ISSN Print: 2394-7489
ISSN Online: 2394-7497
IJADS 2019; 5(1): 28-32

www.oraljournal.com
Received: 11-11-2018
Accepted: 15-12-2018

Dr. Sonal Jindal
Assistant Professor,
Department of Microbiology,
Subharti Medical College,
Meerut, Uttar Pradesh, India

Dr. Molly Madan
Ex-Prof & HOD, Department of microbiology, Subharti Medical College, Meerut, Uttar Pradesh, India

Dr. Deepa D
Prof, Department of Periodontology, Subharti Dental College, Meerut, Uttar Pradesh, India

Correspondence
Dr. Sonal Jindal
Assistant Professor,
Department of Microbiology,
Subharti Medical College,
Meerut, Uttar Pradesh, India

Study showing the major association of obligate anaerobic gram negative bacilli as cause of chronic periodontitis

Dr. Sonal Jindal, Dr. Molly Madan and Dr. Deepa D

Abstract
Introduction: Chronic periodontitis is the major cause of tooth loss in the adult population. The bacteria that are involved in periodontitis accumulate in the sub-gingival plaque that comprises predominantly of Gram-negative strict anaerobic rods. Clinical interests in these organisms are linked to the therapeutic problems usually encountered in treating mixed orodental infections.
Aims and Objectives: The purpose of this study was to look at the frequency of strict anaerobic bacteria in patients with chronic periodontitis and healthy subjects without periodontal destruction. Lack of data regarding from this geographical area prompted us to carry out this study.
Material and Methods: Study comprises of 40 patients. 20 apparently healthy subjects without clinical signs and symptoms of Periodontitis constituted the control group and 20 patients with Chronic Periodontitis comprise of study group Sub gingival plaque Samples were collected into sterile test tubes containing 2 ml of transport media that is Robertson cooked meat (RCM) broth Samples were processed from RCM Broth, for both aerobes and anaerobes bacteria.
Results: Chronic periodontitis is polymicrobial in nature. Anaerobes outnumber the aerobic bacteria by 3-4 folds. Anaerobe isolation was 78.33% in Chronic Periodontitis and 48.33% in healthy subjects, which shows increased rate of isolation of anaerobe has strong association with Chronic Periodontitis. Obligate Gram negative anaerobes (70.21%) were predominantly isolated than the gram positive (14.89%) in patients suffering from Chronic Periodontitis. Most common isolate was *Prevotella* spp. (Chronic Periodontitis= 60%, Healthy= 30%) followed by *Fusobacterium* sp. (Chronic Periodontitis= 50%, Healthy= 15%), *Porphyromonas* sp. (Chronic Periodontitis= 30%, Healthy= 10%) and *Bacteroides* sp. (Chronic Periodontitis= 25%, Healthy= 10%).
Conclusion: Significant decrease in aerobic-anaerobic ratio suggesting that anaerobes play a major role in orodental infections and their proportion is markedly increased in Chronic Periodontitis with respect to healthy subjects. Cultural methods are still economical and gold standard. Diversity of anaerobic bacteria in Chronic Periodontitis should be considered in the treatment strategy of the patients.

Keywords: Anaerobic gram, chronic periodontitis, polymicrobial

Introduction
Chronic periodontitis is the major cause of tooth loss in the adult population. The etiology of the disease is multifactorial and bacterial deposits play an essential role in the pathogenesis. The bacteria that are involved in periodontitis accumulate in the sub-gingival plaque that comprises predominantly of Gram-negative strict anaerobic rods [1]. Anaerobic gram-negative bacteria (AGNB) belonging to the genera *Bacteroides*, *Prevotella* and *Porphyromonas* are most commonly encountered in clinical infections [2, 3].
The currently recognized key periodontal pathogens include Gram negative anaerobes which are: *Porphyromonas gingivalis, Prevotella intermedia, Bacteroides* sp., *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum* [4].
However, some anaerobic, Gram positive microorganisms such as *Peptostreptococcus micros* and *Eubacterium* species have recently been implicated in chronic periodontitis [1].
It is a disease that affects the tooth supporting tissues (periodontium) and is characterized by loss of periodontal attachment including the alveolar bone [5]. The disease prevalence is 13-57% worldwide and 65-80% in India accounting to a major health problem [6, 7]
It is generally accepted that the oral biofilm in association with anaerobic bacteria is the main etiological factor in periodontal disease [8].

The proportions of strict anaerobic, Gram negative organisms increase significantly in accordance with increasing severity of disease.

Clinical interests in these organisms are linked to the therapeutic problems usually encountered in treating mixed orodental infections.

Data on anaerobic periodontal microflora in the Indian population is very scarce.

The purpose of this study was to look at the frequency of strict anaerobic bacteria in patients with chronic periodontitis and healthy subjects without periodontal destruction. Lack of data regarding from this geographical area prompted us to carry out this study.

Material and Methods

The present study was carried out in Post Graduate Department of Microbiology, Subharti Medical College in collaboration with the Department of Periodontics, Subharti Dental College, Meerut for a period of one year.

Adult patients of either sex, attending the OPD of Periodontology department and diagnosed clinically as Chronic Periodontitis were included in the study. Patients who have taken any antibiotic, anti-inflammatory medications in past 6 months, or on any dental treatment or with any systemic illnesses were excluded from study.

The study has been approved by the research and ethical committee of Subharti Medical College.

Present study comprises of 40 patients. 20 apparently healthy subjects without clinical signs and symptoms of Periodontitis constituted the control group and 20 patients with Chronic Periodontitis comprise of study group.

Informed and written consent of all the 40 subjects was taken for the study.

The culture media, reagents and chemicals used in the study were procured from Himedia Laboratories Private Limited, Mumbai, India.

Sub gingival plaque Samples were collected into sterile test tubes containing 2 ml of transport media that is Robertson cooked meat (RCM) broth [9]. (HIMEDIA REF 149) with complete aseptic precautions.

Samples were processed from RCM Broth, for both aerobes and anaerobes bacteria:

For isolation of aerobes, samples were inoculated on Blood Agar, Chocolate Agar and MacConkey agar culture plates. For microaerophilic bacteria, plates were incubated under 5-10% carbon di oxide at 37 degree C for 24-48 hours. Aerobic isolates were identified on the basis of their gram staining, culture characteristic and biochemical reactions as per standard protocol [10, 11, 12, 13].

For isolation of anaerobes, sample was inoculated on Brain heart infusion Blood (BHIB) agar supplemented with vitamin K. Metronidazole disc (5 microgram) was placed on the well of sample inoculation to look for anaerobic growth away from it. Inoculated plates were placed in McIntosh Fildes jar with gas pack (Himedia Anaerogas pack) and incubated at 37 ^{o}C for 5 days. Anaerobic isolates so obtained were identified on the basis of their gram staining, culture characteristics, aerotolerance test and biochemical reactions as per standard protocol [10, 11, 12, 13, 14, 15, 16] for identification of gram negative anaerobes special potency discs test was done. Antibiotic discs Vancomycin (5 microgram), Kanamycin (1000 microgram) and Colistin (10 microgram) were placed on Brain heart infusion Blood agar (supplemented with vitamin K) plate with lawn culture of anaerobic isolates [15, 16].

Identification of both aerobic and anaerobic bacteria were also confirmed by the Automated Vitek 2 Compact System using GP card, GN card and ANC card (Bio Merieux, Marcy I' Etiole, France).

Interpretation and analysis of the obtained results were carried out using standard statistical tests of significance.

p Value of <0.05 was considered a statistically significant.

Results and Discussion

Important feature of chronic periodontitis is that it is polymicrobial in nature, with mixed aerobic and anaerobic bacteria present. However, the anaerobes outnumber the aerobic bacteria by 3-4 folds. [17]In our study, more number of aerobic flora was isolated from healthy individuals that is 88.33% as compared to chronic periodontitis that is 65%. (Table 1) Similarly Mane *et al*. [17], Benachinmardi *et al* [18] and Daniluk *et al*. [19], isolated 14%, 7.43%, & 42.9% of aerobes in chronic periodontitis

Table 1: Aerobic/facultative anaerobes isolated were

Aerobic Flora	Healthy Group	Chronic Periodontitis
Samples processed (n) = 40	**20**	**20**
Gram Positive Cocci		
• *Streptococcus viridans*	5 (25%)	3 (15%)
▪ *Subspecies mutans*	6 (30%)	1 (5%)
▪ *Subspecies salivarius*	2 (10%)	5 (25%)
▪ *Subspecies mitior*	2 (10%)	6 (30%)
▪ *Subspecies sanguis*	6 (30%)	2 (10%)
▪ *Subspecies milleri*	-	-
• *Staphylococcus aureus*	14 (70%)	12 (60%)
• *Staphylococcus epidermidis*		
• *Streptococcus pyogenes*	-	-
Gram Negative Cocci		
• *Moraxella catarrhalis*	13 (65%)	9 (45%)
Gram Negative Bacilli		
• *Escherichia coli*	2 (10%)	-
• *Klebsiella pneumoniae*	2 (10%)	-
• *Pseudomonas aeruginosa*	1 (5%)	1 (5%)
Total Isolates	53	39
Percentage Total	88.33%	65%

Isolation of Anaerobic flora was higher in Chronic Periodontitis as compared to healthy group. Anaerobe isolation was 78.33% in Chronic Periodontitis and 48.33% in healthy subjects, which shows increased rate of isolation of anaerobe has strong association with Chronic Periodontitis. (Table 2)

Table 2: Obligate anaerobes isolated were

Anaerobic Flora	Healthy Group	Chronic Periodontitis
Samples processed (n) = 40	**20**	**20**
Gram Positive Cocci		
• *Peptoniphilus asaccharolyticus*	-	3 (15%)
• *Peptostreptococcus anaerobius*	1 (5%)	2 (10%)
• *Parvimonas micra*	-	1 (5%)
• *Anaerococcus prevotii*	-	1 (5%)
Gram Negative Cocci		
• *Veillonella* sp.	5 (25%)	3 (10%)
Gram Negative Bacilli		
• *Prevotella species*	6 (30%)	12 (60%)
• *Porphyromonas species*	2 (10%)	6 (30%)
• *Bacteroides species*	2 (10%)	5 (25%)
• *Fusobacterium species*	3 (15%)	10 (50%)
Gram Positive Bacilli		
• *Bifidobacterium* sp.	1 (5%)	2 (10%)
• *Actinomycetes*	5 (25%)	-
Sub* sp. *naeslundii	1 (5%)	2 (10%)
Sub* sp. *Odontolyticus		
• *Clostridium* sp.	1 (5%)	-
Total isolates	29	47
Percentage total	48.33%	78.33%

Various studies from India and other countries showed an isolation rate of strict anaerobes ranging from 42% to 100% in Periodontitis cases. Two different studies conducted in France, detected 91.42% and 80.77% of anaerobic isolates. In a study by Mane *et al*. [17], Salari and Kadkhoda [20], Daniluk *et al*. [19], Nonnenmacher *et al*. [21], Saini *et al*. [7] & Benachinmardi, *et al*. [18] detected 83%, 41.22%, 57.1%, 53.84% and 64.25% & 91.4% of anaerobes respectively.

In the present study obligate Gram negative anaerobes (70.21%) were predominantly isolated than the gram positive (14.89%) in patients suffering from Chronic Periodontitis

In our study most common isolate was *Prevotella* spp. (Chronic Periodontitis= 60%, Healthy= 30%) followed by *Fusobacterium* sp. (Chronic Periodontitis= 50%, Healthy= 15%), *Porphyromonas* sp. (Chronic Periodontitis= 30%, Healthy= 10%) and *Bacteroides* sp. (Chronic Periodontitis= 25%, Healthy= 10%).

p- Value for *Prevotella* sp., *Fusobacterium* sp., *Porphyromonas* sp. & *Bacteroides* sp. *were* 0.026, 0.018, 0.114, 0.211 for Chronic Periodontitis which shows strong association of these anaerobic GNB with Chronic Periodontitis. (Table 3 & 4)

Table 3 & 4: Comparative spectrum of aerobe/facultative anaerobes & obligates anaerobes in patients with Chronic Periodontitis & healthy control group subjects & and their statistical analysis.

Aerobic Flora	Healthy Group	Chronic Periodontitis	p Value*	
Streptococcus viridans sub sp. *mutans*	25%	15%	0.429	Not Significant
Streptococcus viridans sub sp. *salivarius*	30%	5%	0.037	Significant
Streptococcus viridans sub sp. *mitior*	10%	25%	0.212	Not Significant
Streptococcus viridans sub sp. *sanguis*	10%	30%	0.114	Not Significant
Streptococcus viridans sub sp. *milleri*	30%	10%	0.114	Not Significant
Staphylococcus aureus	0%	0%		--
Staphylococcus epidermidis	70%	60%	0.507	Not Significant
Streptococcus pyogenes	0%	0%		--
Moraxella catarrhalis	65%	45%	0.203	Not Significant
Escherichia coli	10%	0%	0.146	Not Significant
Klebsiella pneumoniae	10%	0%	0.146	Not Significant
Pseudomonas aeruginosa	5%	5%	1	Not Significant

Anaerobic Flora	Healthy Group	Chronic Periodontitis	P-Value*	
Peptoniphilus asaccharolyticus	0%	15%	0.071	Not Significant
Peptostreptococcus anaerobius	5%	10%	0.548	Not Significant
Parvimonas micra	0%	5%	0.311	Not Significant
Anaerococcus prevotii	0%	5%	0.311	Not Significant
Veillonella sp.	25%	15%	0.429	Not Significant
Prevotella sp.	30%	65%	0.026	Significant
Porphyromonas sp.	10%	30%	0.114	Not Significant
Bacteroides species	10%	25%	0.211	Not Significant
Fusobacterium sp.	15%	50%	0.018	Significant

Bifidobacterium sp.	5%	10%	0.548	Not Significant
Actinomycetes sub sp. *naeslundii*	25%	0%	0.016	Significant
Actinomycetes sub sp. *odontolyticus*	5%	10%	0.548	Not Significant
Clostridium sp.	5%	0%	0.311	Not Significant

The increased rate of isolation rate of *Prevotella* sp. and *Fusobacterium* sp. showed statistically significant results with Chronic Periodontitis with significant p value of 0.001 & 0.003 respectively, proving *Prevotella species* and *Fusobacterium species* as a causative agent for Chronic Periodontitis. Other studies have also found these bacteria to be strongly associated in orodental infections. Studies by Siani *et al*. [7], Mane *et al*. [17], Salari and Kadkhoda [20], Nonnenmacher *et al*. [21] Socransky *et al*. [22], Benachinmardi, *et al*. [18], Sixou *et al*. [23], Beena *et al*. [24], Chakraborti *et al*. [25] have reported them as causative agent for Chronic Periodontitis. The finding of other studies in comparison to our study has been discussed in Table 5 as follows

Table 5: Comparison of spectrum of gram negative anaerobes in Chronic Periodontitis with various other studies

Gram negative anaerobes	Mane *et al.* [17]	Salari and Kadkhoda [20]	Saini *et al.* [7]	Socransky *et al.* [22]	Nonnenmacher *et al.* [21]	Benachinmardi *et al.* [18]	Present study
Fusobacterium sp.	-	-	70.4	14.01	-	32.2	50%
Bacteroides sp.	05	-	-	3.10	-	22.98	25%
Porphyromonas sp.	48	24.5	14	5.26	6.4	24.48	30%
Prevotella sp.	26	13	23.4	15.78	10.2	19.54	60%
Veillonella sp.	09	-	-	-	-	-	10%

Polymicrobial nature of the chronic periodontitis has been brought out in this study. In our study, all the samples belonging to control as well as the study groups yielded microbes. (Table 6)

Table 6: Comparative evaluation of Aerobic/Facultative Anaerobic isolates in various groups showing Polymicrobial nature of oral microflora

Aerobic	Healthy group (Total = 20)	Chronic periodontitis group (Total = 20)
Positive culture	20 (100%)	18 (90%)
Negative culture	0	2 (10%)
Monomicrobial	2 (10%)	0
Polymicrobial	18 (90%)	18 (100%)
2 isolates	6 (33.33%)	11 (61.11%)
3 isolates	8 (44.44%)	7 (38.89%)
4 isolates	3 (16.66)	0
5 isolates	1 (5.55%)	0

Similar studies of Chakraborti *et al.* [25], Salari *et al*. [20], Saini *et al.* [7], Mane *et al.* [17], Sixou *et al*. [23] Nonenmacher *et al*. [21] have brought out the polymicrobial nature of flora in orodental infections in their studies.

In our Study, the ratio of aerobes and facultative anaerobe to anaerobes was 1.828 and 0.830 in cases of Healthy group and Chronic Periodontitis. This depicted that aerobes and Facultative anaerobes were more in normal healthy gingiva while anaerobes predominated in Chronic Periodontitis. This sharp decrease in Aerobic-Anaerobic Ratio was found to be statistically significant (p value 0.06) inferring that the number of aerobes decreases proportionally as compared to number of anaerobes in chronic periodontitis. (Table 7) Similar studies by Chakraborti *et al.* [25], Saini *et al.* [7] also shows that aerobic anaerobic ratio decrease in case of orodental infections.

Table 7: Comparative evaluation of Aerobic-Anaerobic Ratio in various groups

	Healthy Group	Chronic Periodontitis
Samples processed (n) = 40	20	20
Aerobes	53	39
Anaerobes	29	47
Aerobic Anaerobic Ratio	1.828	0.830

Conclusion

Chronic Periodontitis. Was found to be polymicrobial in nature. Anaerobic gram negative bacilli *Prevotella* and *Fusobacterium species* were found to be the commonest organism associated with periodontal destruction in our study. Aerobic/facultative anaerobe like *Streptococcus viridians subspecies sanguis, mitior, mutans,* and Anaerobes like *Porphyromonas* sp., *Bacteroides* sp., *Peptoniphilus asaccharolyticus, Peptostreptococcus anaerobius, Parvimonas micra, Anaerococcus prevotii, Actinomyces odontolyticus* have increased isolation in this group and they might play a role in Chronic Periodontitis. In our study, statistically significant decrease in aerobic –anaerobic ratio suggests that anaerobes play a major role in Chronic Periodontitis Cultural methods are still economical and gold standard. However many bacteria in the oral cavity cannot be cultured, it is likely that these still uncharacterized bacteria might play a role in the initiation and progression of periodontal disease. Diversity of anaerobic bacteria in Chronic Periodontitis should be considered in the treatment strategy of the patients. Considering the scarce data on microbial flora in the Indian population, further studies for assessment of microbial profile in various forms of Chronic Periodontitis should be carried out. Since the concept of dental infections has been bacteriologically non-specific and offers no rationale for antibacterial treatment, careful assessment and isolation of both aerobes and anaerobes is of utmost importance in the treatment of Chronic Periodontitis.

References

1. Nonnenmacher C, Mutters R, Flores de Jacoby L. Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects. Clin Microbiol Infect. 2001; 7:213-217.
2. Duerden BI. Virulence factors in anaerobes. Clin Infect Dis. 1994; 14:S253-9.
3. Botta GA, Arzese A, Minisini R, Trani G. Role of structural and extracellular virulence factors in gram negative anaerobic bacteria. Clin Infect Dis. 1994; 18:S260-4.
4. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol. 2000,

1994; 5:78-111.
5. Van Winkelhoff AJ, Loss BG, Van Der Reijden WA, Van Der Velden U. *Porphyromonas gingivalis*, Bacteroides forsythus and other periodontal pathogens in subjects with and without periodontal destruction. J Clin Periodontol. 2002; 29:1023-1028.
6. Rylev M, Kilian M. Prevalence and distribution of principal periodontal pathogens worldwide. J Clin Periodontol. 2008; 35:346-61.
7. Saini S, Aparna Gupta N, Mahajan A, Arora DR. Microbial flora in orodental infections. Indian J Med Microbiol. 2003; 21:111-4.
8. Moore WEC. Microbiology of periodontal disease. J Periodontol Res. 1987; 22:335-341.
9. Amel Y, Bouziane D, Leila M, Ahmed B. Microbiology study of periodontitis in West of Algeria. Adv in Med Dent Sci. 2010; 3(3):80-85.
10. Collee JG, Marr W, Watt B, Miles RS. Culture of Bacteria and Tests for Identification of Bacteria. In: Collee JG, Fraser AG, Marmion BP, Simmons A, Eds. Mackie and McCartney's Practical Medical Microbiology, 14th edition. New York: Elsevier Churchill Livingstone, 2012, 113-49.
11. Betty A Forbes, Daniel F Sahm, Alice S Weissfeld. Overview and General Considerations, Laboratory Considerations. In: Bailey & Scott's Diagnostic Microbiology, 12th edition, 2007, 216-253.
12. Winn W, Allen S, Janda W, Koneman E, Procop G *et al*. Gram positive cocci Part II: Streptococci, Enterococci and Streptococci Like Bacteria. In: Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th edition, Lippincott Williams and Wilkins, Baltimore, 2006, 674-745
13. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing.22nd Informational Supplement. CLSI document M100-So24. Wayne. PA: Clinical and Laboratory Standards Institute, 2014.
14. Jousimies-Somer HR, Summanen P, Baron EJ, Citron DM, Wexler HM, Finegold SM. Wadsworth-KTL anaerobic bacteriology manual, 6th edition, Belmont, CA, Star Publishing, 2002, 49-70.
15. Mahon CR, Lehman DC, Manuselis G, Engelkirk GP, Engelkirk DJ. Chapter. 23 Anaerobes of Clinical Importance. In: Textbook of Diagnostic Microbiology, 3rd edition, 2007, 587-639.
16. Betty A Forbes, Daniel F Sahm, Alice S Weissfeld. DJ. Chapter 43. Overview and general considerations of Anaerobic of Bacteriology. In: Bailey & Scott's Diagnostic Microbiology, 12th edition, 2007, 455-77.
17. Mane AK, Karmarkar AP, Bharadwaj RS. Anaerobic bacteria in subjects with Chronic Periodontitis and in periodontal health. J Oral Health Comm Dent. 2009; 3(3):49-51.
18. Benachinmardi KK, Nagamoti J, Kothiwale S, Metgud SC. Microbial Flora in Chronic Periodontitis: Study at a Tertiary Health Care Center from North Karnataka. Journal of Laboratory Physicians. 2015; 7(1):49-54.
19. Daniluk T, Tokajuk G, Cylwik-Rokicka D, Rozkiewicz D, Zaremba ML, Stokowska W. Aerobic and anaerobic bacteria in subgingival and supragingival plaques of adult patients with periodontal disease. Adv in Med Sci. 2006; 51(1):81-5.
20. Salari MH, Kadkhoda Z. Rate of cultivable subgingival periodontopathogenic bacteria in Chronic Periodontitis. J Oral Sci. 2004; 46:157-61.
21. Nonnenmacher C, Mutters R, De Jacoby LF. Microbiological characteristic of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects. Clin Microbiol Infect. 2001; 7:213-7.
22. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25:134-44.
23. Sixou JL, Magaud C, Jolivet-Gougeon A, Cormier M, Bonnaure-Mallet M. Evaluation of the mandibular third molar pericoronitis flora and its susceptibility to different antibiotics prescribed in France. J Clin Microbiol. 2003; 41:5794-7.
24. Beena VK, Francis J, Bhat M, Kotion M, Shivananda PG. Anaerobic bacteria in periodontal infections. J Indian Dent Assoc. 1992; 63:215-9.
25. Chakraborti CK, Chatterjee BD, Kundu D, Banerjee KL. Role of anaerobes in advanced adult periodontitis. In: Proceedings of the first Asian congress on anaerobic bacteria in health and disease. Mehta A, Kochar N, eds., 1987, 202-4.