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PREVALENCE OF THE BACTERIAL FLORA IN THE ORAL CAVITY OF DIABETIC INDIVIDUALS IN PONDICHERRY

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ABSTRACT

Our aim is to determine the bacterial flora in the oral cavity of diabetic individuals in Pondicherry. In this study 200 buccal swabs from Diabetic individuals from age group 35±30 were included in the study. Blood Agar was used for the primary isolation of the bacterial isolates. Morphological, biochemical and phylogenetic classification were used for the identification of bacteria. Gram positive bacteria were predominant organisms in the oral cavity of diabetic individuals. Disturbances caused by the changes in the normal flora may lead to changes in the oral cavity, this may cause pathogenic micro flora to multiply and further lead to diseases. The normal flora of every individual is not same and depends on the oral hygiene. The prevalence and isolation of bacteria from the oral cavity of diabetic individuals was studied. *Staphylococcus aureus* and *Streptococcus* species were the predominant micro flora in this study.

KEYWORDS: **Diabetes, oral cavity, bacteria, *Staphylococcus aureus, Streptococcus***

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INTRODUCTION

Diabetes Mellitus is a metabolic disease that is characterized by chronic hyperglycemia due to insufficient secretion and or action of insulin with long term damage, dysfunction and failure of different organs like the eyes, kidney, nerves, heart and blood vessels. There are several complex processes associated with the evolution of diabetes. The extent of diabetes mellitus effects varies from autoimmune destruction of beta cells of the pancreas which result in deficiency of insulin to abnormalities that occur because of insulin action. The deficiency of insulin action results from an inadequate insulin secretion and /or a reduction in tissue responses to insulin in the pathway of hormone action. The co-existence of defects in insulin secretion and action in the same patient, often present an unclear picture of the defect, if either alone, is the main reason for hyperglycemia.[1]One of the major health problems in the world is Type 2 Diabetes. The change in lifestyle and diet preference apart from the genetic predisposition has enhanced the developing countries to become epicenters of cardio metabolic disorders.[2] Diabetes is becoming a potential epidemic in India.[3] In 2000, India stood first with the largest number of people with diabetes mellitus around 31.7 million and China stood second with around 20.8 million people with diabetes, followed by United States of around 17.7 millions. It was predicted that there would be a maximum increase in the incidence of diabetes which would double globally from 171 million in the year 2000 to 2030.[4] Due to the development of molecular biological techniques , many non – cultivable organisms are being discovered besides the oral flora that contains more than 300 known species of bacteria.[5] The oral flora consists of the normal flora that belongs to a diverse group of microorganisms and the oral infections are the most frequently occurring chronic disease in the world.[6] Poor oral hygiene in diabetes had been linked to the predominance of oral infection such as periodontitis , dental caries , gingivitis and candidiasis. Elucidation of whether oral hygiene influences the oral microbial colonization is not fully understood.[7]It was reported that the severity of diabetes increased the prevalence of both gram negative and gram positive bacteria. There are several studies on the microbiota in diabetic patients as well as other patient populations.[8] There are studies on the aerobic flora in diabetes but the priority was provided to different anatomical sites rather than the oral cavity and less was known on the role of aerobic bacterial flora in oral infections in diabetic patients.[9] The aim of the present study is to find the prevalence of the aerobic bacterial flora in the oral cavity of the diabetic individuals attending a diabetic clinic in Pondicherry.

MATERIALS AND METHODS

Approval for the present study was provided by the Institutional Ethics Committee of Madras Diabetes Research foundation. Samples were collected from the volunteers attending Dr. Mohan's Diabetes and Endocrine specialties in Pondicherry. Considerations on the inclusion criteria for this study included volunteers in the age group of 35±30, volunteers with uncontrolled type II diabetes , either male or female, denture wearers or non - denture wearers, with or without oral lesions. Exclusion criteria considered volunteers who were on antibiotic or corticosteroid therapy during the period of 4 weeks of study. All the volunteers included in the study were explained about the nature and purpose of the study. A questionnaire was developed to fill in the history and details of the volunteer. Informed consent was obtained from each volunteer and then the buccal swabs were collected.

(i) Sample collection

The oral cavity was examined before the collection of buccal swab.[10]After obtaining the informed consent, buccal swabs were collected aseptically from 200 diabetic volunteers. The 200 diabetic volunteers were either male or female belonging to the age group from 20 to 65 years. The buccal swabs were directly inoculated onto Blood agar plates.[11]

(ii) Processing of Sample, Isolation and Identification of Bacteria

The blood agar plates[11] were incubated aerobically at 37ºC for 24 hours. After 24 hours of incubation, the plates were examined for the growth of the bacteria. The bacterial isolates recovered were identified based on morphology, biochemical and phylogenic properties. Morphological characterization included the appearance of the colony on blood agar, gram staining which was performed to differentiate the bacteria into 2 distinct groups.[12] Further, Hanging drop method was performed to determine the motility of the bacteria. Bacterial characterization included Catalase test, Oxidase test, Indole production test, Methyl Red test, Voges-Proskauer test, Citrate Utilization, Urease and the Triple Sugar Iron Agar test. Additional test included growth of the bacterial culture on Mannitol Salt Agar, Coagulase test, *Lactobacillus* MRS Agar, Bismuth Sulphite Agar, Eosin Methylene Blue Agar and Bacitracin Sensitivity.[13] One bacterial isolate which was not identified through conventional biochemical tests was subjected to phylogenetic characterization using 16S rRNA gene sequencing technique[14]

(iii) Phylogenetic characterization of Bacteria

One of the isolate that was not successfully identified morphological and biochemically (L5) was further subjected to phylogenetic analysis using 16S rRNA gene sequencing.[14] The template DNA of the isolate was prepared. Colonies were picked up with a sterilized toothpick and suspended in 0.5 ml of sterile saline in a 1.5 ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). It was incubated at 56℃ for 30 min and then heated at 100℃ for 10 min. After heating, the supernatant was used for PCR. PCR: 1 µl of template DNA in 20 µl of PCR reaction solution was added. 518F/800R primers was used for bacteria, and then 35 amplification cycles

at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec were performed. DNA fragments were amplified about 1,400 bp in the case of bacteria. A positive control (*E. coli* genomic DNA) and a negative control in the PCR were included. Purification of PCR products: Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). Sequencing: The purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

RESULTS

A total of 200 buccal swabs were collected from Diabetic volunteers who were attending a diabetic clinic in Pondicherry. The distribution of various factors between the male and female diabetic individuals was recorded. (Table 1)

Table 1
Distribution of various factors between male and female diabetic individual

Sl.No	Factors	Gender	
		Male	Female
1	Number of volunteers (n)	101	99
2	Age range (years)	20-65	20 -65
3	Dental carries (n)	42	61
4	Lesions in cavity (n)	Nil	6
5	Fasting Blood glucose level (mg/dl)	>140	>140

Out of the 200 samples screened, all the 200 samples showed significant growth on blood agar. A total of 277 bacterial isolates were obtained.

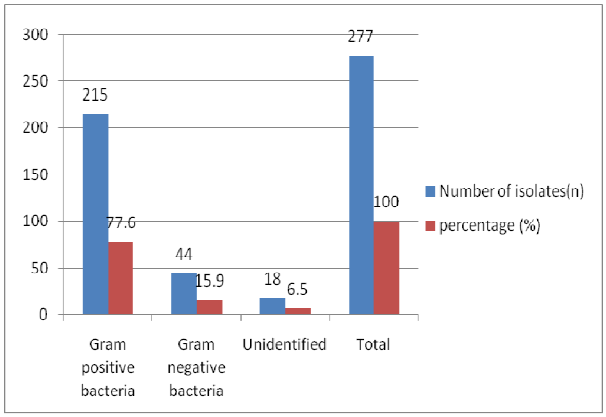
(i) Isolation of Bacteria from buccal swabs of Diabetic individuals

The samples which were swabbed onto blood agar were observed for growth, after 24 hours of incubation. A total of 277 bacterial isolates were obtained of which 18 isolates did not grow when sub cultured and the remaining 259 cultures were identified. Morphological, biochemical and phylogenetic characteristics were used in identification.

(ii) Distribution of gram positive and gram negative bacteria in diabetic individuals.

The numbers of gram positive and gram negative bacteria identified from the oral cavity of diabetic volunteers were 215 and 44 respectively. (Figure 1)

Figure 1
Distribution of Gram positive and Gram negative bacteria in diabetic individuals



(iii) Additional tests for Identification

Mannitol Salt Agar - *Staphylococcus aureus* produced small yellow colonies which were surrounded by clear zones.
Coagulase test - *Staphylococcus aureus* produced coagulase which are needed for the conversion of fibrinogen to fibrin.
Eosine Methylene Blue Agar – *Escherichia coli* produced small dark colonies with a green metallic sheen. *Enterobacter aerogenes* produced large colonies which are pink to buff around dark centres.
Bismuth Sulphite Agar – Bacterial isolates were grown on BSA. Black colonies were observed and identified as *Salmonella typhi*.

Bacitracin Sensitivity test – Bacitracin disc was placed on blood agar streaked with the *Streptococcus* species. Group A Streptococci was inhibited by bacitracin placed on Blood agar.
Triple Sugar Iron Agar test – this media was used for the selective identification of enteric bacteria which were isolated from the oral cavity of diabetic individuals.
MRS Agar – This media was used for selective identification of *Lactobacillus sp.*

(iv) Distribution of bacteria in Diabetic individuals.
The bacterial isolates which were obtained had a maximum 137 isolates of *Staphylococcus aureus* (49.45%), 4 *Streptococcus pyogenes* (1.44%), 55 *Streptococcus sp.* (19.85%),10 *Micrococcus sp.*(3.61%), 9 *Lactobacillus sp.*(3.24%), 2 *Escherichia coli* (0.72%), 6 *Enterobacter sp.*(2.16%), 4 *Klebsiella sp* (1.44%), 1 *Klebsiella pneumoniae subsp. pnuemoniae* (0.36%), 4 *Proteus sp.*(1.44%), 9 *Citrobacter sp* (3.24%), 11 *Serratia sp.*(3.97%). 3 *Salmonella typhi* (1.08%), 4 *Pseudomonas aeruginosa* (1.44%) and 18 unidentified bacterial isolates. (Table 2). *Staphylococcus aureus* isolates were the highest among the bacterial isolates, isolated from the buccal cavity of diabetic individuals. (Figure 2)

Table 2
Distribution of bacteria isolated from the oral cavity of Diabetic individuals.

S.No	Bacterial Isolates	Number (n)	Percentage (%)
1.	*Staphylococcus aureus*	137	49.45
2.	*Streptococcus pyogenes*	4	1.44
3.	*Streptococcus sp.*	55	19.85
4.	*Micrococcus sp.*	10	3.61
5.	*Lactobacillus sp.*	9	3.24
6.	*Escherichia coli*	2	0.72
7.	*Enterobacter sp.*	6	2.16
8.	*Klebsiella sp.*	4	1.44
9.	*Klebsiella pneumoniae*	1	0.36
10.	*Proteus sp.*	4	1.44
11.	*Citrobacter sp.*	9	3.24
12.	*Serratia sp.*	11	3.97
13.	*Salmonella typhi*	3	1.08
14.	*Pseudomonas aeruginosa*	4	1.44
15.	Unidentified isolates	18	6.49
	Total	277	100

Figure 2
Percentage of bacterial isolates obtained from the oral cavity of Diabetic individuals.

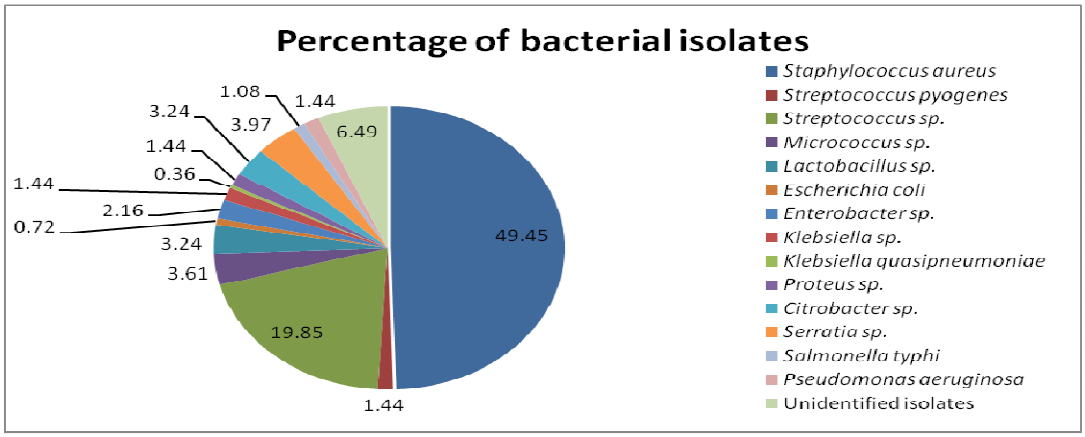
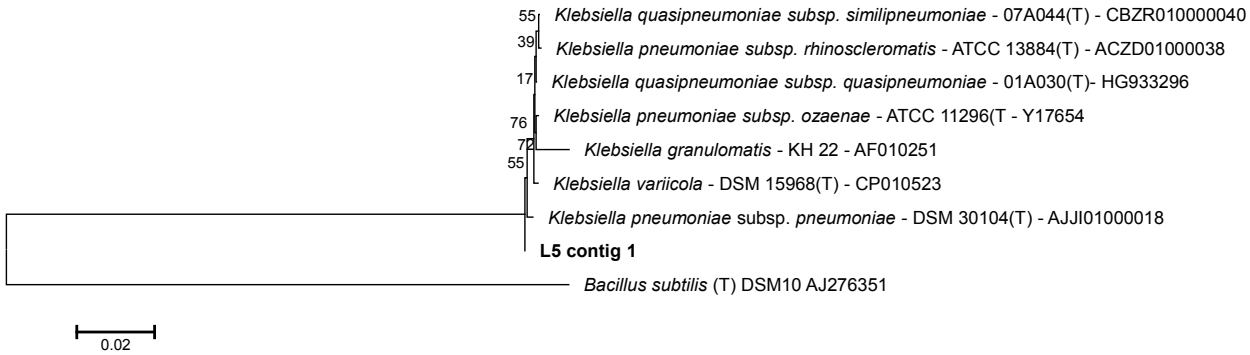


Figure 3
Phylogenetic position of the strain L5, using the neighbor joining analysis.



The strain that was not identified by the conventional biochemical tests, L5 was subjected to phylogenetic identification by sequencing the 16S rRNA gene of that strain (Figure3). The culture sequence obtained was subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetic related sequence were carefully selected from the GenBank and they were subjected to multiple sequence alignment and then the aligned sequences were trimmed to similar length in nucleotides, that were subjected to phylogenetic tree (neighbor joining) construction using MEGA 6. This phylogenic tree is based on a neighbor-joining analysis of 1,000 re-sampled data sets. Bar 0.02 substitutions per site. The phylogenetic analysis clearly showed that the isolate belonged to *Klebsiella pneumoniae subsp. pneumoniae*.

DISCUSSION

Distribution of aerobic bacteria in the oral cavity of diabetic individuals was analyzed in the present study. The distribution of gram positive bacteria and gram negative bacteria were 215 and 44 isolates respectively. This contributed to 77.62% of gram positive bacteria and 15.88% of gram negative bacteria and 6.48% unidentified bacteria. It was reported in a previous study that Gram positive and Gram negative bacteria were fairly involved in dental diseases in diabetic individuals. The bacteria which were present also have its highest incidence based on the severity of the disease, the immunity of the patient and also the effect of predisposing factors. The involvement of gram positive organism was (52.4%) and (47%) respectively in the reported study.[8] One of the previous study reported that due to the increase in concentrations of glucose in the saliva of diabetic individuals and crevicular fluid , there were increase in the micro flora of these individuals.[15] In another study , higher number of bacteria was isolated from diabetic individuals among which the *Streptococcus* species (59.78%) were the most commonest, followed by *Staphylococcus aureus* (21.73) and *Klebsiella* species (7.6%). These were isolated from diabetic individuals who had oral lesions.[16]In the present study, there were 6 diabetic individuals who had oral lesions and *Staphylococcus aureus* had been isolated from each of their samples. This indicates that they might have been suffering from periodontal infections. Proper control of glycemic index is important in maintaining the oral health in diabetic individuals. It has also been reported that *Staphylococcus aureus* was the frequent isolate from the oral cavity and investigation was required to find the role of *Staphylococcus aureus* in several diseases.[17] In a previous study, they reported that the dietary changes[18] and age of the individual[19]also had an influence and change on the bacterial flora of the oral cavity.103 diabetic individuals had dental caries and this reflects to poor oral hygiene in them. Oral infections are common among diabetic individuals and this is mainly associated with poor oral hygiene. The influence on the oral hygiene on microbial colonization is not well understood.[20] The health of the oral cavity depends on the normal oral flora present and this prevents the colonization of pathogenic organisms from colonization. Oral diseases may be caused due to the destruction of the normal flora.[21]Infections like pneumonia are caused by *Staphylococcus* and Gram negative bacteria which are thought to be the transient colonizers of the oral cavity.[22]Many systemic diseases like the bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birth and cardiovascular disease have been caused by specific oral bacterial species.[11] The oral micro flora in diabetic individuals also play important roles.

CONCLUSION

Diabetes mellitus is a chronic metabolic disease and the diabetic individuals suffer from long term complications and there is no cure for them. In the present study, the prevalence of bacteria in the oral cavity of diabetic individuals were *Staphylococcus aureus* 49.45%, *Streptococcus pyogenes* 1.44%,*Streptococcus sp.* 19.85%, *Micrococcus sp.* 3.61%, *Lactobacillus sp.* 3.24%, *Escherichia coli* 0.72%, *Enterobacter sp.* 2.16%, *Klebsiella sp* 1.44%, *Klebsiella pneumoniae subsp. pneumoniae* 0.36%, *Proteus sp.*1.44%, *Citrobacter sp.* 3.24%, *Serratia sp.*3.97%, *Salmonella typhi* 1.08%, *Pseudomonas aeruginosa* 1.44% and 6.49% unidentified

bacterial isolates. 137 isolates of *Staphylococcus aureus* was isolated and identified. Our current knowledge on the prevalence of bacterial flora in the oral cavity of diabetic individuals showed that gram positive organisms are predominant in the oral cavity of diabetic individuals and further changes in the normal oral flora occur in any systemic disease. In order to prevent complications, it is essential for all diabetic individuals to have proper glycemic control and also maintain oral hygiene.

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