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Isolation of the oral aerobic and anaerobic flora in dental caries patients

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Abstract
The study carried out among 30 patients suffering with dental caries, attending the out patients Department of Conservative Dentistry, MGPGI, Pondicherry, and 30 controls attending the students of MGPGI the results revealed as the oral microflora from the dental caries showed prevalence of *Streptococcus mutans* (50%) for 41-50 age groups and 42.2% for 30-40 age groups. *Streptococcus viridans* are more prevalence for 41-50 age groups 40%. *Klebsiella pneumoniae* (21.4%) are more prevalence for 30-40 age groups. *Neisseria catarrhalis* (50%) are more prevalence for 51; 60 age groups. The oral microbial flora of the normal population showed the prevalence of *Neisseria catarrhalis* to a tune of 75%. The other aerobic organism showed as *Streptococcus viridians* isolated (64%) from normal controls. The other most prevalence microflora *Streptococcus mutans* (43%), E.coli (26%), *Enterococcus spp* (20%), *Pseudomonas aeruginosa* (7%), *Staphylococcus aureus* (40%).

Keywords: dental caries, micro flora, aerobic, anaerobic

1. Introduction

Dental caries are an infection caused by a combination of carbohydrate-containing foods and bacteria that live in our mouths. The bacteria are contained in a film that continuously forms on and around our teeth. We call this film as plaque. Although there are many different types of bacteria in our mouths, only a few are associated with cavities (Roger, 2008) [14]. The predominant microbial component of the gastro intestinal tract associated flora of human and mammals (Yuko Ohara *et al*., 2012) [20] During the process of bacterial cultivation, researchers realized that the majority of of these anaerobic bacteria cannot be cultivated anaerobically, or with the conventional anaerobic cultivation technique such as anaerobic culture tube introduced by Laidlaw (Laidlaw, 1915) [9] the vacuum jar designed by Noguchi, and the anaerobic container the “Mclntosh bomb” developed by “Mclntosh and Fildes”. The introduction of anaerobic glove boxes a primitive version of now widely used anaerobic chamber, by Socransky and Manganiello the 1971’s greatly facilitated the isolation and cultivation of anaerobes, particularly those obligate anaerobic microorganism, from human oral cavity (Xue song He *et al*., 2009) [18]. The genetic studies together with the additional bio-informatics information obtained from whole genomic sequences of many oral bacteria (Zhulin, 2009) [21] allow microbiologists to dissect the development and function of oral microbial community at the molecular level. Now a days microbial community analysis are largely based on PCR amplification of 16S rRNA sequences from microbial communities, that are relatively short often conserved but varied enough to differentiate bacteria at species level. Although these approaches can provide us with the microbial community, unless genomic or other research data on those identified species, it reveals very limited information regarding and function they may carry out within the flora. The advances in refinements of DNA amplification, Bioinformatics, and enhanced power of for analyzing DNA sequences have enabled the adaptation of shotgun sequencing such as chip based pyro sequencing to meta-genomic samples. The approach randomly shears DNA sequences many short sequences, reconstructs them into a consensus sequence. The purpose of this study was investigate the incidence of cultivable dental caries pathogenic bacteria in dental caries patients both aerobic and anaerobic by conventional methods.

2. Materials and Methods

2.1 Subjects

About 30 cases of dental caries were selected in the study between the age group of 30-60 years, attending as out patients in the Department of Conservative Dentistry, Mahatma Gandhi Post Graduate Institute of Dental Sciences, Pondicherry after obtaining patients consent. About 30 students were selected as controls with no signs of Dental caries.

2.2 Sample Collection and Transport

2.2.1 Aerobic Sample Collection

Unstimulated Saliva sample was collected from the dental caries patients attending the OPD Conservative Dentistry Department in to a sterile container. Saliva sample also collected from students and staff working in MGPGI with no signs of Dental caries and them were considered as controls.

2.2.2 Anaerobic Sample Collection

Saliva sample was collected from dental caries patients and control sample in directly to the Robertsons Cooked Meat Medium. All the samples were properly labeled and transported to the Microbiology Laboratory, MGPGI, Pondicherry for analysis as soon as possible to prevent

overgrowth of contamination at microorganism and death of potential pathogens. All the samples collected and processed as follows.

2.3 Method of Isolation

2.3.1 Aerobic Isolation

The saliva sample was streaked on to Nutrient Agar, Blood Agar, MacConkey, Agar plates for aerobic isolation. All the plates incubate 37ºc for 24 hours. After overnight incubation Nutrient Agar, Blood Agar, MacConkey Agar plate colonies was examined. For the further identification smear was made from the colonies for Gram staining and further biochemical tests was made from the colonies. After incubation biochemical tests were interpreted to the identification of organism.

2.3.2 Anaerobic Isolation

The saliva sample was directly collected to Robertson's Cooked Meat Medium and incubate all the RCM tubes for 37ºc for 48 hours. After 48 hours incubation colonies picked from the RC Medium and streaked on to Blood Agar, Trypticase Soy Agar, Brain Heart Infusion Agar plates and inoculate Thioglycolate broth for anaerobic isolation. All the plates incubate 37ºc for 48hours at anaerobic condition. Anaerobic jar with anaerobic Gas pack used to create an oxygen free environment for the growth of anaerobic microorganism. After the 48 hours incubation all the plate was examined. Smear was made from the colonies for Gram staining method to determine the morphology and further biochemical processed as per standard methods. Isolated colonies was first identified depending on their Gram staining for microscopic examination, oxidase and catalase test.

3. Results

Isolation of both aerobic and anaerobic pathogenic organisms among the dental caries patients and comparison to that the normal population was conducted in the Department of Conservative Dentistry, Mahatma Gandhi Post Graduate Institute Dental Sciences, Pondicherry. 30 patients with dental caries and 30 controls were used in the study. The sample collected saliva sample in dental caries patients. The patients under study were divided into Group I and II.
Group I includes dental caries patients.
Group II includes normal healthy control subjects.

Table 1: Study Group and Age Distribution

Groups	Patient characteristic feature	No. of cases	Age
Group I	Dental caries patients	30	30-60
Group II	Controls	30	18-30

Table 2 shows the isolation aerobes from (group I) individuals and their distribution according to their age group is shows in table 2. And also percentage of aerobic organism for group I showed in table 2.
The prevalence of the aerobic organism isolated among the dental caries based on the age group shows that *Neisseria catarrhalis* and *Streptococcus mutans* to be more prevalent all the age groups 50% for 40-50 age groups, 42.8 % for 30-40 age groups followed by *Staphylococcus aureus* 42.8% among the age group 30-40 and 40% for 40-50 age groups. *E.coli* 40% for the age group of 40-50, 21.8% for 30-40 age group and 16.6 for 50-60 age groups. Isolation of Streptococcus viridians 21.4% for 30-40 age groups, 30% for 40-50 age groups, 33.3% for 50-60 age groups. *Enterococcus spp* 14.4% for 30-50 age groups. *Klebsiella pneumonia* 21.4% for 30-50 age groups. *Pseudomonas aeruginosa* 7.1% for 30-50 age groups.

Table 2: Number of Organism and Percentage of Organism (Aerobic) Isolated From Group I

Organism isolated	Number and % of organism isolated		
Age groups	30-40	41-50	51-60
Number of patients	14	10	6
Neisseria catarrhalis	6(42.2%)	5(50%)	3(50%)
Streptococcus mutans	6(42.2%)	5(50%)	2(33.3%)
Staphylococcus aureus	6(21.4%)	4(30%)	2(16.6%)
Streptococcus viridians	3(42.8%)	3(40%)	1(33.3%)
E. coli	3(21.4%/	4(40%)	1(16.6%)
Enterococcus spp	2(14.2%)	2(14.2%)	0
Klebsiella pneumonia	3(21.4%)	3(21.4%)	0
Pseudomonas aeruginosa	1(7.1%)	1(7.1%)	0

Table 3 shows the isolation anaerobes from (group I) individuals and their distribution according to their age group is shows in table 3. And also percentage of aerobic organism for group I shows in table 3. The anaerobic organisms *Porphyromonas spp* and *Lactobacillus spp* were most prevalent 42.8% for the age groups of 30-40, 40% for the age groups of 40-50. *Actinobacillus spp* isolated 28.5 % for 30-40 age groups, 50% for 40-50 age groups and 16.6% for 50-60 age groups. *Fusobacterium spp* were isolated 28.5% for 30-40 age groups, 20% for 40-50 age groups, 33.3% for 50-60 age groups. *Eubacterium spp* isolated 14.2% for 30-40 age groups, 30% for 40-50 age groups. *Porphyromonas spp* were isolated 14.2% for 30-40 age groups, 10% for 40-50 age groups, 16.6% for 50-60 age groups.

Table 3: Number of Organism and Percentage of Organism (Anaerobic) Isolated From Group I

Anaerobic Microorganism			
Organism Isolated	**Number and % of Organisms**		
Age Group	30-40	41-50	51-60
Number of Patients	14	10	6
Peptostreptococcus spp	6(42.2%)	4(40%)	2(33.3%)
Lactobacillus spp	6(42.2%)	4(40%)	2(33.3%)
Actinobacillus spp	4(28.5%)	5(50%)	1(16.6%)
Fusobacterium spp	4(28.5%)	2(20%)	2(33.3%)
Eubacterium spp	2(14.2%)	3(30%)	0
Porphyromonas spp	2(14.2%)	1(10%)	1(16.6%)

Table 4 shows the isolation of aerobes including both gram positive and gram negative organisms among Group II control subjects and their distribution according to age groups. And also percentage distribution of each type of organism including gram positive and gram negative aerobes according to their age groups among Group II individuals are shows in table 4. Its shows the prevalence of oral microflora isolated among Group II control healthy subjects. *Neisseria*

catarrhalis was found to be more prevalent among all the age groups, 85.7% for the age 18-20, 69.2% for the age group of 21-25, 70% for the age group of 26-30. *Staphylococcus spp* isolated for 71.4% for 18-20 age group, 70% for the age group of 26-30. *Streptococcus viridans* was found in 71.4% for the age group of 18-20, 61.5 % for 21-25, 60% for the age group of 26-30.*Diptheroids* were isolated 14.2% to 20% for all the age groups. *Pseudomonas aeruginosa* was isolated 15.3% for 21-25, 20% for the age group of 26-30.

Table 4: Number of Organism and Percentage of Organism Isolated from Group I

Name of the organisms isolated	% of organism		
Age groups	18-20	21-25	26-30
Number of patients	7	13	10
Neisseria catarrhalis	6(85.7%)	9(69.2%)	7(70%)
Staphylococcus spp	5(71.4%)	8(61.5%)	7(70%)
Streptococcus viridians	5(71.4%)	8(61.5%)	6(60%)
Diptheroids	1(14.2%)	2(15.3%)	2(20%)
Pseudomonas aeruginosa	0	2(15.3%)	2(20%)

Anaerobic organism isolated in group II normal controls
No organism isolated

Table 5 shows the comparitive data of the isolation and identification of both aerobic and anaerobic organisms isolated among the dental caries patients and control subjects.

Among the dental caries (group I) there has been mixed infection with isolated *Neisseria catarrhalis* (46.6%), *Streptococcus mutants* (43.3%.), *Staphylococcus aureus* (40%), S*treptococcus viridians* (30%), *Klebsiella pneumonia* (13.3%,) *Pseudomonas aeruginosa* (6.6%), E.coli (26%), *Enterococcus spp* (20%). Among the dental caries (group I), it was seen that the anaerobic organisms like *Peptostreptococcus spp* (40%), *Lactobacillus spp* (36.6%), *Actinobacillus spp* (33.3%), *Fusobacterium spp* (26.6%), *Eubacterium spp (*16.6%), *Porphyromonas spp* (13.3%). Played an active role either individually or mixed infection to cause dental caries. Among the control subjects (group II) seen in *Neisseria catarrhalis* (75%), *Staphylococcus aureus* (67%), *Streptococcus viridans (64%), Diptheroids (16%), Pseudomonas aeruginosa* (10%).

Table 5 also shows the statistical analysis of Group I and Group II. Based on the statistical analysis Mean number of bacteria is found to be 28.12±1.88 in Group I aerobic bacteria and Mean number of bacteria found to be 27.13±0.4 in Group I anaerobic bacteria. And also shows the statistical analysis of Group II Mean number of aerobic bacteria is found to be 27.6±0.6.

Table 5: % Distribution of Organisms among All Groups

S. No	Organism Isolated	Group I	Group II
1	*Neisseria catarrhali*	46%	75%
2	*Streptococcus mutans*	43%	0
3	*Staphylococcus spp*	40%	67.3%
4	*Streptococcus viridians*	30%	64%
5	*Escherichia coli*	26%	0
6	*Enterococcus spp*	20%	0
7	*Klebsiella pneumoniae*	13%	0
8	*Pseudomonas aeruginosa*	7%	10.6%
		Mean=28.12±1.8	Mean=27.13
S.NO	Anaerobic Organism Isolated	Group I	Group II
1	*Peptostreptococcus spp*	40%	0
2	*Lactobacillus spp*	36.6%	0
3	*Actinobacillus spp*	33%	0
4	*Fusobacterium spp*	27%	0
5	*Eubacterium spp*	16%	0
6	*Porphyromonas spp*	13%	0
		Mean=27.6±0.4	Mean=0

Table 6 shows the number of cases and their percentage prevalence of the only aerobes isolated, only anaerobes isolated and mixture of both aerobes and anaerobes isolated among group I and II individuals.

On analyzing the data it was found that isolation of aerobes among controls constituted to 100% showing no anaerobes infection and percentage of both aerobes and anaerobes (mixed infection) isolated was 57%. only aerobes was found in 33% and only anaerobes was found in (10%) for group I.

The above data is represented as bar diagram in figure 6 showing the high prevalence of mixed infection among the dental caries (group I).

Table 6: % of Individuals Organisms and Mixed Organisms

Groups	No. of cases only aerobic were isolated	No. of cases only anaerobic were isolated	No. of cases both aerobic and anaerobic were isolated
I	10(33%)	3(10%)	17 (57%)
II	30(100%)	0	0

4. Discussion

The present study shows the prevalence of oral microbial flora in the normal subjects and the comparison of the flora among the dental caries patients attending the out patients Department of Conservative Dentistry, Mahatma Gandhi Post Graduate Institute of Dental sciences, Pondicherry. Dental caries and periodontal diseases may come into existence due to an alteration of the equilibrium of the oral bacterial population by many factors such as antimicrobial factors or the inhibitory substances of the human saliva. Salivary peroxidase system, immunoglobulins, bacteriocins, and other inhibitory substances of saliva have been reported to exhibit a relation with caries prevalence (Goyette*, et al*., 1995) [6].

The pathogenic examination from the saliva sample in the present study have been shown to be important in the etiology of dental caries. The effect of age on development of dental caries was found to be significant in the present study. This is in acceptable with the studies of (Behnaz Yalda *et al*., 2000) [1]. Dental caries is commonly known as oral microbial communities is one of the most complex bacteria flora associate with human body. so far more than 700 different bacterial species identified from human oral cavity and the majority of them are associate with dental caries. Most of these stains are through to be commensals and a smaller numbers, potential opportunistic pathogens (Packey and Sartor, 2009) [3]. In our study frequently from dental caries of 30 patients compared to normal control subjects. Moreover, there was more isolation of Gram positive bacteria than Gram negative bacteria in dental caries patients. Gram positive (40%) more frequently were isolated than Gram negative (15%). And also more frequently isolation of Gram positive bacteria than gram negative bacteria in normal controls subjects.

Enterococcus faecalis in saliva (45.5%) and subgingival biofilm samples (47.8%) from periodontitis patients compared to periodontally healthy controls (14.6 and 17.1, respectively) (Souto and Colombo, 2008) [16]. However very few studies have evaluated the correlation between the prevalence of *Enterococcus faecalis* and dental caries disease Rams *et al*., (1992) detected that *Enterococcus faecalis* in 1% of early onset dental caries and 5.1% of dental caries patients using culture methods, whereas Souto and Colombo, (2008) [16] found a much higher prevalence of this species (80%) in a large number of subgingival biofilm sample from dental caries patients. In addition, these authors observed this bacterium was much more prevalent in healthy sites from dental caries patients as compared to sites in dental caries healthy individuals. *Streptococcus mutans* levels correlate with caries incidence at the population level, but not necessarily at the individual level. *Streptococcus mutans* counts in saliva and plaque are not linearly associated with caries incidence in an individual patient, despite evidence for a linear caries progression over time.8 *Streptococcus mutans* negative individuals with coronal or root surface caries can be found, albeit at low rates (typically 2 percent). *S. mutans* was found at low frequency even in infants with caries, but was isolated more often from those infants with caries compared to those who were caries-free (29.7 *vs* 9.8%),however differences in the isolation frequencies of *S. sobrinus* (2.7 *vs* 1.3%) were not significant. In our study (43.3%) isolated from 30 dental caries patients saliva sample.

The micro flora of dental caries is characterized by a high proportion of facultative anaerobic Gram-negative bacteria (30%). In our study also isolated *Klebsiella pneumoniae* (13.3%), *Pseudomonas aeruginosa* (6.6%), and *Escherichia coli* (20%) isolated from saliva sample in 30 dental caries patients. The genus *Fusobacterium* is frequently reported in infections of the dental caries with reports indicating that *Fusobacterium* species can be detected in up to 52% of specimens (Gill & Scully, 1990) [5]. In our study (26.6%) was isolated from the saliva sample in dental caries patients. (Uematsu *et al.,* 1993) [17] isolated and identified 422 strains from the seven patients with dental caries. They showed that 42% of these isolates were asaccharolytic *Eubacterium* species or closely related strains. (Hoshino *et al*., 1992) [7] reported that a saccharolytic *Eubacterium* species are the predominant bacteria of infectious lesions in smooth surface decay and of the infected layers of dentin, suggesting that these bacterial species are involved in the progression of dental caries. In our study also showed prevalence of *Eubacterium spp* (16.6%) isolated in saliva sample in 30 dental caries patients

A correlation also exists between Lactobacillus rates in dental plaque and in saliva. If bacteria from the genus *Lactobacillus* represent 0.1% of the total salivary flora, a critical concentration of 105 CFU/ml of saliva is necessary for the detection of lactobacilli on the surface of enamel. *Lactobacilli* absent from the oral cavity of newborns appear during the first year of the life. (Mc Carthy *et al.,* 1965) [11] observed the presence of this species in 50% of newborns during their first year with a rate from 200 to 30000 bacteria. In children without caries, the rate of salivary *Lactobacilli* varied among the different studies. (Carlsson *et al.,* 1975) [2] considered that *Lactobacilli* became regularly present in 50% of children and only since the age of 2. Later, (Kohler and Bjarnason, 1987) [8] indicated that 40% of a population of 3-year old children harboured *Lactobacilli* in rates varying from 2.103 to 4.104 CFU/ml of saliva. For older children (from 6 to 16 years old), this rate is slightly bigger (54.6%). On the other hand, other authors reported the presence of *Lactobacilli* in 100% of sampled children. One factor that could influence the rate of salivary *Lactobacilli* during childhood is the carbohydrate intake. In our study is also isolated more frequently (36.6%) *Lactobacillus spp* isolated in saliva sample at 30 dental caries patients.

Gram positive anaerobic bacteria, especially *Peptostreptococcus spp* were isolated with high rates in dental caries patients (Daniluk *et al*., 2006) [4]. In also our study *Peptostreptococcus spp* isolated in high rates. Its was isolated (40%) in saliva sample at 30 dental caries patients. *Actinomycetes spp* are abundant in the human mouth and induce root surface caries in hamsters and gnotobiotic rats. They are also carbohydrate users, but are not powerfully acidogenic or acid tolerant. *Actinobacillus suis* is not easy to routinely diagnose (Minton 2008) [12], as it can be isolated along with other bacteria, and may be present in chronic cases (MacDonald *et al.,* 1976, Yaeger 1996) [10, 19]. In our study *Actinobacillus spp* isolated for conventional method and also in this study showed *Actinobacillus spp* was isolated more prevalence (33.3%) from saliva sample in 30 dental caries

patients. The microflora of severe, moderate and minimal lesions in young adults with rapidly progressing dental caries, and have observed microbial complexes associated with severe and moderate lesions, while in small lesions species *Actinomyces, Capnocytophaga ochracea, Haemophilus segnis* and *Veillonella parvula* were identified. Veillonella species *Fusobacterium and P.gingivalis* have all been associated with dental caries infection. (Daniluk *et al*., 2006) [4]. In our study *Peptostreptococcus spp (40%), Actinobacillus spp (33.3%), Fusobacterium spp (26.6%), and Porphyromonas spp* (16.6%) was isolated in saliva sample at 30 dental caries patients.

In this study, we concluded the aerobic and anaerobic microflora from patients with dental caries and without dental caries. Our data demonstrated that these species showed a trend to be more frequently detected in association with tooth surface and inner surface compared to controls. Further molecular studies are required for a better understanding of this association.

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