Research Article

Molecular Detection of Porphyromonas gingivalis in COVID-19 Patients

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Abstract: Background:SARS-CoV-2 infection has caused a global pandemic that continues to negatively impact human health. A large group of microbial domains including bacteria co-evolved and interacted in complex molecular pathogenesis along with SARS-CoV-2. Evidence suggests that periodontal disease bacteria are involved in COVID-19, and are associated with chronic inflammatory systemic diseases. This study was performed to investigate the association between bacterial loads of *Porphyromonas gingivalis* and pathogenesis of SARS-CoV-2 infection. Fifty patients with confirmed COVID-19 by reverse transcriptase-polymerase chain reaction, their age ranges between 20-76 years, and 35 healthy volunteers (matched accordingly with age and sex to the patients) participated in this case control study. Oral hygiene status was determined by the simplified oral hygiene index. Blood and saliva samples were obtained from patients and controls, *Porphyromonas gingivalis* quantification from extracted DNA of blood and saliva samples performed by means of real-time polymerase chain reaction. The present result revealed that the quantity of salivary *Porphyromonas gingivalis* was significantly higher (p=0.003) in the patients' group than in the controls group, while there was no significant difference in the number of bacteria in the blood samples between the two groups. Moreover, the number of bacteria in severe cases was higher than that in moderate and mild with no significant differences, and there was a significant increase in the number of bacteria among patients with poor oral hygiene compared to patients with good oral hygiene. This study demonstrated that the high level of salivary *Porphyromonas gingivalis* in patients increases in number with disease severity, which may indicate that bacterial infections contribute to the spread of the disease.

Keywords: *COVID-19, Porphyromonas gingivalis, co-infection.*

Introduction

COVID-19 caused by SARS-CoV-2, has affected most countries all over the world since its first case in end of 2019. The genomic characteristics of SARS-CoV-2 was initially reported by Lu and colleagues, suggesting this coronavirus had enveloped RNA, resembling severe acute respiratory syndrome coronavirus (SARS-CoV) in both structural and homological ways[1]. During this COVID-19 attack, besides the primary infection of SARS-CoV-2, many other complications are emerging, contributing greatly to the mortality. Among these, co-infection plays a crucial role, threatening many COVID-19 patients' lives [2]. As reported by researchers, the prevalence of co-infection was variable, being found occurred in half of the non-survivors[3]. The pathogens of respiratory co-infection could be many, either common or rare, including bacteria, virus, fungus, etc. Bacteria were reckoned to be one of the most commonly isolated[4]. Poor oral hygiene is considered to be a major ecological shift that steers complex microbial communities in the mouth to dysbiosis. Ecological shifts in a dysbiotic ecosystem favour an increased prevalence of pathogenic oral bacteria. Daily activities such as mastication, flossing and tooth brushing can induce bacteraemia, which facilitate haematogenous dissemination of oral bacteria and inflammatory mediators inducing

systemic inflammation in some patients. Individuals with periodontal disease show micro-ulcerated sulcular epithelia and damaged periodontal tissues, and thus seem more susceptible to bacteraemia[5].

Metagenomic analyses of patients infected with COVID-19 have frequently reported high reads of cariogenic and periodontopathic bacteria such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia*[6], endorsing the notion of a connection between the oral microbiome and COVID-19 complications. Evidence suggests that periodontopathic bacteria are involved in the pathogenesis of respiratory diseases and are associated with chronic inflammatory systemic diseases including type-2 diabetes, hypertension and cardiovascular disease. These diseases are frequently reported comorbidities associated with an increased risk of severe complications and death from COVID-19[7]. Therefore, the current study aims to establish a link between the presence of oral bacteria *Porphyromonas gingivalis* and SARS-CoV-2 infection.

Some studies have suggested that patient sex my help to predict the patient cooperation during the treatment as females appears to be more adaptable to the treatment than males. Nevertheless, the satisfaction level with the appearance is lower in females than males, thus this feature could negatively affect the use of special appliances needed during the treatment [1]. Additionally, the socio-economic status may have an effect on patient's cooperation. It has been suggested that patients with high socio-economic level cooperate better than patients with low socio-economic level [2].

The aim of the study was to evaluate the compliance of patients with Class III malocclusion to orthodontic treatment using different types of orthopaedic appliances.

Materials and Methods

Subjects: Fifty patients with COVID-19 were enrolled in this study, (23 males and 27 females), their age ranges between 20-76 years. All cases were collected from hospitalized patients at Ibn AL-Khateeb and AL-Ataa hospital in Baghdad governorate. Control group included 35 healthy volunteers, where age and sex matched to those in the study group, they tested negative RT-PCR for COVID-19, and didn't have medical history or clinic evidence of any chronic or acute diseases.

Inclusion and Exclusion Criteria: inclusion criteria included; patients with signs and symptoms of COVID-19 infection (fever, generalized malaise, cough and shortness of breath) and positive RT-PCR for COVID-19. Exclusion criteria included; pediatric and pregnant patients, patients with chronic viral infection and systemic diseases, allergic rhinitis and chronic sinusitis and patients who could not give informed consent.

Oral hygiene Index: was determined by the simplified oral hygiene index "Oral Hygiene Index = Debris Index + Calculus Index"[8].

Sample size calculation: To calculate sample size, *Porphyromonas gingivalis* was used as primary outcome of the study, which was used to calculate sample size using online tool EPITOOLS (https://epitools.ausvet.com.au/casecontrolss) at 95% confidence interval, 5% error margin

Samples Collection: After obtaining ethical approval from College of Dentistry, University of Baghdad Committee (ID No. 203), Two ml of venous blood was drawn from each subject under aseptic technique. Blood added in EDTA tube (1.5mg/ml), then kept at -70 ºC until use for the genetic bacterial detection. Three ml of unstimulated saliva was collected from forty subjects in a sterile container. Then it was transferred to Eppendorf tubes and separated by an Eppendorf centrifuge at 3000 rpm for 10 min, then the supernatant was discarded and the pellet was kept at -70 ºC until use for the genetic bacterial detection.

Quantification of *P. gingivalis* RTPCR

I- DNA Extraction (Blood and Saliva Samples)

Genomic DNA was isolated from blood or saliva samples according to the protocol ReliaPrep™ Blood gDNA Miniprep system, Promega.

II- Reaction Setup and Thermal Cycling Protocol Optimization

For specific detection and quantification of *P. gingivalis* bacteria, primers were designed for the species-specific region on the 16S rRNA. The primers used for detection of *P. gingivalis* 16S rRNA gene were designed by software program and approved by Primer Quest from Integrated DNA Technology, Table 1.

Table 1: Primers of *P. gingivalis.*

	Seq.	**Annealing Temp. (°C)**	**Prouct Size (bp)**
***P. gingivalis* 16srRNA-F**	**5`-AGAATAA-GCATCGGCTAACTCC-3`**	55	99
***P.gingivalis* 16srRNA-R**	**5`-GAACAACCTAC-GCACCCTTTA-3`**		

These primers were supplied by the Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

RT-PCR amplifications were performed by Magnetic Induction Cycler Real Time PCR (Mic RT-qPCR), with 10µl volumes containing 5 µl GoTaq Green Master Mix (2X); 0.5 µl for each primer (10 µM); 0 µl nuclease free water; and 4µl of template DNA. RT-qPCR system cycling was performed with the following temperature program: denatured at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20 seconds; annealing at 55°C for 20 seconds; and extension at 72°C for 30 seconds, Table 2.

Table 2: Real Time PCR Program

Steps	°C	M: s	Cycles
Initial Denaturation	95	10:00	1
Denaturation	95	00:20	40
Annealing	55	00:20	
Extension	72	00:30	

Absolute quantification by the standard curve

The standard curve method employs a dilution series of known template copy numbers in the qPCR assay (Figure 1). Linear regression of log concentration (copy µl-1) versus CT gives the standard curve, and this is then used to calculate the template concentration (copy µl-1) of the sample. Eight of 0.2 ml tubes were prepared, 90 µl of Nuclease Free Water was added to each tube then 10 µl from a sample of 129*109 copy µl-1 was added to the first tube and a serial dilution was made by transferring 10 µl from the first tube to the second tube and so on. The standard curve reaction started from the tube of 129*107 copy µl-1 to the tube of 129 copy µl-1.

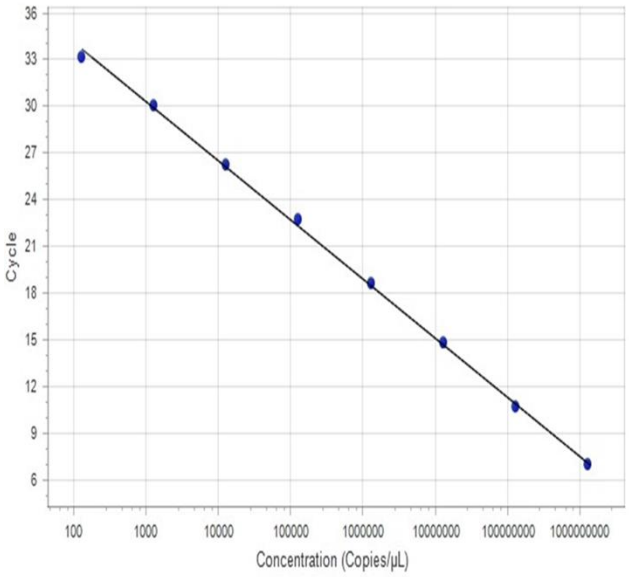

Figure 1: Amplification plot of Serial dilutions of genomic DNA from P. gingivalis were used as templates for real-time PCR.

Statistical Analysis: Data description, analysis and presentation have been performed using a computerized software statistical package for social science (SPSS version 21). The Shapiro Wilk test was used to test the normality distribution of the quantitative variable. Both descriptive and inferential statistics were used, Analysis of variance student t-test, Mann-Whitney test and Chi-square test. The statistical significance of the difference of mean between 2 groups was calculated by T-test, Mann-Whitney test and Chi-square test. Correlation among different parameters was calculated by the Spearman and Pearson correlation coefficient test. $P<0.05$ was considered significant.

Results

The demographic characteristics of the patients group and controls group included in this study are presented in Table 3. The mean age of patients was (51.04 ± 13.25) years and (47.08 ± 11.45) years for the controls' group. The most age group frequency was (40+ years) which comprised 80% of the patients, whereas the age group (<40 years), constituted 20% of the patients. Moreover, this study showed that 54% of COVID-19 patients were females, while 46% were males. There were no significant differences (P>0.05) in age and gender between the two study groups.

Table 3: Case-control differences in gender and age.

Gender and Age	**Study groups**				**P-value**
	Patients group N=50		Controls group N=35		
	N	%	N	%	
Gender					0.774NS
Female	27	54	20	57	
Male	23	46	15	43	
Age group (years)					
<40	10	20	9	25.7	
40+	40	80	26	74.3	0.103NS
Range	(20 - 76)		(24 - 72)		
Mean ± SD	51.04±13.25		47.08±11.45		

NS: Non–Significant, SD: Standard Deviation, No.: Number, %: Percentage.

Based on the severity of COVID-19, the current study showed that 11 (22%) of patients have mild disease, 29 (58%) have a moderate disease and 10 (20%) have severe disease, as illustrated in Figure 1. It is presented that out of 50 patients participating in this study 18 (36%) had good oral health status and 32 (64%) of patients had poor oral health status, Table 4.

The results showed that there were significant differences (p<0.05) in the state of oral health among three groups of patients. 7 (70%) in severe cases, 18 (62%) in moderate and 7 (64%) of mild cases had poor oral hygiene. Whereas 3 (30%) of severe cases, 11 (38%) for moderate and 4 (36%) of mild cases had good oral health status, Table 5.

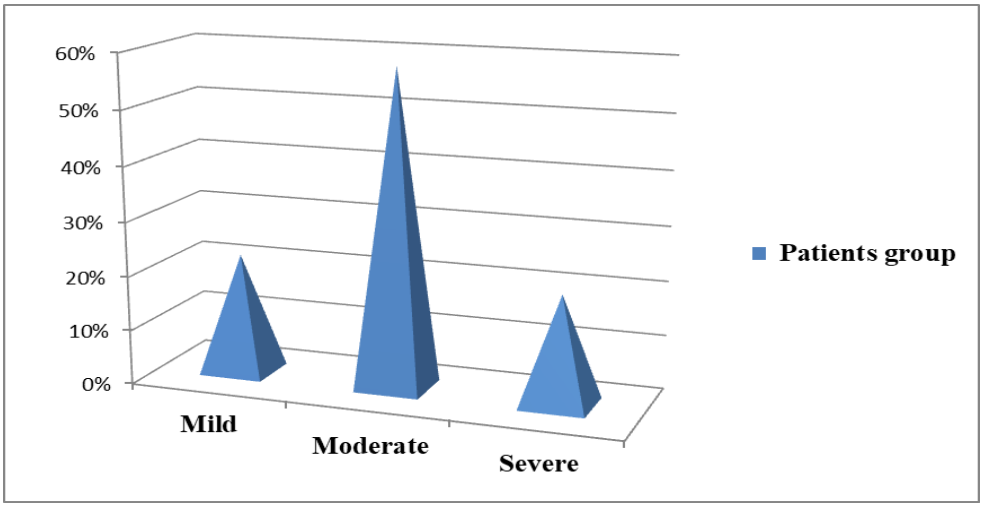

Figure 1: Frequency distribution of patients according to disease severity.

Table 4: Frequency distribution of patients according to oral hygiene.

Oral health status	Number	Percentage
Good	18	36%
Poor	32	64%

Table 5: Distribution of oral hygiene according to severity of COVID19.

Oral Hygiene		Severity of COVID19			
		Severe	**Moderate**	**Mild**	**P-value**
Good	N	3	11	4	0.000 (HS)
	%	30%	38%	36%	
Poor	N	7	18	7	
	%	70%	62%	64%	

The current results revealed a highly significant difference ($p<0.05$) in the copy number of salivary *P. gingivalis* between patients and controls. Table 6 shows that the median number of bacteria in the patient group was higher (11954) than the median number of bacteria in the healthy control group (211). Regarding the bacterial DNA present in the blood of patients and controls, the findings of this study conclude that there are only five cases with bacteria in the blood and all of them were severe and that there was no significant difference in the median number of bacteria in patients and controls, as shown in Table 7.

Table 6: Descriptive and Analytical Statistics of Median Level of *P. gingivalis* in Saliva of Patients and Controls.

Bacterial DNA	Patients group N=30	Controls group N=10	Mann-Whitney	P-value
Minimum	45	17		
Maximum	9576841	7774		
Median	11954	211	2.888	0.003**

Table-7: Descriptive and Analytical Statistics of Median Level of P. gingivalis in Blood of Patients and Controls.

P. gingivalis	Patients group N=30	Controls group N=10	Mann-Whitney	P-value
Minimum	0	0		
Maximum	128	151		
Median	0	0	0.633	0.528NS

The findings of this study state that there is no significant difference (p> 0.05) in the salivary median number of bacteria in the patients group according to severity of disease among three groups of patients. However, the median number of bacteria in severe cases was higher (20278) than that in moderate (18330) and mild (3384) cases Table 8. Furthermore, the median number level of *P. gingivalis* in poor oral hygiene was (25086), compared to that in patients with good oral hygiene (3546), as shown in Table 9.

Table 8: Salivary levels of *P. gingivalis* in patients according to disease severity.

P. gingivalis	Patients group			
	Severe N=5	Moderate N=18	Mild N=7	P-value
Min	1209	848	45	
Max	2799807	974105	9576841	0.737NS
Median	20278	18330	3384	
Mean Rank	7	6.14	12.89	
Severe group vs. moderate group				0.631NS
Severe group vs. mild group				0.417NS
Moderate group vs. mild group				0.787NS

Table 9: Salivary levels of P. gingivalis in patients according to oral hygiene.

P. gingivalis	Good N=15	Poor N=15
Min	45	848
Max	2799807	9576841
Median	3546	25086
Mean Rank	13.20	17.80
P-value	0.158 NS	

Discussion

The co-infection of the SARS-CoV-2 with other microorganisms is a very important factor in COVID-19 pathogenesis that may complicate the accurate diagnosis, treatment, and prognosis of COVID-19 and even increase the mortality rates[9]. Previous studies report that *P. gingivalis* can facilitate the reactivation of latent Epstein-Barr and Human Immunodeficiency Virus-1[10, 11]. So a synergistic relationship between SARS-CoV-2 and periodontal bacteria cannot be excluded.

In the present study, RT-PCR method was successfully used to detect and count *P. gingivalis* in saliva and blood samples. The results demonstrated that *P. gingivalis* was detected in both COVID-19 patients and healthy subjects with a significantly higher detection rate in the saliva of patients. Co-infection may be caused by decreased lymphocytes and host immune function as it is well known that SARS-CoV-2 infection can damage lymphocytes, especially B cells, T cells and NK cells, which will lead to the immune system's impairment during illness[12,13]. These findings are consistent with a recent study[14], which found that many periodontopathogenic bacterial genera (Porphyromonas, Prevotella and Aggregatibacter) were significantly elevated in COVID-19 patients as compared to control subjects. Likewise, Chakraborty (2020) reported that metagenomic analyzes of patients infected with SARS-COV-2 have frequently reported high reads of cariogenic and periodontopathic bacteria in line with the concept of a connection between the oral microbiome and COVID-19 complications[6]. In addition, a previous study conducted by Herrera et al, (2020), elucidates that there is an association between oral diseases like periodontitis and a higher risk of increased gravity of COVID-19 patients[15]. The co-presence of SARS-CoV-2 with periodontal bacteria may exacerbate periodontal tissue damage, however, the nature, extent and consequences of this interaction are currently unknown. In periodontitis patients, it can be speculated that i) a viral-bacterial synergy might facilitate penetration of SARS-CoV-2 through the pocket epithelium, ii) such an interaction can help viruses evade the immune response, thus enabling its entrance to gingival capillaries and endovascular transmission directly to the pulmonary vessels[16], which coincides with the current finding of the presence of *P. gingivalis* in the blood of COVID-19 patients. On the other hand, autopsy studies show a surprising lack of bacterial super-infection in those who have died from COVID-19[17]. Moreover, another study of critical care patients found no evidence of bacterial co-infection in blood, sputum or bronchoscopy sampling upon admission to intensive care[18].

This study furthermore showed that the number of bacteria in severe cases were higher than that in moderate and mild with no significant differences, and that there was a significant increase in the number of bacteria among patients with poor oral hygiene compared to patients with good oral hygiene. This may attribute to the limited number of patients investigated in this study, and also the low number of patients after subdivision may result in the absence of such an association. Unfortunately, no previous studies with such comparisons were found. Anyhow, Chakraborty et al. (2020) showed that improper oral hygiene increases the risk of inter-bacterial exchange between mouth and lungs, thus increasing the incidence of respiratory infections and post-viral bacterial complication[6]. This study concluded that the high level of salivary *Porphyromonas gingivalis* in patients increases in number with disease severity, which may indicate that bacterial infections contribute to the spread of the disease.

Conflict of interest: None.

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الكشف الجزيئي عن البورفيروموناس اللثوية في مرضى فايروس كورونا-2019

الباحثون: هيفاء حمود كريم, د. بتول حسن الغرابي, د. سناريا البدري

المستخلص

الخلفية: تسببت عدوى فايروس كورونا -٢ المتلازمة التنفسية الحادة الوخيمة في حدوث جائحة عالمية تستمر في التأثير سلبًا على صحة الإنسان. المجالات الميكروبية بما في ذلك البكتيريا تفاعلت في التسبب الجزيئي المعقد جنبًا إلى جنب مع متلازمة الجهاز التنفسي الحادة الوخيمة فايروس كورونا -٢.تشارك بكتريا اللثة في مرض فايروس كورونا ٢٠١٩ ، وترتبط بالأمراض الجهازية الالتهابية المزمنة. تم أجراء هذه الدراسة لبحث وتحديد نسبة البورفيروموناس اللثوية في مرضى فايروس كورونا ٢٠١٩ والاصحاء ، وكذلك لتحري ارتباط الحمل البكتيري مع شدة المرض ونظافة الفم. شارك خمسون مريضًا مصابًا بمرض فايروس كورونا ٢٠١٩ المؤكد عن طريق تقنية أنزيم النسخ العكسي- تفاعل البلمرة المتسلسل ، تتراوح أعمارهم بين (٢٠-٧٦) عامًا, و خمسة وثلاثون متطوعًا ممن يتمتعون بصحة جيدة تمت مطابقة أعمارهم وجنسهم مع المرضى في هذه دراسة. تم تحديد حالة نظافة الفم من خلال مؤشر نظافة الفم المبسط. تم الحصول على عينات الدم واللعاب من المشاركين ، وتقدير البورفيروموناس اللثوية من الحمض النووي المستخرج من عينات الدم واللعاب التي تم إجراؤها عن طريق تفاعل البلمرة المتسلسل في الوقت الحقيقي. كانت كمية البورفيروموناس اللثوية في اللعاب أعلى معنويا (**p = 0.003**) في المرضى مقارنة بمجموعة الاصحاء، بينما لم يكن هناك فرق معنوي في عدد البكتيريا في عينة الدم بين المجموعتين.علاوة على ذلك كان عدد البكتريا في الحالات الشديدة اعلى منه في الحالات المتوسطة والخفيفة مع عدم وجود فروقات احصائية (**p> 0.05**) أظهرت هذه الدراسة أن مستوى البكتيريا اللعابية لدى المرضى يزداد في العدد مع شدة المرض ، مما قد يشير إلى أن الالتهابات البكتيرية تساهم في انتشار المرض.