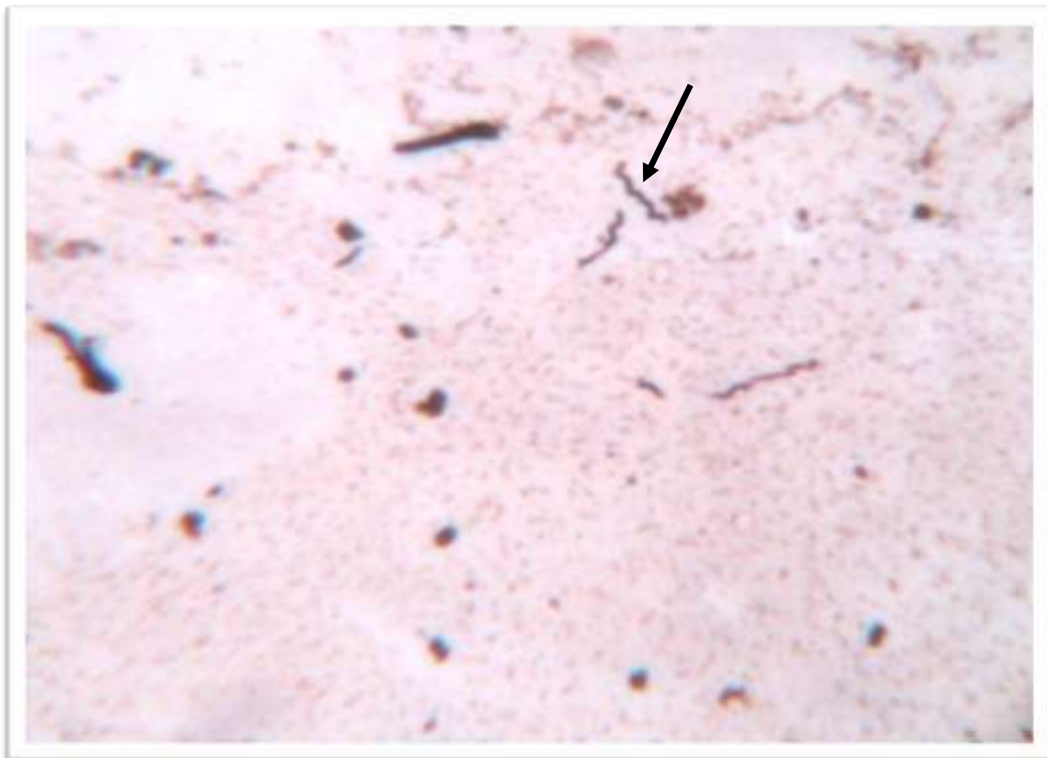


Photograph No. 1: Armamentorium for sample collection

Photograph No. 2: Sample collection

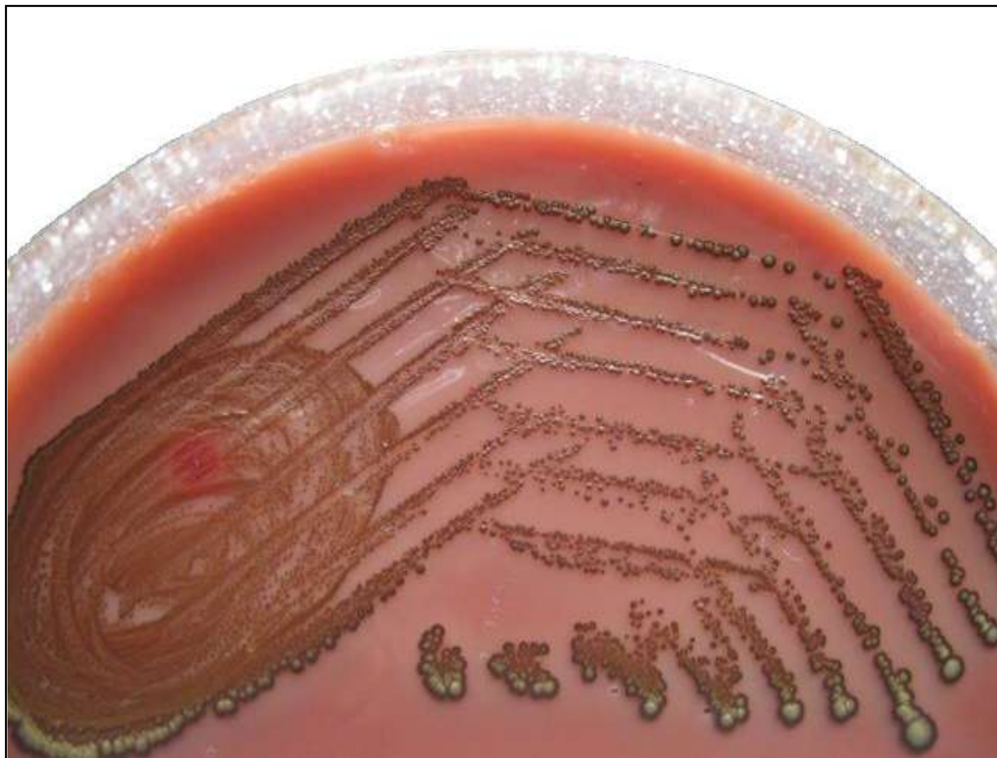
Photograph No 3: Fluid thioglycolate medium

Photograph No.4: McIntosh Fildes jar



Photograph No. 5: Modified Fontanna stained direct plaque sample smear showing spirochetes

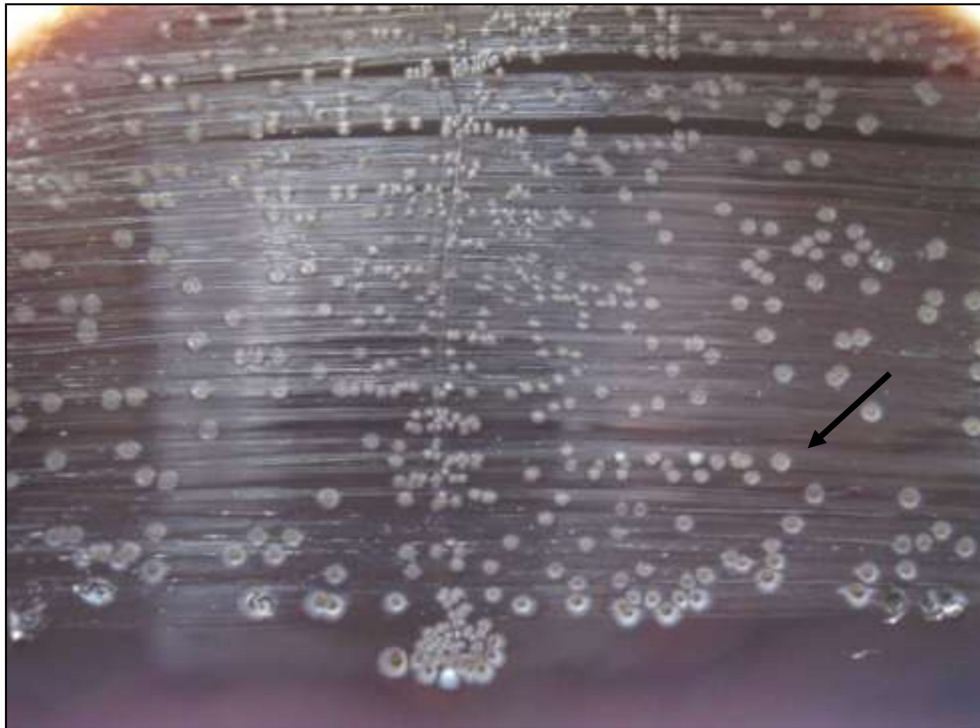
Photograph No. 6: Growth on primary blood agar plate



Photograph No. 7: Growth of Prevotella intermedia on Brucella blood agar

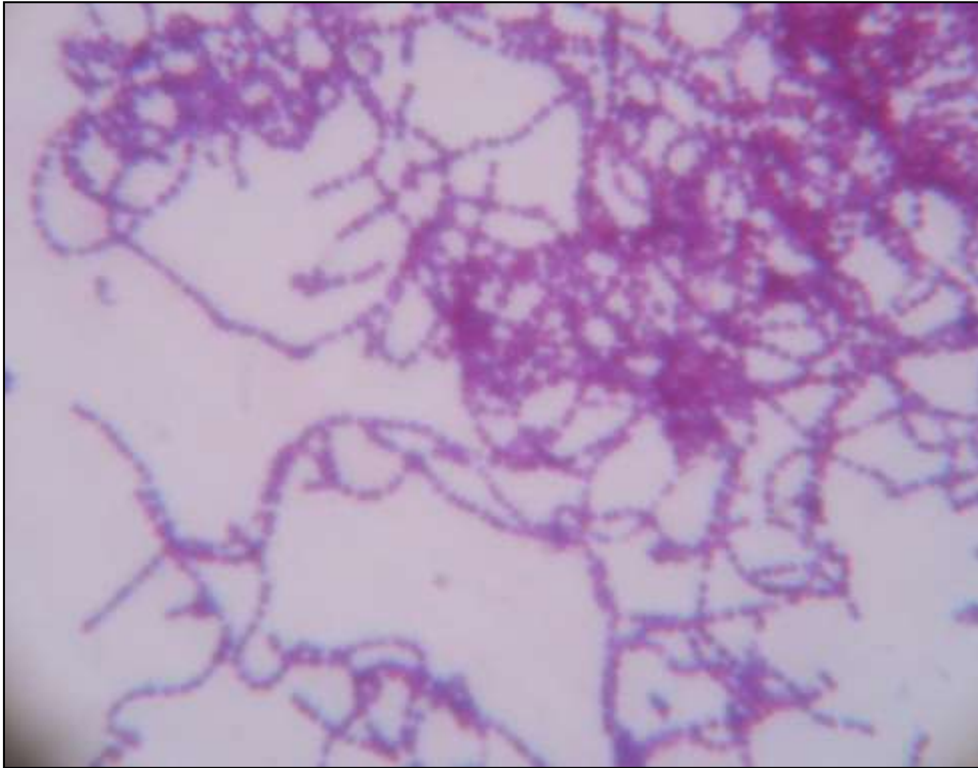
Photograph No. 8: Bacteroides fragilis showing resistance to Kanamycin, Vancomycin, and Colistin discs

Photograph No. 9: Peptostreptreptococcus anaerobicus showing sensitivity to SPS disc on blood agar

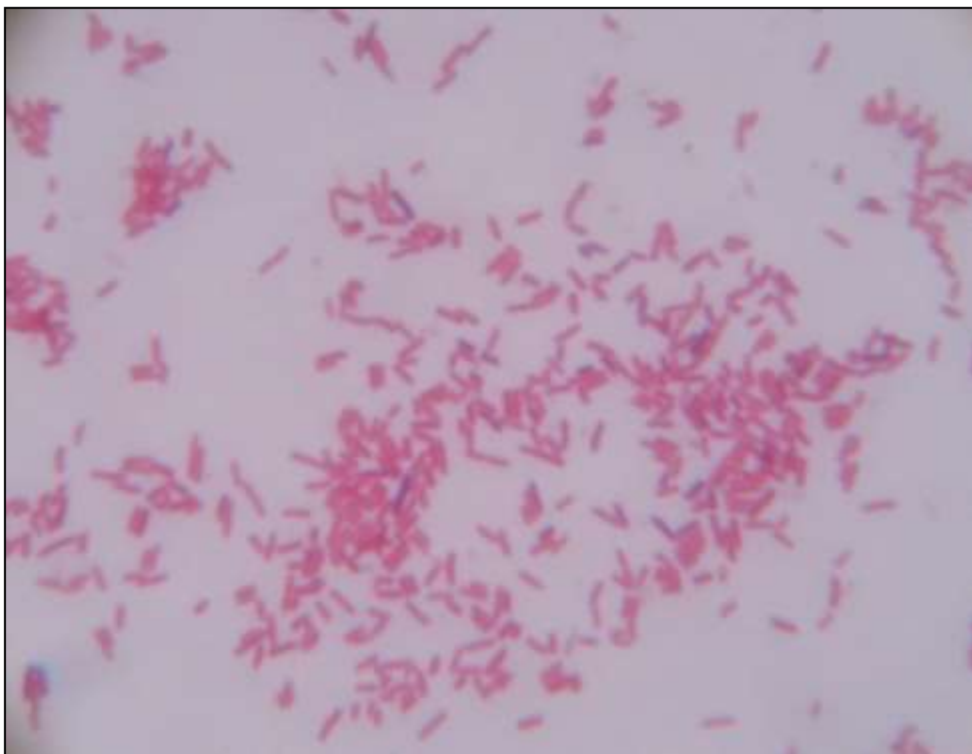


Photograph No. 10: Bacteroides urealyticus on BBE agar (→ Pitting colony)

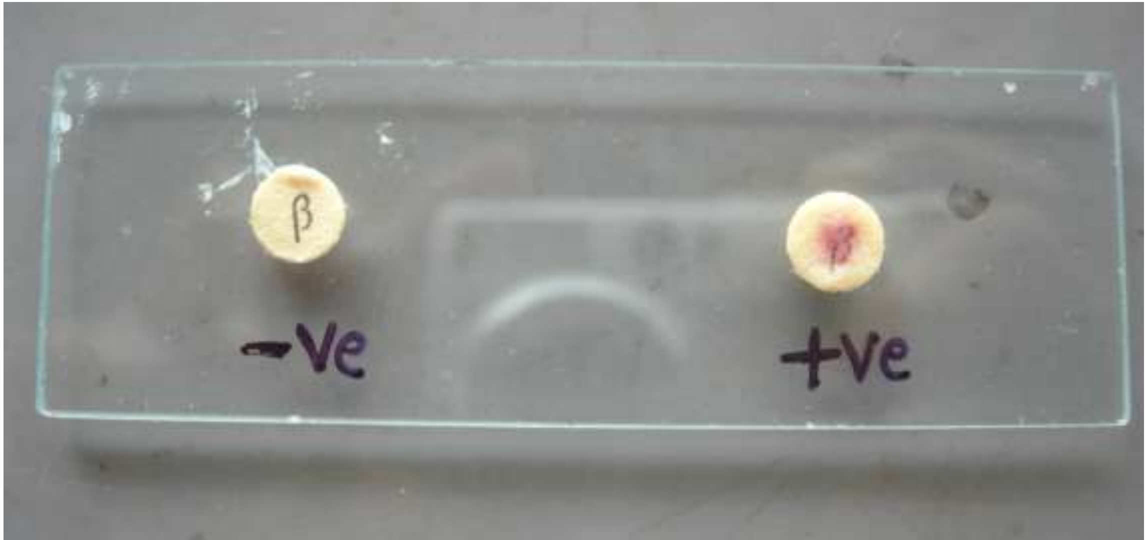
Photograph No. 11: Low power microscopic picture of A. actinomycetemcomitans showing star shaped colony



Photograph No. 12: Gram stain showing Gram positive cocci in chains of Streptococcus mutans



Photograph No. 13: Gram stain showing GNB of Bacteroides fragilis



Photograph No.14: Nitrocefin disc test showing positive reaction

RESULTS

A total of 120 samples (60 pre-treatment and 60 post-treatment) were collected from 60 patients.

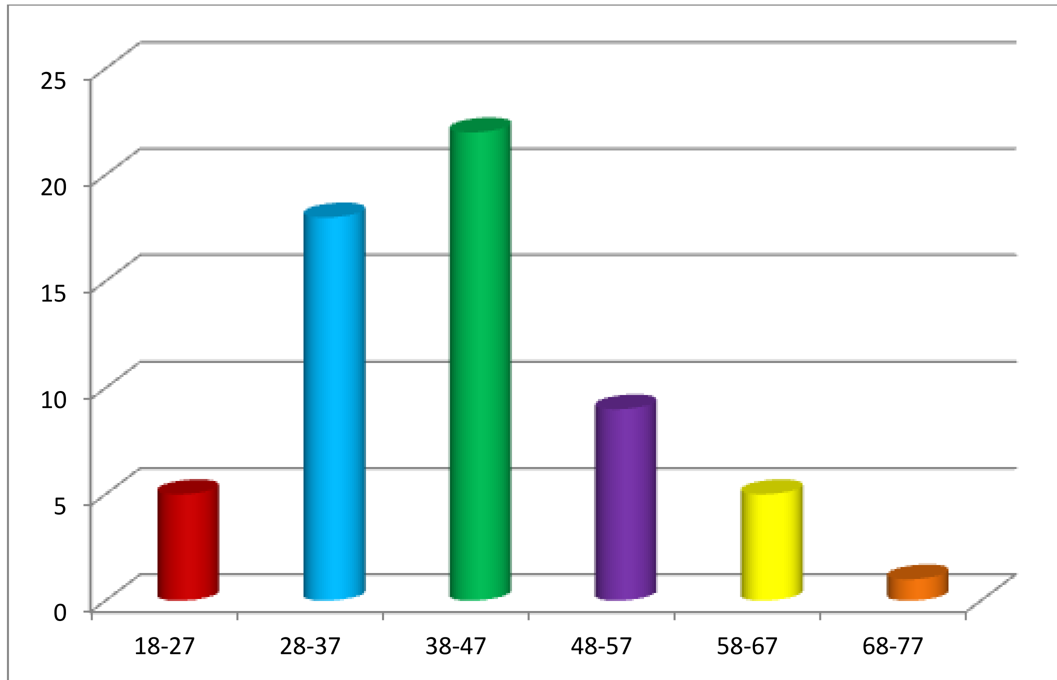
AGE DISTRIBUTION:

Our cases ranged between 22 to 70 years. Maximum number of cases (22) were in the age group of 38-47 years and minimum number of cases (01) seen in 68-77 years. The age distribution is shown in Table 1 and Figure 1.

Table 3: Age distribution of cases. N=60

Age (years)	Number of cases	Percentage
18-27	05	8.33
28-37	18	30.00
38-47	22	36.67
48-57	09	15.00
58-67	05	8.33
68-77	01	1.67

Figure 3: Age distribution of cases



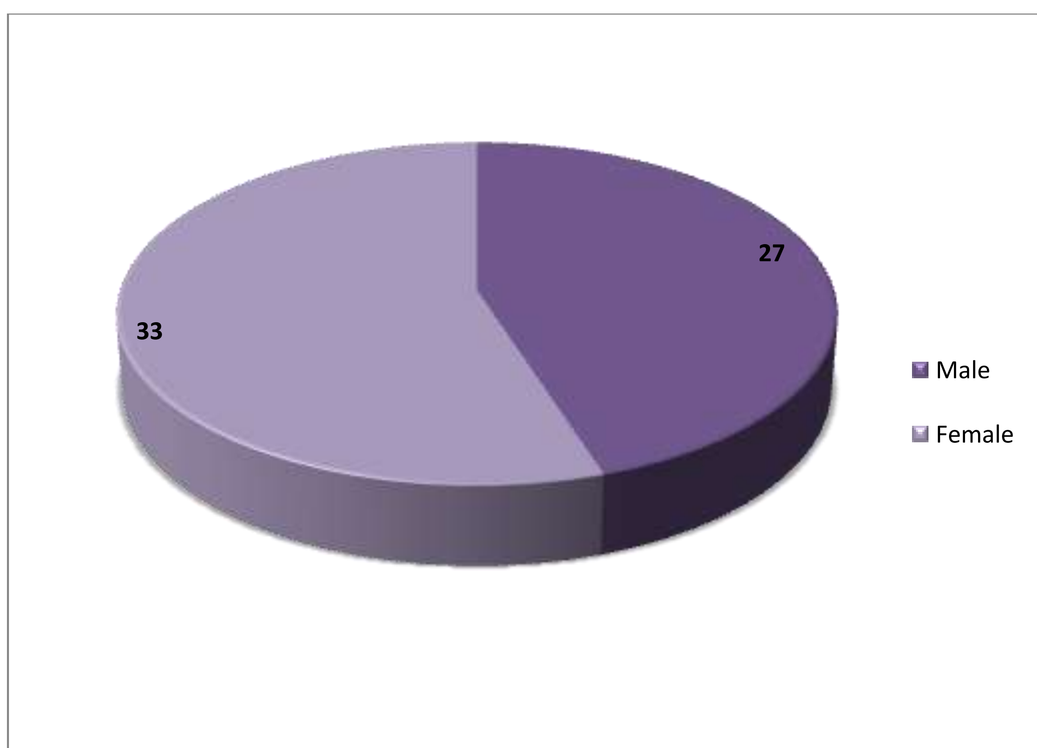
SEX DISTRIBUTION:

Out of 60 cases, 27 (45%) were male and 33 (55%) were female.

Table 4: Sex distribution of cases

Male (%)	Female (%)
27 (45%)	33 (55%)

Figure 4: Sex distribution of cases



ISOLATES OBSERVED

60 samples yielded 121 isolates of which 47 (78.34%) were polymicrobial, 7 (11.66%) were monomicrobial and oral commensals were grown in 6 (10%) cases.

Table 5: Distribution of isolates

Type of isolates	Number of cases	Percentage
Polymicrobial	47	78.34
Monomicrobial	7	11.66
Oral commensals	6	10

Out of 47 polymicrobial isolates 27 (57.45) were combination of 2 isolates and 20 (42.55) were combination of 3 isolates.

Table 6: Polymicrobial isolates

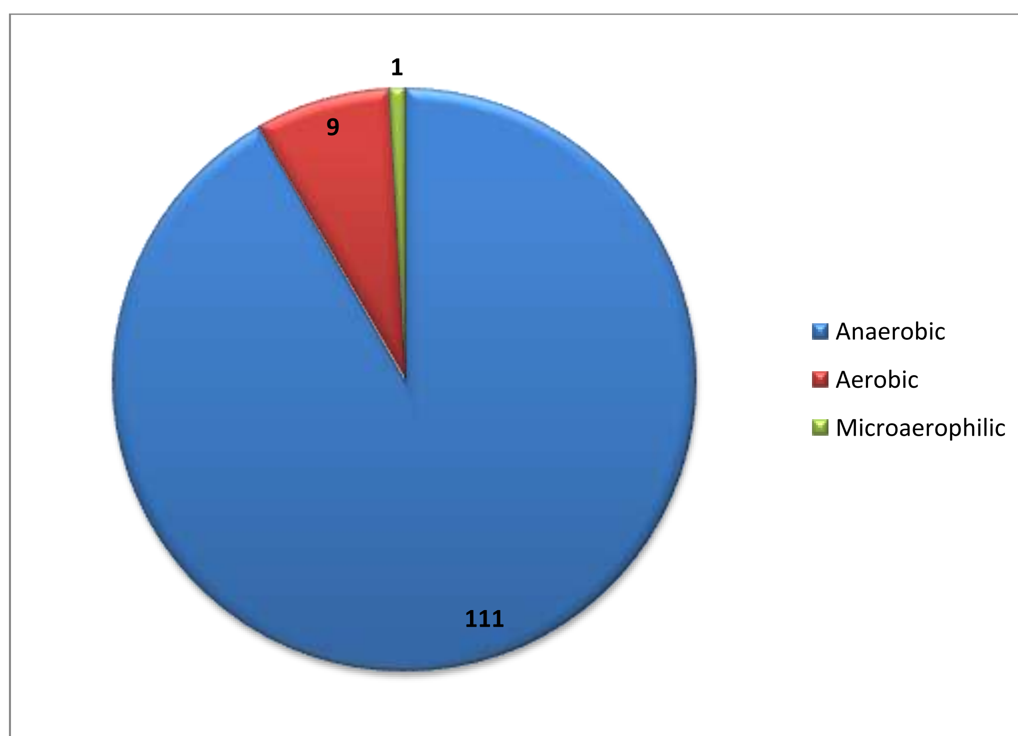
Type of isolates	Number of cases	Percentage
2 isolates	27	57.45
3 isolates	20	42.55

Out of 121 isolates 111 (91.74%) were anaerobic, 9 (7.43%) were aerobic and 01 (0.83%) was microaerophilic.

Table 7: Distribution of type of isolates

Type of isolates	Number of cases	Percentage
Anaerobic	111	91.74
Aerobic	09	7.43
Microaerophilic	01	0.83

Figure 5: Distribution of type of isolates



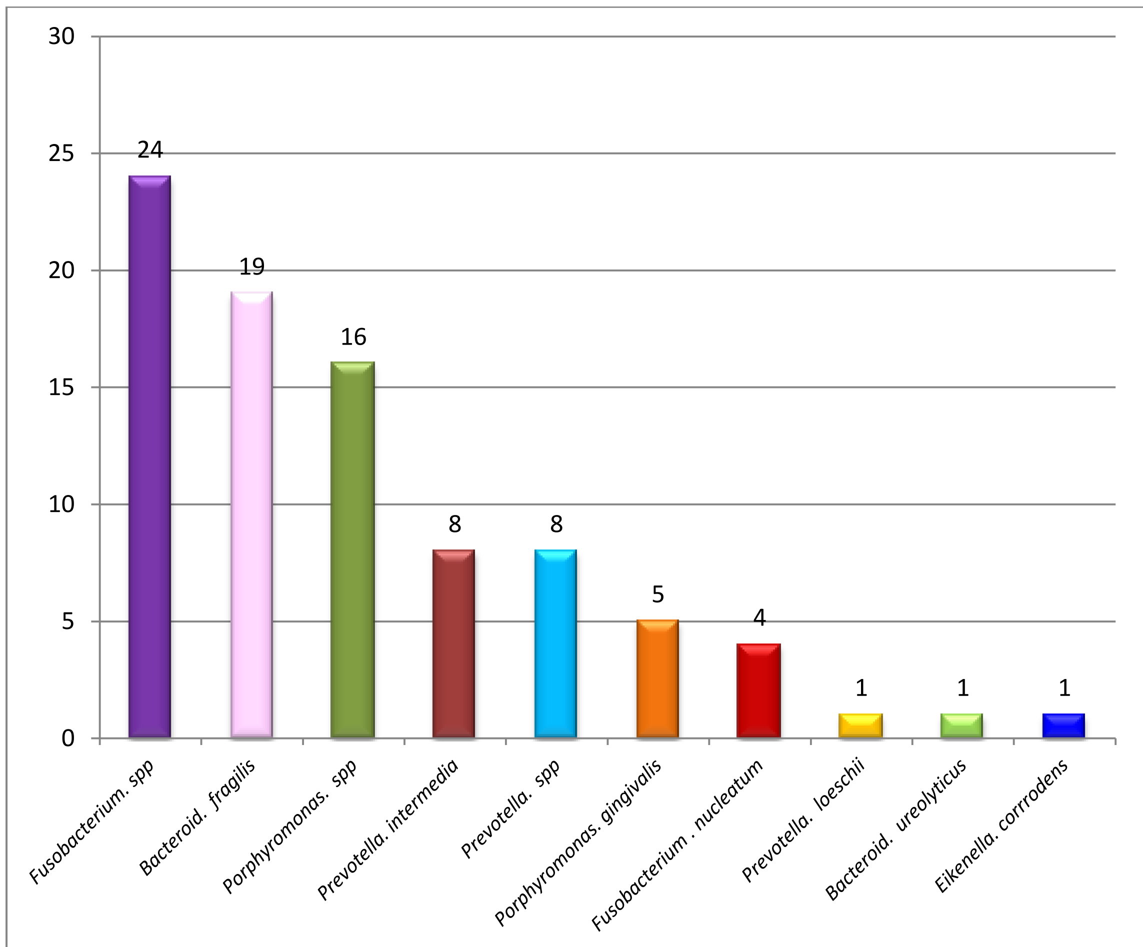
Anaerobic isolates:

Out of 111 anaerobic isolates 87(78.4%) were GNB and 24 (21.6%) were GPC. Among GNB *Fusobacterium spp* was the most common isolate.

Table 8: Anaerobic GNB isolates

Type of isolates	Number of cases	Percentage
Fusobacterium. spp	24	27.60
Bacteroides. fragilis	19	21.84
Porphyromonas. spp	16	18.74
Prevotella. intermedia	8	9.20
Prevotella. spp	8	9.20
Porphyromonas. gingivalis	5	5.74
Fusobacterium . nucleatum	4	4.60
Prevotella. loeschii	1	1.14
Bacteroides. ureolyticus	1	1.14
Eikenella. corrrodens	1	1.14

Figure 6: Anaerobic GNB isolates

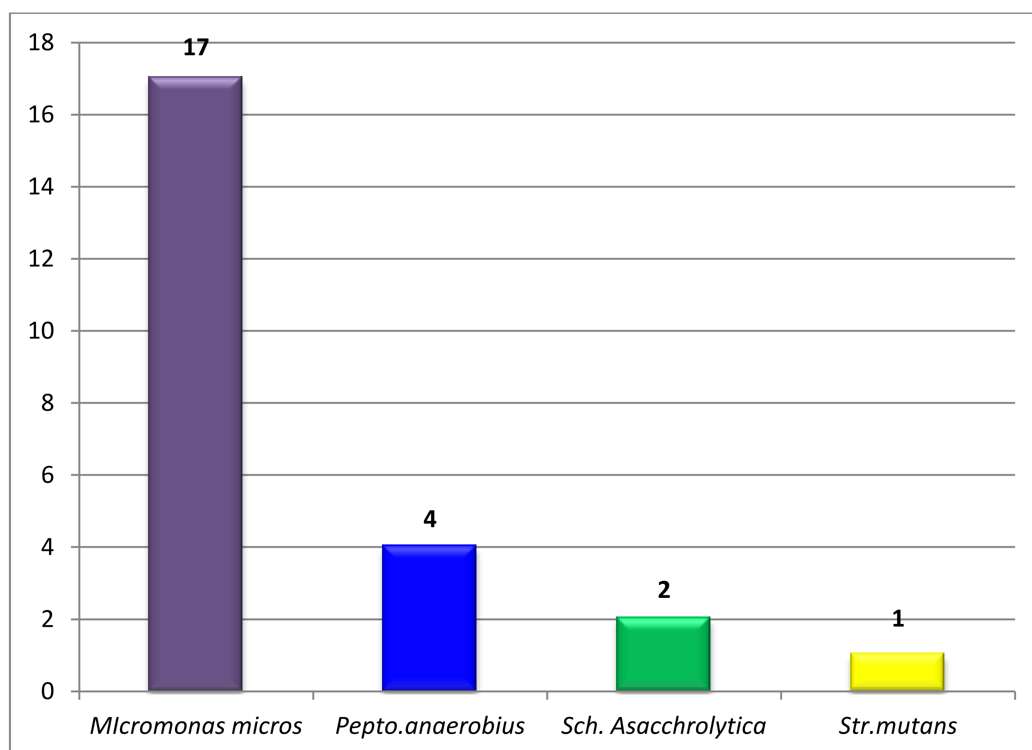


Out of 24 GPC, most common isolate was *Micromonas. micros*

Table 9: Anaerobic GPC isolates

Type of isolates	Number of cases	Percentage
Micromonas. Micros	17	70.83
Peptostreptococcus. Anaerobius	4	16.67
Schleiferella. Asacchrolytica	2	8.33
Streptococcus. Mutans	1	4.17

Figure 7: Anaerobic GPC isolates.



Aerobic isolates:

Table 10: Aerobic isolates

Type of isolates	Number of cases	Percentage
Staphylococcus. aureus	03	33.33
Streptococcus. pneumoniae	03	33.33
Klebsiella. pneumoniae	03	33.33

Microaerophilic:

The only microaerophilic organism isolated was *Aggregatibacter.actinomycetemcomitans.*

Spirochetes:

Spirochetes (*Treponema denticola*) were observed in 19(31.66) cases by modified Fontana staining from the direct plaque sample smears.

Extended spectrum beta-lactamase production:

Out of 121 isolates 16 (13.22%) showed ESBL production and all of them were anaerobic GNB. None of the aerobic isolates were ESBL producers. Five isolates showed ESBL production by double disc diffusion test, 12 showed ESBL production by potentiated disc diffusion test. All 16 isolates showed ESBL production by Nitrocefine disc method indicating Nitrocefine disc method is the best method.

Fusobacterium. spp was the most common isolate showing ESBL production

Table 11: Results of ESBL test

Isolates	Double disc diffusion test	Potentiated disc diffusion test	Nitrocefine disc method
Porphyromonas spp	+	+	+
Bacteroides. fragilis	+	+	+
Fusobacterium. spp	+	+	+
Bacteroides. fragilis	-	-	+
Bacteroides. fragilis	-	+	+
Fusobacterium. nucleatum	+	+	+
Fusobacterium spp	-	-	+
Fusobacterium spp	-	+	+
Bacteroides. fragilis	-	-	+
Prevotella. intermedia	-	+	+
Fusobacterium spp	-	+	+
Fusobacterium spp	-	+	+
Fusobacterium spp	-	+	+
Bacteroides fragilis	-	+	+
Bacteroides fragilis	-	-	+
Fusobacterium spp	+	+	+
	05	12	16

Table 12:Beta-lactamase producing anaerobic GNB

Type of isolate	Number of cases	Percentage
Fusobacterium spp	7	43.75
Bacteroides. Fragilis	6	37.5
Fusobacterium. Nucleatum	1	6.25
Porphyromonas spp	1	6.25
Prevotella intermedia	1	6.25

Table 13: Antibiotic susceptibility pattern of aerobic isolates

Isolates	**No. of isolates**	**Antibiotic sensitivity**											
		Cf		**E**		**Ac**		**Ce**		**Ak**		**Ox**	
		S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
Staph. aureus	03	3	0	3	0	3	0	3	0	NT	NT	3	0
Str. pneumoniae	03	2	1	3	0	3	0	3	0	NT	NT	3	0
Kleb. pneumoniae	03	2	1	NT	NT	3	0	3	0	3	0	NT	NT

[S= Sensitive, R= Resistant, NT= Not tested, Cf=Ciprofloxacin, E=Erythromycin,

Ac= Amoxiclav, Ak= Amikacin, Ox= Oxacillin]

Statistical analysis done using “paired t” test

Table No 14: Results of semiquantitative study:

Pre-treatment (n=121)	Post-treatment (n=110)	Mean reduction (n=110)
750160±2267760= $7.5x10^5±2.2x10^6$	123.4±233.76	802530± 2367570 $8x10^5±2.3x10^6$

Paired t = 3.555, P= 0.001

Using “Paired t” test “p” value is significant indicating significant reduction in colony count after phase-I periodontal therapy.

DISCUSSION

The complexity of the subgingival microbiota has been recognized since the First microscopic examination of this ecosystem by Van Leeuwenhoek in 1683.[96] Since that time, numerous studies have evaluated the composition of plaque using light and electron microscopy, cultural techniques and more recently immunologic or DNA probe techniques. All techniques reinforce Van Leeuwenhoek's initial observation that subgingival plaques are comprised of a large complex mixture of bacterial species. Indeed it has been estimated that 400 or more species reside in this area. [62]

The goal of the present investigation was to attempt to understand the nature of the microbial complexes that exist in subgingival plaque.

In the present study, the cases ranged from 20-70 years with maximum number of cases in the age group of 38-47 years. In contrast, in a study conducted in France observed periodontitis cases in age group of 18-52 years (mean age 26.8 ±8.2 years).[81] In an another study conducted in Manipal cases ranged between ages of 30-60 years.[97] In an another study conducted at Greece Athens observed rapidly progressive periodontitis cases in the age group of 14-35 years.[98] It is a good old concept in the literature that the prevalence of periodontitis increases as age increases because of loss of periodontal tissue support, however maximum number of cases in our study were in adult group as observed by the previous authors. [99]

The male to female ratio in our study is 0.8:1. i.e females are more affected than males as observed in studies by Sixou et al, Salari et al, Daniluk et al, Nonnenmacher et al and Socransky et al[6,62,81,100,101] In contrast in a study conducted

in France, the ratio is M:F::1.3:1.[10] However there is no established, inherent difference between men vs women in their susceptibility to periodontitis.[99]

Table 15: Comparison of sex distribution

Sixou et al [81]	Sixou et al [102]	Kamma et al [98]	Salari et al [100]	Daniluk et al [6]	Nonnenmacher et al [101]	Socransky et al [62]	Present study
0.625:1	1.33:1	1:1	0.82:1	0.61:1	0.70:1	0.75:1	0.81:1

A 78.34% of our isolate were polymicrobial in nature in comparison to 11.06% of monomicrobial isolates. Our observations are consistent with the literature. Polymicrobial nature of periodontal plaque has been well established. In a study conducted in France, Germany, Pune and Haryana observed 97.14%, 100%, 72.28% and 97.3% of their isolates to be polymicrobial respectivly.[4,81,101,103] In contrast in a study conducted in Iran, observed 18.28% of their isolates to be polymicrobial. [100] The evidence concerning coaggregation of oral bacteria was first reported by Gibbons & Nygaard.[104] Further studies have shown possible bacterial aggregation between gram-positive and gram positive gram positive and gram negative as well as gram negative and gram negative bacteria. [104] Different types of surface components including carbohydrates, fimbriae and outer membrane proteins have the ability to participate in coaggregation reactions between oral bacteria. [105] A second mechanism of bacterial interactions involves the production of growth-stimulating factors which may contribute to bacterial successions in periodontal sites. [106-09,]

Out of 78.34% of polymicrobial isolates 57.44% were two isolate, 42.55% were three isolates. In a study conducted in Pune India, observed two isolates in

91.66% and three in 8.2%.In an another study conducted in Haryana, India observed two isolates in 8%, three & four isolates 40% each, five isolates in 12%.[4,103]

The results of present study show the diversity of anaerobic bacteria in chronic periodontitis. Anaerobic bacteria were isolated in 91.74% of periodontitis cases. Various studies from India and other countries showed an isolation rate of strict anaerobes ranging from 42-100% in periodontitis cases.[4,100,103,110] In the two different studies conducted in France, detected 91.42% and 80.77% of anaerobic isolates. In a study by Mane et al detected 83% anaerobes in their study. Whereas studies conducted in Iran, Poland, Germany, and Pune detected competitively lower number of anaerobes i.e 41.22%, 57.1%, 53.84%, 42% and 64.25% respectively.[4,6,8,100,101,103] The varying recovery rates of isolation can be due to varying criteria of patient selection, culture method employed, geographical differences and molecular technique used for identification. [4,103]

Table 16: Comparison of isolates in different studies

	Sixou et al [81]	**Sixou et al[102]**	**Salani et al [100]**	**Nonnenmcher Et al [101]**	**Mane et al [103]**	**Saini et al [4]**	**Daniluk et al [99]**	**Present study**
Anaerobic(Values in percentage)	80.77	91.42	41.22	53.84	83	64.05	57.1	91.74
Aerobic	-	-				35.95	42.9	7.43
Capnophilic	-	-	58.77	46.16	-	-	-	0.83

In the present study gram negative anaerobes (78.4%) were predominantly isolated than the gram positive (21.6%) in the periodontitis cases. And our observations are comparable to the literature.[4,6,8,100,103] *Fusobactrium spp* (27.60%), *Bacteroids fragilis* (21.84%), *Porphyromonas spp* (24.48%), *Prevotella intermedia* (9.20%), *Fusobactrium nucleatum* (4.60%) and *Prevotella spp* (9.20%) were the

commonest organisms isolated. Other workers have isolated similar anaerobes but in varying proportions. In an Indian study at Pune, reported a much higher number of these isolates *Prophyromonas spp* 40%, Prev intermedia 12%, *prev spp* 14%, *Fusobactrium nucleatum* 24% and *Bacteroids* 5%. Another Indian study in Haryana reported higher number of *Prevotella. intermedia* (17.6%). *Fusobacterium nucleatum* (41%), *Fusobacterium spp* (29.41%) were in accordance with our results. A study at France reported higher number of *Prevotella. intermedia* (15.78%) and *Fusobacterium nucleatum* (7.01%). While studies conducted at Iran and Germany reported much lower number of these isolates.[4,6,8,100,101-103] comparision of spectrum of anaerobes in different studies is shown in Table 17.

Beena et al concluded from their study on periodontal pathogen that as the infection progress the proportion of anaerobes especially gram negative bacilli like Prevotella, Porphyromnas and fusobacterium increases.[111]

Table 17: Comparison of spectrum of gram negative anaerobes in the literature

	Mane et al [103]	Salari et al [100]	Saini et al [4]	Sixou et al [62]	Nonnenmacher et al [101]	Present study
Fusobacterium Spp	-	-	29.4	7	-	27.60
Bacteroids	-	-	-	-	-	21.84
Porphyromonas spp	48	24.5	14	5.26	6.4	24.48%
Prevotella intermedia	12	7	17.4	15.78	10.2	9.2
Prevotella Species	14	5	6	-	-	9.2
Fusobactrium nucleatum	24	-	41	7.01	1.3	4.6
Prevotella	-	-	-	-	-	1.14
Bacteroids	05	-	-	3.10	-	1.14
Eikenella cordons	-	22	-	-	-	1.14
Veillonella Spp	09	-	-	-	-	-

The gram positive anaerobic cocci constitute 21.6% of total anaerobic isolates in our study. Out of which *Micromonas micros* (70.83%) were the commonest isolates other GPC isolated in our study are *Peptostreptococcus anaerobius* (16.67%), *Schleiferella asachrolytica* (8.33%) and *Streptococcus mutans* (4.17%)

A study at Pune and Germany reported *Micromonas micos* in 23% and 51% respectively, which is much lower than our study. Sixou et al observed 52.94% of *Micromonas. micros,* 5.88% of *Peptostreptococcus. anaerobius*. In another study by Sixou et al, there were 14.28% each of *Micromonas micos* and *Peptostreptococcus. anaerobius.* Salari et al reported 2.9% and winkellhoff reported 70% which is

accordance with our study. *Micromonas micros* is considered to be a pathogen in the etiology of mixed anaerobic infections more often and in increased percentage from patients with periodontitis, especially in diseases active subjects. [112-116] Our results are in accordance with previous observation suggesting that *Micromonas micos* may be associated with periodontitis.

In the present study group, aerobes constituted 7.43%, while the studies conducted in Poland and Haryana iolated 42.9% and 14% of aerobes respectively. The aerobes isolated are *Stphylococcus aureus* (33.33%), *staphylococcus aureus* (33.33%), *Streptococcus pnemoniae* (33.33%) and *Klebsiella pneumoniae* (33.33%) and our results are in accordance with studies of Daniluk et al who reported *Staphylococcus species* (16.66%) and *Streptococcus species* (38.88%). Saini et al reported 42.85% of *stphyloccous aureus* and 44.44% of *Klebsiella spp*. They also reported *Escherichia coli, Pseudomonas spp* (14.28%) and non fermenters (28.5%) which are not observed in our study. [4,6]

The only capnophilic (microaerophilic) bacteria isolated in the present study is *Aggregatibacter actinomycetemcomitans* which accounts for 0.83% of total bacterial isolates. The isolation rate of *A. actinomycetemcomitans* from adult periodontitis was reported to be 20.9% by Zambon et al, 53% by Antony et al, 9% by Nonnenmacher et al, 20.6% by Salari et al and 14.6% by Saini et al. These results show that *A. actinomycetemcomitans* seems to play a significant role in periodontitis along with the other anaerobes. [97,100,101,111]

Spirochetes such as *Trepanoma denticola* were observed by modified Fontana staining in 31.66% of cases.

The present study showed 13.66 of anaerobic isolates as ESBL producers. Among them *Fusobacterium spp* (43.75%) was most common isolate showing ESBL production. Others are 37.5% of *Bacteriod fragilis*, 6.25% each of *Fusobactrium nucleatum, Porphyromonas species and Prevotella intermedia.*

Of the selected putative periodontal species, strains of *P. intermedia, B. fragilis and F.nucleatum* were beta-lactamase-positive which is much lower than the 52% that has been reported earlier in strains from the USA. The difference may be explained to the fact that Appelbaum et al (1991) have tested also non-oral *P. intermedia* strains; it may however also be related to previous antibiotic use. The same explanations may be true for the low number of beta-lactamase positive *F. nucleatum* strains in the present study (13%). In comparison, Appelbaum et al (1991) and Jacobs et al (1990) found a prevalence of 21% and 23%, respectively.[86,117,118]

In the present study, no beta-lactamase activity among *P. gingivalis* strains was observed, which confirms the findings of Pajukanta et al. (1993), Sixau et al and Winkelhoff et al. However these findings are in contrast to results by Jacobs et al. (1990) and Legg & Wilson, (1990). We have no explains for these different observations and this matter should be subject of future research.[110,119-121]

In this study there was significant decrease in bacterial count following scaling and root planning with p value of <0.001, as observed in the literature. Two systemic reviews have suggested that adjuvant systemic antibiotic administration will have better outcome than SRP alone. [122,123]

Since the concept of dental infection has been bacteriologically non-specific and offers no rationale for antimicrobial treatment, careful assessment and isolation of both aerobes and anaerobes is utmost importance in the treatment of orodental infection. [4]

CONCLUSION

This study has shown that anaerobic bacteria are important cause of chronic periodontitis, along with aerobes and microaerophilic organisms. *Fusobacterium. spp, Bacteroides fragilis , Porphyromonas. spp* and *Prevotella. intermedia* are the commonest anaerobic pathogens. Cultural methods are still economical and gold standard in microbiology. Isolation of these anaerobic organisms and their antibiotic susceptibility should be determined to start appropriate antibiotic therapy as early as possible to overcome the morbidity and mortality associated with this disease and to prevent the development of resistant strains. Considering the scarce data on microbial flora in the Indian population, further studies for assessment of microbial profile in various forms of periodontitis should be carried out.

SUMMARY

The present study was conducted in the department of Microbiology, J.N. Medical College, Belgaum for a period of 1 year from January 2010 to December 2010.

The samples were collected from outpatient of Periodontalogy department of V.K. Institute of Dental Sciences, Belgaum.

- A total of 120 samples were collected (60 pretreatment and 60 post treatment)
- Our cases ranged between 22 to 70 years. Maximum number of cases were in the age group of 38-47 years and minimum number of cases seen in 68-77 years.
- Male to Female ratio observed in our study is M:F::0.8:1
- 60 samples yielded 121 isolates of which 78.34% were polymicrobial, 11.66% were monomicrobial and oral commensals were grown in 10% cases.
- Out of 121 isolates 91.74% were anaerobic, 7.43% were aerobic and 0.83% was microaerophilic.
- Out of 111 anaerobic isolates 78.4% were GNB and 21.6% were GPC. Among GNB *Fusobacterium spp* was the most common isolate and among anaerobic GPC *Micromonas micors* was the commonest.
- Aerobes were less commonly associated with periodontitis.
- The only microaerophilic organism isolated was *Aggregatibacter.actinomycetemcomitans*
- Spirochetes were observed in 31.66% of cases.

- Out of 121 isolates 13.22% showed ESBL production and all of them were anaerobic GNB. None of the aerobic isolates were ESBL producers.
- Semiquentitative study was significant with P value of 0.001.

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ANNEXURE – I: CONSENT FOR PARTICIPATION IN RESEARCH

You are requested to enroll yourself in study titled **"CLINICO BACTERIOLOGICAL STUDY OF ADULT PERIODONTITIS WITH SPECIAL REFERENCE TO DETECTION OF BETA-LACTAMASE PRODUCING ANAEROBIC BACTERIA"** conducted by Dr. Kirtilaxmi. K. Benachinmardi, PG student in Microbiology, KLE's Dr. Prabhakar Kore Hospital and Medical Research Centre, KLE University, Belgaum.

You have been requested to participate in research because you are into the study group. During the study you will be asked some questions and you are supposed to answer to the best of your knowledge.

Your participation in research is voluntary. Your decision whether or not to participate in the study will not affect your relationship with KLE's V.K. Institute of Dental Sciences. If you decide to participate you are free to withdraw at any time.

The purpose of research is to isolate and identify the predominant aerobic and anaerobic microflora associated with adult generalized periodontitis. Semiquantitative study of isolated microflora of adult generalized periodontitis in pre-treatment & post-treatment groups. To test the anaerobic isolates for the production of enzyme beta-lactamase in pre-treatment group.

PROCEDURE INVOLVED:

Bacteriological study of the material obtained from dental plaque will be done to detect the aerobic & anaerobic microflora causing periodontitis.

RISKS AND BENEFITS:

There are no extra risks involved and benefits are to be evaluated.

PRIVACY AND CONFIDENTIALITY

The only people to know that you are a research subject are members of the research team. No information about you or provided by you during research will be disclosed to others without your written permission, except:

1. In emergency to protect your rights and welfare.
2. If required by law.

AUTHORIZATION TO PUBLISH RESULTS

When the results of the research are published or discussed, in a conference no information will be displayed that would disclose your identity. Any information that is obtained in connection with this study and that can be identified with you will remain confidential.

FINANCIAL INCENTIVES FOR PARTICIPATION

You will not be paid/offered any gifts for participating in the research. You will not be reimbursed for expenses.

I undersigned --------------------- have been explained in my vernacular language about the study and my participation in the study is voluntary. If I want, I can withdraw at any time. Also I have been given enough time to clear my doubts and rights as study participant.

In case you have any questions related to the study, you can contact Dr.Kirtilaxmi.K.Benachinmardi(9886358982).

In case you have any questions about your rights as a study participant, you can contact Dr. V. D. Patil.

CONSENT STATEMENT

Signature or left thumb print of participant or legally authorized representative.

Participants name ---------------------------- Signature ---------------

Witness Name Signature ----------------------- Signature ---------------

Experimenters Name Signature ----------------- Signature ---------------

Date :

Place :

ANNEXURE – II: PROFORMA

PRE-TREATMENT-

Sl No. OP No.:

Name: Unit:

Age: Date:

Sex: Specimen:

Adress:

Chief comlaints:

Any other complaints:

Past history:

Treatment history:

Oral examination:

Periodontal pocket depth-

Plaque Index(Silvess & Loe)-

16	12	24
44	32	36

Gingival Index(Loe & Silvess)-

16	12	24
44	32	36

Clinical diagnosis:

Microbiological investigations:

Microscopic examination- Gram stain

Fontana stain

Culture –

Aerobic culture

Growth on MacConkey agar:

Processing:

- Indole test:
- Citrate utilization:
- H2S production:
- Mannitol fermentation
- Urea hydrolisation;
- TSI media:

Tests:

- Oxidase test: - Motility:
- Catalase test: - OF :
- Coagulase test- Slide:

  Tube :

Antibiotic sensitivity testing:

- Ciprofloxacin (Cf) :
- Erythromycin(E) :
- Amoxycillin/ Clavulinic acid (Ac) :
- Cefotaxime (Ce) :
- Amikacin (Ak) :
- Oxacillin (Ox) :

Microaerophilic culture

Growth on 5% Blood agar:

BHI agar:

Gram staining of each colony:

Observation of colony under low power microscope:

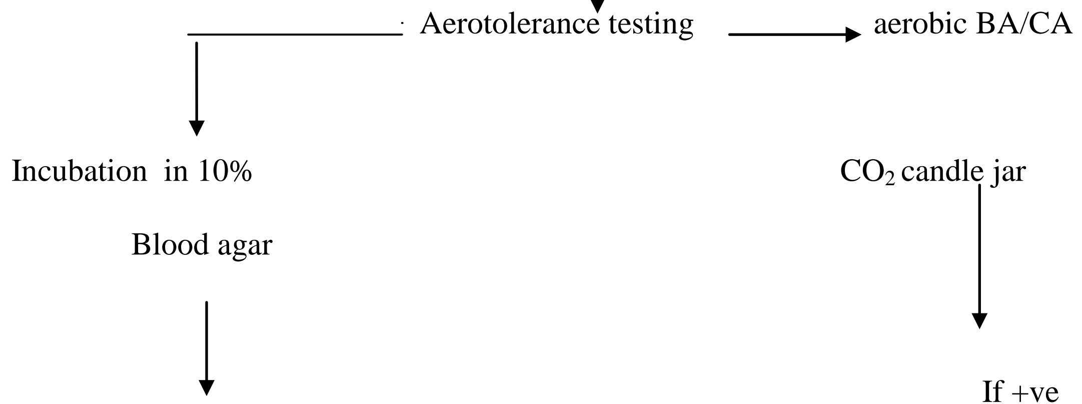
(From BHI agar plate)

Anaerobic culture

Growth on blood agar supplemented with hemin & vitamin k:

KVLB:

BBE:



Proceed as aerobic

Colony morphology

Pigment

Haemolysis

Fluoresence

Pitting

Antibiotic identification disc-SPS disc

Gram stain of individual colony

Spot indole test

Catalase test

Nitrate test

Susceptibility to-Vancomycin:

Kanamycin:

Colistin:

Tests for beta-lactamase-

Sl.No.		Positive	Negative
1.	Double disc diffusion test		
2.	Potentiated disc diffusion test		
3.	Nitrocefin disc method		

POST-TREATMENT-

Sl No. OP No.:

Name: Unit:

Age: Date:

Sex: Specimen:

Adress:

Chief comlaints:

Any other complaints:

Past history:

Treatment history:

Oral examination:

Periodontal pocket depth-

Plaque Index(Silvess & Loe)-

16 12 24

44 32 36

Gingival Index(Loe & Silvess)-

16 12 24

16 12 36

Clinical diagnosis:

Microbiological investigations:

Microscopic examination- Gram stain

Fontana stain

Culture –

Aerobic culture

Growth on MacConkey agar:

Processing:

- Indole test:
- H2S production:
- Urea hydrolisation;
- Citrate utilization:
- Mannitol fermentation
- TSI media:

Tests:

- Oxidase test: - Motility:
- Catalase test: - OF :
- Coagulase test- Slide:

  Tube :

Antibiotic sensitivity testing:

- Ciprofloxacin (Cf) :
- Erythromycin(E) :
- Amoxycillin/ Clavulinic acid (Ac) :
- Cefotaxime (Ce) :
- Amikacin (Ak) :
- Oxacillin (Ox) :

Microaerophilic culture

Growth on 5% Blood agar:

BHI agar:

Gram staining of each colony:

Observation of colony under low power microscope:

(From BHI agar plate)

Anaerobic culture

Growth on blood agar supplemented with hemin & vitamin k:

KVLB:

BBE:

Single colony of each distinct type

↓

Aerotolerance testing → aerobic BA/CA

Incubation in 10%

CO_2 candle jar

Blood agar

If +ve

Proceed as aerobic

Colony morphology

Pigment

Haemolysis

Fluoresence

Pitting

Antibiotic identification disc-SPS disc

Gram stain of individual colony

Spot indole test

Catalase test

Nitrate test

Susceptibility to-Vancomycin:

Kanamycin:

Colistin:

ANNNEXURE – III: MASTER CHART

Sl no	Hospital no	Age in Yrs	Sex	Aerobic organism Isolated	Semiquantitation CFU/ml		Antibiotic sensitivity						Gram stain	Fontana stain(Spirochete)	Anaerobic organism isolated	Semiquantitation CFU/ml		Test for β-lactamase		
					Pre treatment	Post treatment	AC	AK	Ce	Cf	E	OX				Pre treatment	Post treatment	DD	PDD	Nitrocefin disc test
1	V-3385	22	M	S. aureus	10^3	NG	S	NT	S	S	S	S	GPC GNB	Not seen	P. intermedia	10^5	10^2	-	-	-
2	V-2889	58	M	S. pneumoniae	10^5	10^2	S	NT	S	S	S	S	GPC	Not seen	NOGC	-				
3	W-3819	25	F	NOGC									GNB	seen	Porphyromonas spp B. fragilis	10^4 $2x10^5$	10^2 10	+ -	+ -	+ -
4	V-3818	30	F	S. aureus	10^3	NG	S	NT	S	S	S	S	GPC GNB	Not seen	P. intermedia	10^6	10^2	-	-	-
5	W-3784	65	M	NOGC									GPC GNB	Not seen	M. micros Fusobacterium. spp	10^5 $1.8x10^5$	10 10^2	- -	- -	- -
6	w-3794	20	F	NOGC									GPC GNB	Not seen	Fusobacterium spp M. micros Schleiferella asacharolytica	10^4 $2x10^5$ 10^4	10^2 10^2 10	- - -	- - -	- - -
7	B-245	40	M	K. pnemoniae	10	NG	S	S	S	S	NT	NT	GNB	seen	Bacteroid. fragilis	10^5	10^2	+	+	+
8	B-254	33	M	NOGC									GNB	Not seen	Fusobacterium spp	$2x10^5$	10^2	-	-	-
9	B-260	42	M	NOGC									GPC GNB	Not seen	P. anaerobius Prevotella spp	10^4 $2x10^6$	10 10^2	- -	- -	- -
10	B-204	45	F	NOGC									GNB	seen	B. fragilis Porphyromonas. gingivalis	$1.6x10^5$ $1.2x10^6$	10^2 10^2	- -	- -	- -
11	A-2301	45	F	NOGC									GNB	seen	Porphyromonas spp	$2.2x10^5$	MFU	-	-	-

12	B-2200	38	M	NOGC									GPC	Not seen	Schleiferella asacharolytica	10^5	10^3	-	-	-
13	B-2556	70	M	NOGC									GPC GNCB	Not seen	M. micros Prevotella. spp	$2x10^5$ 10^4	10^2 10	- -	- -	- -
14	B-2518	30	M	NOGC									GPC GNB	Not seen	Fusobacterium. spp M. micros Prevotella spp	$3x10^4$ $2x10^6$ $1.2x10^4$	10^3 10^2 10	+ - -	+ - -	+ - -
15	B-3712	50	F	S. pnemoniae	10^2	NG	S	NT	S	S	S	S	GPC GNB	Not seen	B. fragilis	$2.2x10^5$	10^2	-	-	-
16	B-3590	57	M	NOGC									GPC GNB	Not seen	M. micros Fusobacterium spp	10^3 $3.2x10^6$	NG 10^3	- -	- -	- -
17	C-287	35	M	NOGC									GNB	Not seen	B. fragilis P. intermedia	$2.1x10^4$ $2x10^4$	10^2 10^2	- -	- -	+ -
18	C-289	42	M	S. pnemoniae	10^5	10^2	S	NT	S	R	S	S	GPC	Not seen	P. anaerobius	10^4	10	-	-	-
19	C-1269	45	F	Oral commensals									GPC	Not seen	NOGC					
20	C-1275	25	F	NOGC									GNB	seen	F. nucleatum Porphyromonas spp	10^5 $1.2x10^6$	10^2 10^2	- -	- -	- -
21	C-1307	65	M	NOGC									GPC GNB	seen	Porphyromonas. spp B. fragilis P. anaerobius	10^4 $1.4x10^6$ 10^5	10^2 10^2 10	- - -	- - +	- - +
22	C-1315	42	F	NOGC									GNB	seen	B. fragilis Porphyromonas gingivalis	10^5 $2x10^6$	10 10^2	- -	- -	- -
23	B-1138	46	F	Oral commensals									GPC	Not seen	NOGC					
24	C-308	51	F	NOGC									GNB	seen	Porphyromonas spp B. fragilis F. nucleatum	$2x10^3$ $2.2x10^5$ $1.2x10^6$	10 10^2 10^2	- - +	- - +	- - +

25	B-1650	43	F	NOGC									GPC GNB	Not seen	Fusobacterium. spp Prevotella spp Micromonas. micros	$2x10^4$ $2x10^6$ 10^5	10^2 10^2 10	- - -	- - -	- - -
26	C-1573	30	M	NOGC									GNCB	Not seen	Aggre. actinomycetemcomitans	10^3	MFU	-	-	-
27	C-2672	64	F	NOGC									GPC GNB	seen	M. micros Fusobacterium spp Porphyromonas spp	10^3 $1.8x10^6$ 10^7	10 10^2 10^2	- - -	- - -	- + -
28	C-2630	57	M	NOGC									GPC	Not seen	Peptostreptococcus. anaerobius	$2x10^4$	10^2	-	-	-
29	C-123	42	F	NOGC									GPC	Not seen	Streptococcus. mutans	10^5	10	-	-	-
30	C-278	45	M	Oral commensals									GPC	Not seen	NOGC					
31	C-2996	36	M	NOGC									GPC GNB GNCB	seen	M. micros Fusobacterium spp Porphyromonas spp	10^5 10^5 $1.2x10^6$	10^2 10 10^2	- - -	- + -	- + -
33	C-3498	28	F	NOGC									GPC GNB	seen	Porphyromonas spp Bacteroid fragilis Eikenella. corrodens	10^2 10^4 10^4	NG 10^3 10^3	- - -	- - -	- - -
32	C-3000	29	M	Oral commensals									GPC	Not seen	NOGC					
34	C-3062	55	F	Oral commensals									GPC	Not seen	NOGC					
35	C-3500	35	F	Oral commensals									GPC	Not seen	NOGC					
36	C-3251	38	F	NOGC									GNB	seen	Bacteroid. ureolyticus Porphyromonas gingivalis Fusobacterium spp	10^2 10^5 10^6	NG 10^2 10	- - -	- - -	- - -
37	C-3493	50	F	NOGC									GPC GNB	Not seen	Fusobacterium. spp M. micros	$2x10^4$ $1.6x10^4$	MFU MFU	- -	- -	- -

38	C-3495	39	F	NOGC									GNB	Not seen	Fusobacterium. spp Prevotella intermedia	10^5 $2x10^6$	10 <10 colony	- -	- -	- -
39	V-1340	36	F	S. aureus	$3x10^6$	10^2	S	NT	S	S	S	S	GPC GNB	seen	Porphyromonas spp B. fragilis	$2x10^5$ $2x10^5$	10^2 10	- -	- -	- +
40	V-1352	29	M	NOGC									GPC GNB	Not seen	P. intermedia M. micros Fusobacterium spp	10^5 $2x10^5$ $1.8x10^6$	10^2 10^2 10^2	- - -	+ - -	+ - -
41	W-1243	30	M	NOGC									GNB GNCB	seen	Porphyromonas spp Fusobacterium. spp	$2x10^4$ 10^5	NG 10^2	- -	- -	- -
42	W-1246	36	F	NOGC									GPC GNB	Not seen	Fusobacterium. spp M. micros	10^5 $2x10^6$	10 10^2	- -	- -	- -
43	W-1350	48	M	NOGC									GNB	seen	Porphyromonas spp Bacteroid fragilis Fusobacterium. spp	10^4 10^5 $1.2x10^5$	10^2 10 10^2	- - -	- - -	- - -
44	W-1354	38	F	NOGC									GNB GNCB	Not seen	B. fragilis Prevotella spp Porphyromonas spp	10^5 $2x10^6$ $1.2x10^5$	10^2 10^2 10	- - -	- - -	- - -
45	B-126	45	M	NOGC									GNB	seen	B. fragilis Porphyromonas gingivalis	10^5 $2.2x10^6$	10 10^2	- -	- -	- -
46	B-195	32	M	NOGC									GPC GNB	Not seen	Prevotella spp Fusobacterium. spp M. micros	$2x10^4$ $1.4x10^6$ $1.2x10^5$	10 10^2 10^2	- - -	- + -	- + -
47	C-145	38	F	NOGC									GPC GNB	Not seen	Fusobacterium. spp P intermedia M. micros	10^4 $2x10^4$ $1.8x10^6$	MFU MFU MFU	- - -	- - -	- - -
48	C-158	29	F	NOGC									GNB	seen	Porphyromonas. spp Fusobacterium. spp	10^5 10^2	10 NG	- -	- +	- +
49	D-912	26	M	NOGC									GNB	Not seen	Bacteroid fragilis Porphyromonas gingivalis	$2x10^3$ $2x10^5$	10^2 10^2	- -	- -	- -
50	X-3212	36	F	NOGC									GPC GNB	Not seen	Prevotella. loeschii M. micros Fusobacterium spp	10^3 $1.4x10^6$ $2.2x10^6$	10^2 10^2 10^2	- - -	- - -	- - -
51	X-3215	42	F	K. pneumoniae	$1.8x10^2$	NG	S	S	S	R	NT	NT	GNB	Not seen	Fusobacterium spp	$2x10^4$	10	-	+	+

52	B-1251	45	F	S. aureus	$2x10^5$	10	S	NT	S	S	S	S	GPC GNB	seen	B. fragilis Porphyromonas spp	10^5 $2x10^6$	10^2 10^2	- -	- -	- -
53	B-1342	52	F	NOGC									GNB	seen	B. fragilis Fusobacterium nucleatum Porphyromonas spp	$3x10^4$ $2.2x10^7$ $1.8x10^6$	10^2 10^2 10^2	- - -	+ - -	+ - -
54	C-987	62	M	NOGC									GNB	Not seen	B. fragilis Fusobacterium spp	$2x10^4$ $1.2x10^6$	10^2 10^3	- -	- -	- -
55	C-1112	55	F	K. pneumoniaee	$2x10^2$	NG	S	S	S	S	NT	NT	GNB	Not seen	B. fragilis	10^5	10^2	-	-	+
56	C-1255	33	M	NOGC									GNB	seen	Porphyromonas spp Fusobactrium spp	$2x10^4$ $2.2x10^5$	MFU MFU	- -	- -	- -
57	C-1260	46	M	NOGC									GPC GNB	Not seen	M. micros F. nucleatum Prevotella spp	10^5 $1.8x10^6$ $1.2x10^5$	10^2 10^3 10	- - -	- - -	- - -
58	D-380	37	M	NOGC									GPC GNB	Not seen	Fusobacterium. spp Prevotella spp M. micros	10^4 $2x10^6$ $1.8x10^5$	10 10^2 10	+ - -	+ - -	+ - -
59	D-893	40	F	NOGC									GNB	Not seen	P. intermedia Fusobacterium spp	$2x10^5$ $2x10^6$	10^2 10^3	- -	- -	- -
60	D-899	40	F	NOGC									GPC GNB	Not seen	P. intermedia M. micros	10^4 $1.4x10^5$	MFU MFU	- -	- -	- -

KEY TO MASTER CHART

NOGC – No organism grown in culture

CFU/ml – Colony forming unit per ml