Prevalence of Periodontopathic Bacteria in Generalized Aggressive Periodontitis (GAP) Subjects in the Central Indian Population

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Abstract

Aims and Objective: Qualitative and quantitative analysis of periodontopathic bacteria in Generalised Aggressive Periodontitis (GAP) patients in Central Indian population.

Materials and Methods: Plaque samples were collected from thirty patients of GAP and transferred to a vial containing fluid enriched with vitamin K1 and Heminthio glycollate medium. In the laboratory, Dentaid-1 culture media was used for detection and quantification of *Aggregatibacter actinomycetemcomitans*. Kanamycin blood agar media and supplemented blood agar plates were used for *Porphyromonas gingivalis, Tannerella forsythia*, *Prevotella intermedia* and *Fuscobacterium nucleatum*.

Results and Observations: Quantitative analysis revealed predominance of streptococci in highest quantity followed by *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum* and *P. intermedia*. With respect to prevalence, streptococci prevailed in all the subjects, followed by *P. gingivalis*, *F. nucleatum*, *P. intermedia*. *A. action mycetemcomitans* prevailed only in 10 subjects that were lowest among all.

Conclusion: In this study the mean percentage of prevalence of bacteria was above the minimal threshold in aggressive periodontitis patients. *P. gingivalis*, *F. nucleatum* and *P. intermedia* had higher levels of prevalence in the GAP. The prevalence of *A. action mycetemcomitans* was much lower than that of *P. gingivalis*.

Key Words: Generalised aggressive periodontitis, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, Fuscobacterium nucleatum, Prevotella intermedia

Introduction

The current concept concerning the aetiology of periodontitis considers 3 factors which determine whether active periodontitis will occur in a subject: a susceptible host, the presence of pathogenic species and the absence of so called "beneficial bacteria" [1-3]. The infectious etiology of periodontitis is well documented, as is the role of the host immune response in the pathogenesis of the disease [4,5]. Dental plaque is a complex microbial biofilm comprised of as many as 500 different bacterial species organized in the supragingival and subgingival locations [6]. As opposed to the supragingival plaque that is attached to the tooth surface and dominated by *streptococci*, *actinomyces* and gram-positive facultative species [7], the subgingival plaque is tooth and tissue associated and contains a great variety of Gram-negative anaerobic bacteria [8]. From supra to subgingival plaque, there is a significant decrease of *streptococci* and *actinomyces* species accompanied by an increase of *Aggrigatebacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) Which is said to result into periodontal disease progression and poor prognosis [9].

The consensus report for the new classification [10] confirmed that *A. actinomycetemcomitans* can cause aggressive periodontitis, and is present in an elevated proportion in aggressive periodontitis (AP). *P. gingivalis* was also mentioned as an etiologic agent for AP, although its importance in the pathogenesis of this disease has been generally thought to be less than that of *A. actinomycetemcomitans*. In a previous study in Chile, low prevalence of *A. actinomycetemcomitans* was observed in AP patients [11]. The role of these bacteria in the pathogenesis of human periodontal disease is determined by their high frequency of isolation and pathogenic potential that includes a myriad of virulence factors that enable them to subvert the host defense systems. These include the ability to attach to epithelial cells and extracellular matrix proteins, proteases, collagenases, endotoxins (LPS), antibiotic resistance, bacteriocins, the production of chemotactic inhibitors, leukotoxins, cytotoxins, toxic metabolic substances (H_2S, putrecins), immunosuppressive proteins, etc [12-14]. *P. gingivalis* has the ability to invade human gingival fibroblasts [15] and endothelial cells [16] in cell culture whereas *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia*, have the ability to invade human oral epithelial cells in cell culture [17]. The effect of this bacterial infection on the general health has also been emphasised by recent papers which reported a close relationship between periodontitis and the occurrence of coronary heart disease [18] and other vascular infections, even the risk for preterm labor low-birth weight babies and miscarriage [19]. Under the above mentioned conditions pathogens may constitute an intracellular reservoir in some

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individuals and may lead to recolonization of the periodontal pocket after treatment. The Generalised Aggressive periodontitis (GAP) usually affects men and women over the age of 30, although patient may be older. Intraoral periapical radiographic examination reveals that the disease process involves generalised interproximal bone loss affecting three permanent teeth other than the first molars and incisors. Clinical features include soft tissue inflammation, attachment loss, periodontal pocket formation and active bone resorption [20].

In the present study, we investigated the prevalence and proportions of periodontopathic bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia/nigrescens and F. nucleatum*) in GAP patients in the Central Indian population. As described by Yano-Higuchi K et al. 2000, bacterial culture technique was applied as a gold standard in the qualitative and quantitative analysis.

Materials and Methods

Thirty patients presenting with signs and symptoms of GAP were selected from the out -patient department Of Periodontics, Modern Dental College and Research Centre, Indore, India. The patients were explained about the study protocol and informed consents that were approved by institutional reviewer board were obtained. The protocol stated that, within 1 week of the detection of disease, all patients would be provided with periodontal treatment and the ethical clearance was obtained from the registered body of ethical committee. Patients with minimum of 20 teeth present and seeking periodontal treatment for the first time formed the study group. Further, only GAP patients having pockets of ≥5 mm and clinical attachment loss of ≥4 mm, associated with two or more first molars and / or incisors; and three or more cuspids or premolars or second molars were selected for the study. Subjects who were already been prescribed antibiotics or who have taken the same within six months, presence of systemic diseases, smokers or tobacco chewers and teeth with pockets extending up to root apex were excluded from the study.

Subgingival plaque samples

Subgingival plaque samples were collected from the undisturbed subgingival area around both single and multi-rooted teeth. In each patient, four sites (each one representing a quadrant), with pocket depth of ≥5 mm and/or less of attachment of ≥4 mm were selected. Teeth associated with furcation involvement were excluded [21]. Supragingival deposits were carefully removed with supragingivalscalers *(Figure 1)*. Subgingival plaque samples were collected and pooled from four sites per patient by means of a Hu-friedy, Gracey curette *(Figure 2)* [22]. These pooled samples were transferred to a vial *(Figure 3)* containing 1 ml fluid enriched with vitamin K1, heminthioglycollate medium and PR II (pre-reduced medium in tubes) which are general-purpose media for the cultivation of a wide variety of microorganisms, particularly obligate anaerobes. Thioglycollate medium with calcium carbonate and enriched thioglycollate broth with sodium bicarbonate and fildes enrichment are also recommended for the maintenance of stock cultures. It is used in qualitative procedures for the cultivation of fastidious as well as nonfastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical and nonclinical materials. The medium is prepared with an anaerobic head space and is provided in screw-capped tubes, in accordance with Centre for Disease Control (CDC) recommendations [23]. Vitamin K1 and hemin have been shown to be required by certain anaerobes for growth [24,25].

Microbiological analysis

In the laboratory, samples were dispersed with a vortex mixer for 30 s and serially diluted in 10-fold steps in pre-reduced Phosphate Buffered Solution (PBS). For, *A. actinomycetemcomitans* appropriate dilutions were plated in parallel on Dentaid-1[26], prepared using Brain Heart Infusion Agar (BHIA) to which the following compounds were added: 5 g of yeast extract, 1.5 g of sodium fumigate and 1 g of sodium formate per litre. This medium was autoclaved for 15 min at 121°C. The final pH attained was 7.2 ± 0.2. Once the medium got cooled to 50°C, vancomycin was added to a final concentration of 9 µg/ ml]. After incubation for 72 h at 37°C in a 5% CO_2 incubator, the plates were examined for the presence and enumeration of *A. actinomycetemcomitans*. The Counts on clinical samples were numbered as CFU/millilitre. An *A. actinomycetemcomitans* presumptive identification was first made on the basis of colonial morphology. The morphology at 72 h on Dentaid-1, showed smooth, circular, spherical colonies with an enhanced dark border and an incipient star inner structure *(Figure 4)*. For the detection and quantification of *P. gingivalis*, *T. forsythensis*, *P. intermedia*, *F. nucleatum*, kanamycin [27]*(Figure 5)* and supplemented blood agar media [28] were used trypticase soy 5% sheep blood agar plates were used for the detection of streptococci. CDC anaerobes laked sheep blood agar media with kanamycin andvancomycin is an enriched (5 ml of reduced enriched thioglycollate medium containing vitamin K1 and hemin) selective culture medium for the selective isolation of obligatory anaerobic gram negative bacilli. Culture was handled quickly to avoid prolonged exposure to oxygen, total time of exposure not to exceeding 20 min; also using an 18 – 24 h broth culture of the facultative anaerobic organisms. Using a volumetric pipettor or equivalent method, 0.05 ml of the appropriate inoculums were delivered to the plated media samples and streaked for isolation. Plates were incubated anaerobically in a jar with modified gas pack at 35 ± 2ºC. All inoculated plates were examined at 48 and 72 h for amount of growth, colony size, pigmentation and haemolytic reactions. *Porphyromonas and prevotella* strains were observed under UV light (365 nm) for fluorescence. For other anaerobic strains supplemented blood agar plates were incubated at 37ºC in 80% N_2, 10% $CO_2$and 10% H_2. After 7-14 days of incubation, total anaerobic counts were assessed. Bacterial identification was based on colony morphology under a stereoscopic microscope, pigment production, gram staining and bio-chemical test (including N-acetyl-β-D-glucosaminidase, α-galactosidase, α-glucosidase, α-fucosidase, esculine, indole and trypsineactvity) in order to identify anaerobic bacteria.

Results

Microbiological data of subjects from the sites selected for bacterial sampling are summarized in *Table 1*. As

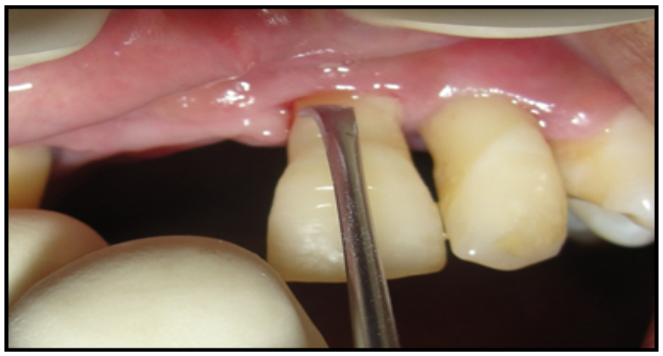

Figure 1. *Supragingival surface scaler used to remove supragingival plaque & calculus.*

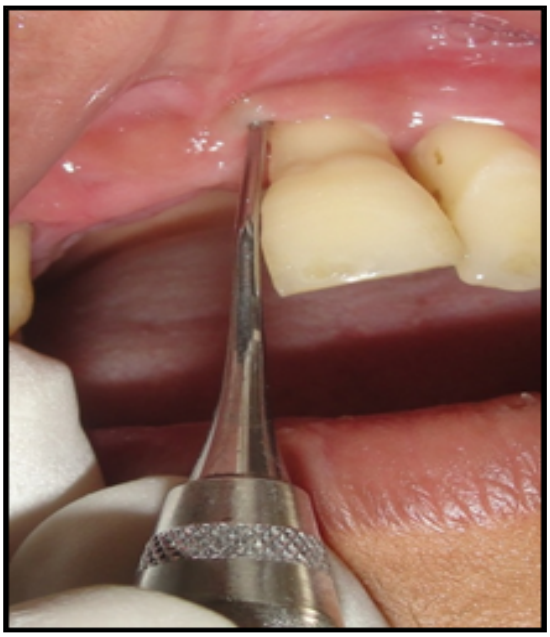

Figure 2. *Sub-gingival plaque sample collected with Hu-friedy, Gracy curette (no. 1-2) from 21 after supra-gingival plaque removal.*

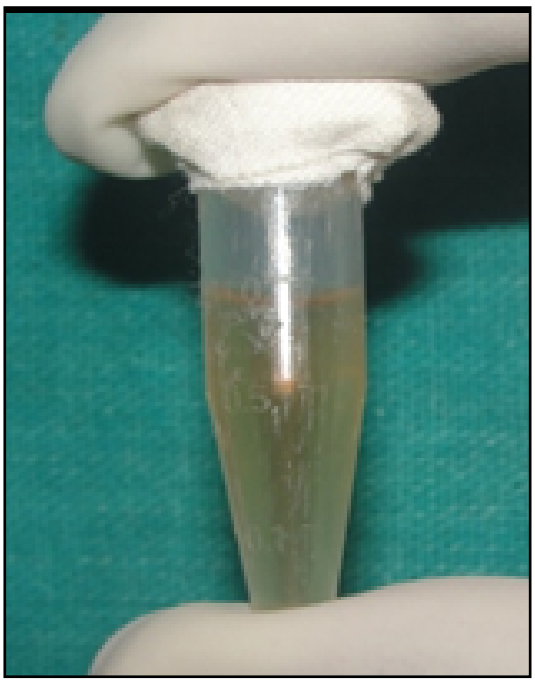

Figure 3. *Transport medium containing thioglycollate growth with hemin and vitamin K1.*

evidenced from the table, quantitative analysis revealed predominance of *Streptococci* in highest quantity followed by *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum* and*P. intermedia*.

Even w.r.t prevalence as shown in *Table 2* (determined in terms of isolation frequency) Streptococci prevailed in all the subjects, followed by *P. gingivalis*, *F. nucleatum*, *P. intermedia*, *A. actinomycetemcomitans* prevailed only in 10 subjects, accounting for lowest prevalence among all.

Discussion

Present study evidenced that streptococci (69567 ± 27393 CFU/ml) predominated the pathologic flora, followed by *A. actinomycetemcomitans* which was 7100 ± 12361 CFU/ml, *P. gingivalis* (6807 ± 8802 CFU/ml), *F. nucleatum* (6617 ± 8755 CFU/ml) and*P. Intermedia* (4624 ± 7651 CFU/ml). Similar observations were made in a study by Faveri et al. (2008). Whereas prevalence pattern of pathogens as determined by isolation frequency, *(Table 2)* was quite contrary to predominance pattern. Although streptococci prevailed in all 30 subjects indicating 100% prevalence, next in order were, *P. gingivalis* (86.67%), *F. nucleatum* (60%), *P. intermedia* (46.666%). Surprisingly, *A. actinomycetemcomitans* exhibited lowest prevalence of 33.33% (detected among 10 subjects only.)

Gibbons RJ et al 1966 demonstrated that streptococci caused severe bone loss in experimental animal [29]. *P. gingivalis* is associated with increased IL-8 plasma levels which dysregulate L-selectin shedding and increased basal

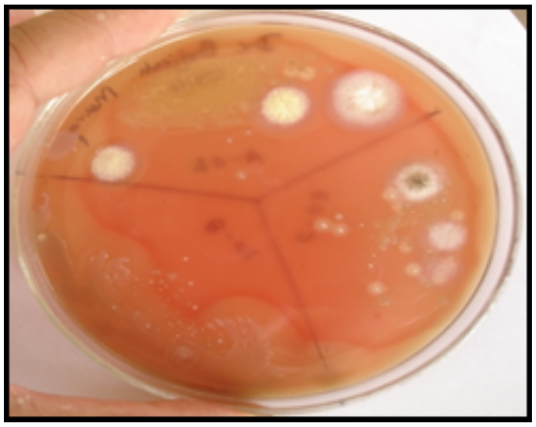

Figure 4. *Dentaid-1 selective media showing translucent colony of A. actinomycetemcomitans with internal star.*

Figure 5. *Kanamycin blood agar media showing black pigmented colonies of P. gingvalis.*

Table 1. *Quantitative analysis of periodontopathogens.*

Pts S. ttNO.	Pg (CFU/ml)	Aa (CFU/ml)	Pi (CFU/ml)	Fn (CFU/ml)	Streptococci (CFU/ml)
	7500	0	5000	0	60000
	1500	0	0	0	23000
	6500	0	0	0	90000
	8000	0	0	15000	80000
	24000	0	16000	10000	86000
	40000	30000	30000	10000	90000
	10000	20000	0	3000	100000
	800	40000	0	1000	100000
	15000	12000	0	20000	90000
	600	0	10000	0	100000
	0	0	20000	0	50000
	10000	0	0	0	40000
	6000	0	0	0	50000
	2000	0	0	0	80000
	2400	11000	7600	4000	60000
	0	0	0	10000	100000
	600	0	0	8500	90000
	800	0	1200	4000	56000
	1200	0	8500	30000	80000
	7500	0	5000	0	90000
	12000	2000	0	0	40000
	0	15000	0	0	10000
	6000	25000	800	0	22000
	800	0	6000	25000	50000
	5600	0	20000	24000	40000
	400	0	0	0	50000
	12000	18000	8000	14000	100000
	3000	40000	0	10000	100000
	0	0	0	10000	60000
	20000	0	600	0	100000
Mean ± SD (CFU/ml)	6807 ± 8802	7100 ± 12361	4624 ± 7651	6617 ± 8755	69567 ± 27393

Table 2. *Isolation frequency (%) of periodontopathogens.*

Bacteria	Isolation	Frequency
	N	%
A. actinomycetemcomitans	10	33.33
P. gingivalis	26	86.66
P. intermedia	14	46.66
F. nucleatum	16	53.33
Streptococci	30	100

H_2O_2 production that contribute to defective bactericidal activity, further allowing easy infiltration of larger number of pathogens (in addition to *P. gingivalis*) in GAP. These abundant pathogens could also amplify alterations of PMN functions and contribute to vascular and tissue damage, pointing to a correlation between PMN abnormalities and the clinical form of periodontitis [30]. The differences between *A. actinomycetemcomitans* and *P. gingivalis* levels were also demonstrated by Botero et al. [31], who demonstrated that mean proportions of *A. actinomycetemcomitans* frequency were as low as 8.3% in AP where as that of *P. gingivalis* were 91.6%. Imbronito et al. [32] demonstrated a 90% frequency of *P. intermedia* among 30 subjects of GAP, while frequency of *A. actinomycetemcomitans* was lower (50%) than that of P. gingivalis (73.3%).

In the present study, pooled samples of four affected sites per patient were analyzed by bacterial culture. Although opinions differ as to whether microbiological data should be analyzed on individual sites or using the patient as one study unit, we chose the latter approach based on the criterion that patients are being treated as a whole. Mombelli et al. [33] reported that four individual subgingival samples, each from the deepest periodontal pocket in each quadrant, should be included to detect *P. gingivalis*. In our study, the presence of *A. actinomycetemcomitans* was determined from a pooled subgingival sample of the four deepest periodontal pockets per patient and therefore, the periodontal occurrence of this bacteria may have been underestimated [34]. Other reason might be ethnicity and geographic and cultural factors could influence the colonization of *A. actinomycetemcomitans* in Western, African and Asian populations in contrast to North Americans and Europeans, where *A. actinomycetemcomitans* has been proposed as a frequently recovered microorganism from AP patients [35]. In this study *T. forsythensis* was not detected because of fastidious growth demonstrated by this bacterial species. *T. forsythensis* is a strict anaerobe, which grows very slowly in common media. The blood agar plates used in this study are not probably the most suitable medium for its growth, since it requires N-acetyl muramic acid used for its detection [28]. In the study by Gainet Jean et al. [30] only one sample was positive for *T. forsythensis* among 10 samples of Rapid Progressive Periodontitis. They also used supplemented blood agar media for culture analysis. In this study the only higher statistical difference that was found for *P. gingivalis* and*A. actinomycetemcomitans*. These results indicate that, as a whole, the subgingivalmicrobiota differ in GAP subjects.

According to the criteria that a bacterium has to be above a certain minimal threshold with respect to the total cultivable sub-gingival microbiota to be associated with periodontal disease, it has been suggested that this threshold is 0.1% for *P. gingivali s*[36], 0.01% for *A. actinomycetemcomitans* [37], 2.5% for *P. intermedia* [36,38,39], 5% for *F. Nucleatum* [38,39]. Bacteria are essential, but insufficient to cause disease; a susceptible host is also essential and host factors are determinative [40]. Periodontitis is a family of related diseases that differ in aetiology, natural history, disease progression, and response to therapy, but with common shared pathways of tissue destruction [41]. The shared events in the pathobiology are influenced by disease modifiers (also known as risk factors and indicators), both genetic and environmental or acquired, which may differ from one stage and form of disease to another. The modifying factors are major determinants of the differences observed in different periodontal conditions [40].

Conclusion

The results presented here, on the basis of mean percentage of total counts indicate that, *P. gingivalis* (86.66%), *F. nucleatum* (53.33%) and *P. intermedia* (46.66%) were strongly associated with GAP. In this study *P. gingivalis*, *F. nucleatum* and *P. intermedia* posted higher mean percentages above the minimal threshold in aggressive periodontitis. In turn, the prevalence of *A. actinomycetemcomitans* was much

lower than that of *P. gingivalis*. There is a general tendency among the clinical practitioners to diagnose the GAP case based the clinical and radiographic presentation alone. The microbial assays are skipped and treatment strategies are quickly decided assuming that elimination of lesions is an ultimate goal irrespective of the type of microorganisms. However one cannot deny that the knowledge of approximate bacterial load in GAP patients helps in early diagnosis and determination of prognosis, which further dictates precise treatment planning. Microbial culturing could also be an effective tool for educating and motivating the GAP patients towards better compliance.

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