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Isolation, Identification and Characterization of Bacteria Occurring In the Oral Cavity of Diabetic and Non Diabetic Individuals.

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ABSTRACT

The largest diabetic population in India is around 50.8 million that could reach an epidemic proportion by 2030. The most common chronic oral diseases encountered worldwide are the oral infections seen in diabetes like the dental caries, gingivitis and periodontitis. The aim of the present study is to investigate the prevalence of oral bacterial flora in the buccal cavity of Diabetes mellitus individuals and control. A total of 400 buccal samples were collected from diabetic and non diabetic individuals (200 buccal samples each). Nutrient agar was used for the primary isolation of bacterial species. Morphological, Biochemical and Phylogenetic characterization along with additional tests using selective and differential media were used for identification. Results revealed that the normal oral flora in diabetic individual were higher , compared to non diabetic individuals. *Staphylococcus aureus* was the most predominant bacteria followed by *Streptococcus* species in diabetic individuals. In-vitro anti microbial susceptibilitytesting was performed using Amikacin (30mcg), Amoxicillin (30mcg), Erythromycin(15mcg), Gentamicin (30mcg) and Tetracycline (30mcg). Results showed that the antibiotics used in the test were effective against the isolated bacterial species. In-vitro antimicrobial susceptibility testing is needed for proper management of the disease.

Keywords: Diabetes, bacterial flora, buccal swabs, antimicrobial susceptibility test.

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INTRODUCTION

Diabetes is described by a marked increase in blood sugar levels in the body and is often referred to as hyperglycaemia. There are two types of diabetes which includes Diabetes insipidus and Diabetes mellitus. More than 171 individuals worldwide are affected by diabetes and the epidemic status for diabetes had been attained. Diabetes mellitus is described as a group of metabolic diseases in which an individuals body neither secretes the needed amount of insulin nor responds to the insulin that is produced. Hence a person suffering from Diabetes mellitus has increased blood sugar levels and this produces various symptoms of polyuria, polydipsia, and polyphagia. Several complications are caused by diabetes mellitus and this is one of the common and growing global health problem. Hyposalivation [1] and salivary dysfunction [2] are experienced by the people with diabetes.

Diabetes mellitus makes an individual highly susceptible to bacterial and fungal infections. The warmth, the moisture, the constant influx of nutrients through saliva and food intake through the mouth provides a favourable environment for the growth of microorganisms [3]. About 700species of bacteria inhabits the oral cavity and this has contributed to the health and physiological status of the oral cavity. Colonization of the bacteria within the oral cavity occur on 2 types of surfaces, the hard surface of the teeth and the soft tissues of the oral mucosa. [4] Oral health decides the health of the individual. Many varieties and number of microorganisms are present in the oral cavity. The human body provides a habitat for the microorganisms, similarly the oral cavities also provides unique sites for the multiplication of microorganisms. [5] Due to prolonged period of poor glycemic control in diabetic individuals, they are at a greater risk of developing oral conditions such as gingivitis, periodontal disease, dental caries, xerostomia, alveolar bone loss. [6] Inadequate salivary flow and composition , greater number of cariogenic bacteria and other factors contribute to oral caries. Caries are linked with the persons lifestyle and behavioural factors. [7] Not much research has been carried out and the factors that underlie the potential association of dental caries with diabetes. [8]

MATERIALS AND METHODS

The present study was accepted and approved by the Institutional Ethics Committee of Madras Diabetes Research Foundation. The study was planned and executed in collaboration with Dr. Mohans Diabetes and Endocrine specialities, Pondicherry. Volunteers in the age group 35±30 with type II Diabetes and controls that were healthy individuals without diabetes, male and female, denture wearer or non- denture wearer, with or without oral lesion were included in the study. Fasting blood glucose level was also recorded. Those volunteers with steroid or antibiotic use in the last 4 weeks of study were excluded. Volunteers were asked to complete a questionnaire bearing information on demographics (age, gender) and medical history. An informed consent was obtained from them after explaining the nature and purpose of study. Samples were collected from diabetic and non diabetic individuals after oral examination and when these subjects were fasting.

Collection of Sample

The buccal swabs [9] were collected aseptically and swabbed onto Nutrient Agar plates [10] (Himedia Laboratories, Mumbai, India) and incubated for 24 hours at 37^{o}C. By repeated streaking onto Nutrient Agar, pure cultures of each bacteria were obtained.

Isolation and Identification of bacterial isolates

The isolated bacteria were identified based on colony morphology, gram staining, motility, biochemical test which includes catalase test, oxidase test, indole production test, methy red test, Voges – Proskauer test, urease test, Triple sugar iron agar test and carbohydrate fermentation test. Phylogenetic characterization using 16S rRNA technique was performed. [11] Selective and differential media were used for further identification of bacterial isolates. These includes Mannitol Salt Agar, coagulase test, MRS media, Bismuth Sulphite Agar, Mac Conkey, Blood Agar, Eosin Methylene Blue Agar (Levine).

Antibiotic Sensitivity test

Disc diffusion method [12] was employed to find the sensitivity of the bacterial isolates against commonly used antibiotics like Amikacin (30mcg), Amoxycillin (30mcg), Erythromycin (15mcg), Gentamicin (30mcg) and Tetracycline (30mcg). The islolates were scored as resistant or susceptible based on CLSI guidelines. [13] Mueller Hinton Agar was used to determine the antimicrobial sensitivity. Inoculum was prepared by transferring 4 or 5 similar colonies obtained from pure culture to Tryptone Soya Broth and incubated at 35-37^{o}C for 2 to 4 hrs. The inoculums turbidity was compared and adjusted to standard 0.5 McFarland. A sterile cotton swab was dipped into the standard inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface was streaked with the swab three times, turning the plate at 60^{o} angle between each streaking. The inoculum was allowed to dry for 5 -15 minutes. The antibiotic discs were applied aseptically. The plates were incubated immediately at 35±2^{o}C and examined after 20-24 hrs of incubation. The zones of inhibition were measured and recorded using a calibrated instrument.

Phenotypic and phylogenetic characterization of Bacteria

One of the morphological and biochemically identified isolate was further subjected to phylogenetic analysis using 16S rRNA sequencing.

The template DNA was prepared from the pure cultured strain CS3. 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). Incubated at 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant can be use for PCR.

PCR 1 µl of template DNA in 20 µl of PCR reaction solution was added. 518F/800R primers for bacteria were used, 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 bpin the case of bacteria. a positive control (*E. coli* genomic DNA) and a negative control in the PCR was added.

Purification of PCR products. Unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore) were removed.

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 were 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 AND DISCUSSION

Buccal swabs were collected from 400 individuals (age 35±30), 200 had Type II Diabetes and the other 200 were non- diabetic healthy controls. The study showed that out of 200 diabetic individuals, 101 were male and 99 were female age ranging from 20 - 65, 28 were denture wearers and 6 individuals had lesions in the oral cavity, 103 individual had dental caries.

Out of the 200 healthy non – diabetic individuals, 73 were male and 127 were female, age ranging from 18 -55, 6 were denture wearers, none had oral lesions, 62 had dental caries. (Table 1)

The incidence of dental caries was higher in diabetic individuals than non- diabetic individuals. Around 103 diabetic individuals had dental caries (51.5%) whereas only 62 had dental caries (31%). In one of the previous studies, it was reported that periodonitis is common among diabetic individuals and dental caries is common among non – diabetic individuals. This is controversial to the present study. [14] It was reported in a study that there could be a risk of development of new and recurrent dental caries since the patients with diabetes are more susceptible to oral sensory, periodontal, and salivary disorders. [8] Receding of gums and exposure of the root surfaces makes the elderly people more susceptible to root caries [15].

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Table 1: Distribution of various factors between diabetic and non – diabetic individuals

S. No	Factors	Diabetic individuals	Non – diabetic individuals
1.	Number of Volunteers (n)	200	200
2.	Male (n)	101	73
3.	Female (n)	99	127
4.	Age range (years)	20 – 65	18 -55
5.	Presence of oral lesions (n)	6	0
6.	Denture wearers (n)	28	6
7.	Dental caries (n)	103	62

n – number

Table 2: Number of bacterial isolates obtained from diabetic and non- diabetic healthy individuals.

S. No	Bacterial Isolates	Diabetic Individuals		Non- diabetic individuals	
		Number of Isolates (n)	Percentage of isolates (%)	Number of Isolates (n)	Percentage of isolates (%)
1.	*Staphylococcus aureus*	105	39.47	95	46.34
2.	*Streptococcus sp.*	80	30.45	29	14.15
3.	*Micrococcus*	25	9.39	16	7.80
4.	*Lactobacillus*	17	6.3	28	13.66
5.	*Escherichia coli*	2	0.75	0	0
6.	*Klebsiella.sp*	12	4.5	9	4.39
7.	*Proteus mirabilis*	5	1.8	3	1.46
8.	*Proteus vulgaris*	3	1.13	0	0
9.	*Salmonella typhi*	2	0.75	0	0
10.	*Enterobacter sp.*	2	0.75	3	1.46
11.	*Citrobacter sp.*	5	1.88	2	0.97
12.	*Serratia sp.*	2	0.75	16	7.80
13.	*Pseudomonas sp*	5	1.88	4	1.95
14.	*Acinetobacter baumannii*	1	0.38	0	0
	Total	266	100	205	100

n – number, % - percentage

One of the previous study reported that at least one lesion or abnormality is found in most diabetic patients, the most likely occurring abnormalities were lingual varicosity and erythematous candidiasis. [16] In the present study, out of 200 samples collected from diabetic individuals, 6 individuals had manifestations of the oral lesions and none of the healthy non- diabetic individuals had oral lesions.

A total of 266 and 205 bacterial species were isolated and identified from diabetic and non – diabetic healthy individuals respectively. (Table 2) The bacterial isolates were identified by performing morphological characterization, biochemical characterization and phylogenetic characterization. There was difficulty in identification of an isolate and it was identified by phylogenetic characterization that is 16S rRNA sequencing. (Figure 1) This CS3 isolate was identified as *Acinetobacter baumannii.* The frequency of isolation of different kinds of aerobic bacteria from the oral cavity of diabetic individuals was higher when compared to non-diabetic individuals using Nutrient Agar medium. The number of bacterial isolates obtained were also higher. The reasons for higher number of bacteria and kinds of bacteria may be associated to hyperglycaemia in diabetic individuals.

In the present study, the number of gram positive bacteria and gram negative bacteria isolated from diabetic individual were 227 and 39 making up to 85.3% and 39% respectively. (Figure 2). In the same way , the number of gram positive and gram negative bacteria isolated from non – diabetic individuals were 168 and 37 making up to 81.9% and 18% respectively. (Figure 3). The percentage distribution of the bacterial isolates from diabetic and non- diabetic individuals have been shown. (Figure 4 and Figure 5*) Staphylococcus aureus* was the predominant bacteria in both diabetic individuals and non – diabetic individuals. They constituted about 105 isolates from diabetic and 95 isolates from non- diabetic individuals respectively. In one of the previous study *Streptococcus* was found to be 59.8% followed by Staphylococci to 21.73% and then *Klebsiella* species to 7.6%. Gingivitis, dental caries and periodontitis in diabetic individuals were mainly caused by Gram positive bacteria

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which plays a major role in causing these infections. [14] In previous studies, it was reported that there was a shift in the composition of the oral bacteria due to dietary changes in addition to poor hygiene. [17] Age also has an influence on the oral microflora. [18]

Figure 1: Phylogenetic tree for the isolate CS3 which was not successfully identified by biochemical method and was successfully identified using the sequencing of the 16S rRNA gene sequence, this tree is neighbor joining tree generated in MEGA6.

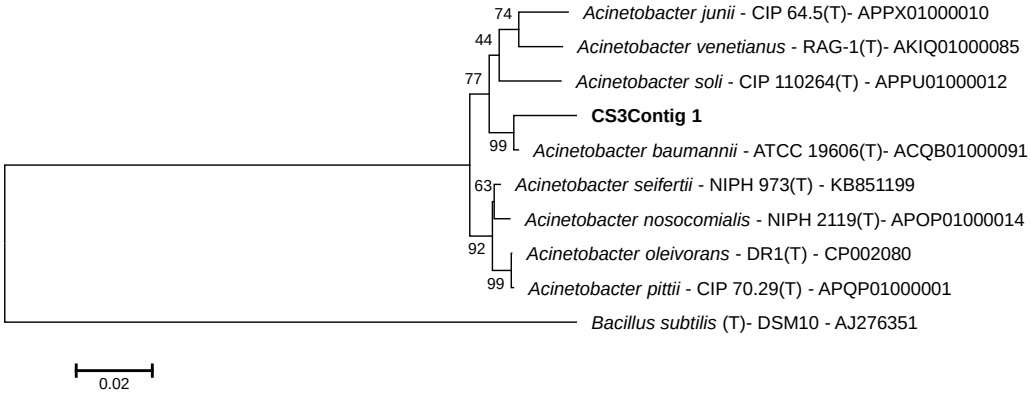

Figure 2: Distribution of Gram positive and gram negative organisms in diabetic individuals and percentage (%)

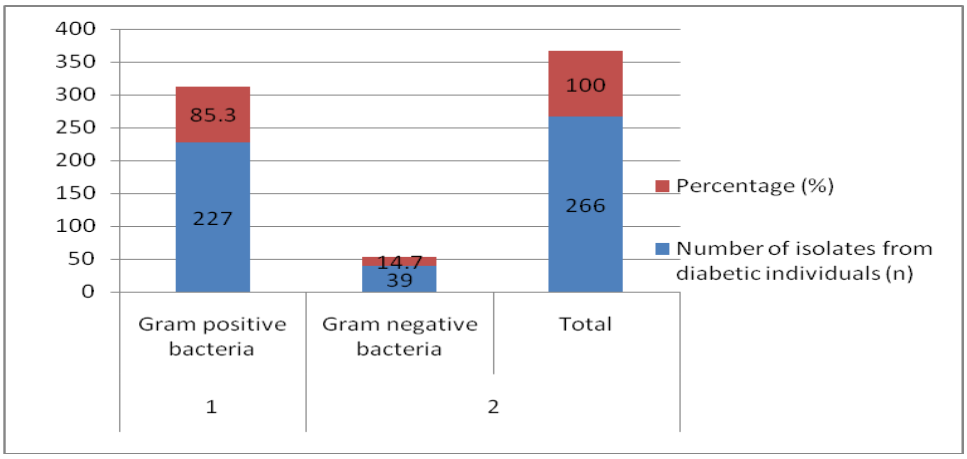

Figure 3: Distribution of Gram positive and gram negative organisms in non - diabetic individuals and percentage (%)

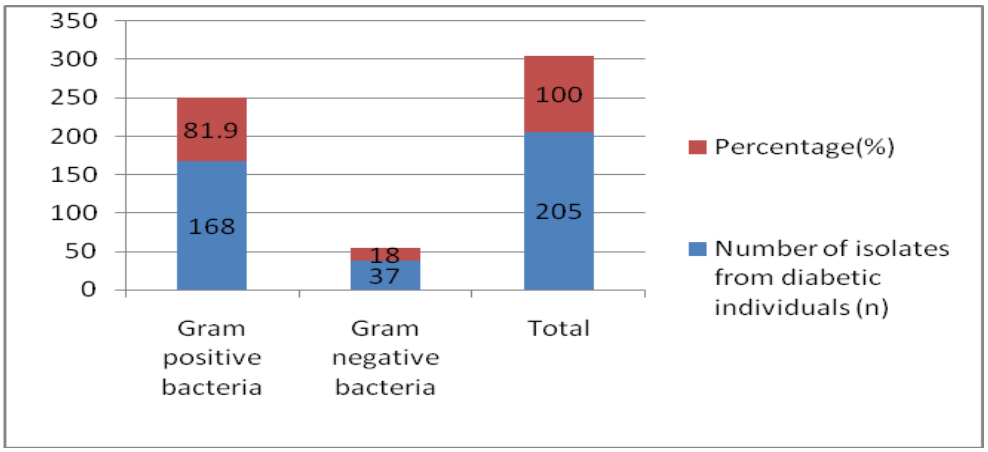

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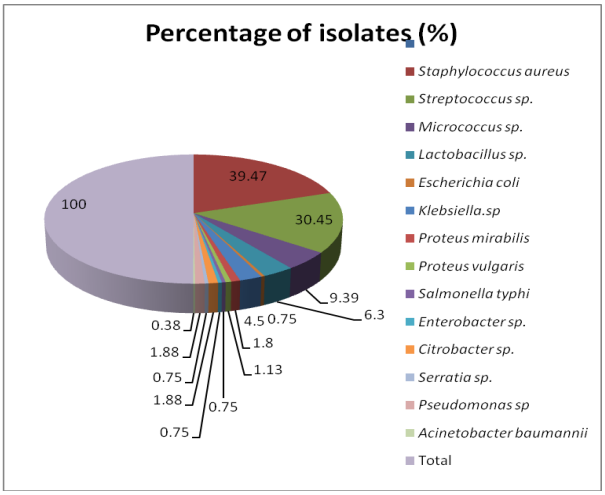
Figure 4: Percentage distribution of bacterial isolates in diabetic individuals



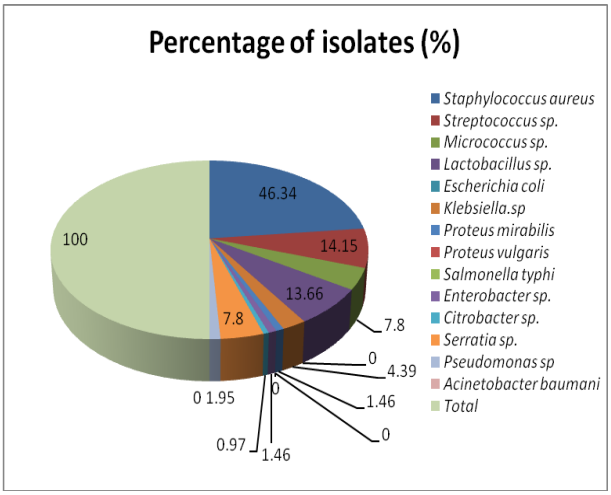


Figure 5: Percentage distribution of bacterial isolates in non - diabetic individuals

The antibiotic sensitivity test was performed for all the isolated organisms (471 isolates) against 5 different antibiotics , Amikacin (30mcg), Amoxicillin(30mcg), Erythromycin (15mcg), Gentamicin (30mcg) and tetracycline (30mcg) for 266 isolates from diabetic individuals and 205 isolates from non- diabetic individuals. All the Gram positive and Gram negative bacteria showed varying sensitivity and resistance to the antibiotics. (Table 3 and Table 4) The 200 isolates of *Staphylococcus aureus* were sensitive to amikacin, 195 isolates sensitive to amoxicillin, 192 isolates sensitive to erythromycin and gentamicin and 153 isolates sensitive to tetracyclin. Among the 109 isolates of *Streptococcus sp,* 100 isolates were sensitive to amikacin and Amoxicillin, 68 isolates were sensitive to erythromycin and 83 isolates were sensitive to gentamicin and 98 isolates were sensitive to tetracycline. Among the 41 isolates of *Micrococcus sp.* 18 isolates were sensitive to amikacin, 28 isolates sensitive to amoxicillin, 23 isolates were sensitive to erythromycin and 20 isolates were sensitive to gentamicin and 38 isolates were sensitive to tetracycline. Among the 45 isolates of *Lactobacillus sp,* 40 isolates were sensitive to amikacin , 38 were sensitive to Amoxicillin and all the isolates were resistant to erythromycin, 40 isolates were sensitive to gentamicin and 43 isolates were sensitive to tetracycline. Among the Gram negative bacteria, 2 isolates of *E.coli,* were sensitive to amikacin and 1 isolate to Amoxicillin, 2 isolates were sensitive to erythromycin, gentamycin and tetracycline, (100% sensitivity) Among 21 *Klebsiella* isolates, 18 were sensitive to amikacin, 6 to Amoxicillin,11 to erythromycin, 17 to gentamicin and 21 to Tetracycline. Among 8 isolates of *Proteus mirabilis,* 7 were sensitive to amikacin, 2 to amoxicillin, 5 to erythromycin, 8 to gentamicin and resistant to Tetracycline. Among 3 *Proteus vulgaris* isolates, 2 were sensitive to amikacin, 3 to Amoxicillin, erythromycin and gentamicin and 1 to Tetracycline. The *Salmonella typhi* isolate showed 100% sensitivity to all the 5 antibiotics. Among 5 *Enterobacter sp.* , 5 isolates were sensitive to amikacin, 1 to Amoxicillin and erythromycin, 5 to gentamicin and 5 to tetracycline. Among 7

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Citrobacter sp. which were isolated, 5 were sensitive to amikacin, 2 to amoxicillin, 6 to erythromycin, 5 to gentamicin and 3 to Tetracycline. Among the 18 isolates of *Serratia,* 15 were sensitive to Amikacin, 2 to amoxicillin, 17 to gentamicin and 3 to tetracycline and they are resistant to erythromycin. Further , 1 isolate of *Acinetobacter baumannii* showed sensitivity to amikacin and reistance to amoxicillin, erythromycin, gentamicin and tetracycline. *Acinetobacter baumanii* is seen on the skin surface and it is the most difficult antimicrobial resistant gram negative bacilli to control and treat. This bacteria is seen in immunocompromised people and also in the case of diabetes.

Table 3: Antibiotic sensitivity pattern of Gram positive bacterial isolates from both diabetic and non – diabetic individuals

SNo	Gram positive bacteria	Total no. of Isolates	AK (30mcg)		AMC (30mcg)		E (15mcg)		GEN (30mcg)		TE (30mcg)	
			S	R	S	R	S	R	S	R	S	R
1.	*Staphylococcus aureus* (n)	200	200	0	195	5	192	8	192	8	153	47
	%		100	0	97.5	2.5	96	4	96	4	76.5	23.5
2	*Streptococcus sp.* (n)	109	100	9	100	9	68	41	83	26	98	11
	%		91.7	8.25	91.7	8.25	62.4	37.6	76.1	23.9	89.9	10.1
3	*Micrococcus sp.* (n)	41	18	23	28	13	23	18	20	21	38	3
	%		43.9	56.1	68.3	31.7	56.1	43.9	48.8	51.2	92.6	7.3
4.	*Lactobacillus sp* (n)	45	40	5	38	7	0	45	40	5	43	2
	%		88.9	11.1	84.4	15.6	0	100	88.9	11.1	95.6	4..4
Total		395										

S – Sensitive R – Resistance N - Number of bacterial isolates % - Percentage of bacterial isolates AK –Amikacin AMC – Amoxicillin E – Erythromycin GEN – Gentamicin TE - Tetracycline

Table 4: Antibiotic sensitivity pattern of Gram negative bacterial isolates from both diabetic and non – diabetic individuals

SNo	Gram negative bacteria	Total no. of Isolates	Ak (30 mcg)		AMC (30 mcg)		E (15 mcg)		GEN (30 mcg)		TE (30 mcg)	
			S	R	S	R	S	R	S	R	S	R
1.	*Escherichia coli* (n)	2	2	0	1	1	2	0	2	0	2	0
	%		100	0	50	50	100	0	100	0	100	0
2	*Kiebsiella sp.* (n)	21	18	3	6	15	11	10	17	4	21	0
	%		85.7	14.3	28.6	71.4	52.4	47.6	80.9	19.0	100	0
3	*Proteus mirabilis* (n)	8	7	1	2	6	5	3	8	0	0	8
	%		87.5	12.5	25	75	62.5	37.5	100	0	0	100
4.	*Proteus vulgaris* (n)	3	2	1	3	0	3	0	3	0	1	2
	%		66.7	33.3	100	0	100	0	100	0	33.3	66.7
5	*Salmonella typhi* (n)	2	2	0	2	0	2	0	2	0	2	0
	%		100	0	100	0	100	0	100	0	100	0
6.	*Enterobacter sp.* (n)	5	5	0	1	4	1	4	5	0	5	0
	%		100	0	20	80	20	80	100	0	100	0
7	*Citrobacter sp*. (n)	7	5	2	2	5	6	1	5	2	3	4
	%		71.4	28.6	28.6	71.4	85.7	14.3	71.4	28.6	42.9	57.1
8.	*Serratia sp*. (n)	18	15	3	2	16	0	18	17	1	3	15
	%		83.3	16.7	11.1	88.9	0	100	94.4	5.5	16.7	83.3
9.	*Pseudomonas sp*. (n)	9	7	2	1	8	0	9	7	2	3	6
	%		77.8	22.2	11.1	88.9	0	100	77.8	22.2	33.3	66.7
10	*Acinetobacter baumannii* (n)	1	1	0	0	1	0	1	0	1	1	0
			100	0	0	100	0	100	0	100	100	0
	Total	76										

S – Sensitive R – Resistance N - Number of bacterial isolates % - Percentage of bacterial isolates AK –Amikacin AMC – Amoxicillin E – Erythromycin GEN – Gentamicin TE - Tetracycline

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CONCLUSION

In the present study , it can be concluded that the oral cavity of diabetic and non-diabetic individuals harbor a diverse group of aerbic bacteria. These bacteria play a major role in altering the oral health of the individual. Comparing the bacterial flora, it is evident that the number and kinds of bacteria isolated from the diabetic individuals are much higher than the non- diabetic individuals. The antifungal susceptibility testing showed that some of the bacterial isolates were sensitive to the 5 antibiotics used (Ampicillin, Amoxicillin, Erythromycin, Gentamycin and tetracycline).

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REFERENCES

[1] Field EA, Longman LB, Bucknall R, Kaye SB, Higham SM, Edgar WM. Br J Oral Maxillofac Surg. 1997;35: 96.
[2] Chavez EM, Borrell LN, Taylor GW, Ship JA. Oral Surg Oral Med Oral Pathol Oral Radial Endod 2001.
[3] Loesche WJ. Microbiol Rev 1986;50: 353.
[4] Zaura E, Keijser BJ, Huse SM , Crielaard W. BMC Microbiol 2009; 259:12
[5] Aas JA, Paster BJ, Strokes LN, Olsen I and Dewhirst FE. J Clin Microbiol 2005;43: 5721.
[6] Grossi SG. Ann Periodontol 2001;6:138.
[7] Malicka B, Kaczmarck U, Zietek M. J Stoma 2011; 64:9.
[8] Ship JA. J Am Dent Assoc 2003;134:45-105.
[9] Theilade E, Fejerskov O, Karring T, Theilade J. Infect Immun 1982;36:977.
[10] Al-Muala HD, Sami SM, Al- Shimirity I. J Kerbala Uni 2014;12 :257.
[11] Levin JA, Muzyka BC, Glick M. Compendium 1996;1:82.
[12] Bauer AW, Kirby WWM, Sherris JC , Turck M. Am J ClinPathol 1966; 45: 493
[13] CLSI. Performance standards for Antimicrobial disk susceptibility tests: approved standard- Eleventh Edition. CLSI document M 02 – A11. Wayne PA, Clinical and Laboratory standards Institute.
[14] Suryaprabha P, Rani DM, Illamani V , Lakshmipriya R. Research Journal of Pharmaceutical, Biological and Chemical Sciences 2014;5: 721
[15] Gupta ,MaryaV , Juneja K and Dhaliya. The Internet J Dental Sciences 2006; 5.
[16] Vasconcelos BC, Novaes M, Sandrini FA, Filho AW Coimbra LS. Rev Bras Otorrinolaringol 2008 ;74: 423
[17] Al-Ahmad A, Roth D, Wolkewitz M, Al-Ahmad WM, Follo M, Kruger RP. Clin Oral Investig.2010; 14:391
[18] Berezow AB, Darveau RP. Periodontol 2000;2011; 55:36.