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RESEARCH ARTICLE

Quantification of *Porphyromonas gingivalis* in Plaque of Severe Chronic Periodontitis Patients Associated with Interleukin-12 Serum Level Using Real-Time Polymerase Chain Reaction

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Abstract

Background: Periodontitis is a multifactorial polymicrobial infection in which the bacteria play a fundamental role in its initiation and progression. *Porphyromonas gingivalis* has been implicated as a major etiological agent causing tooth loss. Aim of the study: This study was carried out for molecular identification and quantification of *Porphyromonas gingivalis* in subgingival plaque samples of severe chronic periodontitis (CP) patients and healthy controls and its association with interleukine-12 serum levels. Materials and Methods: Fifty one CP patients and 45 healthy controls with age ranged from (30-50) years old, were recruited. Periodontal status was assessed based on plaque index (PLI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL). Real time-Polymerase chain reaction-SYBR green technique was used to quantify *Porphyromonas gingivalis* from extracted DNA of dental plaque samples, while enzyme-linked immunosorbent assay (ELISA) was carried out to estimate the serum level of interleukin-12. Results: The present finding revealed that *Porphyromonas gingivalis* count was higher in the patients compared to the healthy controls and positively associated with all clinical parameters. As well significant increase of interleukin-12 serum level was found to be associated with severe chronic periodontitis. Interestingly, positive significant correlation between bacterial count and serum interlukin-12 level was noticed. Conclusion: A quantitative real time-polymerase chain reaction is sensitive and efficient method which permits to study specific organisms and their role in periodontal disease pathogenesis. The results of this study confirmed the earlier finding of *Porphyromonas gingivalis* present in significantly higher levels in CP patients. The positive association between bacterial count and interlukin-12 appear to be indicative of interactive relationship and may play a crucial role in pathogenesis of CP.

Keywords: *Periodontitis, Porphyromonas gingivalis, Real time-polymerase chain reaction, Interlukin-12, SYBR green.*

Introduction

Periodontal disease is a highly prevalent, multifactorial, chronic inflammatory disease of periodontium eventually leading to destruction of supportive tissues of teeth and tooth loss [1, 2]. Chronic periodontitis is associated with plaque and calculus accumulation and has a slow to moderate rate of disease progression; however, periods of rapid destruction are also observed. The increase in the rate of disease development and clinical manifestations of periodontal disease is caused by the impact of local, systemic or environmental factors that influence the normal host- bacterial interaction [3, 5].Among subgingival plaque bacterial species, *Porphyromonas gingivalis (P.gingivalis)*, a Gram-negative black-pigmented anaerobe, is a key etiologic agent of periodontitis, especially its chronic form [6]. *P. gingivalis*, and/or its components, is capable of inducing a strong destructive pro-inflammatory cytokine response in the

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gingival epithelial and periodontal ligament cells in-vitro, which is correlated with the adhesive/invasive potential of *P. gingivalis* [7, 9]. It is noteworthy that *P. gingivalis* can escape the host's innate immune response and internalize to the host cells, as well as invade into deep tissue [10]. The severity of the disease depend on the balance between the aggressiveness of the subgingival plaque biofilm and the individual host immune response [11] further modulated by the genetic and by environmental factors like age, gender, smoking and oral care [12].

Interleukin-12 (IL-12) is a heterodimeric proinflammatory cytokine produced mainly by antigen-presenting cells. IL-12 has been shown to stimulate natural killer cell (NK) production. In addition, it bridges the early nonspecific innate resistance and the subsequent antigen-specific adaptive immunity through promotion of CD4+ T cell differentiation from T- helper (Th1) cells to (Th2) cells and can also stimulate the bacterial clearance function of macrophages [13, 14].

IL-12 also activates the production of other cytokines, including tumor necrosis factor-α (TNF-α) and interferon gamma (IFN-γ) by activated T cells and NK cells. Generation of cytotoxic T cells is also promoted by the action of IL-12. These different activities support the role of IL-12 in the host immunopathology of periodontal disease [15]. This study was carried out for molecular identification and quantification of *P. gingivalis* in subgingival plaque samples of CP patients and healthy controls and its association with interleukine-12 serum levels.

Materials and Methods

This is a case-control study and subjects were selected from patients attending the department of periodontics in the teaching hospital of College of Dentistry /University of Baghdad or attendants to the Iraqi blood bank / Baghdad Medical city, for blood donation. This study was approved by the Ethics Committee of College of Dentistry/ University of Baghdad. Sixty nine subjects with age range of 30-50 years old were enrolled in this study. Samples collection was started from February to July 2018. All participants were informed about the aims of the study and subjected to questionnaire about name, age, past periodontal treatment, medical history, medication used, smoking or alcohol consumption. The diagnosis for severe CP was done by assessing and recording at least ≥2 interproximal sites with CAL ≥6 mm (not on same tooth) and ≥1 interproximal site with PD ≥5 mm according to the criteria of Centers for Disease Control and Prevention (CDC) and American Academy of Periodontology (AAP) [16]. Periodontal health status was recorded through the examination of clinical periodontal parameters PLI, GI, BOP, PPD and CAL by using Michigan O probe with William's markings at 1,2,3,5,7,8,9 and 10 mm.

The subjects were divided into 2 groups: - Group I (Study Group): Consist of 51 patients (37 males and 14 females) with severe CP their age range (35-50) years. Group II (Control Group): Consist of 45 subjects (34 males and 11 females) with clinically healthy periodontium, their age range (30- 50) years with gingival index ≤ 0.5, gingival sulcus depth < 3 mm and attachment loss = 0 mm. The age and gender of healthy control group were matched with patients group.

Subgingival plaque samples were collected from four deepest periodontal pockets in each subject of CP group and gingival sulcus in control group. The selected sites were randomized in different quadrants. After the clinical parameters had been recorded, the sampling area was isolated with cotton rolls, carefully scaled supra-gingivaily with a sterile curette, cleaned with sterile cotton pellets to prevent the contamination of the samples with saliva or supragingival plaque according to [17, 18] and the samples were taken with sterile Gracey curettes the curette was inserted slightly as deep as possible into the pocket without applying any pressure on the tooth surface, in order to avoid a dislocation of subgingival plaque with the curette into the depth of the pocket.

As soon as the curette met tissue resistance at the apical part of the pocket, subgingival sampling was performed with one single vertical stroke immediately placed in eppendorf tube containing 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), [18] by vigorously agitating the tip of instrument in the solution. Then the sample stored at (-40˚C) until the DNA extraction. Then 3 ml of venous blood were withdrawn from each subject under aseptic technique and putted in serum separating gel tubes and leave the

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samples to clot for 30 minutes, after that centrifugation was performed at 1000 x g (3500 rpm) for 10 minutes; then the serum samples were separated and placed in eppendrof tubes and stored at deep freeze -40˚C in plastic containers for later analysis by Enzyme Linked Immunosorbent Assay (ELISA) sandwich technique, using kit for quantitative determination of (IL-12) serum levels (Komabiotech /Korea).

DNA Extraction and Real Time-PCR Assay

Genomic DNA was isolated from dental plaque sample according to the protocol of (QIAamp DNA Mini Kit, QIAGEN/ Germany**)** according to the manufacturer's protocol (QIAamp Mini spin column), then the extracted DNA quantified by Quantus Florometer. The primers used in this study were specific for 16s rRNA -encoding gene for detection of *P.gingivalis* bacteria, table-1, they were designed by software program and approved by Primer Quest from Integrated DNA Technology. RT-PCR (SYBR green)performed by Magnetic Induction Cycler RT- PCR (Mic RT qPCR), (Bio Molecular System(BMS)/Australia), 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 20 seconds.

Known amounts of bacterial specific DNA were used to determine a standard curve. The standard curve method employs a dilution series of known template copy number in the qPCR assay. A standard curve was generated from serial dilutions versus CT (CT is the cycle number at which the fluorescence generated within a reaction crosses the threshold line) by linear regression analysis and used as a basis for further quantification of target DNA from the clinical samples, the R^2 was (0.9989), Figure (1, 2).

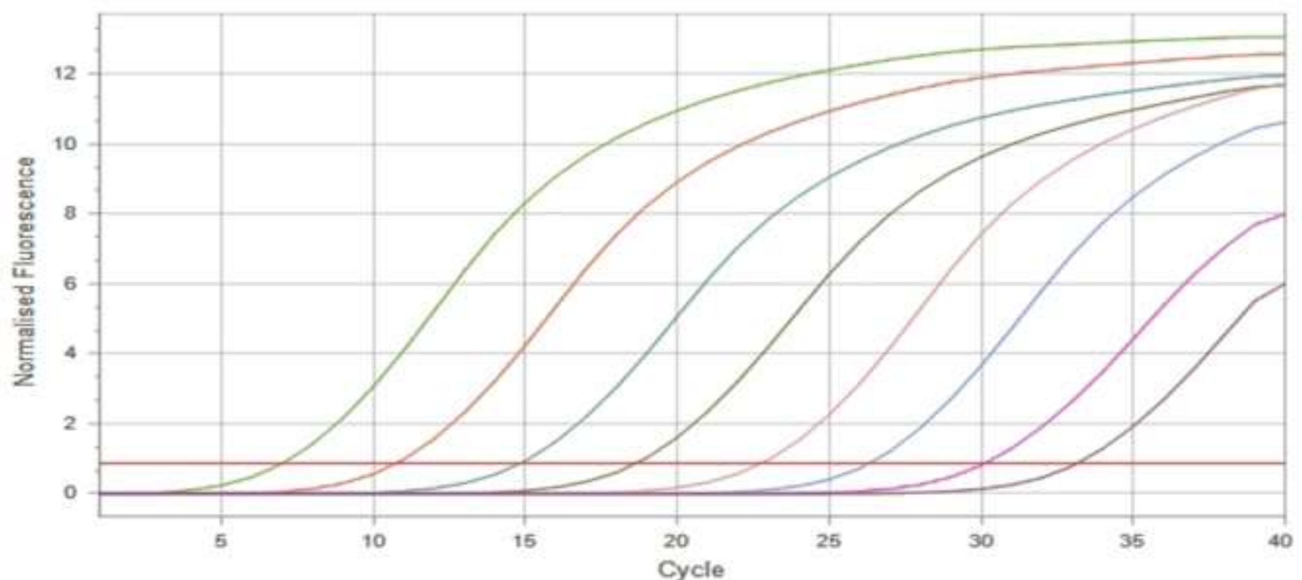

Figure 1: Amplification plot of Serial dilutions of genomic DNA from *P. gingivalis* were used as templates for real-time PCR

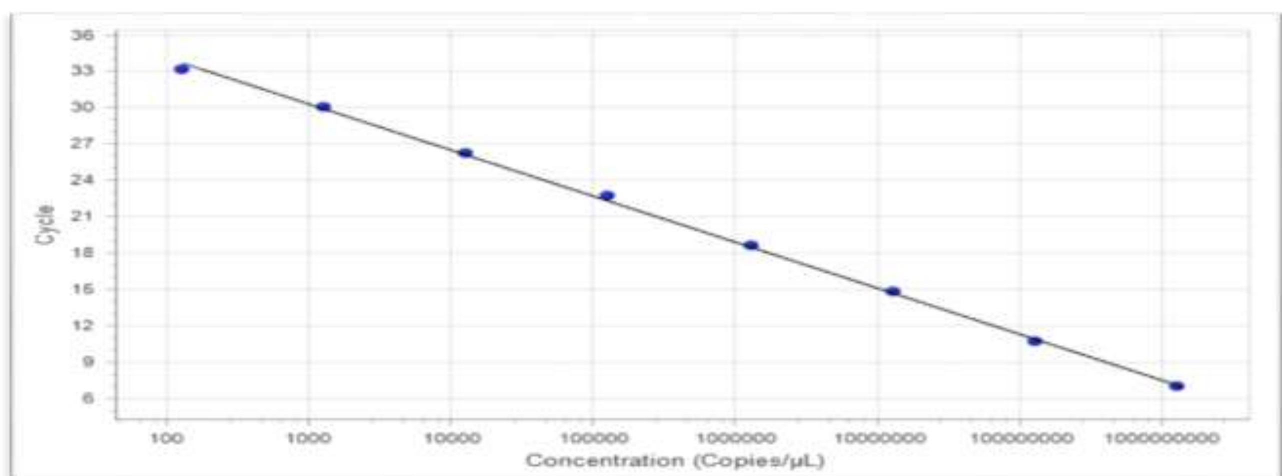

Figure 2: *P. gingivlais* Standard Curve

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Statistical Analysis

Statistical analysis was accomplished by using computerized statistical analysis SPSS software version 24 (statistical package for social science). Semirnov Kolmogorov to test the normality of distribution of the data, test mean, median, SD, SE, percentages, T-test, Mann-Whitney U test, Spearman's rank correlation coefficient test (r), Chi-square test was used to compare the frequencies of BOP in the disease and control groups. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference.

Results

The demographic variables and periodontal parameters of the 96 subjects enrolled in this study are illustrated in Table-2.

Table 2: Demographic and clinical variables in the study and control groups

Characteristics		CP n= 51	Controls n= 45	P-value
Age (years) †		42.78±3.73	40.98±6.38	0.10#
Gender††	Male	37 (72.5%)	34 (75.6%)	0.73##
	Female	14 (27.5%)	11(24.4%)	
PLI†		2.53±0.43	0.35±0.16	<0.001#
GI†		1.96±0.25	0.31±0.15	<0.001#
BOP sites (%) ††		3976 (77.9%)	17 (0.3%)	<0.001##
PPD(mm) †		5.67 ±0.64	-	-
CAL(mm) †		5.70 ±0.74	-	-

† Values are given as mean ± SD; ††Values are given as n (%) of subjects; # Two-sided unpaired t-test; ##Two-sided Pearson's chi-square test (χ2)

The results of this study found that there was a significant elevation in the median serum levels of IL-12 among CP group (406.2 pg/ml) when compared to controls group (152.5 pg/ml), ($P< 0.05$), as observed in table-3. By using coefficient of spearman's rank correlation (r), this study found that there were non-significant correlations between serum level of IL-12 and clinical parameters (PLI, GI, BOP, PPD and CAL), as demonstrated in Table-4.

Table 3: Descriptive and Analytic statistics of median values of IL-12 parameter for the CP &Control groups with comparison of significance

IL-12	CP n= 51	Controls n= 37	Mann-Whitney U	p- value
Median	406.2	152.5	679.5	0.02*
Minimum	139.2	17.8		
Maximum	2000	1944.4		

* S: Significant

Table 4: Spearman's Correlation between IL-12 Serum Levels and Clinical Parameters in Study Groups

Parameter	IL-12			
	CP		Control	
	r	P	r	P
PLI	0.117	0.414	0.069	0.680
GI	0.055	0.696	0.064	0.703
BOP 1	0.076	0.592	0.071	0.673
PPD	0.195	0.168	-	-
CAL	0.180	0.207		

R: Spearman's rank correlation

Table-5 shows the results of quantification of *P. gingivalis* assessed by real-time PCR absolute quantification method. *P. gingivalis* bacteria were detected in all samples from patients group, whereas only 18 subjects in controls were positive for the presence of target pathogen. It was clearly shown that median number of bacteria in patients group was higher (405886) than median number of bacteria in healthy controls group. This study revealed a highly significant difference in the copy number of *P. gingivalis* between CP patients and controls ($P< 0.01$). The current study revealed a highly significant correlation between the *P.gingivalis* counts and clinical parameters in both study groups

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(P< 0.01). The results showed that there were non-significant correlations between *P. gingivalis* counts and serum levels of IL-12 in CP group (P>0.05). While demonstrated positive significant correlation (r=0.381, P=0.020) in controls, Table-6.

Table 5: Analytic Statistics of Median Value of *P. gingivalis* for the CP and Control Groups with Comparison of Significance

P. gingivalis	CP n= 51	Controls n= 45	Mann-Whitney U	p- value
Median	405886	0	10.000	0.000**
Minimum	57063	0		
Maximum	3215583	91115		

**: Highly Significant

Table 6: Correlation of *P.gingivalis* Counts with Clinical Parameters and Serum Biomarkers

Parameter	*P. gingivalis*			
	CP group		Controls group	
	r	P	r	P
PLI	0.781	0.000**	0.846	0.000**
GI	0.456	0.001**	0.811	0.000**
BOP 1	0.690	0.000**	0.833	0.000**
PPD	0.760	0.000**	-	-
CAL	0.723	0.000**	-	-
IL-12	0.050	0.727NS	0.381	0.020*

NS: Non-Significant; *: Significant; **: highly significant; r: Spearman's rank correlation

Discussion

In this study, there was significant increase of serum IL-12 level in the CP group as compared to healthy group. This indicates that a relation exists between the level of periodontal tissues destruction and the increase in serum IL-12. The increase in the level of IL-12 follows the stimulation of dendritic cells by *P.gingivalis* and *A. actinomycetemcomitans* bacteria. This increase plays an important role in the response of Th1 and production of INF-γ. INF-γ affects the monocyte-macrophage cells and causes the production of IL-12, IL-1, and INF-γ [19].

These findings were in harmony with studies [20, 23], whereas opposed the findings of Orozco et al. [24] who reported that the level of IL-12 in the serum obtained from patients with periodontitis was extremely low. This difference was may be due to the small number of the individuals who had participated in the research of Orozco and co-workers (10 patients with periodontitis) or the difference in the kits used for IL-12 analysis.

Concerning the correlation of IL-12 serum level with periodontal parameters, this study showed that no correlation between clinical periodontal parameters and IL-12 was found, the findings are suggestive of a destructive role of IL-12 on periodontal tissues. These results were similar with (Yu¨cel et al. [21], Tarannum & Faizuddin [23] and Sharma [25]. Whereas, contradicted with other study [19].Yet, the exact role of IL-12 in the development and progress of periodontitis has not been clarified. Several studies have demonstrated a positive correlation between the level of IL-12 and the severity of periodontal destruction; indicate the involvement of this cytokine in the development of periodontal disease [15].

Real-time PCR is a DNA amplification technique that allows accurate determination of nucleic acid levels by scanning fluorescent signals at a cycle-to-cycle rate [26], bringing remarkable contribution to the detection of pathogenic microbiota in mixed oral infections [27-29]. In the present study, RT-PCR method was successfully used to detect and count of *P. gingivalis* in subgingival microbial samples.

The result demonstrated that *P.gingivalis* was detected in both periodontally healthy subjects and those with chronic periodontitis with significantly higher detection rate in the latter. Several researches [30, 32] confirm that the use of bacterial quantitative data rather than dichotomous data (presence/absence or positive/negative) provides more statistical power for detecting any predictive correlation with disease severity. These results were consistent with other previous studies of Tomás et al. [32], Avila-Campos [33], Al-hebshi et al. [34] and Abdulaziz et al [35].

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Who found that the number of this bacterium was higher among CP patients and inconsistent with study of Avila-Campos [33] who reported that *P.gingivalis* were prevalent in both CP and control groups with non-significant difference between them. Generally, the presence of *P. gingivalis* in control group implies that this bacteria exists in commensal harmony with other organisms present in the individuals with healthy periodontium, this is supported by other studies [36, 38] which showed the presence of *P. gingivalis* in healthy subjects and concluded that this organism may also be a normal inhabitant of a periodontally healthy dentition.

Almost all the correlations between *P.gingivalis* level and clinical periodontal parameters values were positive. The interpretation is that subgingival sites with periodontal destruction were associated with higher increments of *P.gingivalis*. This is in accordance with results of Tomás et al. [32], Abdulaziz et al. [35], Kawada et al. [39] and Vajawat et al. [40]. Our results showed that the presence of bacteria was not correlated with serum levels of IL-12 in CP group and this may attributed to small samples number, while in control group *P. gingivalis* counts were associated positively with IL-12 levels suggesting that the reduction in *P.gingivalis* counts in control group were associated with accompanied decrease of serum IL-12 levels. Multiple studies have demonstrated that *P. gingivalis* and *A. actinomycetemcomitans* induce IL-12 expression in dendritic cells [41-43].

Yun et al. in their *in-vitro* study reported that *P. gingivalis* LPS synergistically enhances IFN-γ stimulation of monocytes to secrete IL-12 [44].A quantitative RT-PCR is sensitive and efficient method which permits to study specific organisms and their role in periodontal disease pathogenesis. The positive association between *P. gingivalis* counts and IL-12 serum level appear to be indicative of interactive relationship and may play a crucial role in pathogenesis of CP.

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