**ISSN: 0067-2904**

# Screening of periodontal disease related Bacteria in Iraqi patients and their relationship with salivary TLR2 and IL-6 level

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Received: 23/6/2022 Accepted: 23/9/2022 Published: 30/7/2023

**Abstract**

The oral cavity has the second major and most diverse microflora after the gut. Since it is the starting point of digestion; the oral microbiota is essential for maintaining oral and systemic health. Thus, this study aimed to find the bacterial isolates which act as a causative agent of periodontal diseases in Iraqi patients and their relation with some immune oral parameters. Saliva specimens and oral swabs of 91 patients (51) and control individuals (40) were collected in this study. The specimens of patients were collected from Al-Amirya and Almaamon Specialized Dental Centers in Baghdad for a period from November/2021 to February/2022. The microbiological results revealed, that the most prevalent bacterial isolates were related to the genus, which was descending graduated into *Staphylococcus spp.*74.5%> *Enterobacteriaceae spp.*62.7% > *Streptococcus spp.* 13.7% and *Acinetobacter spp.*13.7% > *Neisseria spp.*9.8% > *Bacillus spp.*5.8% > *Corynebacterium spp*. 3.9% and *Pseudomonas spp.*3.9%, and in the same context, the acidic pH of the patient's or control's mouths is more conducive for bacterial development than other pH items. As well by using the ELISA technique, the soluble form of TLR2 7.384 ± 4.031 ng/ml elucidated a significant increase in patients than the control group 5.313 ± 3.106 ng/ml, while interleukin 6 shows a strange increase in both patients and the control group saliva 50161 ± 63869, 52087 ± 62756 ng/ l respectively.

**Keywords**: oral microbiome, TLR2, salivary levels, IL6, oral diseases

# الصورة البكتيرية لأمراض الفم الخمجية وعلاقتها ببعض المعايير اللعابية

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**الخلاصة**

يحتوي تجويف الفم على ثاني اعلى محتوى من البكتيريا والأكثر تنوعًا بعد القناة الهضمية. فهو يشكل نقطة بداية عملية الهضم. تعد الميكروبات الفموية أمر بالغ الأهمية في الحفاظ على صحة الفم وعموم الجسم. لذا هدفت هذه الدراسة إلى إيجاد العزلات البكتيرية التي تعمل كعامل مسبب لأمراض اللثة في المرضى العراقيين وعلاقتها ببعض المعايير المناعية الفموية. جمعت عينات اللعاب ومسحات فموية من 91 فردا منهم (51)مريضا و(40) كمجموعة سيطرة في هذه الدراسة. جمعت عينات المرضى من مراكز العامرية والمامؤن التخصصية

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لطب الاسنان في بغداد لفترة من 2021/11الى 2022/2 . أوضحت النتائج الميكروبيولوجية أن أكثر العزلات البكتيرية انتشارا كانت مرتبطة بالجنس الذي تتدرج تنازليا الى *Staphylococcus spp.*74.5% > *Streptococcus spp.*13.7% < *Enterobacteriaceae spp.*62.7% < *Acinetobacter* و *Corynebacterium spp.*3.9% < 5.8% *Bacillus spp.* <9.8% *Neisseria spp.* < *spp.*13.7% و *Pseudomonas spp.*3.9%، وفي نفس السياق ، كان الرقم الهيدروجيني الحامضي لفم المرضى و مجموعة السيطرة أكثر ملائمة لنمو البكتيريا من عناصر الأس الهيدروجيني الأخرى. كذلك باستخدام تقنية الاليزا ، أوضحت النتائج ان الشكل الذائب من TLR2 4.031 ng/ml ± 7.384 زيادة ملحوظة في المرضى مقارنة بمجموعة السيطرة 3.106 ng/ml ± 5.313 ، بينما أظهر إنترلوكين 6 زيادة غريبة في كل من لعاب المرضى والمجموعة السيطرة , على التوالي 63869 ng/l ± 50161, 62756 ± 52087.

## 1. Introduction

The oral cavity is occupied by a wide diversity of microorganisms, the majority of which belong to the normal microbiota. However, it can also be inhibited by other opportunistic microorganisms that have a role in the development of systemic and oral illnesses [1]. Periodontal diseases are multifactorial infections caused by specific invasive oral bacteria that live in biofilms of dental plaque on the tooth surface. Bacteria are considered as the most common cause of ~~the~~ periodontal disease [2,3]. These bacteria play a crucial role in the progression of the periodontitis, by contributing to the development of the periodontal pocket, breakdown of connective tissue, and alveolar bone resorption through an immunological pathological process [4]. Dental health is defined by a predominating Gram-positive bacterial population in the subgingival biofilm, whereas in disease is characterized by a predominating Gram-negative bacterial population in the subgingival microbiota. Periodontal disease does not think to be caused by a single organism; rather, numerous important bacteria seem to be crucial in changing the subgingival plaque's microbial population into a dysbiotic state, which causes this chronic inflammatory illness [5]. Toll-like receptors (TLRs) are known to be produced by host immune cells in order to identify harmful bacteria by detecting different microbial components known as pathogen-associated molecular patterns (PAMPs) and to start inflammatory responses [6]. According to reports, TLR2 can identify the widest variety of PAMPs, including lipoprotein/lipopeptide, lipoteichoic acid, and lipopolysaccharide [7]. Interestingly, TLR2 also has been implicated in the inflammatory response triggered by number of periodontal pathogenic bacteria [8,9], indicating that TLR2 plays a key role in starting periodontal inflammation. According to one study, periodontal bacteria can activate the TLR2 pathways, which causes monocytes to produce pro-inflammatory cytokines and chemokines [10]. Interleukin-6 is a well-known pro-inflammatory cytokine released by Numerous cells, including monocytes, macrophages, endothelial cells, epithelial cells, and B and T cells [11]. IL-6 modulates multiple physiological processes including cell proliferation, apoptosis, differentiation, and survival [38]. IL-6 is one of the most studied inflammatory markers in periodontal disorders, which is the main regulator of acute-phase protein and synthesizes during the acute-phase response. [12,13]. Interleukin-6 levels in saliva were shown to be the same in both periodontal disease and periodontal health, according to studies [14].

Saliva is a readily available biologic fluid that contains a variety of vital proteins that are created locally or sourced from the vascular beds in the gingival tissues [15]. Therefore, saliva has become an emerging tool for the diagnostic assessment of various oral and systemic diseases, particularly periodontal disease [16]. In the oral cavity, saliva helps maintain the pH level around neutral (6.78±0.04) [17]. Bacteria found in the mouth cavity digest carbohydrates and create acids. On the other hand, the metabolism of silane and urea releases ammonia which elevates pH. Due to the inclusion of bicarbonate, peptides, proteins, and phosphates, saliva

functions as a buffer and aids in pH maintenance. Acids build up in dental plaque as a result of the sluggish passage of saliva through it. Sugar consumption significantly lowers the pH of tooth plaque, making it more acidic. [18].

Smoking has been recognized as a major risk factor for periodontal disease, affecting the prevalence, severity, progression, and treatment response of the disease. According to epidemiological research, smokers have a much higher chance of developing the periodontal disease compared to non-smokers, and this risk is proportionate to how often and for how long they smoke. [19, 20, 21]. Therefore, this study aimed to understand the effect of oral microbiota on health and illness and will give additional directions to find the functional changes associated with some immunopathological states.

## 2. Materials and Methods

### Patients and control collection

Fifty-one patients (28 male and 23 female) with Periodontal disease in their age range (13-70) years and 40 (15 male and 25 female) ~~apparently~~ healthy individuals as control their ages were coordinated with the patients enrolled in the study. The patients were attendants seeking treatment in the Amirya Specialized Dental Center and Almaamon Specialized Dental Center in Baghdad for the period November/2021 to February/2022. All cases of gingivitis were diagnosed by a specialized Dentist, based on the clinical characteristics in comparison to the control group.

### Isolation and identification of bacteria

Swab samples were collected for isolation of bacteria from each patient and control individual using a sterile cotton swab. Each swab sample was directly cultured using brain heart infusion broth/ Himedia- India and 24 hours of incubation at 37 °C. then primary identification was done using selective and differential media including; Blood agar, Mannitol salt agar, and MacConkey agar/ Himedia- India, and incubated for 24 hours at 37°C. These media were used primarily to distinguish and select the pure colonies for each specimen [22]. Each isolate was identified according to the colony morphology, colony color, gram staining reaction, and biochemical tests (catalase test, oxidase test, coagulase test, indole test, Voges-Proskauer (VP) Test, Methyl Red Test, Urease Test, and Simon Citrate Test) [23].

### Saliva collection

Unstimulated whole saliva approximately (2ml) was collected for analysis from each participant, after being informed that must not have eaten or drunk at least 2h before sampling collection. Dependent on procedure instruction [24], they were instructed to rinse their mouth several times with sterilized water and eliminated it outside, then the saliva specimens were collected. Using a pH Test Strip (Cybow/China), the salivary pH was determined after collection by dipping the strip into the saliva for approximately 2 seconds until a color change occurred and comparing it to the colored standard indicator. Each sample of collected saliva was centrifuged at 10,000 rpm for 10 minutes, after which the clear supernatant was collected and kept at -20 °C until used.

### Immunological assay

By using the ELISA technique, following the manufacturer's protocol instructions (BT LAB/China), TLR-2 and IL-6 levels in saliva were measured in a microplate reader at a wavelength of 450nm.

### Statistical analysis

The data were analyzed using the following software, Microsoft Excel, Minitab v17, and IBM SPSS V26. The results reported in this study were expressed as mean ± SD., Z-test was used to compare two proportions. The Pearson correlation evaluates the linear relationship between two continuous variables, and if the categorical variable has two levels, a point-biserial correlation was used. Probability differences (*P*-value) were smaller than 0.05 and were considered to be significant.

## 3. Results and Discussion

The oral cavity, with its numerous functions, is a remarkably complex habitat, where microbes colonize the soft tissues of the oral mucosa and the hard surfaces of the teeth. This study was focused, to find the bacterial isolates which act as a causative agent of periodontal diseases in Iraqi patients and their relation with some immune oral parameters. A total of 51 oral swab specimens were from outpatients diagnosed with periodontal disease, alongside other 40 individuals who were ~~apparently~~ healthy mouth environments. The results revealed that there were at least 8 different genera isolated from patients, which were ascendingly graduated into *Staphylococcus spp.> Enterobacteriaceae spp. > Streptococcus spp. and Acinetobacter spp. > Neisseria spp. > Bacillus spp. > Corynebacterium spp. and Pseudomonas spp*. The result did not record significant variation between patients and control. It seemed that these genera are predominantly found in health and disease and form an oral microbiome Table 1.

**Table 1:** The most prevalent bacterial genus in the oral cavity

| Bacteria | Group | | | | P-value¥ | Total | |
|---|---|---|---|---|---|---|---|
| | **Patient** | | **Control** | | | | |
| | **N** | **%** | **N** | **%** | | **N** | **%** |
| *Staphylococcus spp.* | **38** | **74.5%** | **32** | **80.0** | **0.532** $^{N.S}$ | **70** | **40.7%** |
| *Bacillus spp.* | **3** | **5.8%** | **8** | **20.0%** | **0.048** * | **11** | **6.4%** |
| *Streptococcus spp.* | **7** | **13.7%** | **2** | **5.0%** | **0.141** $^{N.S}$ | **9** | **5.2%** |
| *Neisseria spp.* | **5** | **9.8%** | **3** | **7.5%** | **0.696** $^{N.S}$ | **8** | **4.7%** |
| *Enterobacteriaceae spp.* | **32** | **62.7%** | **27** | **67.5%** | **0.636** $^{N.S}$ | **59** | **34.3%** |
| *Corynebacterium spp.* | **2** | **3.9%** | **1** | **2.5%** | **0.699** $^{N.S}$ | **3** | **1.7%** |
| *Acinetobacter spp.* | **7** | **13.7%** | **3** | **7.5%** | **0.328** $^{N.S}$ | **10** | **5.8%** |
| *Pseudomonas spp.* | **2** | **3.9%** | **0** | **0.0%** | **0.149** $^{N.S}$ | **2** | **1.2%** |
| Total | **96** | **100.0 %** | **76** | **100.0 %** | | **172** | **100.0%** |

¥: Z-test were used to test between two groups

These bacterial prevalent is caused by various oral surfaces in the mouth being colonized especially by the oral bacteria due to unique adhesions on their surface that attach to corresponding receptors on oral surfaces as **[25]** mentioned. As well as, the expanded Human Oral Microbiome Database is developed with the aim of providing the scientific community with comprehensive curated information on the bacterial species present in the human aerodigestive tract (ADT), which encompasses the upper digestive and upper respiratory tracts, pharynx, nasal passages, sinuses and esophagus and the oral cavity **[26].** And they concluded that the healthy mouth cavity categorized the inhabitant bacteria into six major phyla, namely, *Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Bacteroidetes, and Spirochaetes* constituting 96% of the total oral bacteria. However, the appropriate functioning of these bacteria in oral can be harmful to the human oral cavity if the conditions are unsuitable such as the pH of a site, the results in Tables 2 and 3 elucidated that, the acidic pH of the mouth of either patient or control is favorable for bacterial growth than other pH items, and results were

compatible with [27] whom they demonstrated the importance of oral cavity pH in maintaining mouth health, throughout directly affects the health of teeth and gums. According to studies, having a lower or more acidic pH level puts you at higher risk for developing significant health issues including type 2 diabetes, heart disease, and obesity. [28] [29] reported that pH was found to be minimal for alkaline pH levels and to be strain and species-dependent. And concluded that pH may be useful as an ecological determinant or as
an indicator of the carcinogenicity of oral streptococci.

**Table 2:** Most prevalent bacterial isolates isolated from periodontal patients

| Bacteria | pH saliva | | | | | | P-value¥ | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | Acidic | | Neutral | | Alkaline | | | | |
| | N | % | N | % | N | % | | N | % |
| *Staphylococcus spp.* | 23 | 60.5% | 10 | 26.3% | 5 | 13.2 % | 0.001** | 38 | 100% |
| *Bacillus spp.* | 2 | 66.6% | 1 | 33.3% | 0 | 0.0% | 0.564 N.S | 3 | 100% |
| *Streptococcus spp.* | 3 | 42.9% | 2 | 28.6% | 2 | 28.6 % | 0.286 N.S | 7 | 100% |
| *Neisseria spp.* | 3 | 60.0% | 2 | 40.0% | 0 | 0.0% | 0.655 N.S | 5 | 100% |
| *Enterobacteriaceae spp.* | 21 | 65.6% | 8 | 25.0% | 3 | 9.4% | 0.001** | 32 | 100% |
| *Corynebacterium spp.* | 1 | 50% | 1 | 50% | 0 | 0.0% | 1.00 N.S | 2 | 100% |
| *Acinetobacter spp.* | 4 | 57.1% | 2 | 28.6% | 1 | 14.3 % | 0.368 N.S | 7 | 100% |
| *Pseudomonas spp.* | 1 | 50.0% | 1 | 50.0% | 0 | 0.0% | 1.00 N.S | 2 | 100% |
| Total | 58 | 100% | 27 | 100% | 11 | 100% | | 96 | 100% |

¥: Chi-square test goodness of fit was used

**Table 3:** Most prevalent bacterial isolates isolated from control individuals

| Bacteria | pH saliva | | | | | | P-value | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | Acidic | | Neutral | | Alkaline | | | | |
| | N | % | N | % | N | % | | N | % |
| *Staphylococcus spp.* | 22 | 44.9 % | 4 | 25.0 % | 6 | 54.5 % | 0.001** | 32 | 100% |
| *Bacillus Spp.* | 4 | 8.2% | 2 | 12.5 % | 2 | 18.2 % | 0.717 N.S | 8 | 100% |
| *Streptococcus spp.* | 2 | 100% | 0 | 0.0% | 0 | 0.0% | 1.00 N.S | 2 | 100% |
| *Neisseria spp.* | 1 | 33.3 % | 1 | 33.3 % | 1 | 33.3 % | 1.00 N.S | 3 | 100% |
| *Enterobacteriaceae spp.* | 18 | 66.7 % | 7 | 25.9 % | 2 | 7.4% | 0.001** | 27 | 100% |
| *Corynebacterium spp.* | 0 | 0.0% | 1 | 100% | 0 | 0.0% | 1.00 N.S | 1 | 100% |
| *Acinetobacter spp.* | 2 | 4.1% | 1 | 6.3% | 0 | 0.0% | 0.564 N.S | 3 | 100% |
| *Pseudomonas spp.* | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 1.00 N.S | 0 | 100% |
| Total | 49 | 100% | 16 | 100% | 11 | 100% | | 76 | 100% |

Increasing acidic pH significantly among control individuals may reflect the susceptibility to infection with periodontal diseases.

On another hand, the environment in the mouth may also reflect the host's defensive mechanisms and the availability of antimicrobial drugs **[27].** In this study, two main immune

parameters are measured including TLR2 and IL6 in the saliva of the patient, and compared with the control group.

**Table 4:** Independent t-test: to test means across groups

| Variables | Patients N=51 | Control N=40 | *P-value*¥ |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | |
| IL-6 ng/l | 50161 ± 63869 | 52087 ± 62756 | 0.886 |
| TLR-2 ng/ml | 7.384 ± 4.031 | 5.313 ± 3.106 | 0.009** |

The results in Table 4 revealed that increasing TLR2 significantly in the patient than in the control group. [30] found that periodontitis may enhance the chance of developing insulin resistance and cardiovascular disease through a mechanism involving an increase in salivary TLR stimulants. As well as [31] mentioned the soluble TLR-2 content in saliva with active caries was substantially greater than in saliva without active caries Figure 1. Since this result was compatible with previous results related to increasing acidic pH and bacterial prevalence among patients. [32] established in his study, that the interaction of many factors between acid-producing bacteria, fermentable carbohydrates, and host factors cause tooth caries, a complicated infectious illness. Despite being mostly avoidable, it continues to be the most common chronic illness globally.

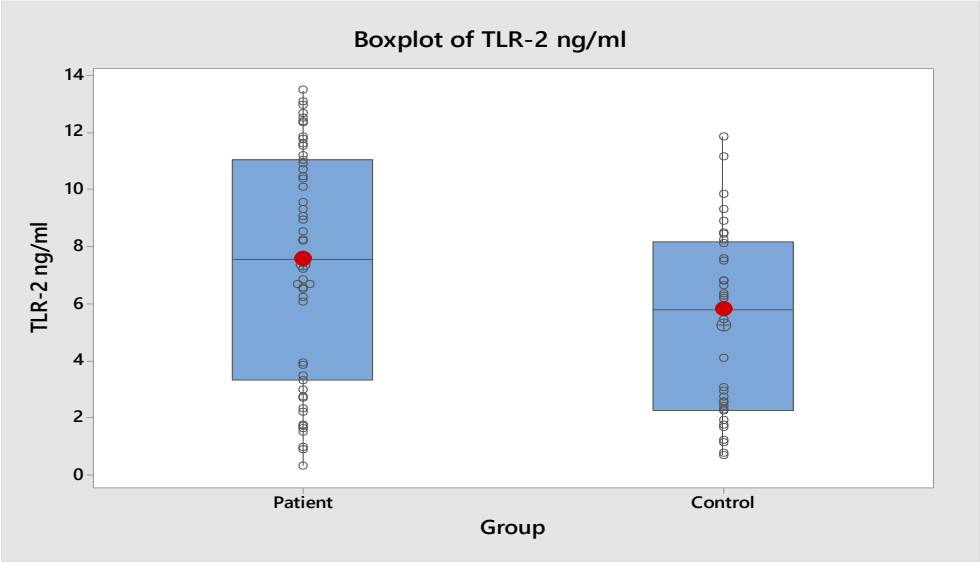


**Figure 1:** A comparable boxplot of TLR2 levels between patients and control

IL6, another immunological parameter evaluated in this study, the results do not significantly differ between the patient and control groups, according to the results in Table 4. This interleukin was revealed to a strange level in saliva, it is so high in both patients and control when compared with globally recorded cases of periodontitis which reached 12.1±10.2 pg/mL as [33] documented, and they considered this cytokine as a biomarker to periodontitis infection. Also, each [34] and [35] demonstrated the level of salivary IL-6 was higher significantly in calculus-associated chronic periodontitis patients as compared to healthy controls and these levels increased with the progression of chronic periodontitis. However, Interleukin-6 is considered to have a function in maintaining the homeostasis of periodontal tissues through the orchestration of the host response [36]. On another hand, an increase in the IL6 in both experimental groups may be due to the late effect of the coronavirus outbreak as [37]

documented that, IL-6 levels were more than three times higher in patients with COVID-19 than in others.

## Conclusion

This study concluded that concurrently TLR-2 activation synergistically induces IL-6. Localized IL-6 production that is elevated as a result of infection activity and the spread of periodontopathogenic bacteria locally causes tissue injury by enhancing the pro-inflammatory cascade**.**

## Ethical Clearance

The Biotechnology Department's local ethics committee approved the trials in this thesis and all of the volunteers signed off on them by hand. Patients were also given information and benefits based on the research

## Conflict of Interest

There are no conflicts of interest between authors. And the research depends on Special financial support for the authors only.

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