Detection of Herpes Simplex Virus in Chronic Generalized Periodontitis via Polymerase chain Reaction –A Pilot Study

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ABSTRACT

Introduction: As bacterial etiology could not support and explain various aspects of periodontal disease, herpes virus is now proposed to be one of the causative factors responsible for the periodontal destruction. The purpose of present study is to detect the presence of Herpes simplex virus (HSV) in patients with chronic generalized periodontitis.

Material and Methods: A total of 11 patients were consecutively selected for the study, out of which 3 were diagnosed with moderate chronic generalized periodontitis and 8 with severe chronic generalized periodontitis. Subgingival material was taken from the deepest pocket of the dentition from every study subject, before the commencement of any procedure and polymerase chain reaction(PCR) assay were done to detect the presence of HSV-1 and -2.

Results: HSV-1 & -2 DNA was not detected in any of the samples.

Conclusion: This study is in conflict with previous studies and questions the proposed pathogenic role and clinical relevance of herpes virus in periodontitis.

Keywords: Chronic Generalized Periodontitis, Herpes Viruses, Polymerase Chain Reaction

INTRODUCTION

According to American Academy of Periodontology (AAP)-International workshop for classification of periodontal diseases, 1999 - Chronic periodontitis is defined as an inflammatory disease of the supporting tissue of the teeth caused by specific microorganisms or groups of specific microorganism, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both.[1] The term microorganisms in its full sense include not only bacteria but also viruses, fungi, protozoa etc. Currently, the bacterial etiology is the only approved and accepted concept. Until now, it has not been able to substantiate various aspects of periodontal disease such as site specificity,[2] rapid periodontal tissue breakdown with minimal plaque,[3] phases of disease activity and quiescence,[4] and the reason for progression to advanced periodontal destruction in some and not in others in a given population.[5]

It is assumed that periodontitis is prevalent in those individuals who have a genetic or environmental predisposition, with distinct immune responses and are infected with virulent infectious agents and reveal persistent gingival inflammation. Based on this concept, various herpes viruses are found to be associated with several types of periodontal disease. It is suggested that herpes viruses may exert its deleterious effect on periodontal tissues via one of the following mechanism: 1) Direct cytopathic effect on fibroblasts, keratinocytes, epithelial cells and inflammatory cells. 2) By hampering the cells involved in host defense. 3) By up regulating the growth of periodontopathic bacteria, 4) Altering the inflammatory pathway and response to cytokines or 5) By inhibiting cell mediated immunity by suppression of MHC Class I molecules.

Many recent studies have isolated herpes viruses from gingival biopsies, gingival crevicular fluid, supragingival and subgingival plaque samples.[6-8] It has also been found out that there is increase in herpes virus count with increase in severity of periodontitis. Now its demonstrated that herpes simplex virus (HSV-1 and -2), human cytomegalovirus (CMV) and Epstein –Barr virus (EBV), have more association with periodontitis than other herpes viruses.[6,7] Recently,it has been demonstrated that phase-1 therapy leads to short term elimination of viruses in diseased states.[7] But the prevalence of herpes viruses in periodontitis can vary according to the age, ethnicity, type of periodontal disease, immune status and genetic predisposition of patients.[9] So further studies in different region should be done to confirm virus as contributing factor for periodontitis

Thus, the purpose of our present study is to detect the presence of HSV in periodontal pockets of patients with chronic periodontitis in population of south Kerala, using conventional polymerase chain reaction.

MATERIAL AND METHODS

Study Population

A total of 11 subjects - 6 females and 5 males in the age range of 25-70 years were consecutively selected from the outpatient Department of Periodontics, Pushpagiri Dental College, Thiruvalla. All study subjects were given a detailed verbal description of the study and signed an informed consent form at the commencement of study. The study was approved by the ethical committee of Pushpagiri College of Dental Sciences.

Study Population was diagnosed with chronic generalized periodontitis, and severity was classified according to Clinical Case Definitions proposed by the CDC Working Group for use in Population –Based Surveillance of Periodontitis:[10] (Table-1)

Subjects with history of systemic diseases, those having or had taken any antiviral therapy or underwent any form of periodontal treatment in the past 6 months, pregnant or lactating females and smokers were excluded from the study. The clinical parameters

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Disease Category	Clinical Definition		
	CAL		PD
Severe Periodontitis	≥2 interproximal sites with ≥6mm	and	≥1 interproximal site with PD ≥5mm
Moderate Periodontitis	≥2 interproximal sites with CAL ≥4mm	or	≥ 2mm interproximal sites with PD ≥ 5mm
No/Mild Periodontitis	Neither 'moderate' nor 'severe' periodontitis		

Table-1: Clinical Definition

Gender	MChP	SChP	Total
Male	0	5 (62.5%)	5 (45.5%)
Female	3 (100%)	3 (37.5%)	6 (54.5%)
Total	3	8	11

MChP – Moderate chronic periodontitis, SChP – Severe chronic periodontitis

Table-2: demographic data

and the subgingival sample were taken in the first visit before the start of any procedures. Periodontal evaluation included Gingival index(GI), plaque index(PI), clinical attachment loss (CAL) and probing pocket depth (PPD) using a UNC-15 probe.

Subgingival Sample Collection

Supragingival plaque and saliva were gently removed from the sample site adjacent to the deepest pocket of the dentition with sterile cotton pellets and was air-dried prior to sampling. Subgingival material was then collected from the bottom of the periodontal pocket using a sterile periodontal curette in a single stroke. The collected specimen was then immediately transferred and suspended in 500µl of TE buffer (10mm Tris Hydrohloride, 1mm EDTA, pH -8) and stored at -20° C for further processing

Nucleic Acid Extraction

DNA was extracted from the sample material using an alkali phenol-chloroform-isoamyl alcohol method. In brief, 20g of sample is added to the 500µl of lysis buffer and is incubated in water bath at 30°C for 30 min. Following centrifugation, 500µl supernatant is added to 500ul (1vol) of phenol choloroform isoamyl alcohol (P:Cl:I -25:24:1) which is again centrifuged. Carefully 400ul supernatant (aqueous layer) is added to 2 vol of ice cold 100% ethanol (Molecular Biology Grade). It is incubated at -20°C overnight and is spinned for 20min at maximum speed. To it 50µl of 70% ethanol is added and spinned again. Supernatant is removed and the pellet is dried and is then dissolved in 50µl sterile MiliQ water, and is stored in -20°C

Conventional PCR Amplification of DNA

It is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands or millions of copies of a particular DNA sequence.

Reagents standardized: Qiagen Blood DNA kit, OrionX Hot start Taq PCR mix. Primer sequence for:

HSV : 5' – ATGGTGAACATCGACATGTAC – 3'

HSV 1 : 5' –CCTCGCGTTCGTCCTCGTCCT – 3'

HSV 2 : 5' – CCTCCTTGTCGAGGCCCCGAA – 3'

A single round PCR was done for all 11 samples, 1 positive control and 1 negative control, each of which contains H Taq-PCR mix, primer for HSV, HSV-1 and HSV-2, Sterile water and respective sample DNA. 35 amplification cycles were used along with various annealing temperatures of 96°C for 45 sec, 58°C for 30sec and 72°C for 30 sec. After the last cycle, the samples were incubated at 72°C for 5 min followed by storage at 4°C. Each amplified product were loaded on a 2% agarose gel with Syber Safe dye and read under ultraviolet light.

STATISTICAL ANALYSIS

Data was analyzed and categorical variables were expressed as numbers and percentage and quantitative variables as mean, standard deviation, minimum and maximum and median. Comparison between severe periodontitis and moderate periodontitis group were done using Mann-Whitney U test for quantitative variables and Fisher's exact test for categorical variables. P-value of less than 0.05 was considered as statistically significant.

Out of the 11 study subjects, 3 (27.27%) were diagnosed with moderate chronic periodontitis and 8 (72.72%) with severe chronic periodontitis. All the moderate periodontitis patients were females while 62.5% were males and 37.5% were females in severe periodontitis.

There was no statistical difference in age between both the groups but the total number of teeth present is statistically significant, which shows that there is more tooth loss in severe periodontitis than that of moderate periodontitis which may be due to increased periodontal destruction present in former than in latter group (Table-3).

There was no significant difference in Oral hygiene index (OHI), Gingival index (GI), Pocket depth (PD) and Clinical attachment loss (CAL) between the groups. But there is significant difference in Plaque index between the groups which may indicate plaque is more important causative agent inducing the periodontal destruction than others or all the other indices are the product of periodontitis rather than the cause for it (Table-4).

Conventional PCR assay showcased no positive findings in terms of HSV -1 and -2 in any of the samples tested.

DISCUSSION

Periodontitis is an inflammatory disease of the periodontium which is triggered by the presence of microorganisms in the subgingival crevice. Recently, herpes virus is suspected among the possible periodontopathogens. Evidence for this comes from various epidemiological studies, which supports the presence of herpes viruses in patients with periodontitis.

Contreras A and Slots J in 2000 reported herpes viruses as emerging putative pathogens in various types of periodontal diseases. In particular, Epstein Barr Virus (EBV-1) and Human Cytomegalovirus (HCMV) seem to play important roles, the presence of Herpes simplex virus -1 and -2 (HSV-1 and -2), Varicella zoster(VZV),Epstein Barr Virus (EBV) and Human Herpes Virus-8 (HHV-8) were also attributed to oral diseases.[11] Herpes virus co-infections can give rise to severe immunosuppression that might trigger proliferation of periodontopathic bacteria and other pathological events associated with destructive periodontal diseases.[12] In 2005,

	MChP			SChP			Total			P value
	Mean± SD	Min-Max	Median	Mean±SD	Min-Max	Median	Mean± SD	Min-Max	Median	
Age	42.7+6.0	37-49	42	51.6+12	27-68	52.5	49.2+11.2	27-68	52	0.102
No: of teeth	30.3+1.5	29-32	30	23.0+2.6	20-28	22.5	25.0+4.1	20-32	24	0.014
MChP – Moderate chronic periodontitis, SChP – Severe chronic periodontitis										

Table-3: Severity of periodontitis

	MChP			SChP			Total			P value
	Mean± SD	Min-Max	Median	Mean± SD	Min-Max	Median	Mean± SD	Min-Max	Median	
OHI	1.5+0.4	1.1-1.8	1.7	2.3+0.8	1-.1-3.4	2.3	2.1+0.8	1.1-3.4	2.1	0.125
PI	0.9+0.1	0.8-0.9	0.9	1.7+0.4	0.9-2.2	1.8	1.5+0.5	0.8-2.2	1.7	0.023
GI	1.2+0.3	0.8-1.4	1.3	1.6+0.6	0.7-2.6	1.5	1.5+0.6	0.7-2.6	1.3	0.473
PD	6.0+1.0	5.0-7.0	6.0	6.3+1.3	5-9	6.0	6.2+1.2	5.0-9.0	6.0	0.914
CAL	7.0+1.7	5.0-8.0	8.0	7.6+1.6	5-10	8.0	7.5+1.6	5.0-10.0	8.0	0.592
MChP – Moderate chronic periodontitis, SChP – Severe chronic periodontitis, OHI-Oral hygiene index, PI-Plaque Index, GI-Gingival Index, PD –Pocket Depth, CAL- Clinical Attachment Loss										

Table-4: Clinical Parameters

Slots et al stated that similar to medical infections, in which Herpes virus can reduce host defense and facilitate overgrowth of pathogenic microorganisms, herpes virus infected periodontal sites seem to be associated with increased levels of periodontal pathogens.[13] Kubar A et al reported that herpes viruses contribute to periodontal pathosis by impairing local host defenses resulting in increased virulence of resident bacterial pathogens or by inducing the release of cytokines and chemokines from inflammatory or connective tissue cells.[14] Contrary to all these, Nibali L et al in 2009 showed very low prevalence of subgingival herpes viruses in periodontal lesions and concluded that high prevalence of subgingival virus in periodontitis cases is not common, but may depend on the study population.[15]

In present study, we attempted to analyze the presence of HSV- 1 and 2 in periodontal phenotypes ranging from severe to moderate generalized chronic periodontitis. Not a single sample was tested positive for the herpes viruses. And this study stands out in clear conflict with the results of previous studies. The reason may be the difference in the population studied or differences involved in the study methods. Besides, small sample size may be another possible reason for the negative outcome.

Patients from different geographical area may have difference in the oral micro biota present in them due to difference in prevalence of infections present in various ethnic groups.[15] Many of the previous studies have been conducted in populations from South America, China, Turkey with lack of study in Indian populations. Also study subjects may not be an exact representation of population studied as it is impossible to make sure whether periodontal disease was active in the study population at the time of study or whether they were present at an earlier stage of periodontal destruction.

Previous studies using Real time PCR / Nested PCR has yielded positive results with regard to presence of HSV, which might be due their higher sensitivity. However, conventional PCR analysis yielded significant results in some of the previous studies, which are different from our study. B.Parra and J Slots, 1996, conducted PCR analysis on the crevicular fluid samples of American population who were diagnosed with advanced periodontitis and gingivitis, 78% of periodontitis patients and 31% of gingivitis patients showed positive for at least one herpes virus.[9] However our study was carried out in the Indian population which has a different pattern of oral microbiota. Besides the sample size for our study was much smaller, which may also be the reason for difference in results.

CONCLUSION

Within the limitations of our study, we found a very low prevalence or absence of subgingival presence of herpes viruses in periodontitis patients in population of south Kerala. Thus we may conclude that the presence of herpes virus in subgingival area is not universal, it may depend on the study population and the study methods used.

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