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Original Research Paper

Isolation and Identification of Bacteria from Diabetic and Non-diabetic Patients with Periodontitis

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This study included isolation and identification of periodontopathogenic bacteria from periodontal pocket with depth ≥ 4mm from diabetic patients (both insulin dependent and non-insulin dependent) and non-diabetic patients coming under the age group of 15-73 years and all having periodontitis from AL-Shaheed Qais Specialized dental teaching center, AL-Juniana Specialized dental teaching center, Dental teaching clinics in the college of dentistry, AL-Juniana center for primary health care, 60 street center for primary health care and AL-Nahda center for primary health care in the center of Basrah governorate of Iraq. The study was conducted during the period from September 2015 to May 2016. The sample chosen for this study, gingival crevicular fluid (GCF) was collected by absorbent paper points from a total of 46 patients which comprised 21(45.65%) diabetic patients and 25(54.35%) non-diabetic patients, including 29 males and yielded the isolation of 97 bacterial isolates divided into to 44 bacterial isolates that represented (47.72%) from diabetic patients and 53 bacterial isolates that represented (47.16%) from non-diabetic patients. Bacteriological investigation of 92 bacterial isolates belonging to 26 species of 14 genera led to the identification of anaerobic bacteria 6(13.6%) in the diabetic patients and 4(7.5%) in the non-diabetic patients and 85 aerobic bacteria confirmed by automated microbiological system Vitek2. The results of this study showed that *Staphylococcus* spp was more common among isolates from two groups and represented 13(29.5%) in the diabetic group and 27(50.9%) in non-diabetic group, followed by Enterobacteriaceae that represented (27.3%), (13.2%) in diabetic and non-diabetic patients respectively. While *Streptococcus* spp represented 8(18.2%) in diabetics and 4(7.5%) in non-diabetic, *Leuconostoc* spp represented 4(9.1%) in diabetics and 6(11.3%) in non-diabetic.

Keywords: Periodontitis, Oral flora, Diabetes mellitus.

INTRODUCTION

Periodontal disease is considered as one of the most common local diseases of the oral cavity (Offenbacher, 1996) the major forms of periodontal disease are gingivitis and periodontitis. Gingivitis is a mild stage of periodontal disease and does not comprise any loss of bone and tissue that carry the teeth in place. When gingivitis is not treated it can lead to periodontitis in susceptible individuals which means inflammation around the tooth. Periodontitis is a polymicrobial infection caused by various types of bacteria that include resident aerobic and anaerobic bacteria found in the subgingival plaque.

Some types of these bacteria are characterized by a potential pathogenic nature (Armitage, 1999) that can access the deeper periodontal tissues and cause infection. In general, periodontal disease is thought to be endogenous in contrast to an exogenous infection (Kobayashi *el al*., 2008). In periodontitis, gums withdraw away from the teeth and form spaces called pockets (NIH, 2013) that are characterized by depths more than 3mm that become infected (Khan *et al*., 2015), the host immune system attacks the bacteria as the plaque deploys and grows below the gum line. Bacterial toxins and the body's natural response to infection start to break down the bone and connective tissues that carry teeth in place. If not treated, the bones, gum, and tissues that support the teeth are damaged (NIH, 2013). In fact, numerous risk factors are associated with periodontitis and the most important is diabetes mellitus which is known as the most common endocrine turmoil and it exists in two types: type (1) which is caused by damage to pancreatic beta cells that produce insulin and is therefore referred to as insulin dependent diabetes mellitus (IDDM).

Type (2) which means target tissues do not sensitive to insulin and it's called non-insulin dependent D.M (NIDDM) (Grossi, 2001) Periodontitis has been reported as the sixth complication of diabetes (Abass and Omer, 2011). Periodontitis is a disease with microbial etiology (Grossi and Robertson, 1997), and diabetic patients have the similar oral flora that found in non-diabetic patients but their immunological response to infection is different (Mealy, 2006 and Emrich *et al*., 1991), that resulted from these endocrine disorders and change the response of periodontal tissues to the local factors

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that produce anatomical changes in the gingiva that may prefer plaque aggregation and progression of disease. For more explanation, the relationship between D.M and periodontitis is that the increased susceptibility of diabetic patients to infection is caused by lack of adequate number of polymorphic nuclear cells and indigent function of PMNs, monocytes/macrophage resulting in impaired chemotaxis, phagocytosis, or impaired adherence.

As a result, the primary defense line against periodontal pathogens is diminished, and bacterial proliferation is increased, in addition to chronic hyperglycemia that impairs collagen structure and function which may directly impact the integrity of the periodontium. Also, there is decreased collagen synthesis and extracellular matrix, osteoporosis and reduction of the alveolar bone (Carenza *et al*., 2010), the inflammatory response is mainly caused by the chronic effects of hyperglycemia and lead to the formation of biologically active glycelated proteins and lipids that stimulate inflammatory responses and it's called accumulated glycation end products (AGEs) (Wautier and Schmidt, 2004 and Basta *et al*., 2005)

The formation of AGEs occurs at normal glucose levels but in higher levels of glucose, it is formed in excessive amounts. Collagen is cross-linked by AGEs formation, decreased solubility and less likely to be normally repaired or replaced. As a result, collagen in the tissues of patients with poorly controlled diabetes is older and more susceptible to pathogenic breakdown (Carenza *et al*., 2010).

AIMS OF THE STUDY

1. To find out the prevalence of different bacterial species in the gingival crevicular fluid (GCF) of both diabetic and non-diabetic patients.

2. To identify the predominance of various types of bacterial species in diabetic and non-diabetic patients

MATERIALS AND METHODS

A total of 46 patients were selected for this study divided into two groups diabetic patients and non-diabetic patients, the patients belonged to the age category of 15 to 73 years and having periodontitis from AL-Shaheed Qais Specialized dental teaching center, AL-Juniana Specialized dental teaching center, Dental teaching clinics in the college of dentistry, AL-Juniana center for primary health care, 60 Street Center for primary health care and AL-Nahda center for primary health care in the center of Basrah governorate of Iraq during the period from September 2015 to May 2016.

After getting permission from the patients who had not received antibiotics, questionnaire form was fully completed. Clinical examination was performed by examining the oral cavity, including teeth and gingiva for signs of periodontitis, the individual tooth site was isolated and cleaned with sterile gauze, and supragingival plaque was removed carefully. Sample of gingival crevicular fluid was collected with assistance of dentist by sterile absorbent paper point (size 30-45). Then paper strips were placed into the pocket with depth ≥4mm until mild resistance was sensed and left it in place for 60 s to obtain sufficient absorption of GCF sample, as shown in figure (1). (Shimada *et al*., 2013).

Culture of sample

GCF samples were directly inoculated into 5ml of thiogllycollate broth. All samples were transferred to the laboratory of microbiology within 2hr of collection and incubated at 37 °C for 24-48 hr after incubation, from the broth culture, each sample was cultured on different agar media; blood agar, chocolate agar and MacConkey agar by direct streaking method incubated aerobically at 37 °C for 24-48 hr and inoculated blood agar and tryptone soya agar supplemented with 5% (v/v) human blood, heamin solution (5 mg/ml) vitamin K1 solution (1 mg/ml) and L-cysteine (1g) (Nerandzic and Donskey, 2009), was used to quantify cultivable facultative and anaerobic bacteria all plates incubated anaerobically in anaerobic culture jar with N_2 gas pack (Thermo, Japan) at 37°C for 4-6 days (Dowell *et al*., 1977 and Harley and Prescott, 2002).

Aerotolerance test

An aerotolerance test was performed on each isolate that was acquired from the primary anaerobe medium plate. The number of each different colony type from positive culture was reported and each different colony type from positive cultures was subcultured for purity and identification (WHO, 1987 and Skucaite *et al*., 2010).

Bacteriological identification of bacterial isolates

Microorganisms were identified based on culture traits, gram staining properties and confirmed with the automated microbiological system vitek2 by using gram-positive ID kit and gram-negative ID kit (biomerieux, franch) (Skucaite *et al*., 2010)

Processing of bacterial isolates with vitek 2

Suspension of 87 bacterial isolates is Prepared with sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure young culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a clear plastic tube 0.5- 0.63 by using a turbidity meter called the DensiChek TM. Identification cards are inoculated with microorganism suspensions. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube present (David, 2007).

Statistical analysis

Statistical analysis was performed by Chi-square (X2) test. (P-value ≤ 0.01) were considered statistically significant and (P-value>0.05) were considered statistically not significant.

RESULTS

Our study yielded the isolation of 97 bacterial isolates from 46 samples of both groups .97 divided to 44 (47.72%) bacterial isolates that were isolated from diabetic patients and 53 (47.16%) bacterial isolates from non-diabetic patients .97 bacterial isolates comprised aerobic bacterial isolates and anaerobic bacterial isolates. Aerobic bacteria represented 38(86.4%) and 49(92.5%) in diabetic and non-diabetic patients respectively.

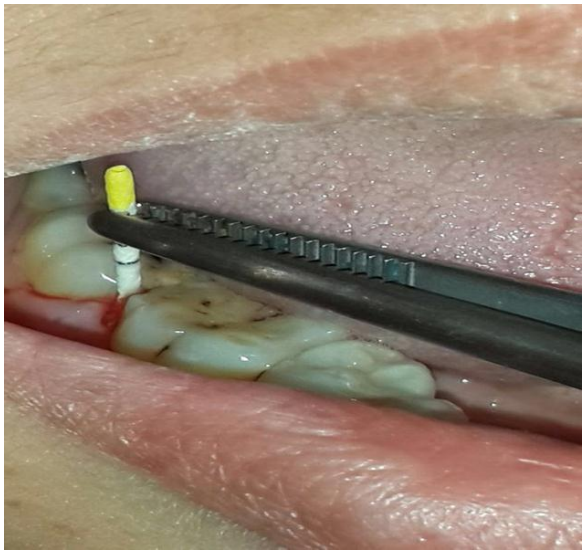

Figure 1: Sampling of GCF by using paper point

Table 1: Distribution of total samples in two groups diabetic patients and non-diabetic patients.

Samples No. of diabetic patients	Samples No. of non-diabetic patients	Total samples
21 (45.65%)	25 (54.35%)	46

Table 2: Number and percentage of diabetic patients and non -diabetic patients according to sex

Sex	Patients No.	Diabetic patients	Non-diabetic patients
Male	29 (63.04%)	10 (47.61%)	19 76%)(
Female	17 (36.95%)	11 (53.38%)	6 24%)(
Total	46	21	25

Table 3: Number and percentage of diabetic patients and non-diabetic patients according to age group

Age (Years)	Total patients	Number of diabetic patients	Number of non-diabetic patients
20 or less	2		2 (100%)
21-30	5	1 (20%)	4 (80%)
31-40	11	2 (18.18%)	9 (81.81%)
41-50	11	5 (45.45%)	6 (54.54%)
51-60	5	2 (40%)	3 (60%)
61-70	10	9 (90 %)	1 (10%)
71-80	2	2 (100%)	
Total	46	21	25

While anaerobic bacteria represented 6(13.6%) and 4(7.5%) in diabetic and non-diabetic patients respectively. Table (1) shows the distribution of a total 46 samples divided into two groups diabetic patients included 21(45.65%) and non-diabetic patients included 25 (54.35%), but the difference was statistically not significant (P value>0.05).

Frequency of bacterial isolates according to the sex

Table (2) shows the sex distribution of the patients from both groups. Of the total, 29(63.04%) males, 17(36.95%) females but the difference was statistically significant ($P \leq 0.01$).

Frequency of bacterial isolates according to the age

Table (3) shows the age group distribution of the patients from diabetic group and non-diabetic group. The highest detection rates of patients (100%) was recorded in the age group 20 or less years, statistically the differences in all age groups were significant ($P \leq 0.01$) except age group (41-50) and (51-60) shows statistically the differences were not significant.

Bacteriological investigation of bacterial isolates

The bacteriological analysis of 46 samples led to the isolation of 97 bacterial isolates, the number of bacterial species per sample varied from one to six with a large variety of

morphological types (the total 97 bacterial isolates missed 2 unidentified bacterial isolates and 3 bacterial isolates of black pigmented bacilli without knowledge to any genus is belong to) for this a total of 92 comprising 26 species of 14 genera were identified with bacteriological methods. Gram stain test was performed to primary to confirm the identification of microbial isolates from both groups, and the results of this test included (67) isolates of gram-positive cocci, (26) isolates of gram-negative bacilli and (4) isolates of gram-positive bacilli in addition to the five isolates of *Candida* spp from diabetic and non-diabetic patients. Most of the isolates were partially identified by using gram stain reaction, from microscopic examination, a large compatibility was noticed with identification of bacterial isolates with vitek2 system.

Results of identification of bacterial isolates with vitek2

Automated microbiological vitek2 system was used to confirm identification of 87 aerobic bacterial isolates. The results revealed the identification of 85 bacterial isolates in addition to the unidentified two bacterial isolates.

Frequency distribution of bacterial isolates in diabetic and non-diabetic patients

The results of the present study shows *Staphylococcus* spp recorded the highest rates in both groups that were represented as shown in table (4).

DISCUSSION

There is a broad agreement on the etiological role of microbiota linked with periodontitis, studies that were made in this scope have revealed a wide diversity in the composition of the subgingival microflora (Winkelhoff and Graaff, 1991). In fact, more studies carried out in Europe and USA about the role of anaerobic bacteria in adult periodontitis and suggested this bacteria is considered key pathogens of periodontitis. While in the present time, the periodontal microflora have been examined in some third world countries and the results refers to differences both qualitatively and quantitatively when compared to those perceptible in the west (Joshi and Vandana, 2007).

The present study showed *Staphylococcus* spp. more common isolates from two groups, its represented 13(%29.5) in diabetic group while represented 27(%50.9) in non-diabetic group the second rate concur with (Rams *et al*., 1990) that reported %50 of periodontal lesions which harbored *Staphylococcus* spp. (Cuesta *et al*., 2010) reported %42.7 of *Staphylococcus* spp. isolated from periodontal pocket and (Slots *et al.*, 1990) found *Staphylococcus* spp. in 28.3% of individuals that were between 25 and sixty years of age, but contrary to (Loberto *et al*., 2004) reported %37.5 of Staphylococci in their study.

The difference of proportions between these two studies may be due to the sample processing. The current study shown *Staph. vitulinus* and *Staph. sciuri* were the most prevalent species in both groups while another related studies showed *Staph. epidermidis* and *Staph. aureus* as most prevalent species, the difference can be due to the geographical differences, clinical history of the patients or drugs administration. While *Viridans streptococci* reported low proportion than Staphylococci in both groups that represented 8(%18.2) in diabetic group and 4(%7.5) in non-diabetic. *Streptococcus thoraltensis,* the commonest species was isolated from diabetic group whereas *Streptococcus anginosus* the most common species was isolated from non-diabetic group. However, this study is in line with (Piret *et al*., 2005) that reported (%15.2) and (AL-Badah *et al*., 2015) that reported (%17.7). Whilst disagreement with (Bissong *et al*., 2014) that recorded (%99.4) and (Abass and Omer, 2011) which recorded (%43). The discrepancy can be due to the higher population that was selected for these studies. In the same time, other studies by Zambon suggested *St. intermedius* was associated with periodontal disease while (Kumer *et al*., 2012) suggested *St. sanguis* was associated with periodontitis, based on the results of these studies *Viridans streptococci* in general, can be implicated in periodontal disease.

Leuconostoc spp reported nearly the same proportions in both groups 4(%9.09) in diabetic group and 6(%11.3) in non-diabetic group. In this study, *Leuconostoc mesenteroides* is the most prevalent species in both groups. However, little studies have reported this genus but the current study is in agreement with (AL-Badah *et al*., 2015) that reported (%9) isolated from root canal abscess while (Robertson *et al*., 2000) documented in his study (%0.9) that was isolated from immunocompromised patients Papillon-Lefevre syndrome.

Enterobacteriaceae too showed difference in the proportions of isolation between the two groups in this study. Where it represented 12(%27.3) in diabetic patients and 7(%13.2) in non-diabetic patients. *Enterobacter* spp. was the most prevalent genus in diabetic group it reported 6(%13.6) while *Klebsiella spp* the commonest genus was isolated from non-diabetic group that represented 5(%9.4), these results agree with (Bossing *et al*., 2014) that documented in his study (%5.2) of *Klebsiella* spp. in diabetic group and (%6.4) from non-diabetic group and (Sharma *et al*., 2011) that reported (%5) of *Klebsiella* spp isolated from gingivitis and dental caries.

Whilst the ratio of anaerobic bacterial isolates represented 6 (%13.6) from diabetic group, this result is in near agreement with (Kumer *el al*., 2012) that documented in his study (%6.7). Conversely, anaerobic bacteria that represented 4(%7.5) from non-diabetic group, this result did not show agreement with (Mane *et al*., 2009) that reported (%83) of anaerobic bacteria that was isolated from chronic periodontitis, varying recovery rates of anaerobic bacteria can be due to varying criteria of patients selection, geographical differences, socioeconomic status, molecular technique used for identification, sampling method, probing depth or culture technique and selective media used such as scheduler agar and non-selective media like brucella agar and available typical culture conditions by using anaerobic cabinet that supply H_2, N_2, CO_2 all these gases made full keeping away of O_2 and preserve the viability of bacterial cell or prolonged incubation period that may reach up to 10 days.

In spite of the current study employed two enrichment, non-selective media blood agar and tryptone soya agar supplemented with some additives (L-cystine, vitamin K1 and heamin) that enhance growth of strict anaerobic bacteria when added to the cultivated media and isolated it from primary subculture but fails to preserve the viability of this bacteria in further subcultures that required for purification and done of confirmed tests. Indeed, microbial culture is considered Golden standard method but the cultivation of strict anaerobic is still a difficult process, for this molecular method was more recommended for recovery of strict anaerobic bacteria.

Table 4: Frequency distribution of bacterial isolates in diabetic and non-diabetic patients

Bacterial isolates	Diabetic patients %	Non-diabetic patients%
Staphylococcus vitulinus	5(11.3%)	9(16.9%)
Staphylococcus sciuri	3(6.8%)	9(16.9%)
Staphylococcus saprophyticus	0	3(5.6%)
Staphylococcus worneri	0	3(5.6%)
Staphylococcus epidermidis	2(4.5%)	1(1.88%)
Staphylococcus lentus	2(4.5%)	1(1.88%)
Staphylococcus haemolyticus	0	1(1.88%)
Staphylococcus xylosus	1(2.2%)	0
Streptococcus anginosus	1(2.2%)	2(3.7%)
Streptococcus thoraltensis	3(6.8%)	1(1.88%)
Streptococcus gordonii	0	1(1.88%)
Streptococcus mutans	1(2.2%)	0
Streptococcus constellatus spp *pharyngis*	1(2.2%)	0
Streptococcus sanguinis	1(2.2%)	0
Streptococcus parasanguinis	1(2.2%)	0
Facklamia hominis	0	1(1.88%)
Aerococcus viridans	0	1(1.88%)
Pediococcus pentosaceus	0	1(1.88%)
Gemella morbillorum	0	1(1.88%)
Leuconostoc mesenteroides spp *cremoris*	4(9%)	4(7.5%)
Leuconostoc mesenteriodes spp *dextranicum*	0	1(1.88%)
Leuconostoc citreum	0	1(1.88%)
Klebsiella oxytoca	2(4.5%)	4(7.5%)
Klebsiella pneumonia spp *pneumonia*	2(4.5%)	1(1.88%)
Enterobacter cloacae	5(11.3%)	0
Enterobacter aerogenes	1(2.2%)	0
Pantoea spp	0	2(3.7%)
Proteus mirabilis	1(2.2%)	0
Pseudomonas aeruginosa	1(2.2%)	0
Fusiform spp	2(4.5%)	1(1.88%)
Black pigmented bacilli	2(4.5%)	1(1.88%)
Actinomyces spp	2(4.5%)	2(3.7%)

On the other hand, the difference in the rate of isolation of anaerobic between both groups in this study can be due to the high level of glucose in the GCF of diabetic patients glucose characterized by high nutritive value that stimulates growth of bacteria. This study too showed isolation of *Candida* spp. in converging few proportions from both groups.

Based on the results of this study, we found the same bacterial species in both diabetic and non-diabetic patients but this results showed differences in the isolates number among two groups according to the diabetics problems that effect on the oral flora and this showed agreement with Mandell suggesting that diabetes may result in weakness of neutrophil functions adherence, chemotaxis and phagocytosis which may facilitate bacterial invasion, persistence and proliferation in the periodontal pocket and significantly worsen the periodontal conditions (Mandell *et al*.,1992).

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