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Evaluation of *Aggregatibacter actinomyctemcomitans* in periodontitis Patients of Metabolic Syndrome and Periodontitis Patients without Metabolic Syndrome - A Dentistry Study

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A B S T R A C T

Oral cavity is said to be the mirror of systemic health. It has long been known, that many systemic diseases first manifest in the oral cavity. On the other hand oral diseases have a potential to act as an independent risk factor for causing systemic disease. Currently, this bidirectional view is quickly gaining acceptance, due to significant findings supporting the association between periodontal disease and systemic conditions such as CVD, T2DM, adverse pregnancy outcomes, osteoporosis, Metabolic Syndrome. Significant effort has brought numerous advances in revealing the etiological and pathological links between this chronic inflammatory dental disease and these other conditions. The role of microorganisms in the causation and pathogenesis of periodontal disease is well documented. Within the past 10 years, many studies have been published indicating a positive or negative relationship between periodontitis and various systemic diseases, including Met.S. The most frequently identified periodontal pathogens include three microaerophilic species (*A. actinomyctemcomitans, Campylobacter rectus*, and *Eikenella corrodens*). The results of the present study confirmed increased colonization of periodontal pathogen, *A. actinomyctemcomitans* in both periodontitis patients without Met.S and Periodontitis patients with Met.S.

Introduction

Oral cavity is an open system exposed to the environment. Furthermore, the possibilities of foreign material entering the system from the oral cavity are heightened due to the constant intake of food and liquids through the mouth. The presence of the large numbers of bacteria can induce tissue destruction indirectly by activating host defense cells, which in turn, produce and release mediators that stimulate the effectors of connective tissue breakdown.[1-13]According to WHO Report 2008, In India, 53% of the deaths were due to Non Communicable Diseases (NCD). Cardiovascular disease (CVDs) alone account for 24 percent of all deaths. These NCD include hypertension, heart diseases, stroke, diabetes, obesity, high cholesterol and diseases associated with tobacco use (smoking and chewing) like chronic bronchitis, Chronic obstructive pulmonary disease (COPD), cancer and excessive use of alcohol. Moreover, a substantial proportion of

these deaths are in the productive age-group and all of them are preventable in nature. But, the rising challenge due to NCDs is that it increasingly affecting the younger populations.[2] Bacteria inhabits the oral cavity from birth to death. They colonize the soft tissue including the gingiva, cheeks and tongue and when teeth are present, bacteria colonize them even below the gingival margin. It is estimated that between 300 to 400 different bacterial species are capable of colonizing mouth, and any individual may typically harbor 150 to 200 different species.[3-15-16`]Bacteria initially colonize and interact with the tooth and then through physical and physiologic interactions among different species with in the microbial mass, there is formation of biofilm and dental plaque. Numerous studies have shown that microbiota of periodontal diseases is significantly different from that of periodontal health.

It is also worth mentioning that different type of periodontal diseases are characterized by a presence of particular group of organisms.[4]In case of periodontitis high percentage of anaerobic (90%), gram negative (75%) bacterial species are present in this type of periodontitis. Bacteria found in the high levels includes *P. gingivalis, T. forsythia, P. intermedia, C. rectus, E. corrodens, F. nucleatum, A. actinomyctemcomitans, P. microns* and *Treponema* and *Eubacterium* spp. Bacteria found to be elevated in active sites *C. rectus, P. gingivalis, P. intermedia, F. nucleatum* and *T. forsythia*. Detectable level of *P. gingivalis, P. intermedia, T. forsythia, C. rectus* and *A. actinomyctemcomitans* are associated with disease progression.[4-14]The term periodontitis refers to an inflammatory disease of the supporting tissues of the teeth caused by specific microorganism or group of specific microorganism resulting in progressive destruction of the periodontal ligament and alveolar bone resulting in pocket formation or recession or both.

The clinical cluster of hypertension, cardiovascular disease, hyperlipemia, hyperuricemia and type 2 Diabetes had been recognized by physicians for many decades, described as syndrome X and finally metabolic syndrome (Met.S)[5] which was the subjects of this study. Metabolic Syndrome (Met.S) it is a cluster of Cardiovascular risk factor that includes Obesity, Diabetes, Hypertension and Dyslipidemia. Subjects meeting 3 of these criteria were classified as having Met.S. (NCEP, 2001).

There is a known association between periodontitis and Met.S which warrants further investigations. The microorganisms associated with periodontitis are diverse. The association between *A.actinomyctemcomitans* and periodontitis is well established but the association between *A.actinomyctemcomitans* and Met.S is not so evident. Studies have also reported presence of *A. actinomyctemcomitans* in periodontitis, but microbiological status in Met.S patients is not yet evaluated. Hence, with this aim in mind, we conducted the present study to evaluate the incidence of *A.actinomyctemcomitans* in periodontitis patients with Met.S and patients with periodontitis without Met.S.

Materials and Methods

The present study was carried out in the Department of Oral Pathology and Microbiology of Sharad Pawar Dental College. The patients were selected from the Outpatient Department (OPD) of the department of Periodontics, Sharad Pawar Dental College and department of General Medicine, Achaya Vinoba Bhave Rural Hospital Sawangi, Meghe Wardha. Before the start of the study, an informed consent was obtained from all the study subjects. The study was approved by the Institutional Ethics Committee, Datta Meghe Institute of Medical Sciences, Wardha (Deemed University) Ref.

No. DMIMS (DU)/ IEC/ 2013-14/ 127 date-30.09.2013. The study was performed on subjects, which were divided into three groups according to their periodontal status and presence or absence of Met.S.

Group I: Periodontally healthy subjects without systemic disorder. (n=50).

Group II: Chronic Periodontitis patients without Met.S (n=50).

Group III: Chronic Periodontitis patients with Met.S (n=50)

The criteria for periodontal healthy sites were

Probing pocket depth (PPD) ≤3mm.

No signs of inflammation or mild inflammation.

Absence of bleeding after probing (BOP).

Gingival index (GI) score ≤ 1.

The criteria for periodontal disease sites were as follows

Probing pocket depth (PPD) > 3mm, with the help of Williams graduated periodontal probe.

Presence of bleeding on probing (BOP).

Presence of clinical attachment loss (CAL).

Gingival Index score (GI ≥ 2)

The criteria for diagnosis of Met.S were as follows

Body Mass Index (BMI), Obesity > 30(mass^{kg}/ height m^2)

Pressure (BP) > 140/90 mmHg

Triglycerides (TG) > 160mg%

Fasting Blood Sugar (FBS) >125mg/dl

High -Density Lipoprotein (HDL) > 75mg%

Detailed clinical history was recorded and clinical assessment was carried out in all the three groups. Subgingival plaque samples were collected and subjected to conventional microbial culture method.

For Group I - Subgingival plaque sample was collected from molar area of periodontally healthy individuals. The sampling site was isolated using cotton rolls and supragingival plaque was removed with the help of sterile cotton. The subgingival plaque sample was then collected using sterile Gracey curettes. For Group II and III- The sampling sites were isolated with cotton roles and subgingival plaque was removed using sterile cotton. Subgingival plaque samples were obtained using sterile gracy curettes from deepest periodontal pocket. Plaque was subsequently transferred into a sterile container (Himedia) with 0.85% of sterile saline that was immediately processed. Serial Dilution was done. A serial dilution is the stepwise dilution of a substance in solution. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. In 100 ml distilled water, 0.85gms Sodium chloride (NaCl - SD fine company) was added. The prepared 9ml of 0.85% NaCl was dispensed into 3 test-tubes (Borosil) each and autoclaved. After autoclaving 5-fold serial dilutions of the sample was made in the test-tube containing 0.85% NaCl. The serial dilution of samples were done immediately and cultured on agar plates. Tryptic Soy-Serum Bacitracin Vancomycin Agar is recommended for the isolation of *A. actinomyctemcomitans* (Table 1 and 2). The prepared culture plate was used for

inoculation. The plaque sample which was already serially diluted was added to each medium using streak culture method. Streak culture method is a routinely employed method for bacterial isolation in pure culture. A platinum or nichrome wire loop of 2-4 nm in internal diameter is used. A loopful of specimen is smeared onto the surface of dried plate near the peripheral area. This is known as primary inoculum. From the primary inoculum, it is spread thinly over the plate by streaking with loop in parallel lines. The streak plate technique is essentially a method to dilute the number of organisms, decreasing the density. This allows for individual colonies to be isolated from other colonies. Each colony is considered "pure," since theoretically, the colony began with an individual cell. Each inoculated petri-plate was sealed with Parafilm (Himedia) to prevent any contamination while incubation, and then placed in an anaerobic jar (Himedia) with AnaeroGas Pack system (Himedia) and incubated at 37°c for 48 hours for *A. actinomyctemcomitans*. All the plates are then placed in the anaerogas jar and anaerogas pack is placed inside the jar to maintain the anaerobic condition. The anaerobic gas jar is then placed inside the incubator at 37^0C for 72 hours. After 72 hours the colonies cultured on Petri plates are subjected to biochemical identification test for confirmation of bacteria intended to be cultured. The identification of a bacterial species is based on factors, including colony morphology, chemical composition of cell walls, biochemical activities, and nutritional requirements. 3 points should be considering during microorganism identification (Table 2). *A. actinomyctemcomitans* is gram negative and catalase test and *Indole Test* were positive. Numbers of colonies of bacteria grown on each plate were counted by colony counter. By working backwards using multiplication with 'dilution factor' (the number of times that you have diluted the bacteria sample with diluents solution), the number of bacteria of the original sample can be determined. To compute the estimated number of bacteria, the following formula was used. B=N/D, B=number of bacteria, N=number of colonies counted on a plate, D=dilution factor (1, 10 or 100).

Results and Discussion

In this study, a quantitative analysis of *A. actinomyctemcomitans* in periodontitis patients with Met.S and periodontitis patients without Met.S done. The results of the present study were subjected to statistical analysis. The comparison of the bacterial counts in periodontitis patients with Met.S (Group III), periodontitis patients without Met.S (Group II) and normal control (Group I) was carried out to find the significant difference between those values. The statistical tests used for the analysis of the results were**:** Chi-square Test, One way ANOVA (F-Test), Tukey HSD and 'Descriptive statistical analysis' (i.e. mean, standard deviation and standard error) was carried out for all the groups in this study.

Among 150 selected patients, *A. actinomyctemcomitans* were present in 2(4%) patients of normal group, 26(52%) patients of periodontitis without Met.S, and 33(66%) patients of periodontitis with Met.S. Therefore, the results of the study revealed that, *A. actinomyctemcomitans* is present in descending order from Group III>Group II>Group I (Table 3A). Among all groups, *A. actinomyctemcomitans* were present in 1(50%) male and 1(50%) female patient of normal group, 14(53.84%) male patients and 12(46.15%) female patients of periodontitis without Met.S (Group II), 26(78.78%) male and 7(21.21%) female patients of periodontitis with Met.S (Group III). Presence of *A. actinomyctemcomitans* in males was more as compared to females in group II and group III, and there was 4:1 distribution of

male: female in Group III and interpreted in Table 3B. In Normal controls (Group I), mean total count for *A. actinomyctemcomitans* was $0.06\pm0.31x10^5$ with range 0.00-2.00. In Periodontitis patients without Met.S (Group II), mean total count for *A. actinomyctemcomitans* was $3.76\pm3.86x10^5$ with range 0.00-10.00. In Periodontitis patients with Met.S (Group III), mean total count for *A. actinomyctemcomitans*was $6.56\pm5.45x10^5$ with range 0.00-20.00. Thus, mean count of *A.actinomyctemcomitans* in periodontitis patients with Met.S was three times more when compared to patients with periodontitis without Met.S. In periodontitis patients without Met.S the count was about three times more than normal control. In periodontitis patients with Met.S the count was about six times more than normal control (Table 4, Graph 1). The statistically significant variations of mean of total count of *A. actinomyctemcomitans* were found among all groups (p=0.000). The total counts of *A.actinomyctemcomitans* were observed in decreasing order in periodontitis with Met. S (Group III) $6.56x10^5\pm5.45x10^5$, periodontitis without Met. S (Group II) $3.76x10^5\pm3.86x10^5$, normal control (Group I) $0.06x10^5\pm0.31\ x10^5$ (Table 5). The statistical significant difference in total count *A. actinomyctemcomitans* was noted between all groups (p=0.000).The statistical analysis revealed that count of *A. actinomyctemcomitans* was highest in periodontitis patients with Met.S (Group III) than periodontitis patients without Met.S (Group II) and normal control (Group I) (Table 6).

The most frequently identified periodontal pathogens include three microaerophilic species (*A. actinomyctemcomitans, Campylobacter rectus, and Eikenella corrodens*) and seven anaerobic species (*P. gingivalis, Bacteroides forsythus, T. denticola, Prevotella intermedia, Fusobacterium nucleatum, Eubacterium, and spirochetes*). Socransky *et al.*, divided the pathogens into two main clusters and deemed them the "red" and "orange" complexes. Furthermore, they defined "green", "yellow", and "purple" complexes as the bacterial colonies that formed on the tooth surface prior to the colonization of the "orange" and "red" complexes respectively. The "red" complex consisted of three tightly related species: *T. forsythensis, P. gingivalis and T. denticola*and were thought to be most pathogenic to humans.[6]

It is known that all Met.S triggering factors play an important role in the onset of oxidative stress, subsequent formation of ROS, and probably also in the activation of the pro-oxidising, pro-inflammatory AGE-RAGE system. Many inflammatory pathways are activated by these conditions. The excess of visceral fat (high waist circumference) is certainly one of the most important factors in activating these signalling molecular cascades through the TNF-alfa pathway. The activation of these pathways is not restricted to limited areas of the body, but their signalling triggers systemic responses, which are also visible at the level of teeth supporting tissues.[7]

Our study results revealed that, the mean count of *A.actinomyctemcomitans* in periodontitis patients was four times more than normal control. The findings of our study were in accordance with the results of the study by Slots *et al.,* 1980; Mandell and Socransky, 1981. Who documented a rise of *A. actinomycetemcomitans* in periodontitis patients than in healthy individuals.[8-9]*A.actinomyctemcomitans* present in the periodontal pocket was associated with preadolescent, localized juvenile and advanced adult aggressive periodontal disease. Several virulence factors are associated with it. Leukotoxin is the most important.

Table.1 Composition of tryptic soy-serum bacitracin vancomycin agar

Sr. No.	Ingredients	Grams/ Litre
1.	Tryptic Soy Agar	40.0g
2.	Yeast Extract	1.0g
3.	Bacitracin	75.0mg
4.	Vancomycin	5.0mg
5.	Horse Serum	100.0ml
6.	Distilled Water	1000.0ml

Final pH (at 25°C) 7.1 ± 0.2

Table.2 Colony morphology (from agar plates)

Shape	Elevation	Edge	Color	Surface
Small, star like	Raised	Irregular	Transparent to light	Smooth

Table.3A Presence of *A. actinomyctemcomitans* colonies group wise

Groups	Present	Absent	Total	ϰ2-value±SD	p-value
Group I	02(4%)	48(96%)	50(100%)		p<0.01
Group II	26(52%)	24(48%)	50(100%)	7.23±1.17	
Group III	33(66%)	17(34%)	50(100%)	9.94±3.30	
Total	61	89	150		

Table.3B Distribution of *A. actinomyctemcomitans* according to gender in different groups

Gender	Group I	Group II	Group III
Male	01(50%)	14(53.84%)	26(78.78%)
Female	01(50%)	12(46.15%)	7(21.21%)
Total	02(100%)	26(100%)	33(100%)

Comparison of total count for *A.actinomyctemcomitans* in three groups (all the values in 10^5)

Table.4 Descriptive Statistics for *A.actinomyctemcomitans* in three groups (All the values in 10^5)

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal	50	0.06	0.31	0.04	-0.02	0.14	0.00	2.00
Periodontitis without Met.S	50	3.76	3.86	0.54	2.66	4.85	0.00	10.00
Periodontitis with Met.S	50	6.56	5.45	0.77	5.01	8.10	0.00	20.00

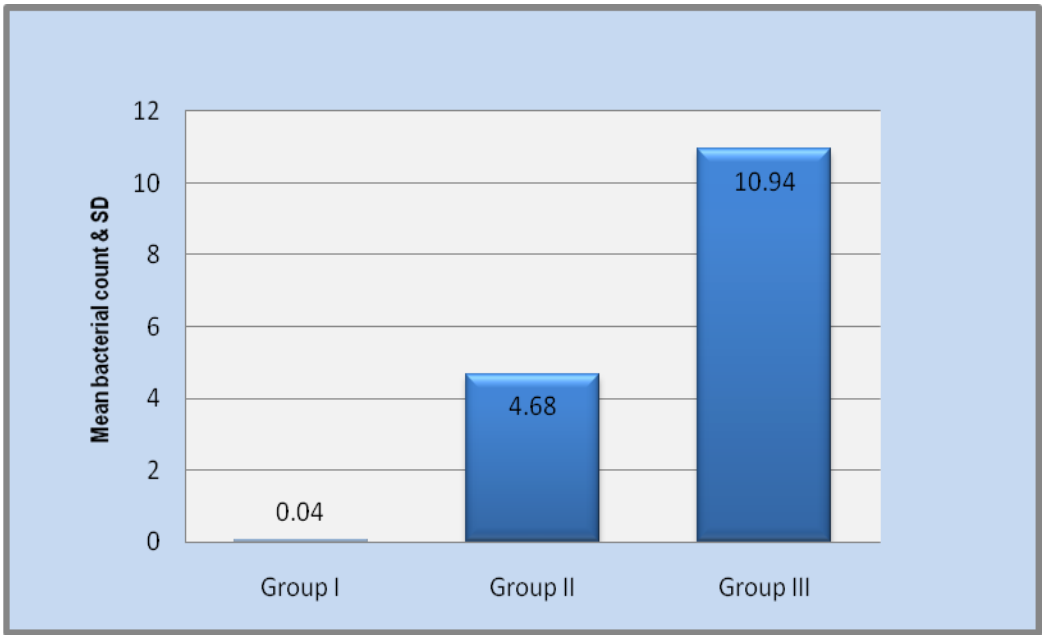
Table.5 One way ANOVA for *A. actinomyctemcomitans* in three groups (All the values in 10^5)

Source of variation	Sum of Squares	Df	Mean Square	F	p-value
Between Groups	1063.00	2	531.56	35.63	0.000 S,p<0.05
Within Groups	2192.26	147	14.91		
Total	3255.26	149			

Table.6 Multiple comparisons: Tukey Test for *A. actinomyctemcomitans* in three groups (All the values in 10^5)

Groups		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal	Periodontitis without Met.S	-3.70	0.77	0.000 S,p<0.05	-5.52	-1.87
	Periodontitis with Met.S	-6.50	0.77	0.000 S,p<0.05	-8.32	-4.67
Periodontitis with Met.S	Periodontitis without Met.S	2.80	0.77	0.000 S,p<0.05	0.97	4.62

Graph.1 Comparison of total count for *A. actinomyctemcomitans* in three groups (All values in 10^5)



Graph.3A Presence of *A. actinomyctemcomitans* colonies group wise

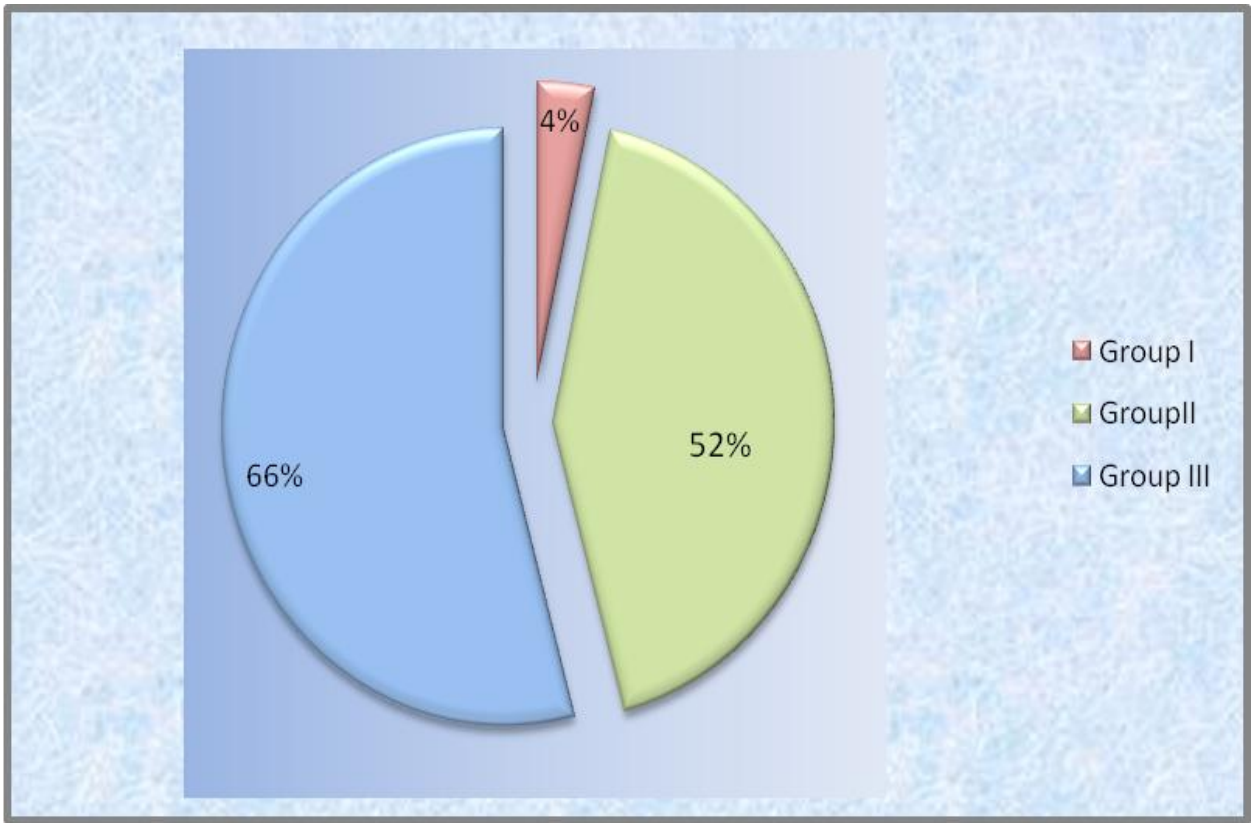
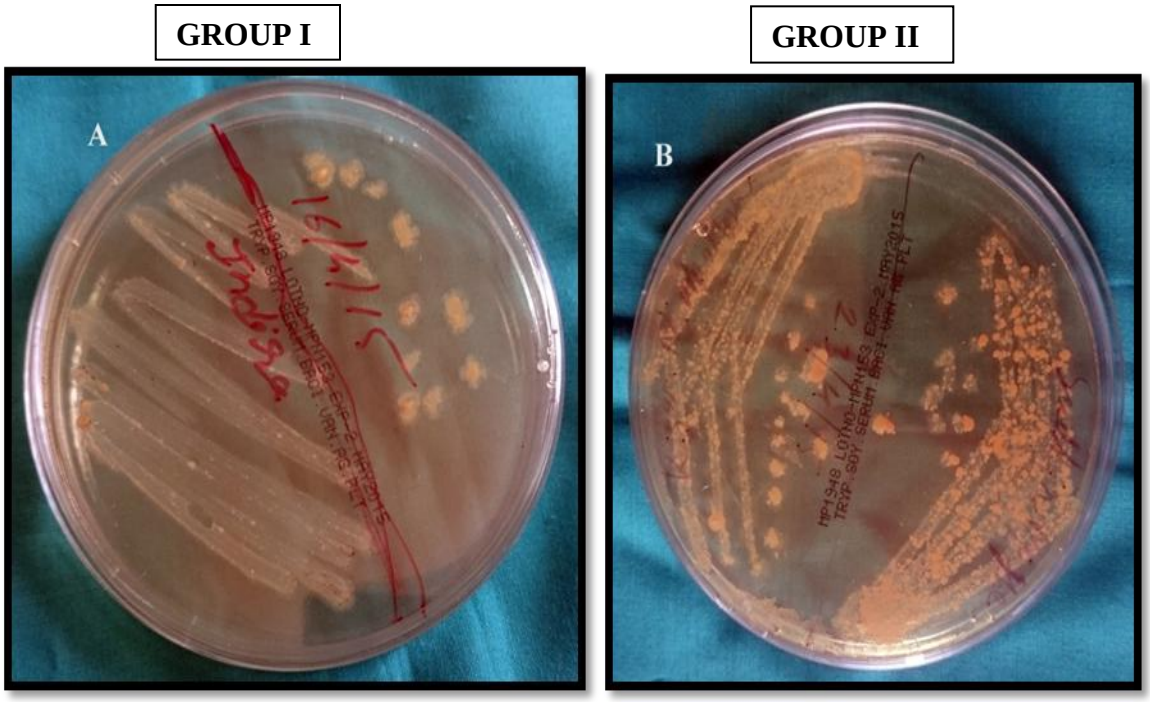


Fig.1 *A. actinomyctemcomitans* Growth in (A) Group I, (B) Group II, (C) Group III



GROUP III

Colour Plates-I

Also cytolethal distending toxin, immunosuppression factors and inhibition of PMNS functions are also seen. Leukotoxin from *A. actinomyctemcomitans* can kill human and non-human primate polymorphonuclear leukocytes and peripheral blood monocyte that are the innate immune response and these could be attacked directly. *A. actinomycetemcomitans* endotoxin has the potential to modulate host responses and contribute to tissue destruction. The ability of the *A. actinomyctemcomitans* lipopoly saccharide to stimulate macrophages to release interleukin IL-1, IL-1β, and TNF is of significance. These cytokines are capable of stimulating bone resorption and thereby contributing to periodontitis.[10]

Our study results also revealed that the mean count for *A. actinomyctemcomitans* in periodontitis patients with Met.S was three times more than periodontitis patients without Met.S. and double than normal control. The results of our study were in accordance with the study done by Sakalauskiene *et al.,* (2014) who reported increased in *A.actinomyctemcomitans* in periodontitis patients with Met.S.

The cause of increase in *A.actinomyctemcomitans* in periodontitis with Met.S could be, T2DM and increased BMI which are components of Met.S. *A.actinomyctemcomitans*was significantly and positively correlated to BMI, and the prevalence of the periodontal pathogen *A.actinomyctemcomitans* was significantly higher in diseased sites than in healthy sites in both type 1 and type 2 diabetes patients. In obesity the increased inflammation, favors oral microbes to disrupt the endocrine function of the adipose tissue which in turn would cause an imbalance in glucose homeostasis. Thus it could be hypothesized that, increased levels of leptin aggravates periodontitis and will contributes to increased in colonies of *A,actinomyctemcomitans*in periodontitis patients with Met.S. [11]

Growth favoring conditions in Met.S could be- *A.actinomyctemcomitans* requires L-cystein, iron and fat soluble vitamins for their

growth, [12] which are thought to be provided in Met.S patients that could be responsible for increases in number of colonies in Met.S patients. However, there is lack of data available regarding this. Also it could be hypothesized that in Met.S both innate and acquired immunity are altered, which likely to be contributing factor of increase in initial colonization of *A.actinomyctemcomitans*.

The results of the present study confirmed increased colonization of periodontal pathogen, *A.actinomyctemcomitans* in both periodontitis patients without Met.S and Periodontitis patients with Met.S. The fact that increases in colonization of periodontal pathogens may be attributed to the assumption that, the common link between periodontitis and Met.S is the action of ROS either acting as a second messenger or directly damaging target molecules as proteins, lipids or DNA. Periodontitis releases proinflammatory cytokines and ROS at the site of inflammation, leading to oxidative stress situation.[13] This will contribute to aggravate existing Met.S. both of which act synergistically is a matter of debate.

Thus, Researchers have hypothesized about the etiologic role of systemic diseases in the pathogenesis of periodontitis. Patients diagnosed with Met.S are thought to be at higher risk due to a compromised immune system. Infectious and opportunistic microbes responsible for periodontal infection may thus bring a burden onto the rest of the body. Furthermore, these microbes can release products that elicit an inflammatory response. Systemic conditions are recognized as continually renewing reservoirs for the periodontitis producing bacterial antigens, Gram-negative bacteria, cytokines, and other proinflammatory mediators.

Therefore, Met.S subjects should be recommended to go for frequent screening and periodontal treatment. Also, chronic periodontitis patients should be considered for multidisciplinary approach by physician, bearing in mind that the periodontal tissues are exposed not only to local bacterial onslaught, but also systemic conditions damaging them through the same mechanisms provoking damage in other tissues. To control the severity of Met.S and restrict the morbidity and mortality attributed to the components of Met.S should be the combined aim of the physician and dentist.

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