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RESEARCH ARTICLE

Detection of Human Herpesviruses in Patients with Chronic Periodontitis in Mosul- Iraq

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Abstract

The aim of the study is to detect human herpesviruses in patients with chronic periodontitis in Mosul, with some immunological and biochemical parameters among patients. The results showed that high percentage of chronic periodontitis was detected between 20-39 years old. Nested PCR assay showed detection of EBV in 44.3%, HSV 26.4% and CMV in 1.9% as a single causative virus in chronic periodontitis. Presence of EBV and HSV in patients pocket showed significance association with chronic periodontitis. This study showed clear association between serum IL-1β, IFN-ɣ, salivary sIgA and total proteins level with the presence of EBV-CMV co infection in periodontitis patients.

INTRODUCTION

Periodontitis is a group of illnesses located in the gingiva and other dental supporting tissues.[(1)] Traditionally, periodontitis has been described as a bacterial infection caused by specific pathogenic species,[(2)]which initiate microbial plaque, that were accumulated on the tooth surface at the gingival margin and in periodontal pockets.[(3)] Moreover, chronic inflammation in gingiva and connective tissues may eventually result in tooth loss.[(1)] Periodontitis is most often seen in maturity, with the majority of adults experiencing some signs and symptoms by the mid-30s.[(4)]

Since the mid1990s, herpesviruses have emerged as putative pathogens in various types of periodontal disease.[(5)] Individual with periodontal lesions may harbor millions of genomic copies of herpesviruses, papillomaviruses, human immunodeficiency virus (HIV), human T-lymphotropic virus type 1, torquetenovirus, hepatitis B and C viruses.[(6)] Recently different types of viruses were discovered in periodontal infections.[(7)] Thus, reactivation of these viruses in periodontal tissue may result from transient immunosuppression.[(8,9)] Therefore, the evolution of periodontal disease depends upon the periodontopathic properties, local host immune responses, oral cavity environmental changes and nutrition.[(10-12)]

MATERIALS AND METHODS

Subject Groups

This study was carried out on total number of 109 subjects, 91 patients with chronic periodontitis with age ranged between ≤15 - 73 years old, 57 males aged 18 to 73 years, mean age, 37.33 years and 34 females aged 15 to 63 years, mean age, 32.14 years. Those were referred to Periodontal Clinic Unit in the College of Dentistry/Mosul University from January 2011 to April 2012.

Control group consisted of 18 clinically healthy subjects with age ranged between 23-35 years old, 15 males aged 23 to 35 years mean age, 26 years and 3 females aged 23 to 30 years, mean age 25 years (students, officials and dentists) working in Mosul University. This group had no signs of systemic disease, gingivitis or any type of periodontitis.

All patients and the healthy individuals underwent a full-mouth examination and selected by a specialist. Patients with chronic periodontitis status have >3-≥7mm pockets depth according to the classification of the periodontal diseases issued by the American Academy of Periodontology in 1999.[13]

Materials (kits) Used for Serological Study

1. CA EBV IgG ELISA kit, Euro Immun ,(Germany).
2. CA EBV IgM ELISA kit, Euro Immun ,(Germany).
3. HSV IgG ELISA kit,VEDA.LAB,(France).
4. HSV IgM ELISA kit,VEDA.LAB,(France).
5. CMV IgG ELISA kit,VEDA.LAB,(France).
6. CMV IgM ELISA kit,VEDA.LAB,(France).

Kits Used For Periodontal Viral Detection

1. Innu PREP Virus DNA extraction kit, REF. 845-KS-4600050, Analytikgena, (Germany).
2. Cytomegalovirus Detection kit, by FAST PCR of IEA region. REF: CL12K (CMV) . CLONIT, Milano, (Italy).
3. Epstein-Barr virus Detection kit, by FAST PCR of Bam HI-W region. REF: CL13K (EBV). CLONIT, Milano, (Italy).
4. Herpes Simplex virus Detection kit by FAST PCR of the gDregion.REF: CL18K (HSV 1 and 2). CLONIT, Milano, (Italy).
5. Electrophoresis hydro gel 3300EB PCR CheckIT Mini with EtBr, Elchrom scientific AG. (Switzerland).

Samples collection:

Prior to sampling, supragingival plaque was removed with sterile cotton pellets and the sample sites were isolated with cotton roles,[14] and a sterile paper point was inserted to the bottom of the selected pocket (more than 3mm) and retained for 30 seconds.[15] Paper point then transferred to sterile Eppendorff tubes containing 0.05 ml of TE buffer (10 mMTris-HCl and 1 mM EDTA, pH 7.5) then DNA extraction was proceeded.

Serum collecting

Five ml blood collected from patients and control groups and serum was separated by centrifugation at 2500 rpm for 10 min, and dispensed in a sterile plastic Eppendorff tubes and stored at -20˚C until the time of analysis.[16] Serological tests for detection of antibody to different viruses

The test was semi quantitative ELISA test for detection of specific IgM or IgG. The test was carried out according to manufacturer instructions.

Nucleic acid extraction

DNA was extracted from the clinical samples using InnuPREP Virus DNA kit. Kit procedure depends upon binding of viral DNA using spin filter membrane. DNA extracts were kept at -20°C prior to detection of CMV, EBV and HSV by nested PCR technique.

Nested PCR technique

Cytomegalovirus Detection by FAST PCR of IEA region

This system is a qualitative test that allows the DNA amplification, by means of FAST PCR, of IEA region of CMV genome, was preceded according to following program .

1-1st FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
25	95 ° C 1 sec	65° C 10 sec
1		72° C 10 sec

2-NESTED FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
30	95 ° C 1 sec	70° C 10 sec
1		72° C 10 sec

Epstein-Barr Virus Detection using FAST PCR of Bam HI-W region

The EBV system is a qualitative test that allows the DNA amplification, by means of FAST PCR, of Bam HI-W region of EBV genome which proceeded according to following program:

1-1st FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
20	95 ° C 1 sec	60° C 10 sec
1		72° C 10 sec

2- NESTED FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
30	95 ° C 1 sec	60° C 10 sec
1		72° C 10 sec

Herpes Simplex Virus (HSV 1-2) Detection using FAST PCR of the gD region

The HSV 1 and 2 system is a qualitative test that allows the DNA amplification, by means of FAST PCR, of gD region of HSV 1 and 2 genome.

1- 1st FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
20	95 ° C 1 sec	55° C 10 sec
1		72° C 10 sec

2- NESTED FAST PCR program:

Cycles	denaturation	annealing/extension
1	95° C 1 min	
30	95 ° C 1 sec	55° C 10 sec
1		72° C 10 sec

Electrophoresis was run at 120 Volts for 15-30 minutes and bands were visualized on transilluminator.

Statistical Analysis

T-Test, Pearson Chi-Square Test , Pos Hoc Test, ANOVA Test, Duncan Test, Correlations, Mean , Std. Deviation , Std. Error .

RESULTS

Chronic Periodontitis and Age Groups

The distribution of the chronic periodontitis patients according to age showed high percentage of chronic periodontitis mainly between 20-39 years old, representing about 58 (52.73%) of the total number of patients, and the lowest percentage was at younger age groups (≤19 years) (Figure 1).

Detection of EBV , CMV and HSV in periodontitis patients Using Nested PCR Technique .

In this study, nested PCR showed detection of EBV in 23(44.3%) patients ,CMV in 1(1.9%) and HSV in 14(26.4%) chronic periodontitis patients pockets(Figures2,3,4), (Table1). These detections were distributions in 34 (37.4%) males and18 (19.8%) females (Figure 5).

Statistical analyses using Chi-Square, revealed significant association between presence of EBV and HSV in chronic periodontitis patients pocket, but there was no significant association with the presence of CMV (Tables 2,3,4) .

Correlation between Nested PCR and ELISA Technique in Virus Detection

The comparison between detection of EBV using nested PCR technique, anti- EBV- CA ELISA IgG and anti- EBV- CA ELISA IgM in periodontitis patients and control groups showed detection of EBV virus in 35 (38.46%) of 91 periodontitis patients using nested PCR technique ,while the investigation of serum IgG anti- EBV using anti- EBV- CA ELISA IgG Kit showed positive reaction with serum samples in 49 (53.84%) and 12 (66.66%) of periodontitis patients and control groups respectively (Figure 6).

Moreover, the detection of CMV using nested PCR technique showed the presence of virus in 7 of 91 of periodontitis patients pocket fluid samples with percentage 7.69%, also the detection of IgG antibody to CMV in human serum using CMV IgG ELISA kit showed positive reaction with serum samples of 78(85.71%) and 18(100%) periodontitis patients and control groups respectively (Figure 7).

In this study, HSV was detected in 25 of 91 periodontitis patients pocket fluid samples using nested PCR technique with percentage 27.47%. Also, the investigation of IgG antibody to HSV in human serum using HSV IgG ELISA Kit showed positive reaction with serum samples of 41(45.05%) and 6 (33.33%) periodontitis patients and control group respectively (Figure 8). Whereas detections of EBV, CMV and HSV IgM in human serum using anti-EBV- CA ELISA IgM , CMV IgM ELISA kit and HSV IgM ELISA kit showed negative reaction with serum of periodontitis patients and control group (Figures 6,7,8). In this study periodontitis patients group showed high percentage of serum IgG positive reaction to three viruses using ELISA assay compared with nested PCR technique (Figures 6,7,8).

Distributions of EBV, CMV and HSV According Periodontal Pocket Depth

In this study, the distributions of viruses according to periodontal pocket depth, showed detection of EBV in 11 (20.4), 6 (11.1), 5 (9.3) and 1 (1.9) patients within 4mm, 5mm, 6mm and 7mm pocket depth groups respectively. While CMV was detected only in 1 (1.9) patient at 4mm pocket depth group. Furthermore HSV was detected in 2 (3.8), 4 (7.4) and 8 (14.8) patients at >3mm, 4mm and 5mm, pocket depth groups respectively. EBV and CMV co infection was detected in 1(1.9) patient within each of >3mm , 5mm and 6mm pocket depth respectively . Also EBV, HSV as a co infection was detected in 3(5.6), 3(5.6), 1 (1.9) and 1 (1.9) patients within 4mm , 5mm, 6mm and 7mm pocket depth groups respectively . CMV and HSV co infection was detected in only in 2 (3.8) patients at 5mm pocket depth group. Finally EBV, CMV and HSV as a triple co infection were detected at 5mm pocket depth group.

According to statistical analysis using Pearson Chi-Square test, showed no statistical association (≤ 0.005) between pocket depth and the distribution of viruses (Table 5).

Relationship between viral detection and some of parameters levels in periodontitis patients.

Results in Figure (9,10) show clear association between IL-1β, IFN-ɣ, sIgA, total proteins level, and presence of EBV-CMV co infection in periodontitis patients.

DISCUSSION

The distribution of the chronic periodontitis patients according to age showed high percentage in 20-39 years old and the lowest percentage was at younger age groups. Age is an important periodontal disease risk factor, therefore older men have more oral health problems than younger men, also younger men have less oral hygiene than girls.[17, 18]

In this study, using Nested PCR technique showed detection of EBV,CMV and HSV in chronic periodontitis patients pockets. These detections were distributed in males more than females. Recently, several studies have noted that pocket area of periodontitis patients from different countries harbors millions of genomic copies of herpesviruses and other viruses.[19,20] However, Contreras and his colleagues suggested a possible role of human viruses in the pathogenesis and destructive of periodontal diseases.[21] Most of these studies agreed with the viral detection result of the presence study, but they vary in percentage of detection.[22, 23] The incompatibility of viral detection percentages could be attributed to the differences in viral strains and study population. The presence of viruses in periodontal pockets does not act as simple bystanders,[24] but they play a critical role as putative pathogens in periodontitis.[8,14] Usually human viral infection is followed by bacterial superinfections, this association between viruses and bacteria usually produces a greater pathogenic effect, and the viral–bacterial synergism could be applied in the oral cavity.[25,26]

This study revealed significant association between the presence of EBV and HSV in chronic periodontitis patient's pocket, but there was no significance association with the presence of CMV. Similar studies showed a significant association between periodontitis and presence of EBV, HSV in chronic periodontal pockets. but there was no significance association with presence of CMV.[27,28] Epstein-Barr virus (EBV), showed a particularly close relationship with human aggressive and chronic periodontal disease.[29,30] Furthermore periodontal human CMV and EBV are associated with major periodontopathic bacteria and with the severity of periodontitis,[31] via inhibition of the immune system response against bacterial challenge.[9, 32]

The comparison between detection of EBV, CMV and HSV using nested PCR technique and ELISA assay in periodontitis patients and control groups showed high percentage of serum IgG positive reaction to three viruses using ELISA assay compared with nested PCR technique. Whereas IgM ELISA kit to three viruses showed negative reaction with serum of periodontitis patients and control group. The increase in serum IgG positive reaction is dependent on the presence of specific serum IgG antibody as a result of prior infection, as IgG antibody stays in serum for long time after latency period with absence of shedding viruses. The presence of viruses in periodontal pocket samples represents primary infection or reactivation status of virus, thus the presence of virus with weak or absence of specific IgM antibody in serum of patients group may depend on infection location site and periodontitis inflammatory reaction which stimulates cellular immune system via releasing cytokines and other mediators.[33]From the other hand serum IgM titer depends on virus concentration. In the present study, high sensitivity nested PCR technique was used and showed positive result in 99.5% with ≤10 virus copies. Thus, reactivation and shedding of low viral number may control by cellular immunity without stimulation of antibody production.

In this study, the distributions of viruses according to periodontal pocket depth showed no statistical association between pocket depth and types of viruses detected. This result is incompatible with Das and his colleagues result who detected HSV in deep sites, followed by EBV, and HCMV.[27] Furthermore, Saygun and his colleagues suggested a strong relationship between the presence of HCMV, EBV, and HSV in crevicular samples of chronic periodontitis lesions with measurement of probing depth, clinical attachment loss, and the severity of the disease.[23] These incompatibility may be due to differences in viral strains and study population.

The present study showed clear association between presence of EBV-CMV co infection in periodontitis patients and IL-1β, IFN-ɣ, sIgA, total proteins level. These observations may support the synergism concept between EBV and CMV. The present results agree with Thomasini, Slots and their colleagues, who noted that EBV and CMV, were in close relationship with human aggressive and chronic periodontal disease.[29, 30] HCMV can infect and replicate in wide range of humans cells, such as the epithelium of salivary gland, fibroblasts, macrophages, microglial cells, and smooth muscle cells, with the exception of lymphocytes and polymorphonuclear leukocytes.[34] On the other hand EBV infects and replicates in the oral cavity, oropharyngeal epithelium and B lymphocytes and primary infection, EBV persists in a latent form in B cells.[35] While the presence of EBV and CMV together can lead to severe periodontal inflammation via inhibits some of functional ability of macrophages respond to periodontopathic bacterial challenge.[9,32] Therefore, it was suggested that the presence of EBV and CMV has a pathogenic role in the development of periodontal disease.[8, 36, 37] HSV is replicated in epithelial cells, and established lifelong latency in the trigeminal ganglion.[38] Therefore, we don't suspect of the presence of clear association between HSV and EBV or CMV because of target cells variation.

Table (1): **Distribution of chronic periodontitis patients into groups according to the type of viral detection in periodontal pocket**

	EBV	CMV	HSV	patients n (%)
Group 1 : EBV	+	-	-	23 (44.23 %)
Group 2 : CMV	-	+	-	1 (1.9 %)
Group 3 : HSV	-	-	+	14 (26.92 %)
Group 4: EBV, CMV	+	+	-	3 (5.76 %)
Group 5 : EBV, HSV	+	-	+	8 (15.38 %)
Group 6 : CMV, HSV	-	+	+	2 (3.8 %)
Group 7 : EBV, CMV, HSV	+	+	+	1 (1.9 %)
Total				52 (100 %)

Table (2): Presence of EBV in periodontal pocket using Pearson Chi-Square test

	EBV		p. value	Chi-Square test	Total patients
	-	+			
Viral infection n(%)	17(18.7%)	35(38.5%)	0.00	Significance	52 (57.1 %)
Other n (%)	39(42.9%)	0(0.0%)			39 (42.9%)
Total n (%)	56(61.5%)	35(38.5%)			91 (100 %)

Table (3): Presence of HSV in periodontal pocket using Pearson Chi-Square test

Table	HSV		p. value	Chi-Square test	Total patients
	-	+			
Viral infection n(%)	27(29.7%)	25(27.5%)	0.000	Significance	52 (57.1%)
Other n (%)	39(42.9%)	0(0.0%)			39 (42.9%)
Total n (%)	66(72.5%)	25(27.5%)			91(100 %)

Table (4): Presence of CMV in periodontal pocket using Pearson Chi-Square test

	CMV		p.value	Chi-Square test	Total patients
	-	+			
Viral infection n(%)	45(49.5%)	7(7.7%)	0.017	Non Significance	52(57.1 %)
Other n (%)	39(42.9%)	0(0.0%)			39 (42.9%)
Total n (%)	84(92.3%)	7(7.7%)			91(100 %)

Table (5): **Relationship between viral detection and periodontal pocket depth using Pearson Chi-Square test**

		Infection Group							P-value	Significance	Total n (%)
		EBV	CMV	HSV	EBV & CMV	EBV & HSV	CMV & HSV	EBV ,CMV & HSV			
Pocket Depth	>3mm	0(0.0)	0(0.0)	2(3.7)	1(1.9)	0(0.0)	0(0.0)	0(0.0)	0.269	non	3(5.7)
	4mm	11(20.4)	1(1.9)	4(7.4)	0(0.0)	3(5.6)	0(0.0)	0(0.0)			19(36.3)
	5mm	6(11.1)	0(0.0)	8(14.8)	1(1.9)	3(5.6)	2(3.8)	1(1.9)			21(40.3)
	6mm	5(9.3)	0(0.0)	0(0.0)	1(1.9)	1(1.9)	0(0.0)	0(0.0)			7(13.4)
	7mm	1(1.9)	0(0.0)	0(0.0)	0(0.0)	1(1.9)	0(0.0)	0(0.0)			2(3.8)
Total n (%)		23(44.2)	1(1.9)	14(26.9)	3(5.7)	8(15.3)	2(3.8)	1(1.9)			52(100)

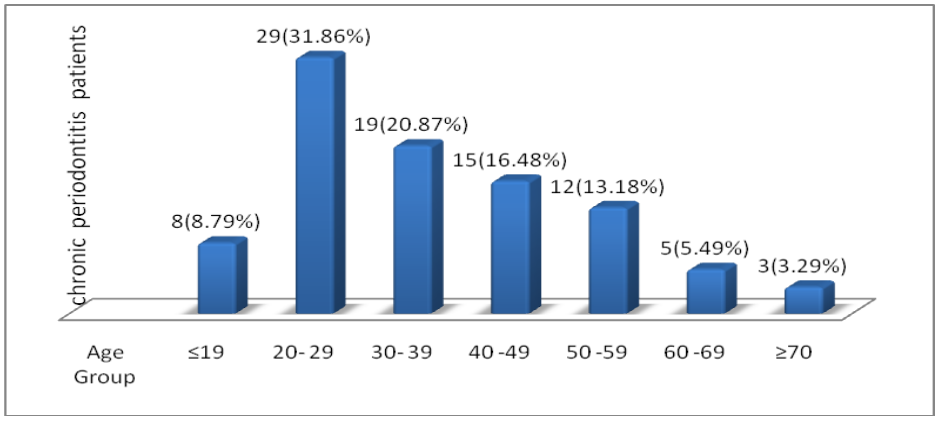

Figure (1): **Frequency and distribution of chronic periodontitis patients according to age groups**

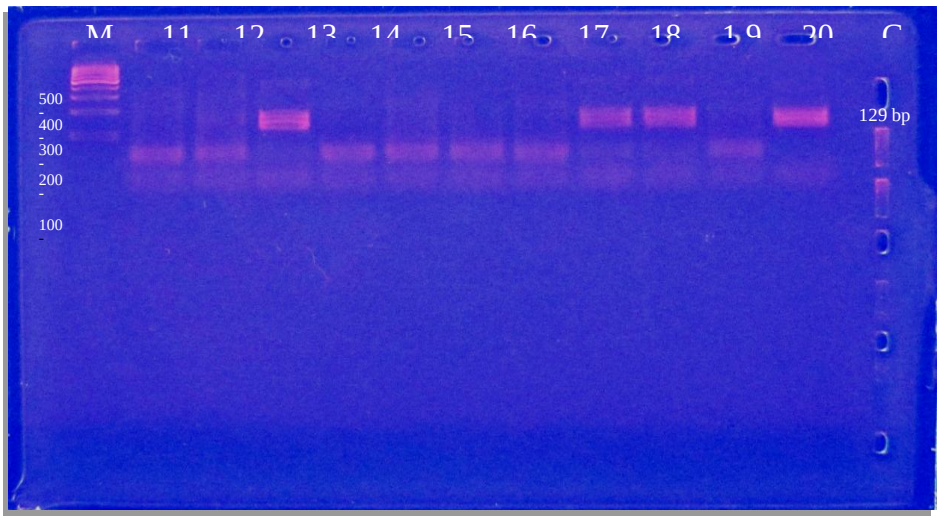

Figure (2): Electrophoreses hydro gel showing Detection of Epstein-Barr Virus using FAST PCR of Bam HI-W region.

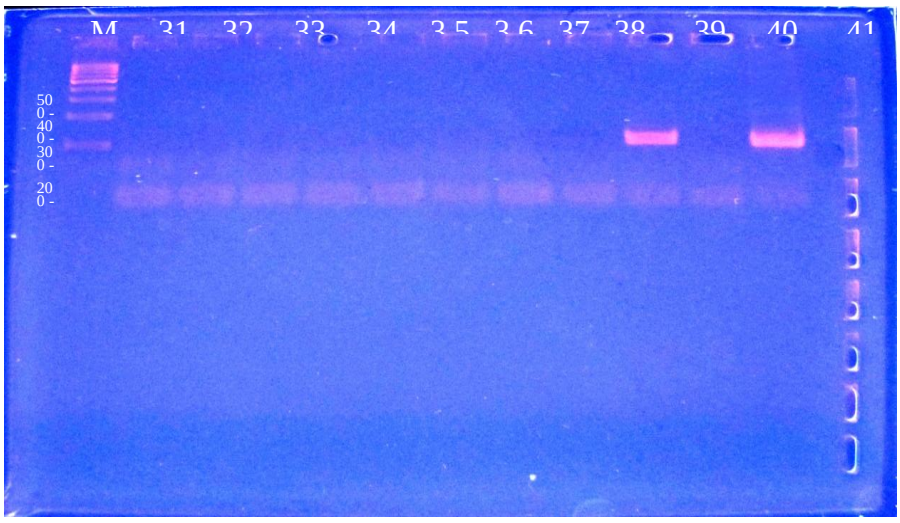

Figure (3): Electrophoreses hydro gel showing Detection of Cytomegalovirus using FAST PCR of IEA region

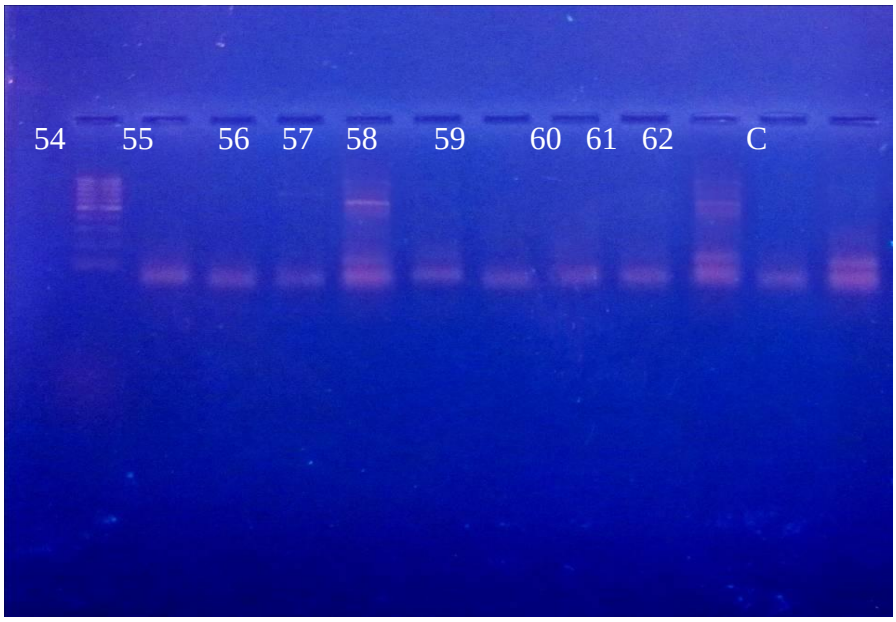

Figure (4): Electrophoreses hydro gel showing Detection of Herpes Simplex Virus (HSV 1-2) using FAST PCR of the gD region.

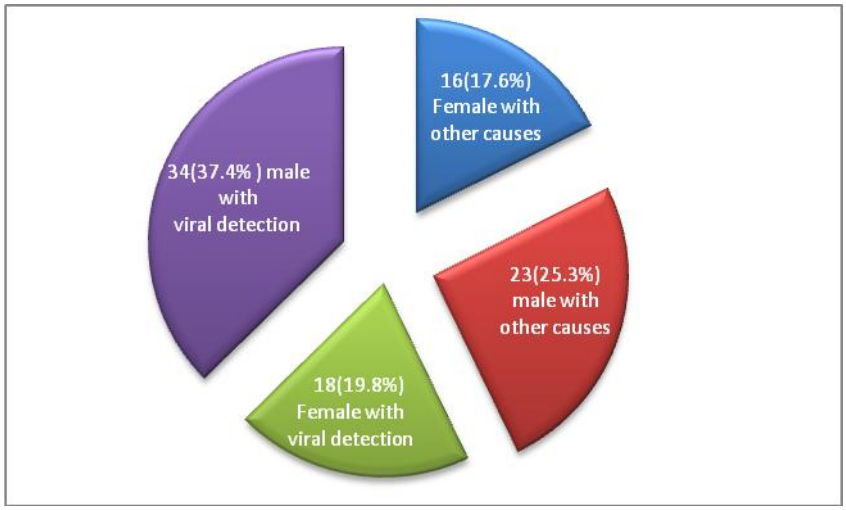

Figure (5): Chronic periodontitis patients percentage distribution according to gender and periodontal pocket viral detection (or other causes).

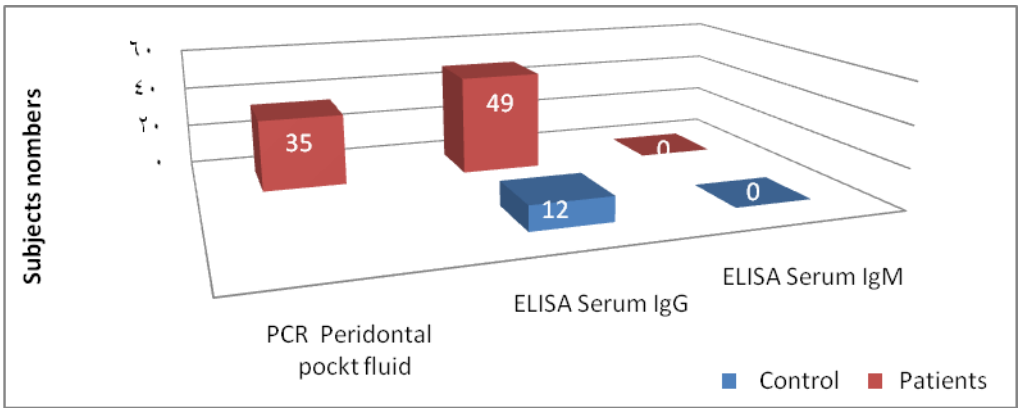

Figure (6): Comparison between EBV detection using nested PCR and ELISA technique

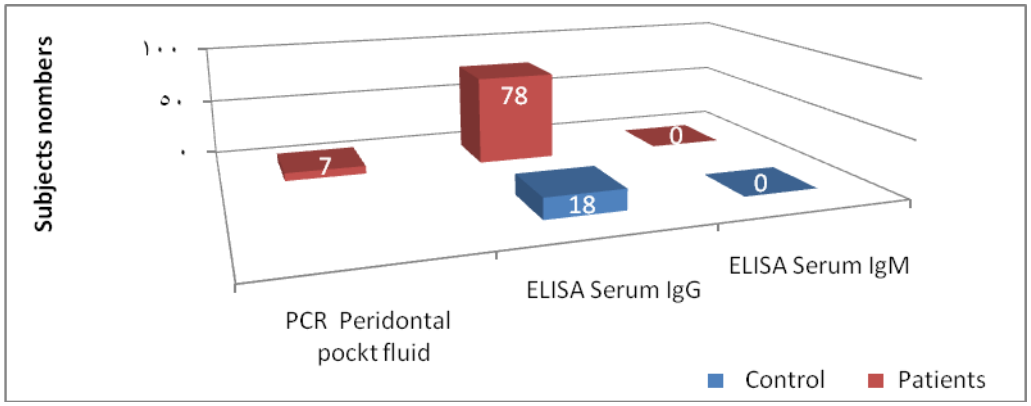

Figure (7): Comparison between CMV detection using nested PCR and ELISA technique

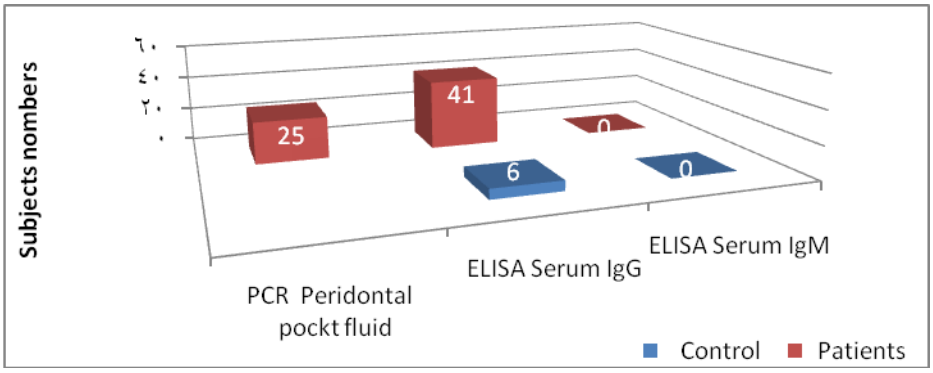

Figure (8): Comparison between HSV detection using nested PCR and ELISA technique

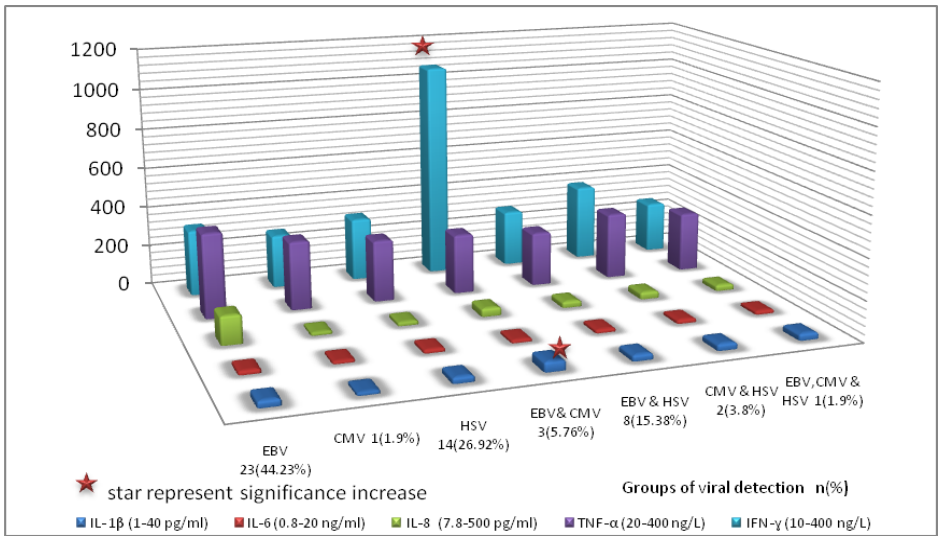

Figure (9): Relationship Between Viral Detection and Some of Serum Cytokines Levels

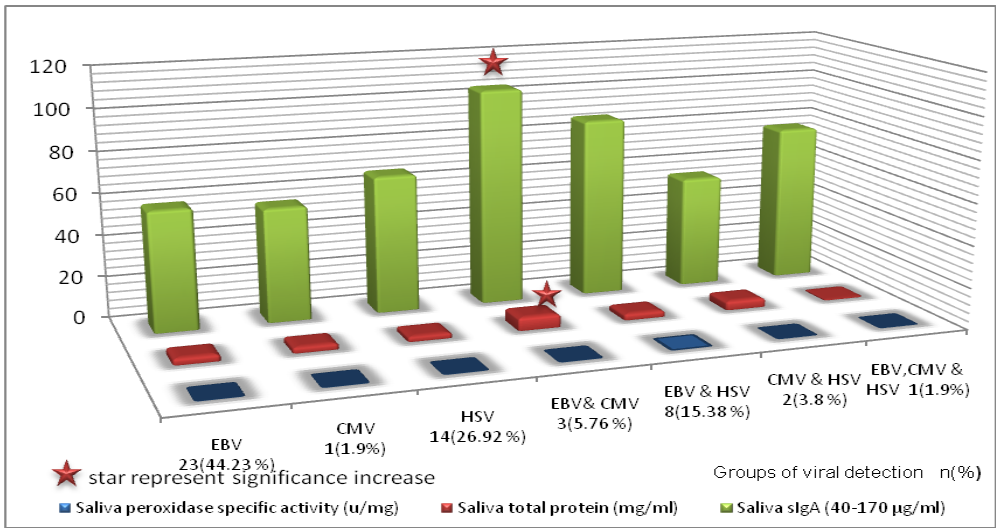

Figure (10): **Relationship between viral detection and some of saliva parameters levels**

Conclusion

In this study, high percentage of chronic periodontitis deteceted between 20-39 years old. Nested PCR showed detection of EBV, CMV and HSV in chronic periodontitis patients pockets . EBV and HSV showed significance association with chronic periodontitis patients pocket, but there was no association with CMV. ELISA assay was unsuitable technique to detect chronic periodontitis viruses. Also no statistical association was detected between pocket depth and type of viruses detected.

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