Quantitative detection and correlation of *Epstein - Barr Virus* in plasma with gingivitis and severity of chronic periodontitis by using real-time polymerase chain reaction technique

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ABSTRACT

Background: This study aimed to detect *EBV* quantitatively in plasma using real-time polymerase chain reaction technique in chronic periodontitis and gingivitis patients and to compare the finding with control subjects (healthy periodontium) and to investigate the relationship between the presence of *EBV* & the severity of periodontal diseases using the clinical periodontal parameters (PLI ,GI , BOP ,PPD and CAL) between each of (chronic periodontitis and gingivitis) patients and compare with control (healthy periodontium) subjects .

Materials and methods: The study sample consisted of (101) individual of both genders, (61) chronic periodontitis patients which were subdivided into (mild, moderate & severe) depending on the scores of clinical attachment level, (20) gingivitis patients and (20) control subjects (healthy periodontium) with age ranged from (30-50) years. All the groups were without any history of systemic diseases. Clinical periodontal parameters used in this study were (PLI ,GI , BOP ,PPD and CAL) .Blood samples were collected from all individuals and examined by Real-Time PCR technique for the detection of *EBV*.

Results: The result of comparison for the occurrence of *EBV* among study and control groups according to sequential responding of *EBV* appears to be highly significant at negative level of *EBV*, significant in (100 - 500 copy/10^5 cells) level and the results of leftover levels appear non significant difference. The result of correlation between the actual occurrence of *EBV* and PPD scores in severe chronic periodontitis subgroup appears to be significant at PPD score (1) and non significant at scores (2&3). The correlations between EBV and PPD scores in moderate and mild chronic periodontitis subgroups appear to be non significant with all scores. The results of correlation between *EBV* and CAL parameter appear to be non significant among all scores of chronic periodontitis subgroup. Concerning plaque index, the correlation appears to be significant in mild chronic periodonttis subgroup and highly significant in control group. In case of gingival index, the correlation appears to be significant in severe chronic periodontitis subgroup and control group .The result of correlation with (B.O.P. score 1) appears to be highly significant in severe subgroup of chronic periodontitis group and significant in gingivitis group, while in case of (B.O.P. score 0), the correlation appears to be significant only in severe chronic periodonttis subgroup.

Conclusions: The present findings revealed that there may be an association between *EBV* infection and the severity of periodontal diseases and thus coinfection with *EBV* may play a role in increase destruction of periodontal tissues.

Keyword: Chronic periodontitis, EBV, Real-Time PCR. (J Bagh Coll Dentistry 2014; 26(4):133-140).

INTRODUCTION

Periodontal diseases (PD) are group of inflammatory diseases caused by pathogenic microflora organized in biofilms surrounding the teeth [(1)].

The causes of infection may include bacterial plaque and herpes virus [(2,3)] . There are two major types of periodontal disease: gingivitis and periodontitis.

Gingivitis involves a limited inflammation of the unattached gingiva and is a relatively common and reversible condition. In contrast, periodontitis is characterized by general inflammation of the periodontal tissues, which leads to the apical migration of the junctional epithelium along the root surface and progressive destruction of the periodontal ligament and the alveolar bone [(4)].

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Bacterial infection alone may not explain the conversion of gingivitis to periodontitis[(5)] ,rapid tissue destruction around teeth exhibiting little plaque, the propensity of periodontitis to proceed with periods of exacerbation, remission and the tendency of periodontal tissue breakdown to advance in a localized and bilaterally symmetrical pattern [(6)] .

However, it has been difficult to unravel the precise role of various putative pathogens and host responses in the pathogenesis of periodontitis.

Recent finding have showed herpes viruses especially *Epstein-Barr virus (EBV)* can infect or alter structural cells and host defense cells of the periodontium [(7)]**.** It's obvious that others factors beyond biofilm are important in the pathogenesis of periodontitis like tobacco smoking and genetically determined variations in inflammatory response patterns and recently, it was suggested that certain viruses might also influence the development and severity of periodontal diseases, though the cause of gingivitis and periodontitis is

credited to bacteria colonizing tooth surfaces and initiating the major mechanisms of periodontal destruction [8].

Viruses can also interfere on immune responses though immune modulators encoded within viral genomes, which include proteins that regulate antigen presentation, function as cytokines or cytokine antagonists, inhibit apoptosis and interrupt the complement cascade [9].

Viral infections may facilitate the destruction of periodontal tissue by lytic activity against periodontal cells, immune mediated tissue destruction and immune suppression, which increase the susceptibility of the host to bacterial attacks [10,11] .Thus, a situation of viral-bacterial interaction could occur in the oral cavity without a denial of the argument for a major etiological role of bacteria in human periodontal disease.

Human Cytomegalovirus (HCMV) and EBV-1 assume a particularly close relationship with human periodontitis while *herpes simplex virus (HSV) , human herpesvirus-6 (HHV-6) and Epstein-Barr virus type-2* seem to exhibit little or no association with most types of periodontitis disease [12] .

Various systems for the detection of oral pathogens have been reported, but most are qualitative [13,14].

Because periodontal pathogens exist not only in infected pockets but also in the healthy sulcus, qualitative detection is not suitable for the diagnosis of periodontitis.

For this purpose, the present study used a quantitative detection system that uses real-time polymerase chain reaction (real-time PCR) methodology.

MATERIALS AND METHODS

Human Sample

The sample in this study consisted of one-hundred & one subjects with an age ranged from (30-50) years, males (73) & females (28), sample collection was started at 16th February 2013 till 20 March 2013. Patients participating in the present study with chronic periodontitis (no=61), which were subdivided according to the severity of disease into (mild, moderate & severe) depending on the scores of clinical attachment level, gingivitis patients (No.=20) with limited inflammation of the gingiva , no probing pocket depth & no clinical attachment loss and control subjects (healthy periodontium) (No.=20) where there was no signs of periodontal disease with clinically healthy gingiva, no pockets, no bleeding on probing and no evidence of bone loss. The sample collected from patients recruited from the Clinic of the Department of Periodontics/ College of Dentistry/ Baghdad University.

Clinical examination

Periodontal examination consisted of plaque index (PLI) [15] ,gingival index (GI) [16], bleeding on probing (BOP) [17], probing pocket depth (PPD) and finally clinical attachment level (CAL) [18] measured at 4 sites for all teeth except 3rd molar on (mesial, vestibular, distal, lingual) using a calibrated periodontal probe (Michigan O probe). Patients with chronic periodontitis had periodontal pockets equal or greater than 3mm with clinical attachment loss [19]. All subjects participated in this study without any systemic diseases, had not received previous periodontal treatment and had not used antibiotics in the past 3 months.

Patients were excluded if pregnant, smokers, menapaused women, female under contraceptive pills and patients with history of herpetic infection (self-reported) during the last six months.

In the present study the laboratory results will be divided into 5 levels according to the quantity of *EBV* with cutoff point will be (100-500 copy/10^5 cell) level, these levels are:

1. Negative level (score-1).
2. % <100 copy/10^5 cells level (score-2).
3. 100-500 copy/10^5 cells level (cutoff point) (score-3).
4. 600-1000 copy/10^5 cells level (score-4).
5. % >1000 copy/10^5 cells level (score-5).

Collection of Blood Sample and Plasma Preparation

The blood was collected from all participants between (9 am-12 pm), the blood samples were taking from their arms from cubital Fossa (cubital vien) , 5cc of venous blood taken from patients & control healthy individual and put it in [Ethylen Diamine Tetra Acetic acid (EDTA)] tubes as anticoagulant tubes used to preserve collected blood samples and then preserved in a cool box containing ice packs until transferring the samples to the laboratory.

Plasma separated from blood samples using centrifuge at speed (1600 R/min) for 20 min according to DNA extraction kit manuals, then plasma samples preserved immediately into other plain tubes and stored in freeze at (- 15 C°) until they were assayed. This was done within 4 hours after sample collection.

DNA Extraction

The kit used is DNA column extraction kit {Sacace-ITALY (Lot number 1306/001)}, for extraction of DNA using column method and

samples preserved in freeze thawed in room temperature for extraction.

Eppendorf tubes used in first step of extraction prepared & numbered according to the samples and then Ribovirus columns used.

After a long series of adding washing solutions, centrifuge and heating, we can get the pure DNA captured at the bottom of each eppendorf tube.

Real-Time PCR amplification

The kit used is *EBV*- Real time PCR kit from {Sacace biotechnology (ITALY) (Lot number 23K13K705)}, which contains (Polymerase, mastermix, controls for quantitation, Internal Control). Smart cycler PCR tubes used that numbered according to the samples, then 12.5 µl of extracted DNA and PCR mix (which contain Taq. Polymerase enzyme) added to each tube and each tube closed well & centrifuged in minispin centrifuge at (8000 R/min) for 20 seconds ,then tubes put in Real-Time PCR for amplification , we notice that Internal control DNA appears in FAM fluorescent signal on PCR ,while DNA samples appear in CY3 fluorescent signal channel on PCR. Quantitation based on formulation of the kit and the results appear as curves of different colors on the monitor of real-time PCR system and the quantitation depends on standard curve.

RESULTS

The goodness-of-fit test ,which tests the mean value of the studied parameters {PLI & GI, BOP (Score 1) and the Sequential Responding}, which was used to determine the normal and non normal distribution of the data , this test reflects that the results of (mean and standard deviation) for the study group was higher than that of control group as seen in table (1).

The descriptive statistics of the clinical periodontal parameters reflect that the results of the study group were higher than that of the control group.

According to sequential responding parameter, we notice that the mean values of positive records increase with increase severity of periodontal diseases & *EBV* levels at different groups and subgroups recorded, the higher mean was at severe subgroup of chronic periodontitis (3.77), while the least appears at control group (1.55) as seen in table (2).

By using (LSD test) ,this test revealed highly significant differences between severe subgroup & other groups and subgroups of the study according to sequential responding and significant difference between moderate subgroup of chronic periodontitis group & control group, while the leftover revealed non significant differences as seen in table (3).

The result of comparison between the groups of the study & the levels of *EBV* appears to be highly significant at the negative level of *EBV*, non significant positive difference at the (%<100 copies/10^5 cells), significant positive difference at (100-500 copies/10^5 cells), non significant positive difference at the (600-1000 copies/10^5 cells) & non significant positive difference at the (% >1000 copies/10^5 cells). The result of comparison between the 3 groups of the study and *EBV* at the cutoff point (100-500 copies/10^5 cells) level appears to be statistically significant as seen in table (4).

Spearman's Correlation Coefficient. Test, that is used for testing the correlation between different periodontitis subgroups & the clinical periodontal parameters {plaque index, gingival index & BOP (score 0& 1)} according to sequential responding of *EBV*. In case of plaque index, a negative significant correlation appears in mild chronic periodontitis subgroup, while it appears non significant in case of (moderate and severe) chronic periodontitis subgroups. A negative significant correlation appears in case of gingival index in severe chronic periodontitis subgroup, while it is non-significant in (mild and moderate) chronic periodontitis subgroups. In case of BOP score 1, the correlation appears to be highly significant and negative in severe subgroup of chronic periodontitis group and non significant in (mild and moderate) subgroups. The correlation of BOP score 0 appears to be significant & positive in severe chronic periodontitis subgroup , while it appears non significant in case of (mild and moderate) subgroups of chronic periodontitis group as seen in table (5) .

The correlation of *EBV* & { PLI, GI, and BOP (score 0&1)} in gingivitis group, which appears non significant, while the correlation of EBV & BOP score (1) appears to be significant and negative as seen in table (6).

The correlation of *EBV* & clinical periodontal parameters{ PLI, GI, and BOP (score 0&1)} in control group appears to be highly significant and negative (strong correlation) with plaque index, negative significant correlation with gingival index , while it appears non significant in case of BOP score (0 &1) as seen in table (7).

The correlation of *EBV* & PPD scores in severe periodontitis subgroup appears to be significant and negative at PPD score (1), while it appears a non significant correlation at PPD score (2&3). The correlation of *EBV* & all CAL scores

appears to be non-significant and negative as seen in table (8).

The correlation of *EBV* and (PPD & CAL) scores in moderate periodontitis subgroup appears to be non significant with all scores of (PPD & CAL) parameters as seen in table (9).

The correlation of *EBV* and (PPD & CAL) scores in mild periodontitis subgroup appears to be non significant with all scores of (PPD&CAL) parameters as seen in table (10).

Table 1: Goodness of Fit test of normal distribution function for the studied parameters (PLI, GI, BOP score 0&1 and sequential responding) in study and control groups

Groups	Statistical Information		Plaque Index	Gingival Index	B.O.P Score 1	Sequential Responding
Study	No.	Parameters				
	81	Mean	1.506	1.452	61.69	2.280
		± SD	0.538	0.452	34.23	1.280
Control	No.	Parameters				
	20	Mean	0.110	0.147	4.800	1.550
		± SD	0.057	0.164	2.840	0.690

Table 2: Summary Statistics of (Sequential Responding) Parameter at the different groups and subgroups

Parameter	Groups and subgroups	No.	Mean	±Std. Dev.	Std. Error
Sequential Responding	Chronic periodontitis - Severe	13	3.77	0.73	0.2
	Chronic periodontitis - Moderate	23	2.26	1.01	0.21
	Chronic periodontitis - Mild	25	2.00	1.22	0.24
	Gingivitis	20	1.70	1.22	0.27
	Control	20	1.55	0.69	0.15

Table 3: Multiple Comparisons (LSD) between all pairs of different groups and subgroups according to sequential responding parameter:

Dependent Variable	Study Groups & subgroups		Mean Difference	Sig.	C.S.
Sequential Responding	Chronic periodontitis – Severe	Chronic periodontitis –Mod.	1.51	0.001**	HS
		Chronic periodontitis - Mild	1.77	0.001**	HS
		Gingivitis	2.07	0.001**	HS
		Control	2.22	0.001**	HS
	Chronic periodontitis - Moderate	Chronic periodontitis - Mild	0.26	0.383	NS
		Gingivitis	0.56	0.078	NS
		Control	0.71	0.026*	S
	Chronic periodontitis - Mild	Gingivitis	0.30	0.334	NS
		Control	0.45	0.149	NS
	Gingivitis	Control	0.15	0.646	NS

(**) HS: Highly Sig. at P< 0.01; (*) S: Sig. at P<0.05; NS: Non Sig. at P>0.05

Table 4: Distribution of the absent & present responding at each group according to different levels of EBV with Contingency Coefficients

Levels	Resp. (+& -)	No. & Percents	Groups			C.S. P-value
			Chronic Periodontitis	Gingivitis	Control	
Negative (score -1)	0	No.	45	7	9	C.C.=0.325 P=0.003 (**) HS
		%Negative	73.8%	11.5%	14.8%	
		%Groups	73.8%	35%	45%	
	1	No.	16	13	11	
		%Negative	40%	32.5%	27.5%	
		%Groups	26.2%	65%	55%	
<100 copy/10^5 cells (score -2)	0	No.	43	16	13	C.C.=0.106 P=0.564 NS
		%< 100 copy/10^5 cells	59.7%	22.2%	18.1%	
		%Groups	70.5%	80%	65%	
	1	No.	18	4	7	
		%< 100 copy/10^5 cells	62.1%	13.8%	24.1%	
		%Groups	29.5%	20.0%	35.0%	
100 - 500 copy/10^5 cells (score - 3)	0	No.	47	20	18	C.C.=0.247 P=0.037 (*) S
		%100 - 500 copy/10^5 cells	55.30 %	23.50%	21.20%	
		%Groups	77.00 %	100.00 %	90.00%	
	1	No.	14	0	2	
		%100 - 500 copy/10^5 cells	87.50 %	0.0%	12.50%	
		%Groups	23.00 %	0.0%	10.00%	
600 -1000 copy/10^5 cells (score - 4)	0	No.	53	18	20	C.C.=0.167 P=0.234 NS
		%500 -1000 copy/10^5 cells	58.20 %	19.80%	22.00%	
		%Groups	86.90 %	90.00%	100.00 %	
	1	No.	8	2	0	
		%500 -1000 copy/10^5 cells	80.00 %	20.00%	0.0%	
		%Groups	13.10 %	10.00%	0.0%	
>1000 copy/10^5 cells (score - 5)	0	No.	56	19	20	C.C.=0.134 P=0.306 NS
		%> 1000 copy/10^5 cells	58.90 %	20.00%	21.10%	
		%Groups	91.80 %	95.00%	100.00 %	
	1	No.	5	1	0	
		%> 1000 copy/10^5 cells	83.30 %	16.70%	0.0%	
		%Groups	8.20%	5.00%	0.0%	

(**) HS: Highly Sign. at P<0.01; (*) S: Sign. at P<0.05 ; NS: Non Sign. at P>0.05
C.C.: Contingency Coefficient

Table 5: Spearman's Correlation Coeff. For testing the correlation between sequential responding of EBV and (PLI, GI, BOP score 0 & BOP score 1) parameters at each chronic periodontitis subgroups:

Clinical Parameters	Spearman's Corr. Coeff.	Chronic Periodontitis - Severe	Chronic Periodontitis - Moderate	Chronic Periodontitis - Mild
Plaque Index	Corr. Coeff.	-0.217	-0.324	-0.387*
	P-value	0.477	0.132	0.05
	No.	13	23	25
Gingival Index	Corr. Coeff.	-0.608*	-0.256	-0.119
	P-value	0.028	0.239	0.572
	No.	13	23	25
B.O.P Score 1	Corr. Coeff.	-0.822**	-0.253	-0.179
	P-value	0.001	0.244	0.392
	No.	13	23	25
B.O.P Score 0	Corr. Coeff.	0.583*	0.26	0.179
	P-value	0.036	0.231	0.392
	No.	13	23	25

(**) HS: Highly Sign. at P<0.01; (*) S: Sign. at P<0.05 ; NS: Non Sign. at P>0.05

Table 6: Correlation Coefficients with their testing of null hypotheses between the actual of virus readings and the studied parameters (PLI, GI, BOP score 0 & BOP score 1) in Gingivitis group

Group	Corr. and P-value	Plaque Index	Gingival Index	B.O.P Score 1	B.O.P Score 0
Gingivitis	Correlation	-0.333	-0.293	-0.383	0.373
	P-value	0.076	0.105	0.048	0.053
	C.S.	NS	NS	S	NS

Table 7: Correlation Coefficients with their testing of null hypotheses between the actual of virus readings and the studied parameters (PLI, GI, BOP score 0 & BOP score 1) in Control group

Group	Corr. and P-value	Plaque Index	Gingival Index	B.O.P Score 1	B.O.P Score 0
Control	Correlation	-0.537	-0.476	-0.274	0.274
	P-value	0.007	0.017	0.121	0.121
	C.S.	HS	S	NS	NS

Table 8: Correlation Coefficients between the actual virus readings and the studied parameters (PPD and CAL) scores in chronic periodontitis – Severe subgroup:

Group	Corr. and P-value	PPD Score-1	PPD Score-2	PPD Score-3	CAL Score-1	CAL Score-2	CAL Score-3
Chronic Periodontitis –Severe subgroup	Correlation	-0.496	-0.175	0.214	-0.362	-0.263	-0.018
	P-value	0.043	0.284	0.241	0.112	0.193	0.477
	C.S.	S	NS	NS	NS	NS	NS

Table 9: Correlation Coefficients between the actual virus readings and the studied parameters in Ch. Periodontitis – Moderate subgroup:

Group	Corr. and P-value	PPD Score-1	PPD Score-2	PPD Score-3	CAL Score-1	CAL Score-2	CAL Score-3
Chronic Periodontitis – Moderate subgroup	Correlation	-0.048	0.312	-0.105	0.102	0.03	0.303
	P-value	0.414	0.074	0.317	0.321	0.445	0.08
	C.S.	NS	NS	NS	NS	NS	NS

Table 10: Correlation Coefficients between the actual virus readings and the studied parameters in Ch. Periodontitis – Mild subgroup:

Group	Corr. and P-value	PPD Score-1	PPD Score-2	PPD Score-3	CAL Score-1	CAL Score-2	CAL Score-3
Chronic Periodontitis –Mild subgroup	Correlation	0.161	-0.150	0.049	0.052	-0.143	0.036
	P-value	0.221	0.237	0.408	0.402	0.248	0.432
	C.S.	NS	NS	NS	NS	NS	NS

DISCUSSION

In this study a significant difference appears when comparison was made between 3 groups of the study and the percentage of *EBV* at the cutoff point of sequential responding parameter. This finding agree with Wu et al [20], where the higher level of *EBV* among study group compare to control group represent the role of these pathogens in destructive periodontal lesion where the virus effect on immune response of such patients. The pathogenic mechanisms of herpes viruses cooperate in exacerbating disease and probably for that reason, a periodontal dual infection with (*HCMV* and *EBV*) tends to occur in severe types of periodontitis [21].

A non significant negative correlation appears when correlation was made between the quantity of *EBV* and plaque index in case of {chronic periodontitis (severe & moderate) subgroups and in gingivitis group}, while there is a significant negative correlation in case of chronic periodontitis (mild subgroup) and a highly significant strong negative correlation appears in healthy control group. These findings appear to be disagree with Saygun et al [22] where the difference in the measurements of plaque index in viral detected & undetected sites were statistically significant .

When the correlation was made between the presence of *EBV* and gingival index, it's found that there is a non significant negative correlation in all groups and subgroups except in chronic periodontitis (severe subgroup) and healthy control group which showed a statistical significant negative correlation with the quantity of *EBV*. These findings are agree with the study done by Charu [23] .The reasons of variation in *EBV* occurrence among studies may include differing *EBV* detection technique, dissimilar periodontal disease states studies and true geographic variation in *EBV* prevalence.

A significant negative correlation was found between the presence of *EBV* and bleeding upon probing for (score 1) in chronic periodontitis-severe subgroup and gingivitis group & a non significant correlation was found in case of BOP score (0) with different groups and subgroups except chronic periodontitis-severe subgroup, which showed a significant strong positive correlation. These findings are disagree with Maryam et al [24] , who found that there were no statistically significant differences in mean of BOP among positive and negative patients for *EBV-1*, *EBV-2* and *HCMV* ,where a higher mean of BOP among patients who were infected with *EBV-1* and *HCMV* did not reach to the level of statistical significance. The coinfection with *EBV* revealed bleeding upon probing, a clinical sign of elevated risk for disease progression [21].

The result of correlations between the three scores of periodontal pocket depth of chronic periodontitis subgroups & *EBV* quantity showed a significant correlation in case of severe chronic periodontitis subgroup at score (1), while there were non significant differences at scores (2&3), also non significant correlations were found in case of (moderate & mild) chronic periodontitis subgroups including all scores. These findings are disagree with Ling [25], who found that the prevalence of virus in chronic periodontitis is very low (4%). These differences between studies could be due to differing in *EBV* detection technique, dissimilar in estimation of (PPD), but the present finding agrees with Saygun et al and Moghim et al [26,27]. Saygun et al observed that periodontal pocket depth was positively correlated with salivary *EBV-DNA* counts. The results agree with Wu et al [28] who found that a higher percentage of *EBV* associated with a deeper probing pocket depth. The prevalence of *EBV-1* found in PD $\geq$ 6 mm was statistically greater than that in PPD $\leq$ 3 mm [24].

A study done by Kubar et al [6] showed that a statistical significance difference was found between *EBV* subgingival counts and periodontal pocket depth at sample sites (Spearman's correlation), where chronic periodontitis patients revealed more than 10,000 copies of *(HCMV or EBV)* in subgingival or gingival tissue samples (Chi-square test. The interaction between *EBV* and *P. gingivalis* is bi-directional with P. gingivalis having the potential to induce *EBV* reactivation and *EBV* reactivation suppressing host defenses and permitting overgrowth of P. gingivalis which having the potential to induce periodontal tissue destruction [29].

A non significant correlation was found between the three scores of clinical attachment level (CAL) of chronic periodontitis subgroups with the actual virus readings of *EBV*, this result disagree with Wu et al [28], which found that a more serious attachment loss associated with the presence of *EBV*. A periodontal dual infection of *EBV* and pathogenic bacteria gives rise to enhanced cytokine release and immune signaling dysregulation and tends to be associated with more severe periodontitis than a periodontal infection involving solely bacteria [30-32].

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