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Detection of HCMV DNA in Chronic Periodontitis Patients by Real time PCR and its relation with the severity of disease

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Abstract:

Chronic periodontitis is an inflammatory disease that extends into the tissues supporting the teeth. Recent studies have demonstrated that various human herpes viruses especially human cytomegalovirus (HCMV) may play a part in the pathogenesis of human chronic periodontitis. The aim of this study is to detect HCMV DNA in saliva of chronic periodontitis patients by real time polymerase chain reaction (PCR) test, measure its copy number and find its relation with severity of the disease. The study sample consisted of fifty – three chronic periodontitis patients of both genders (27 males and 26 females) with ages ranged from 25 to 45 years and all the selected patients have not any systemic diseases.

Periodontal parameters used in this study were [plaque index (PLI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL)]. Unstimulated saliva samples were collected from all subjects and examined by real time PCR test for HCMV DNA detection. The results of this study observed that the percentage of study group who were positive for HCMV DNA was (22.6%). The present study observed that there was no relation between PLI and GI with HCMV DNA salivary level. Concerning BOP and PPD there was no relation found between BOP and PPD with HCMV DNA level. For CAL there was a significant relation found between CAL and HCMV DNA level.

Key words: *Chronic periodontitis, Real time PCR, Copy number of HCMV.*

التحري عن الحامض النووي للراشح المضخم للخلايا البشرية في المرضى المصابين بالتهاب اللثة المزمن باستخدام جهاز PCR (الريل تايم) وعلاقته بشدة المرض

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الخلاصة:

النساغ المزمن هو مرض التهابي، يمتد الى الانسجة السائدة للاسنان. أشارت الدراسات الحديثة الى أن روالشح متعددة وخاصة الراشح المضخم للخلايا البشرية ممكن ان يلعب دورا في شدة المرض.

الهدف من هذه الدراسة هو التحري عن الحامض النووي للراشح المضخم للخلايا البشرية في لعاب المرضى المصابين بالنساغ المزمن بواسطة اختبار سلسلة تفاعل البلمرة نوع الوقت الحقيقي (Real time PCR) وقياس كميته وإيجاد علاقة بين الراشح وشدة المرض.

تكونت عينة الدراسة من ثلاث وخمسين مصابا بالنساغ المزمن من كلي الجنسين (27 ذكرا و26 أنثى) وبمدى عمري يتراوح بين 25-45 سنة. كل المصابين لايشكون من اي مرض جهازي. المعلمات السريرية لماحول الاسنان المذكورة في هذه الدراسة كانت، (مؤشر الصفيحة الجرثومية،المؤشر اللثوي، النزف بالمسبر الطبي، عمق الجيب المسبري والمستوى السريري الرابط). جمعت عينات اللعاب غير المحفز من كل الاشخاص المشاركين في البحث وفحصت باختبارسلسلة تفاعل البلمرة نوع الوقت الحقيقي للتحري عن الحامض النووي للراشح المضخم للخلايا البشرية. نتائج هذه الدراسة اظهرت ان نسبة المصابين بالنساغ المزمن الذين اظهروا تفاعل ايجابي للحاض النووي للراشح الضخم للخلايا البشرية كانت (22.6 %).

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بينت هذه الدراسة انه لايوجد علاقة بين مؤشر الصفيحة الجرثومي، **المؤشر اللثوي، النزف بالمسبر الطبي** وعمق الجيب المسبري وبين المستوى اللعابي للحامض النووي للراشح. **بالنسبة** الى المستوى السريري الرابط، **وجدت** هناك علاقة معنوية ايجابية بين مستوى الحامض النووي للراشح وبين المستوى السريري الرابط.

Introduction:

Periodontal disease is a microbial infection involving a variety of microbes that trigger inflammation, loss of connective tissue attachment and alveolar bone around the teeth. The primary etiologic factor of periodontitis is bacterial plaque. The bacteria involved are largely gram negative species that express pathogenic factors that elicit host defense responses resulting in inflammation and tissue destruction. In fact the propensity of periodontitis to proceed with periods of exacerbation and remission could suggest that the presence of other organisms contributes to the disease [1].

Nonbacterial microorganisms that are found in plaque include viruses, mycoplasma, yeasts and protozoa [2]. The development of human periodontitis may depend upon cooperative interactions among herpes viruses, specific pathogenic bacteria and tissue destructive inflammatory mediators. Various studies have shown that human viruses, especially Human cytomegalovirus (HCMV) seem to play a part in the pathogenesis of periodontal disease [3,4,5].

The presence of HCMV was reported to be associated with the major periodontopathic bacteria and the severity of periodontal disease [6,7]. The hypothesis of a correlation between HCMV and the pathogenesis and progression of periodontitis has been proposed by various studies [8,9,10].

Periodontal destruction may be associated with the coexistence of periodontal herpes viruses, especially HCMV and periodontopathic bacteria.

The HCMV can stimulate the release of cytokines and chemokines from inflammatory and non-inflammatory cells and impair the periodontal immune defense, resulting in more virulent resident bacteria [11]. The association of herpes viruses with periodontitis may be important for diagnosis, treatment and monitoring of the disease [12]. Till date several techniques have been employed for the detection of the viruses, real time PCR is a rapid, accurate and sensitive quantitative technique for the detection of viral DNA sequences [13].

This study aimed to detect HCMV in saliva of chronic periodontitis patients by real time PCR, measure its copy number and find its relation with the severity of chronic periodontitis.

Material and Methods:

Human Samples:

Sample population consisted of fifty three males and females, age ranged from 25 to 45 years. Samples collection was started at 1st of October 2013 till March 2014. Patients participating in the present study with chronic periodontitis (27 males and 26 females) were recruited from the Clinic of the Department of dentistry in Primary health care center in Al- Saydia region in Al-Karkh district of Baghdad city.

Clinical examination:

Periodontal examination consisted of plaque index (PLI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL) at 4 sites for all teeth except 3rd molar on (mesial, midvestibular, distal, midlimgual), using a calibrated periodontal probe (Michigan O probe). Patients with chronic periodontitis had periodontal pockets equal or greater than 4mm with clinical attachment loss. All subjects participated in this study without any systemic diseases, had not received previous periodontal treatment and had not used antibiotics and antiviral drugs in the

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past 3 months. Patients were excluded if pregnant and smokers.

Collection of saliva samples:

All participants were instructed not to eat or drink (except water) at least 1 hour prior to donation of saliva, the subject should sit in a relaxed position and samples containing blood should be discarded. Saliva was collected between 9-12 am. After the subject rinse his mouth several times by sterilized water and then wait for 1-2 minutes for water clearance, 5ml of whole unstimulated mixed saliva was collected into polyethylene tubes using a standardized method [14]. Saliva then stored frozen at (-20 ºC) until they were assayed.

Nucleic acid extraction:

All samples were analyzed at the Department of Molecular biology, Iraqi Center for Cancer and Medical Genetics Research, Al-Mustansyriah University. Frozen saliva samples were allowed to thaw and come to room temperature. Therefore, they were subjected to DNA extraction. DNA was extracted from the saliva samples by using (Norgen's Saliva DNA Isolation Kit/Canada-RU45400).

Real time PCR procedure:

Bosphore®CMV Quantification Kit v1 is based on the real-time PCR principle. 24 µl of the master mix was added into the PCR tubes, and added 16 µl of DNA (sample). The thermal is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, Initial denaturation 95°C 14:30 minutes, and 50 cycles composed of denaturation 97°C 00:30 minutes and annealing and synthesis 53°C 01:30 minutes.

The quantity of virus and Precision data have been calculated for each different samples (on CT value basis and on quantitation basis) were obtained by the analysis of the 1.6x1x104 copies/ml) quantitation control using Agilent real-time PCR machine (Mx3005P q PCR System).

Statistical Analysis of data was carried out using the available statistical package of SPSS-22. Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means The significance of difference of different percentages (qualitative data) were tested using Pearson Chi-square test (χ^2-test) with application of Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value was equal or less than 0.05.

Result:

Table-1 shows the distribution of study group by basic characteristics, according to age and gender. Also this table describes the mean, standard deviation (SD) and the range of plaque index (PLI), gingival index (GI) and number of sites in the study group. The mean and SD of age among periodontitis patients were (37.0±7.8) respectively, while the mean, SD and the range of PLI were [1.5±0.3, (1.00-2.26)] respectively. The mean, SD and the range of GI were [1.3±0.3 (1.02-3.00)] respectively. The mean, SD and the range of number of sites that measured among chronic perio-dontitis patients were [95.8±12.8 (72-112)] respectively. The result of real time PCR for HCMV DNA was shown in figure 1. As seen in this figure the number and percentage of study group who were positive for HCMV DNA were 12(22.6%) respe-ctively, while 41(77.4%) were negative for HCMV DNA. Figure 2 shows the results of real time PCR of detection of HCMV DNA Lane (A) Kapa DNA ladder 6µl; Lanes 4,8,13,17,18, 23, 25, 28, 40, 41, 48, 49 HCMV DNA positive saliva sample from chronic periodontitis patients. The results of real time PCR regarding the copies number and percentage of HCMV DNA were shown in table-2.

As illustrated in this table the mean, SD and therange of copies number of HCMV DNA were 4349.8±469.2 (4007.2-5301.3) respectively. The result of real

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time PCR of HCMV DNA in chronic periodontitis patients and its relation with gender, age, mean plaque index (PLI) and mean gingival index (GI) are shown in table 3. The results illustrates that there was no significant difference between male and female and the presence of HCMV DNA (P>0.05), regarding the age there was no significant difference between the age of study group and the presence of HCMV DNA (P>0.05).

In this table there was no significant difference between mean PLI, mean GI, and no. of sites and the presence of HCMV DNA (P> 0.05). The relationship between the presence of HCMV DNA and clinical periodontal parameter; bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL) are shown in table-4. As seen there was no relation found between BOP, PPD and the presence of HCMV DNA. Regarding CAL there was no significant relation found between CAL scale-1 and the presence of HCMV DNA and there was a significant difference found between the presence of HCMV DNA and CAL scale-2 and 3 as shown in table-4.

Table-1: General characteristics of the chronic periodontitis patients regarding; gender, age, mean PLI, mean GI and No. of sites

		Total
		No(%) Mean±SD(Range)
Gender	Male	27(50.9%)
	Female	26(49.1%)
Age (years)	<30	12(22.6%)
	30---34	7(13.2%)
	35---39	8(15.1%)
	40---44	7(13.2%)
	=>45	19(35.8%)
	Mean Age (years)	37.0±7.8(25-45)
Mean P.I		1.5±0.3(1.00-2.26)
Mean G.I		1.3±0.3(1.02-3.00)
No. of sites		95.8±12.8(72-112)

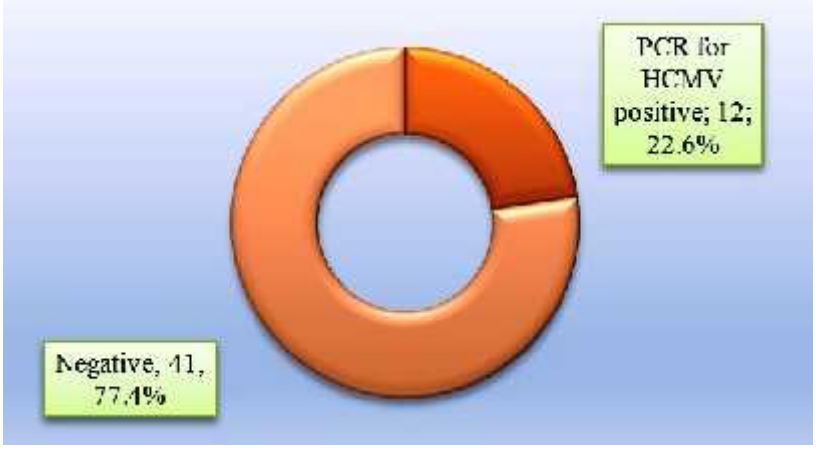

Figure-1: Results of real time PCR for HCMV DNA among chronic periodontitis patients.

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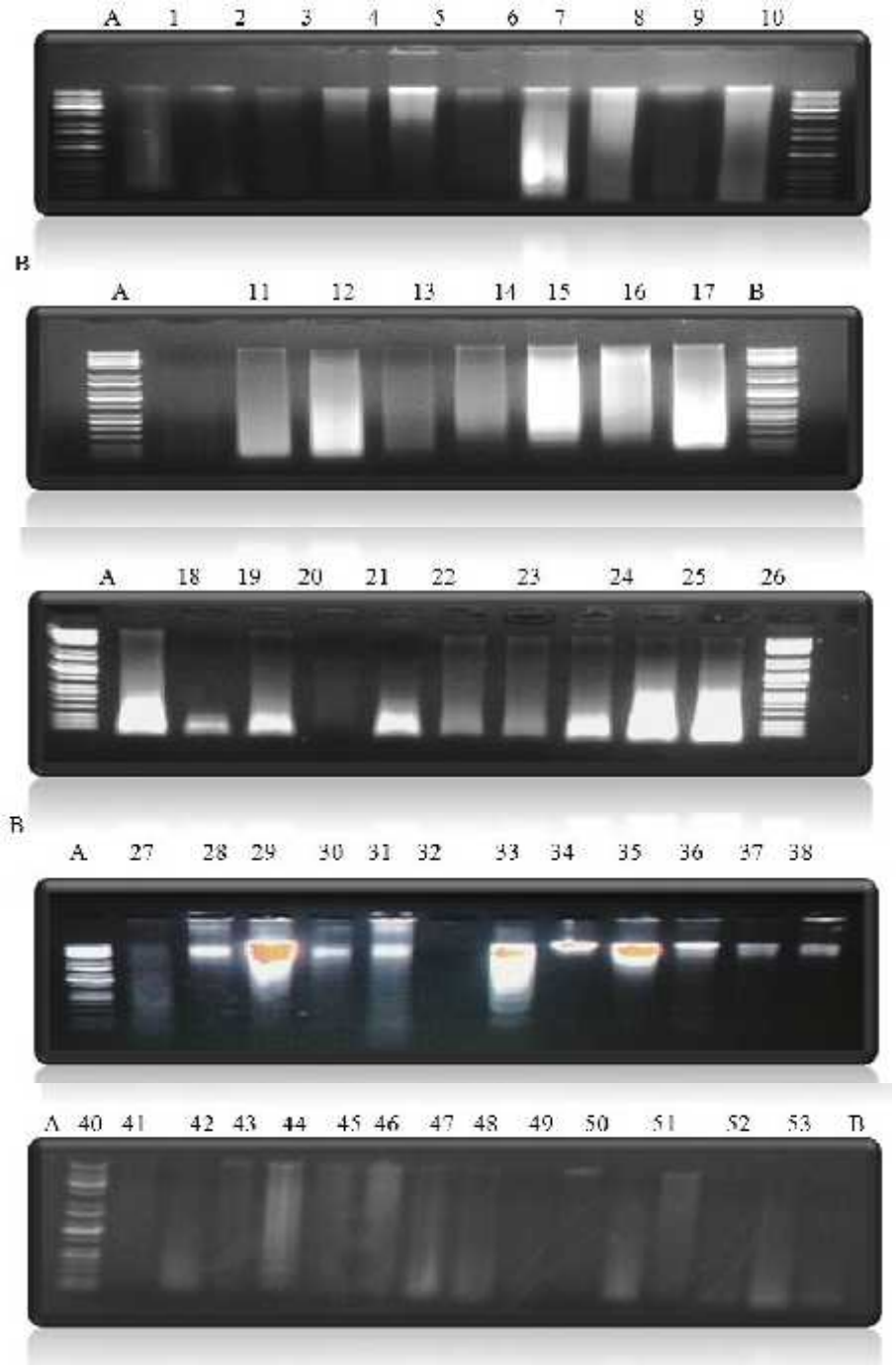

Figure-2: Fifty -three of DNA samples were extracted from saliva of patients using Noragen extraction method. (A and B) 6µl of Kapa DNA ladder and 5µl of (1-53) Saliva DNA samples were loaded in 1% Agarose gel and electrophorized for 45 minutes on 70 Volt.

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Table-2: HCMV DNA copy counts in saliva from chronic periodontitis patients (1.6x104 copies/ml).

No. of Copies CMV DNA 1U/ml	No. (%)
4007.2	1(8.3%)
4010.4	1(8.3%)
4012.3	1(8.3%)
4012.7	1(8.3%)
4093.1	1(8.3%)
4102.9	1(8.3%)
4142.1	1(8.3%)
4207.1	1(8.3%)
4310.5	1(8.3%)
4897.2	1(8.3%)
5101.2	1(8.3%)
Mean ± SD (Range) 4349.8±469.2(4007.2-5301.3**)**	

Table-3: The result of real time PCR of HCMV DNA in chronic periodontitis patients and its relation with gender, age, mean plaque index (PLI), mean gingival index (GI) and number of sites.

		Total	**CMV Positive**	**CMV Negative**	**P value**
		No (%) Mean ± D (Range)	**No. (%) Mean±SD Range**	**No (%) Mean±SD (Range)**	
Gender	**Male**	27(50.9%)	7(58.3%)	20(48.8%)	0.560
	Female	26(49.1%)	5(41.7%)	21(51.2%)	
Age (years)	**<30**	12(22.6%)	1(8.3%)	11(26.8%)	0.429
	30---34	7(13.2%)	1(8.3%)	6(14.6%)	
	35---39	8(15.1%)	3(25.0%)	5(12.2%)	
	40---44	7(13.2%)	1(8.3%)	6(14.6%)	
	=>45	19(35.8%)	6(50.0%)	13(31.7%)	
Age (years)		37.0±7.8(25-45)	40.2±5.7(29-45)	36.1±8.1(25-45)	0.062
Mean P.I		1.5±0.3(1.00-2.26)	1.4±0.4(1.00-2.26)	1.5±0.3(1.00-2.00)	0.276
Mean G.I		1.3±0.3(1.02-3.00)	1.2±0.2(1.04-1.69)	1.3±0.4(1.02-3.00)	0.364
No. of sites		95.8±12.8(72-112)	90.3±12.0(72-108)	97.4±12.7(76-112)	0.093

Non -Significant (P> 0.05)

Table- 4: The relationship between the presence of HCMV DNA and clinical periodontal parameter; bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL).

	Total	**CMV Positive**	**CMV Negative**	**P value**
	No. (%) Mean ± SD (Range)	**No. (%) Mean ± SD (Range)**	**No. (%) Mean ± SD (Range)**	
	53 (100%)	12 (100%)	41 (100%)	
B.O.P Score1	26.6±27.8(2-112)	19.1 ± 19.3(3-72)	28.8 ± 29.6 (2-112)	0.292
P.D Score 1	9.5±7.5(3-34)	9.0±8.9(4-34)	9.7±7.1(3-33)	0.784
P.D Scale 2	5.2±7.3(1-32)	11.0±14.2(1-32)	3.5±2.4(1-9)	0.367
C.A.L Scale 1	8.8±7.1(1-26)	9.1±7.4(3-26)	8.6±7.2(1-26)	0.856
C.A.L Scale 2	9.9±7.5(1-32)	14.4±9.1(1-32)	8.7±6.7(1-32)	0.032*
C.A.L Scale 3	12.0±11.4(1-45)	20.3±13.1(5-45)	8.8±9.1(1-33)	0.021*

***Significant (P<0.05)**

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Discussion:

In this study the real time PCR results revealed that the percentage of HCMV DNA positive of chronic periodontitis patients was 22.6 % while the percentage of HCMV DNA negative was 77.4 %. Saygun *et al* [15] detected HCMV DNA in 65% of chronic periodontitis patients while Imbronito *et al* [11] detected HCMV DNA in 75% of chronic periodontitis patients.

In another study HCMV DNA was detected in saliva of one individual of chronic periodontitis patients (1.5%) at low copy number [16]. Another study done by Bharati *et a* [17] detected HCMV DNA in 41.76 % of chronic periodontitis patients. The reasons for variation in HCMV DNA occurrence among studies might be due to the difference in the methodology and the type of PCR used.

In this study the type of PCR is real time which is a reliable and sensitive technique allowing quantification of the viral load and is being extensively applied for monitoring viral infection [9].

In most of above studies the type of PCR used was nested PCR which is susceptible to contamination and may give rise to false positive results [18] and also this difference might be due to dissimilar periodontitis disease states studied, and true geographic variation in HCMV prevalence, or may be due to diagnostic difficulties and a natural fluctuation of periodontal CMV [19].

The present study showed that there was no difference between males and females regarding the occurrence of HCMV DNA. These results disagree with Bilder *et al* [20], who found that HCMV more frequent in females than in males. Tantivanich *et al* [21], found that females had higher rate of HCMV infection than males. In this study there was no association found between the age of chronic periodontitis patients and the presence of HCMV DNA. These results agree with Tantivanich *et al* [21].

The present study showed that there was no relation between PLI and GI with the presence of HCMV DNA while Saygan *et al* [15] found close relation between PLI and GI with HCMV infection. Ling *et al* [22] found that there was no significant association between PLI and GI with the prevalence of HCMV.

In this study there was no relation found between HCMV DNA and BOP and PPD, these results agree with Ling *et al.*,[22] who found that there was no significant association between the prevalence of HCMV infection and BOP and disagree with Wu *et al* [23] who found that infection with HCMV correlated with BOP. Concerning PPD the results of this study was in agreement with a study done by Saygun *et al* [15] who found that there was no correlation between HCMV DNA counts and PPD. Wu *et al* [23] found that there was association between PPD and HCMV infections. Shivali *et al* [24] found that there was no correlation between HCMV and PPD.

The present study showed that there was a significant relation between CAL scale 2 and 3 and HCMV DNA, this agree with Ling *et al* [22] and disagree with Shivali *et al* [24] who was found that there was no correlation between CAL and HCMV DNA. Chronic periodontitis is a chronic inflammatory condition with multifactorial etiologies, the disease entities have been found to harbor the HCMV.

The HCMV is the most immunodominant antigen which is encountered by the immune system. The virus remains latent in an infected host and its reactivation occurs by immune activation. [24]. Reactivation of virus in periodontal sites comprises an important pathogenic event in the development of periodontitis [25]. Contreras *et al* have detected HCMV in monocytes/macrophages and T lymphocytes [26]. Infected immune cells may not mount a proper immune response against periodontopathogenic bacteria predisposing to

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microbial infection thus increase the risk for destructive periodontal disease [26].

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