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MOLECULAR DETECTION OF VIRUS HERPES SIMPLEX TYPE 1(HSV-1) , VIRUS HERPES SIMPLEX TYPE 2(HSV-2), CYTOMEGALOVIRUS(HCMV) and EPSTEIN-BARRVIRUS (EBV) IN SUPRA-GINGIVAL DENTAL PLAQE IN PATIENTS WITH PERIODONATAL DISEASE

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Abstract: Introduction Chronic periodontitis is as an inflammatory disease of the supporting tissue of the teeth caused by periopatogens microorganisams. New concept about etiology of periodontal disease suggests that herpes viruses may play an important role in the pathogenesis of periodontal disease. The aim of this study was to analyze the presence of herpes simplex virus type 1and 2 (HSV-1, HSV-2) cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), in supra-gingival dental plaque .
Materials and Methods The study comprised a total of 89 patients who were divided in two groups: patients who were diagnosed moderate stage of periodontal disease and patients who were diagnosed with advanced stage of periodontal disease. Clinical examinations included determination of plaque index, gingival index, sulcus bleeding index, probing depth, and clinical attachment level. Supra-gingival dental plaque samples were taken with sterile coton ball scrub on tooth enamel. Molecular detection of HSV1, HSV2 , EBV and CMV was analyzed using polymerase chain reaction (PCR).
Results Molecular analysis of HSV-1 ,HSV-2, EBV and CMV in supra-gingival dental plaque in patients with chronic periodontal disease (a total of 89) showed the presence of EBV in 12,40% of patients , HSV-1 was found in 11,20% of patients, in 7,90% of patients was detected HSV-2, CMV was established in 9,00% of patients. In 3,40% patients in supra-gingival dental plaque was detected combination of viruses HSV1 and CMV, in 3,40% of patient combination of HSV1 and EBV ; and in 2,20% of patient combination ofHSV2,EBV was observed; in 2,20% of patients was found combination of 3 viruses HSV1; HSV2; EBV and in 49,50% patients we found negative finding. Patients with moderate stage of periodontal disease have a 0,78 times(OR = 0,78 / 0,33-1,84/)no significantly lower risk of probability of finding viruses in the supra-gingival plaque compared to patients who had advanced stage of periodontal disease. In the shown cross-culture of the presence of viruses in supra-gingival plaque in patients with moderate and advanced stage of periodontal disease for Fisher, s Exact Test = 5,19 and p >0.05 (p = 0.809 / Monte Carlo sig. (0.799-0.819)there is no significant difference.
Conclusion We can associate the presence of herpes simplex viruses in supra-gingival dental plaque with periodontal disease. Our findings suggest that there is no significant difference in presence of viruses in supra-gingival plaque in patients with moderate and sereve stage of periodontal disease.

INTRODUCTION

Chronic periodontitis is defined as an inflammatory disease of the supporting tissue of the teeth caused by specific microorganisms or groups of specific microorganism, (not only bacteria but also viruses, fungi, protozoa etc.) resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both.] Various risk factors are suggested to influence the progression of periodontal disease, but the most commonly reported etiological factor for this condition are periopatogens microorganisams from dental plaque as

Porphyromonas gingivalis, *Tanerella forsythia* and *Treponema denticola* i. [1] For a very long time the bacterial etiology was the only approved and accepted concept about periodontal disease . However this concept hasn't explain the various aspects of periodontal disease such as site specificity,[2] rapid periodontal tissue breakdown in case with minimal dental plaque[3]phases of disease activity and quiescence, [4] and the reason for progression to advanced periodontal destruction in some and not in others patients[5].

Evidence suggests the presence of many strains of viruses in the periodontal environment so they may play an important role in the pathogenesis of periodontal disease. Several studies have demonstrated a positive association between human cytomegalovirus (HCMV), Epstein Barr (EBV), Herpes Simplex Virus (HSV) and chronic periodontitis [6-8].

As new concept much of the study about microbes in periodontal disease has been studying communities rather than individual pathogens [9,–14]. The coinfection between herpesviruses and periodontopathic bacteria might play a crucial role in the increasing severity of the disease[15],

The aim of this study was to analyze the presence of herpes simplex virus type 1and 2 (HSV-1, HSV-2) cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), in supra-gingival dental plaque , to examine the possible association between the presence of this viruses and periodontal disease .

MATERIAL AND METHOD

This study was performed at the Department of oral pathology and periodontology- University Dental Clinical Centre "St. Panteleimon" Skopje and University Clinic for Children's Diseases -Laboratory for Molecular Genetic in Skopje. Each patient included in the study received a thorough verbal and written explanation about the course of the study, after which he/she signed an informed consent for participation in the study. Institutional Ethical committee approved the study.

The study comprised a total of 89 patients who were divided in two groups:

- Patients who were diagnosed moderate stage of periodontal disease (clinical attachment loss of 2-5 mm) and
- Patients who were diagnosed with an advansed stage of periodontal disease (clinical attachment level ≥ 6 mm).

In this examination we used non-smokers, patients who did not receive antiviral drugs in the previous six months and patients with no systemic diseases such as diabetes and cardiovascular diseases., Patients who received antibiotics or drugs that influence on the periodontium (non-steroidal anti-inflammatory drugs),over the last three months or more, patients with some systemic disease (renal, cardiovascular, respiratory, malignant diseases, diabetes), as well as pregnant and breast-feeding women were excluded from this study.

The study consisted of clinical and laboratory examinations. Clinical examination included determination of: plaque index – PI (Silness-Löe), gingival index of gingival inflammation – GI (Löe-Silness), periodontal bleeding on probing - BOP (Mühlemann-Son), clinical attachment loss – CAL and measurement of periodontal pocket depth – PPD.

Clinical and laboratory examinations consisted of collecting supra -gingival dental plaque samples in both clinical disease stages.

Supra-gingival dental plaque samples were taken with sterile coton ball scrub on tooth enamel from vestibular and oral tooth surface[16] .After collecting the plaque samples, they were put in sterile microbiological plastic tubes – eppendorfs with suspended 1 ml x 1xPBS (phosphate buffered saline) buffer (pH=7.4) (Fig. 2B) and were transported to the Genetic Laboratory at the University Children's Hospital for further analysis.

Dental plaque samples were immediately subjected to the protocol for DNA extraction of the Laboratory. The test tubes with the samples were vortexed and centrifuged (12000 rpm for 5 min), the supernatant was decanted and 300-350 μl buffer for digestion was added to the precipitation (0.05M Tris, 0.001M EDTA, 1% Tween 20, 1% Nonidet 40, 0.3 mg/ml Proteinase K), after which it was incubated in a water bath over night at 56^{o}C. Following digestion DNA was extracted and precipitated by a standard method with phenol-chloroform and ethanol. All extracted DNA samples were stored at -20^{o}C for further analysis.

PCR amplification of viruses was performed with the PCR machine - Veriti Thermal Cycler (Applied Biosystems, California, USA) according to the following protocol: an initial denaturation for 10 minutes at 95^{o}C, followed by 40 amplification cycles of 95^{o}C for 30 sec., 60^{o}C for 2 min. and 72^{o}C for 1 min. and 30 sec., and terminal extension at 72^{o}C for 10 minutes.

The reactive mix with a total volume of 50 μl contains: 1 μl of the isolated DNA, 10 pmol of each prajmer- H1P32 / H1M32 for HSV-1, H2M40 / H2P4 for HSV-2, EP5 / EM3 for EBV and CP15 / CM3 for HCMV (Table 1), 5 μl of

10 x reaction buffer, 0.2mM of each dNTP and 2.5U of cloned pfu DNA polymerase enzyme (G-Biosciences, USA), previously described by Das et al. in 2012[16].

A 10 µl aliquot of the amplified PCR product was taken and analyzed with electrophoresis on a 2.5% agarose gel containing 1 mg/ml ethidium bromide in 1xTBE (Tris/Borate/EDTA) buffer and was visualized under UV transilluminator. Presence of a fragment of 147 bp confirmed the presence of HSV-1 virus in the analyzed sample (Figure 1), presence of a fragment of size 227 bp, 182 bp and 256 bp confirms the presence of HSV-2, EBV and HCMV respectively, in the analyzed sample.

Angiotensinogen served as a control gene for monitoring the success of PCR amplification of HSV-1 virus by using the pair of primers oligo25/oligo26, resulting in PCR product of 165 bp.

The data analysis is performed in a statistical program SPSS Statistics 17.0. The following methods were used: In the analysis of the series with attribute markers (presence of the HSV1, HSV2, CMV and EBV viruses in supra-gingival dental plaque in patients with moderate and advanced stage of periodontal disease), percentage percentages of the structure (%) were determined; The differences in the relative positive and negative viral findings in supra-gingival plaque in patients with moderate and advanced form of periodontal disease were analyzed using the Pearson Chi-square (p); The risk (probability) of finding viruses in supra-gingival plaque in patients with moderate and advanced form of periodontal disease was determined by making Odds Ratio (OR); Differences in the presence of the HSV1, HSV2, CMV and EBV viruses in supra-gingival plaque in patients with moderate and advanced form of periodontitis have been analyzed using the Fisher's Exact Test / Monte Carlo Sig. / (p).The significance is determined by $p < 0.05$.The data is tabulated displayed.

RESULTS

The study examined a total of 89 patients with periodontal disease. In 54 (60.7%) of these patients a moderate clinical stage of the disease was detected, while in 35 (39.3%) an advanced clinical stage was found.

Molecular analysis of HSV-1 ,HSV-2, EBV and CMV in supra-gingival dental plaqe in patients with chronic periodontal disease (a total of 89) showed the presence of Epshtajnbar virus (EBV) in 11(12,40%)patients , Herpes simplex 1 (HSV-1) was found in 10 (11,20%)patients, in 7 (7,90%) patients was detected Herpes simplex 2 (HSV-2), Citomegalovirus (CMV) was established in 8 (9,00%) patients.

In 3(3,40%) patients in supra-gingival dental plaque was detected combination of viruses HSV1 and CMV, in 3(3,40%) patient combination of HSV1 and EBV ; and in 1(2,20%) patient combination ofHSV2,EBV was observed; in 2 (2,20%) patients was found combination of 3 viruses HSV1; HSV2; EBV and in 44(49,50%) patients we found negative finding.

Table 1. Presence of HSV-1, HSV-2, EBV and CMV in supra-gingival dental plaque in patients with periodontal disease.

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	HSV1	10	11,2	11,2	11,2
	HSV2	7	7,9	7,9	19,1
	CMV	8	9,0	9,0	28,1
	EBV	11	12,4	12,4	40,4
	HSV1;CMV	3	3,4	3,4	43,8
	HSV1;EBV	3	3,4	3,4	47,2
	HSV2;EBV	1	1,1	1,1	48,3
	HSV1;HSV2;EBV	2	2,2	2,2	50,6
	Negative	44	49,4	49,4	100,0
	Total	89	100,0	100,0	

The results shown in Table 2 refer to the presence of indicated viruses in supra-gingival plaque in patients with moderate and advanced form of periodontal disease.

In 54 patients with moderate stage of periodontal disease, in 26 (48,10 %) patients a positive finding of (HSV-1,HSV-2, CMV, EBV)viruses was found in supra-gingival dental plaque, and in 28 (51,90%) patients a negative finding of viruses was reported.

In 35 patients with advanced stage of periodontal disease in 19 (54,30%) patients a positive finding of HSV-1,HSV-2, CMV, EBV viruses was found in supra-gingival plaque, and in 16 (45,70%) patients a negative finding of HSV-1, HSV-2, EBV and CMV viruses was detected.
In the shown cross-culture of the presence of viruses in supra-gingival plaque in patients with moderate and advanced stage of periodontal disease for Pearson Chi-Square = 0,32 and p >0.05 (p = 0,57) there is a no significant difference.

Table 2. Induction of viruses in supra-gingival dental plaque in patients with moderate and advanced stage of periodontal disease.

			supra-gingival dental plaque finding		
			Positive	Negative	Total
Group	moderate stage of periodontal disease	Count	26	28	54
		%	48,1%	51,9%	100,0%
	advanced stage of periodontal disease	Count	19	16	35
		%	54,3%	45,7%	100,0%
Total		Count	45	44	89
		%	50,6%	49,4%	100,0%

The results shown in Table 2.1 relate to the estimated risk of finding viruses in supra-gingival dental plaque in patients with moderate and advanced stage of periodontal disease.
Patients with moderate stage of periodontal disease have a 0,78 times(OR = 0,78 / 0,33-1,84/)no significantly lower risk of probability of finding viruses in the supra-gingival plaque compared to patients who had advanced stage of periodontal disease. For the cohort positive finding, the estimated risk is OR = 0,89 (0,59-1,34).
For the cohort negative finding, the estimated risk is OR = 1,13 (0,73-1,77).

Table 2.1 Risk of virus finding in patients with moderate and advanced stage of periodontal disease (Risk Estimate).

		95% Confidence Interval	
	Value	Lower	Upper
Odds Ratio for Group (moderate stage of periodontal disease / advanced stage of periodontal disease)	0,78	0,33	1,84
For cohort supra-gingival finding= positive	0,89	0,59	1,34
For cohort supra-gingival finding = negative	1,13	0,73	1,77
N of Valid Cases	89		

In Table 3, the results show the presence of HSV-1, HSV-2, EBV and CMV in supra-gingival dental plaque expressed as percentage in patients with moderate and advanced stage of periodontal disease.
Of the 54 patients with moderate stage of periodontal disease molecular analysis of HSV-1 ,HSV-2, EBV and CMV in supra-gingival dental plaque showed the presence of HSV1 in 7patient (13,00%), 5 (9,30%)patients had the presence of HSV2, in 4 (7,40%) patients was found the presence of EBV, in 4 (7,40%) patients was found the presence of CMV, in 2 (3,70%) patient we detected the presence of combination of viruses HSV1; CMV; , in 2 (3,70%) patient we detected the presence of combination of viruses HSV1; EBV , in 1 (1,90%) patient we detected the presence of viruses HSV2; EBV; , in 1 (1,90%) patient we detected the presence of combination of viruses HSV1;HSV2; EBV; and in 28 (51,90%) patients did not determine the presence of viruses in the supra-gingival dental plaque.

Of the 35 patients with advanced stage of periodontal disease molecular analysis of HSV-1 ,HSV-2, EBV and CMV in supra-gingival dental plaque showed the presence of HSV1 in 3 (8,60%) patients , in 2 (5,70%) patients there was a presence of HSV-2, 7 (20,00%) patients had the presence of EBV and 4(11,40%) patients had the presence of CMV . In 1 (2.90%)patient we detected the combination of viruses HSV-1,EBV and in 1 (2.90%),patient the presence of HSV1,CMVcombination was determined; and in 1 (2.90%)patient we detected the combination of viruses HSV-1,HSV-2 EBV so in 16 (57.10%) patients, the presence of viruses in the supra-gingival plaque has not been established.

In the shown cross-culture of the presence of viruses in supra-gingival plaque in patients with moderate and advanced stage of periodontal disease for Fisher, s Exact Test = 5,19 and p >0.05 (p = 0.809 / Monte Carlo sig. (0.799-0.819)there is no significant difference.

Table 3. Induction of HSV-1, HSV-2, EBV and CMV viruses expressed as percentage in supra-gingival dental plaque in patients with moderate and in patients with advanced periodontitis

	Induction of HSV-1, HSV-2, EBV и CMV in supra-gingival dental plaque									
	HSV1	HSV2	CMV	EBV	HSV1;CMV	HSV1;EBV	HSV2;EBV	HSV1;HSV2;EBV	Negative	Total
Moderate stage of periodontal disease Count	7	5	4	4	2	2	1	1	28	54
%	13,0%	9,3%	7,4%	7,4%	3,7%	3,7%	1,9%	1,9%	51,9%	100,0%
Advanced stage of periodontal disease Count	3	2	4	7	1	1	0	1	16	35
%	8,6%	5,7%	11,4%	20,0%	2,9%	2,9%	,0%	2,9%	45,7%	100,0%
Total Count	10	7	8	11	3	3	1	2	44	89
%	11,2%	7,9%	9,0%	12,4%	3,4%	3,4%	1,1%	2,2%	49,4%	100,0%

DISCUSION

Chronic periodontal disease is the most common form of periodontal disease which causes periodontal destruction, alveolar bone resorption, occasional pain, and eventual tooth loss [18]. Periodontitis is considered to involve a multifactorial interaction between microbial, host, and environmental modulating factors [19], and microbial agents are the most important in the development of periodontitis. Initial plaque formation starts with formation of dental pellicle (salivary pellicle) on the tooth surface. It is results from the selective absorption of salivary proteins and biomolecules originating from the local oral environment on tooth surface and it is different from bacterial biofilm.

Aggregates of bacterial cells or planktonic cells adhere to this pellicle through specialized adhesins on the bacterial cell surface that recognize pellicle proteins [20] and by non-specific physico-chemical interactions [21]. These results in formation of bacterial deposits [22], [23] composed of initial colonizers like *Actinomyces* sp., *Streptococcus* sp., *Lactobacillus* sp. and *Candida* sp. [24]–[26] and they create it the first layer of supra gingival plaque. Maturation of the biofilm proceeds through co-aggregation of planktonic bacteria to the already adhered biofilm [27] and bacterial growth, of some bacterial species as *Streptococcus sanguinis* [28], make the second layer of supra gingival dental plaque. Listgarten[29] notice that after one week of plaque formation filaments appeared on top of the columns, and after three weeks, the biofilm was predominantly filamentous without any sign of cocci left. It was noticed that after three weeks, undisturbed supra -gingival plaque morphologically is similar with sub gingival dental plaque [30].It has been found that there are viruses in the dental plaque in addition to the bacteria. Millions of genomic copies of human viruses may be found in periodontal lesions [31], with herpes viruses the most commonly researched viruses in periodontology [32]. Studies have shown that the presence of oral viruses cause an immune response to the host, which potentially can play a role in formation of oral immunity and the pathogenesis of the disease [33, 34]. It has also been proven that human oral viruses carry significant gene functions that can be involved in the pathogenic functions of their host bacteria [35], suggesting a more subtle role for viruses as members of human oral microbial.

Our research concerned the presence of herpes simplex viruses : HSV1, HSV2 ,EBV, and CMV In supra-gingival dental plaque, which results showed that herpes viruses are present in supra-gingival dental plaque in patients with periodontal disease . In this study, we detected the presence of combination of viruses in sub gingival dental plaque : in 3(3,40%) patients in supra-gingival dental plaque was detected combination of viruses HSV1 and CMV, in 3(3,40%) patient combination of HSV1 and EBV ; and in 1(2,20%) patient combination ofHSV2,EBV was observed; in 2 (2,20%) patients was found combination of 3 viruses HSV1; HSV2; EBV.

However, in literature we did not find much data and research that dealt with the presence of these viruses in the dental plaque. The datawe found in the literature concerned the examination of the viral communities in the supra-gingival dental plaque, the sub-gingival dental plaque, and the saliva with a particular aspect of the bacteriophages[36]. There is a difference in the viral communities present in supra-gingival and sub-gingival dental plaque in healthy individuals and in patients with periodontal disease.

Herpes viruses are thought to have an effect on the progression of periodontal disease. The results from some study[8,37,38] about that the presence of HSV1,HSV2,CMV, and EBV viruses in sub-gingival dental plaque suggest that the presence of (HSV1,HSV2,CMV, and EBV) herpes viruses in sub-gingival dental plaque in patients with advanced stage of periodontal disease is significantly higher than their presence in sub-gingival dental plaque in patients with moderate stage of periodontal disease.

The results of our study suggest that there is no significant difference in presence of viruses in supra-gingival plaque in patients with moderate and advanced stage of periodontal disease. These results can be linked to differences between sub-gingival and supra-gingival dental plaque.

CONCLUSION

The results of this study showed a presence of HSV1, HSV2, EBV and CMV viruses in supra-gingival dental plaque in patients with chronic periodontal disease , so we can associate the presence of herpes viruses in supra-gingival dental plaque with periodontal disease. It has also been established that that there is no significant difference in presence of viruses in supra-gingival plaque in patients with moderate and sereve stage of periodontal disease

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