

Detection of Herpes Simplex Virus Type 1 in Gingival Crevicular Fluid of Gingival Sulcus/Periodontal Pocket Using Polymerase Chain Reaction

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SUMMARY

Introduction Pathogenesis and some characteristics of periodontitis cannot be fully explained by bacterial etiology alone. Herpes viruses may bridge the gap between clinical characteristics and molecular understanding of periodontal destruction.

Objective The aim of this study was to investigate the prevalence of herpes simplex virus type 1 (HSV-1) in gingival crevicular fluid (GCF) of healthy and damaged periodontium in Serbian population and to explore potential correlation between the presence of this virus and the level of periodontal destruction.

Methods Samples were collected from gingival sulcus/periodontal pockets by sterile paper points and the presence of viral DNA in gingival crevicular fluid was assessed by PCR.

Results There was no statistically significant difference in HSV-1 in presence between periodontitis patients (PG=38.9%) and healthy controls (HC=32.3%), (Chi-square test, with Yates' correction $p=0.7574$). However, HSV-1 positive patients showed significantly higher values of parameters of periodontal destruction (PPD= 7.11 ± 2.52 , CAL= 5.46 ± 2.34) than periodontitis patients without HSV-1 in gingival crevicular fluid (PPD= 4.70 ± 1.79 , CAL= 3.39 ± 2.65) (p values respectively, $p=0.002$ and $p=0.023$, Independent Samples T-Test). HSV-1 occurred more often in deeper (PPD ≥ 6 mm) (69.2%) than in shallow pockets (3 mm<PPD<6 mm) (18.2%) (Chi-square test, with Yates' correction, $p=0.008$). Plaque index was lower in the HSV-1 positive group (0.84 ± 0.69 vs. 1.43 ± 0.76 , $p=0.023$, Independent Samples T-Test).

Conclusion This study demonstrated that the presence of HSV-1 in the gingival crevicular fluid coincides with a higher degree of tissue destruction in patients with periodontitis.

Keywords: periodontitis; herpes simplex; gingival crevicular fluid; periodontal pocket

INTRODUCTION

Plaque-associated periodontal diseases are chronic infections caused by a mixed microbial flora, resulting in an inflammatory process that leads to periodontal attachment loss and ultimately tooth loss [1]. Although bacteria of dental biofilm are known to be the most important etiological factor for periodontal disease, a susceptible host is also needed. Immune-inflammatory reaction that develops in periodontal tissues in response to chronic bacterial presence results in the destruction of structural components of the periodontium [2].

Bacterial etiology has not been able to explain rapid periodontal tissue breakdown in cases with minimal plaque, or low levels of common risk factors [3]. Other aspects of periodontitis that cannot be fully explained by bacterial etiology are disease remission and reactivation [4], periodontitis site specificity [5], progression of periodontal destruction in some patients and not in others [6], evolution of gingivitis to periodontitis, or stable to disease-active periodontitis [7].

Herpes viruses and their biology may provide some answers for better understanding of mechanisms involved in the degradation of periodontal tissues.

Herpes viruses have been found in periodontal tissues and in gingival crevicular fluid in chronic [8], advanced [9] and aggressive [10] periodontitis as well as in the periodontium of HIV patients [11] and patients with the following syndromes: Papillon-Lefèvre [12], Down [13] and Kostmann [14].

Currently, it is believed that the pathogenesis of some types of periodontitis is a multi-step process, involving a complex interaction between the host, bacteria, viruses, and a variety of environmental factors.

OBJECTIVE

The aim of this study was to investigate the prevalence of HSV-1 in gingival crevicular fluid of healthy and damaged periodontium in Serbian population and to explore whether there is a correlation between the presence of this virus and the level of periodontal destruction.

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METHODS

The study, approved by the Ethical Committee of the School of Dentistry, included 67 subjects (25 men and 42 women, age 18-76). The group of patients with periodontitis (periodontitis group -PG) consisted of 36 patients (18 men and 18 women, age 23-76) who had clinical signs of periodontitis and were treated at the Clinic for Periodontology and Oral Medicine, School of Dental Medicine, University of Belgrade. Healthy control group (HC) consisted of 31 volunteers (7 men and 24 women, age 18-33) without clinical signs of periodontitis. Exclusion criteria for the study were: 1) known systemic diseases (cardiovascular, respiratory, renal, malignancy, etc.), 2) presence or history of any severe infections, 3) systemic antibiotic or immunomodulatory treatment in the previous 3 months, 4) long-term treatment with any medication suspected to affect the periodontium (e.g. non-steroidal anti-inflammatory drugs), 5) pregnant or lactating women and 6) less than 20 teeth present, 7) less than 3 teeth from Ramfjord examination model, (8) any therapy of periodontitis 1.5 year period prior to the study.

Anamnestic data included history of oral manifestation of recurrent herpes infection and information regarding smoking.

Clinical examinations included the determination of Plaque index - PI (Silness-Löe) [15], Gingival index - GI (Löe-Silness) [16], bleeding on probing - BOP (Mühlemann-Son) [17], clinical attachment loss - CAL and probing pocket depth - PPD. The probings were done by Williams probe calibrated in millimeters and were assessed on six Ramfjord's teeth [18, 19]. Subjects were assigned to the periodontitis group (PG) if they had at least three sites with probing pocket depth ≥ 3 mm and a clinical attachment loss ≥ 2 mm in at least three quadrants. The PG group was divided into two subgroups according to PPD values. The first subgroup included patients with PPD 3-6 mm and the second patients with PPD ≥ 6 mm. Subjects were assigned to the control group (HC) if they had PPD ≤ 3 mm, CAL=0 and did not have bleeding on probing.

The samples were collected 24 hours after the periodontal examination in order to avoid blood contamination of the samples. All samples were collected from the gingival sulcus/deepest periodontal pocket. The sample site was isolated from saliva with cotton rolls and gently air dried. The supragingival plaque was removed by sterile cotton pellets. Two paper points were inserted in each gingival sulcus/periodontal pocket until a mild resistance for 30 seconds. Paper points contaminated with blood were not used in the analysis. Those points were placed in sterile plastic tubes containing saline. All samples were stored at -70°C until further analysis.

The PCR procedure was carried out at the Laboratory for Molecular Biology, School of Dental Medicine, University of Belgrade. After thawing, the DNA was isolated by boiling at 100°C for 10 minutes.

HSV-1 type-specific oligonucleotide primers (forward 5'- ATA CCG ACG ATA TGC GAC CT and reverse 5'- TTA TTG CCG TCA TAG CGC GG) were used to amplify the 110bp region of thymidine kinase gene, unique for HSV-1.

The PCR was performed in the total volume of 25 μl containing 2.5 μl of 10X PCR buffer (MBI Fermentas, Lithuania), 1.5 μl of MgCl_2 , 0.2 mM dNTPs, 0.375 μM of each primer, 1 unit of Taq DNA polymerase (MBI Fermentas, Lithuania), 3 μl of biological sample and water to the final volume of 25 μl .

The PCR amplification was performed in a thermal cycler (PCR Express, Hybaid, USA). After the initial incubation at 94°C for 10 minutes, the PCR procedure included a 35-round amplification process that was performed in three steps covering denaturation (at 94°C for 1 minute), annealing (at 52°C for 1 minute) and extension (at 72°C for 3 minutes), followed by a final extension at 72°C for 7 minutes.

The PCR products were loaded onto 8% polyacrilamide gels and stained with 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide after electrophoresis. The gels were analyzed and photographed under UV rays on transilluminator (Power Station 300plus, Labnet International, INC, USA). A one-kb DNA ladder digest (MBI, Fermentas, Lithuania) was used as a molecular size marker.

Each gel contained a negative and a positive control; for the negative control, samples were replaced with water while DNA samples obtained from patients with herpes labialis were used as positive controls.

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) for Windows, version 15. The Kolmogorov-Smirnov test allowed for normal evaluation of data distribution. The Independent Samples T-test was used for comparing continuous variables. Statistical differences between frequencies were tested with Chi-square test with Yates' correction. In all analyses the significance level was set at 0.05.

After training and calibration, the samples were collected by the first and second author. For the evaluation of intra- and inter- reliability, 10% of randomly selected subjects were re-examined 2 weeks after the first examination. Reliability was tested by applying the Cohen-Kappa test (performed in SPSS for Windows). The Cohen's Kappa score was determined for each periodontal index in order to test the intra-and inter-observer agreement.

RESULTS

The Kappa scores were 0.5-0.7, representing a very good agreement [20].

The age and sex distribution of study subjects are shown in Table 1. HC and PG subjects were matched for smoking, but not for age. Clinical parameters for both groups are presented in Table 2.

There were no statistically significant differences in the presence of HSV-1 between PG (38.9%) and HC (32.3%) groups (Chi-square test, with Yates' correction $p=0.7574$). The difference in mean age was not found neither between

Table 1. Age and sex distributions of patients

Variable	Healthy control group	Periodontitis group
Number of patients	31	36
Age, years (mean±SD)	18-33 (24.81±4.79)	23-76 (45.83±14.49)
Woman (%)	24 (77.4)	18 (50.0)
Smokers (%)	5 (16.1)	10 (27.8)

Table 2. Clinical parameters of Healthy control (HC) and Periodontitis group (PG)

Parameter	Mean±SD		p*
	HC	PG	
PI	0.74±0.77	1.20±0.78	0.018
GI	1.27±0.76	2.19±0.62	0.000
BOP	1.29±0.80	2.45±1.22	0.000
PPD	2.00±0.00	4.19±2.70	0.000
CAL	0.00±0.00	5.64±2.36	0.000

* p-values determined by Independent-Samples T Test

PI – Plaque Index (Silness-Löe); GI – Gingival Index (Löe-Silness); BOP – bleeding on probing (Mühlemann-Son); PPD – probing pocket depth; CAL – clinical attachment loss

Table 3. Clinical parameters of HSV-1 positive and HSV-1 negative patients with clinical signs of periodontitis

Parameter	HSV-1 patients		p*
	Positive	Negative	
PI	0.84±0.69	1.43±0.76	0.023
GI	2.31±0.41	2.10±0.73	0.333
BOP	2.59±0.60	2.36±1.49	0.524
PPD	7.11±2.52	4.70±1.79	0.020
CAL	5.46±2.34	3.39±2.36	0.023

* p-values determined by Independent-Samples T Test

Table 4. HSV-1 in Periodontitis group (PG) of patients according to the pocket depth (PPD)

PG patients	PPD		p*
	3–6 mm (N=22)	>6 mm (N=13)	
HSV+ (%)	4 (18.2)	9 (69.2)	0.008

* p-values determined by using Chi-Square Test with Yates' correction exact test

the patients with or without HSV-1 nor between genders. Sixteen female (38.1%) and 8 male (32.0%) patients were positive for HSV-1 in gingival crevicular fluid samples (Chi-square test with Yates' correction, $p=0.8105$). Furthermore, the distribution of HSV-1 was similar in smokers (40.0%) and non-smokers (34.6%) (Chi-square test with Yates' correction, $p=0.938$). We also tried to establish a correlation between the presence of HSV-1 and the recurrence of oral herpes infections. Seventeen patients could not recall if they ever had a recurrent herpes infection. For the rest of them, 14 patients noted at least one episode of herpes labialis, and 36 denied it. There was no statistically significant difference in the presence of HSV-1 between patients who had recurrent HSV infection in the oral region (50.0% were HSV positive) and patients who had not (38.9% were HSV positive) (Chi-square test, $p=0.6924$).

However, within the PG group, several clinical parameters differed significantly depending on the presence of the virus (Table 3). Probing pocket depth and clinical attachment loss revealed higher values in the patients with HSV, while plaque index was lower in the HSV-1 positive group than in the HSV-1 negative group. In addition,

HSV-1 occurred more often in deeper pockets (Table 4), and three out of four deepest pockets measuring 11 mm harbored HSV1.

DISCUSSION

As previously mentioned, the etiopathogenesis of the periodontal disease is not completely clarified. The initial event in the development of periodontitis is the formation of dental biofilm followed by gingivitis. T lymphocytes, B lymphocytes and monocytes/macrophages infiltrate can lead to the accumulation of herpes viruses in the periodontal tissue, as these cells are considered to be the source of viruses [21, 22]. Reactivation of herpes viruses can decrease the local host resistance and lead to the overgrowth of periodontal pathogenic bacteria, as *Porphyromonas gingivalis* [7].

Herpes viruses may contribute to the progression of periodontitis through a number of mechanisms. It is assumed that these viruses are able to express cytopathogenic effects, immune evasion, immunopathogenicity, latency, reactivation and tissue tropism [23]. They can infect or alter structural cells and host defense cells in the periodontium, and thereby reduce the ability of periodontal tissues to resist bacterial insults [22].

In the multitude of studies dealing with the presence of viruses from the Herpesviridae family in the periodontium, the majority focused on EBV-1 and HCMV [14, 24, 25, 26]. HSV was investigated to a lesser extent [8, 27, 28]. To the best of our knowledge, this is the first study conducted in Serbian population regarding HSV-1 detection in the gingival sulci in subjects with a healthy periodontium as well as in the periodontal pockets of periodontitis patients.

We decided to analyze the presence of this particular virus because it is most common of all viruses from the Herpesviridae family, and causes well-known and frequent oral pathologies – herpetic stomatitis and recurrent herpetic infections most usually manifested as herpes labialis. Our results showed a high prevalence of HSV-1 in GCF (35.8%), which is in agreement with some other authors who reported a high prevalence of this virus in specimens taken with paper points from gingival crevicular fluid/periodontal pockets [8, 9, 27]. Contrary to our results, Nibali et al. [28] found a low prevalence of all investigated herpes viruses, especially HSV-1 in both patients with periodontitis and healthy controls.

In the present study the hypothesis that the presence of HSV-1 is in correlation with the development of periodontitis could not be confirmed because we did not detect any difference in the presence of this virus between the control group and patients with chronic periodontitis. Although, the subjects with a healthy periodontium were much younger than those with periodontitis, this discrepancy should not have an impact on our results as the peak of the primary herpetic infection occurs until the age of five [29]. However, we can assume that periodontal disease did not develop in younger individuals, or did not lead to clinically

noticeable tissue destruction yet. Consequently, it would be valuable to conduct a follow-up of young patients with HSV-1 detected in their periodontium and periodically make clinical examinations.

As for the different number of male and female individuals in the healthy control group, gender itself is not considered as the predilection factor for periodontal destruction [30]. On the other hand, lactation, pregnancy, oral contraceptives, menstrual period may have an impact on periodontal tissues, which is why we excluded females with any of the mentioned conditions. Regarding the influence of gender on HSV-1 prevalence, no gender differences were found in the study performed in Romania from 2004-2005 [31]. As the prevalence of HSV-1 infection varies among different geographic regions, and Romania borders with Serbia, we consider findings of this study relevant in regard to our population.

Contreras and Slots [27] also failed to detect differences in the presence of HSV-1 between PG and HC groups. On the other hand, Grenier et al. [8] reported a higher prevalence of HSV-1 in subjects with periodontitis than in healthy controls. Parra and Slots [9] also found statistically higher prevalence of HSV-1 in patients with chronic periodontitis than in patients with mild gingivitis. The same results were reached by Contreras et al. [22] in gingival tissue specimens. Surprisingly, Bilichodmath et al. [32] found higher prevalence of HSV-1 in patients with chronic periodontitis than in patients with the aggressive form of the disease, but they explained the results as the influence of their patients' age.

The most important result in our study is the relationship between the presence of HSV-1 and pocket depth. Our results showed a significantly higher prevalence of HSV-1 in deeper pockets than in shallower ones; clinical parameters (CAL, PPD) also showed significantly higher values in HSV+ periodontitis patients than in HSV-, which is in agreement with the results of Slots et al. [7]. Other authors did not find correlation between the depth of periodontal pockets and HSV-1 presence. [8]. Our results also showed lower values for the plaque index in PG HSV+ patients, which speaks in favor of HSV-1 influence on periodontal tissue destruction and confirms the hypothesis that viruses might have influence on periodontitis progression in patients with good oral hygiene [3]. Kamma et al.

[33] detected significantly higher frequencies of HCMV, EBV-1 and HSV in active and progressive periodontitis sites than in stable sites.

Herpes viruses and in particular HSV-1 are considered to have a potential role in the pathogenesis of some oral diseases. There is evidence of a higher presence of HCMV, EBV-1 and HSV in Nigerian malnourished children with acute necrotizing ulcerative gingivitis (ANUG) [34]. The hypothesis is that herpes viruses can affect the host's immune system, facilitating the development of secondary bacterial infections. Sabeti et al. [35] found a clear relationship between symptomatic periapical lesions and the presence of HCMV and EBV. They presume that viral infections contribute to immune impairment, which in turn creates a fertile ground for endodontopathogenic bacterial infections. This model of pathogenesis could be potentially applied to the shifting of gingivitis toward periodontitis. Furthermore, phases of remission and reactivation of periodontitis might coincide with the latency and the reactivation of viruses [36], whilst viral tissue tropism could explain the site-specificity of periodontal destruction in some patients [37].

CONCLUSION

In the present study, we demonstrated that the presence of HSV-1 in the GCF is related to the degree of tissue destruction in the patients with periodontitis. The confirmation of the role of HSV-1 in the pathogenesis of periodontitis will require a larger sample along with a prospective study that would detect the presence of HSV in the periodontium before the onset, at the time of periodontitis initiation, and periodically during its development. Also, future studies demonstrating the role of HSV infection in the pathogenesis of periodontitis should prove that eradication of viral infection can prevent the progression of periodontal destruction.

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Откривање вируса *herpes simplex* тип 1 у гингивалној течности сулкуса или пародонталног џепа ланчаном реакцијом полимеразе

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КРАТАК САДРЖАЈ

Увод Патогенеза и неке клиничке одлике пародонтопатије не могу се до краја објаснити бактеријском етиологијом. Херпесвируси би могли да премосте јаз који постоји између клиничких особина и познавања патогенетских механизма пародонтопатије на молекуларном нивоу.

Циљ рада Циљ ове студије био је одређивање преваленције вируса *herpes simplex* тип 1 (*HSV-1*) у здравом и оболелом пародонцијуму особа у Србији, као и утврђивање могуће корелације између постојања ових вируса и степена оштећења пародонцијума.

Методе рада Узорци су узимани папирним поенима из гингивалних сулкуса или пародонталних џепова, а вирус је откриван реакцијом ланчаном умножавања молекула ДНК (енгл. *polymerase chain reaction – PCR*).

Резултати Није утврђена статистички значајна разлика у преваленцији *HSV-1* између особа с пародонтопатијом (32,3%) и здравим пародонцијумом (38,9%); χ^2 -тест са Јејтсовом (*Yates*) корекцијом: $p=0,7574$. У групи испитаника с

пародонтопатијом параметри који означавају степен оштећења (дубина пародонталног џепа – ДПЦ; ниво припојног епитела – НПЕ) били су значајно већи код оних с откривеним вирусом (ДПЦ: $7,11 \pm 2,52$ mm; НПЕ: $5,46 \pm 2,34$), него код испитаника без вируса у узорцима гингивалне течности (ДПЦ= $4,70 \pm 1,79$ mm; НПЕ= $3,39 \pm 2,65$); Студентов *t*-тест за неvezане узорке: $p=0,002$, односно $p=0,023$. У дубљим пародонталним џеповима (ДПЦ ≥ 6 mm) *HSV-1* је откривен статистички значајно чешће (69,2%) него у плићим џеповима (ДПЦ=3–6 mm) (18,2%); χ^2 -тест са Јејтсовом корекцијом: $p=0,008$. Просечне вредности плак-индекса биле су ниже код испитаника са *HSV-1* ($0,84 \pm 0,69$) у поређењу са испитаницима код којих овај вирус није откривен ($1,43 \pm 0,76$); Студентов *t*-тест за неvezане узорке: $p=0,023$.

Закључак Приказана студија показала је да је постојање *HSV-1* повезано са нивоом оштећења ткива код особа с пародонтопатијом.

Кључне речи: пародонтопатија; *herpes simplex*; гингивална течност; пародонтални џеп