Original Article

Detection and Assessment of *Human CytomegaloVirus*, *Epstein-Barr Virus-1* and *Herpes Simplex Virus* in Patients with Chronic Periodontitis of Varying Pocket Depths

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ABSTRACT

Introduction: Recent microbiological research suggests an important role of herpes viruses in the pathogenesis of periodontal disease. Understanding the role of viruses in periodontal disease will lead to better prevention and treatment of the disease. Thus, the present study was undertaken to detect and assess the presence of *human cytomegalovirus (HCMV), Epstein-Barr virus-1 (EBV-1)* and *herpes simplex virus (HSV)* in chronic periodontitis patients and also to determine the correlation between clinical parameters and presence of herpes viruses.

Methodology: 30 patients with chronic periodontitis participated in the study. Subgingival plaque samples were obtained from one deep and one shallow site of each patient. DNA extractions were done from these samples and polymerase chain reaction (PCR) assay was carried out to detect the viral DNA. Statistical analysis of the results was done using Z - test of proportion.

Results: The detection frequency of *HSV-1* was found to be highest in deep (73.33%) as well as shallow (53.33%) sites among all the viruses followed by *EBV-1*, *HSV-2* and *HCMV*. The detection frequency of all the four viruses was found to be higher in deep sites as compared to shallow sites and the results were found to be statistically highly significant in case of *EBV-1* and *HSV-2*. Statistically highly significant differences were found when comparisons were made between plaque index and presence of viruses in deep (both viral detected and undetected) sites but not in relation to shallow sites. There were no statistically significant differences between gingival index and the presence of viruses in both deep as well as shallow sites, except for *HCMV*, where the results were found to be significant.

Conclusion: Detection frequency of herpes viruses mainly *HSV-1* and *EBV-1* was higher in deep sites as compared to shallow sites.

INTRODUCTION

Periodontitis is defined as "an inflammatory disease of the supporting tissues of teeth caused by specific microorganisms or group of microorganisms resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both". The pathogenic process of periodontitis includes dynamic interactions among various infectious agents and interconnected cellular and humoral host responses. However, despite a long history of research into the pathobiology of periodontitis, a definitive statement about its probable causes on a molecular level remains elusive.

The search for the etiologic agents of periodontal disease has been in progress for more than centuries. At the 1996 World Workshop on Clinical Periodontics, three bacterial species were concluded to be periodontal pathogens namely *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythus.*[1] Bacterial pathogens are causally necessary antecedents for the development of periodontitis but the mere amount of bacterial plaque does not seem to provide a sufficient basis for explaining important clinicopathologic features of the disease. Recently, the paradigm of pathogenesis of periodontitis is expanding. A new concept has emerged during the last decade concerning the contribution of herpes viruses in etiopathogenesis of periodontal diseases. *Human cytomegalovirus (HCMV)*, the largest member of the herpes family of viruses, is responsible for a significant percentage of asymptomatic viral infections worldwide.[2] The first case of intraoral *cytomegalovirus* was presented by Williams et al in 1960.[3] Although Sabiston[4] suggested that there was a relationship between *HCMV* and acute necrotizing ulcerative gingivitis (ANUG), the study could not present experimental evidence to support the hypothesis. Contreras and co-workers[5] showed that there was significantly higher prevalence of *HCMV, Epstein Bar virus (EBV)-1* and *Herpes simplex virus (HSV)* in gingival crevicular fluid samples taken from Nigerian children with ANUG who were malnourished compared to those taken from control groups of children. Rones et al[6] demonstrated the significant sensitivity of both epithelial and fibroblast cells from the gingival sulcus area to *HSV* infection, in vitro. Amit et al[7] suggested that *herpes simplex virus* was present in the latent form in the gingiva. Slots and Contreras[8] have hypothesized that herpes viruses cause local immunosuppression, impair protective immunity, induce proinflammatory cytokine production, alter the structural integrity of periodontium and lead to overgrowth of periodontopathic bacteria. herpes viruses are important DNA viruses in the pathogenesis of oral diseases. The hallmark of herpes infection is immune impairment. Activation of latent herpes virus infection can cause symptomatic or asymptomatic recurrent infection. Physical trauma, psychological or nutritional stress, immunosuppression, immune dysfunction and radiotherapy may trigger viral reactivation.

Data in the literature suggest an increased frequency of detection of specific members of the *Herpes viridae* family, such as *EBV-1, HCMV* and *Herpes simplex virus (HSV)* in various forms of periodontal disease. Studies[8,9,10] have reported that these viruses can infect and alter functions of polymorphonuclear leukocytes, lymphocytes and macrophages. This may potentiate the virulence of periodontopathogenic microbiota and dysfunction of polymorphonuclear leukocytes in periodontal sites can set the stage for overgrowth of periodontopathic bacteria and subsequent progression of destructive periodontal disease.

Inspite of circumstantial evidence of role of herpes viruses in destructive periodontal disease, a cause and effect relationship remains to be established. Therefore, the proposed bacterial-viral etiopathogenesis model of periodontitis needs to be verified by clinical and microbiological studies, as well as by research into molecular aspects of herpes virus infection in the periodontium.

Since, few studies have been done in this context , the present study was undertaken to detect and assess the presence of *HCMV, EBV-1* and *Herpes simplex virus type 1* and *type 2* (*HSV-1* and *HSV-2*) in patients with chronic periodontitis and to correlate the clinical parameters (plaque index and gingival index) with the presence of viruses in order to study the relationship between viruses and clinical severity of periodontal disease.

METHODS

In the present study, a total number of 30 patients of either gender above 35 years of age, were included having chronic periodontitis with probing pocket depths measuring >5mm (deep periodontal sites) and <5mm (shallow periodontal sites). Control group was not selected as comparisons were made between deep and shallow sites in the same patient. Patients with debilitating systemic diseases or co-existing oral infections, if they received antibiotic therapy six months prior to study, who had received periodontal treatment six months prior to the study, pregnant women and smokers were excluded from the study. A special proforma was designed to have a systemic and methodical recording of all the observations and information, which included a detailed case history, clinical examination and radiographic evaluation.

The patients for the present study were selected from the

outpatient department who presented to the Department of Periodontology at KLES Institute of Dental Sciences, Belgaum. A prior written informed consent was taken from all the patients, which was based on the Declaration of Helsinki (1964). Ethical clearance was obtained for the study from institutional ethical committee. The clinical examination included evaluation of age, sex, habits and past medical and dental history. The examination was done for assessment of the following parameters: plaque index (PI), gingival index(GI), clinical attachment loss (CAL). Subgingival plaque samples were collected 24 hours after the clinical examination in order to avoid blood contamination of the samples. Scaling was done to remove supragingival plaque. Subgingival plaque samples were obtained from deep and shallow periodontal sites of chronic periodontitis patients. The samples were collected with the help of sterile curettes in one single stroke and suspended in 500 ml of TE buffer (10 mM Tris-hydrochloride/ mM EDTA, pH 8).

Subgingival specimens were subjected to following procedures in microbiology laboratory:

1. DNA extraction
2. Multiplex polymerase chain reaction
3. Gel electrophoresis

In the present study, multiplex PCR method was used where more than one primer pair was included in the PCR mixture. Nucleotide Sequences of the primers used for the detection of viruses are described in Table1. Statistical analysis of the results was done using Z-test of proportion. Z-values >1.96 was considered to be significant and p-value <0.05 was considered to be significant.

Table 1: Nucleotide sequences of the primers used for the detection of viruses

HSV-1	H1P32	5'-TGG GAC ACA TGC CTTT CTT GG-3'
	H1M32	5'-ACC CTT AGT CAG ACT CTG TTA CTT ACC C-3'
HSV-2	H2M40	5'-GTA CAG ACC TTC GGA GG-3'
	H2P4	5'-CGC TTC ATC ATG GGC-3'
EBV	EP5	5'-AAC ATT GGC AGC AGG TAA GC-3'
	EM3	5'-ACT TAC CAA GTG TCC ATA GGA GC-3'
HCMV	CP15	5'-GTA CAC GCA CGC TGG TTA CC-3'
	Cm3	5'-GTA GAA AGC CTC GAC ATC GC-3'

RESULTS

Comparisons of viruses in deep and shallow sites are shown in Table 2. Results were not found to be statistically significant in the detection frequency of *HSV-1* (73.33%) in deep sites and (53.33%) shallow sites (z-value = 1.6764). *HSV-2* was found in 26.67% of deep sites and 0.00% of shallow sites which was highly statistically significant (z-value = 3.0382).

Table 2: Comparison of viruses in deep and shallow sites

Viruses	Sites		z-value
	Deep	Shallow	
HSV-1	22 (73.33%)	16 (53.33%)	1.6764 (NS)
HSV-2	8 (26.67%)	0	3.0382 (HS)
HCMV	4 (13.33%)	1(3.33%)	1.4013 (NS)
EBV-1	10 (33.33%)	1(3.33%)	3.0028 (HS)

NS – Non significant, HS – Highly significant, Z-values >1.96 Significant

Table 3: Comparison of plaque index with the presence of viruses in deep sites

Viruses	Fair		Poor		z-value
	No.	%	No.	%	
HSV-1	7	23.33	15	50.00	2.1432 (HS)
HSV-2	1	3.33	7	23.33	2.2787 (HS)
HCMV	0	0.0	4	13.33	2.0702 (HS)
EBV-1	1	3.33	9	30.00	2.7713 (HS)
None	5	16.67	3	10.00	0.7596 (NS)

NS - Non significant, HS - Highly significant

Table 4: Comparison of plaque index with the presence of viruses in shallow sites

Viruses	Fair		Poor		z-value
	No.	%	No.	%	
HSV-1	6	20.00	11	36.67	1.4325 (NS)
HSV-2	0	0.00	0	0.00	-
HCMV	0	0.00	1	3.33	1.0084 (NS)
EBV-1	0	0.00	1	3.33	1.0084 (NS)
None	7	23.33	7	23.33	0 (NS)

NS - Non significant

No statistically significant differences were seen in the detection frequency of *HCMV* (13.33%) in deep sites and (3.33%) shallow sites. Z-value was not found to be significant (Z-value = 1.4013). *EBV-1* was detected in 33.33% of deep sites and 3.33% of shallow sites which was highly statistically significant (z-value = 3.0028). Therefore, the results were found to be statistically significant in case of two viruses i.e. *HSV-2* and *EBV-1* when comparisons were made between deep and shallow sites. The results were not statistically significant in case of *HSV-1* and *HCMV*, although the detection frequency of viruses was more in deep sites as compared to shallow sites.

There was statistical significant relation to sites where all

the four viruses were present i.e. *HSV-1, HSV-2, HCMV* and *EBV-1* when compared with plaque index (Table 3). No statistical significant in relation to sites where no viruses were present. Correlation between plaque index and sites where viruses were present was found to be significant only in deep sites (Table 4). No statistically significant relationship was seen in both viral detected and undetected sites with the exception of sites containing *HCMV*. Results were not found to be statistically significant relation in both viral detected and undetected sites. Therefore, the correlation between the presence of viruses and gingival index was not statistically significant in deep as well as shallow sites.

DISCUSSION

There is a diverse body of evidence present regarding role of herpes viruses and periodontal diseases. It is yet to be established whether herpes viruses act as simple bystanders in periodontal disease or actually play an etiologic role in the pathogenesis of periodontal disease. Since the mid 1990s, herpes viruses have emerged as putative pathogens in various types of periodontal disease.[10] *HCMV* and *EBV* seem to play important roles in the etiopathogenesis of severe types of periodontitis. Genomes of the herpes viruses occur at high frequency in progressive periodontitis in adults, localized and generalized aggressive periodontitis, HIV- associated periodontitis, ANUG, periodontal abscesses and some rare types of advanced periodontitis associated with medical disorders.[11,12] The cytopathic changes in the herpes viruses which become infected by periodontal disease along with bacterial overgrowth and increased cytokine production ultimately leads to periodontal disease initiation and progression.[13,14] In the present study subgingival plaque samples were obtained from one deep and one shallow site of each patient. Most of the previous studies have determined the presence of herpes viruses in periodontal pockets by using subgingival plaque samples.[15,16,17,18]

Traditional detection assays for viruses are viral culture and direct antigen testing, which may lack the sensitivity and specificity to detect low level viral titers. Serological tests may not differentiate between past or reactivated infection. Therefore, detection of viruses in this study was done with rapid and sensitive polymerase chain reaction (PCR) assay which was the same technique used for viral detection in previous studies.[19,20,21,22]

HSV-1 was found in highest prevalence among all the four viruses followed by *EBV-1, HSV-2* and *HCMV*. All the four viruses occurred in higher frequency in deep sites as compared to shallow sites. The results were found to be statistically significant in case of *HSV-2* and *EBV-1*. These findings are in accordance with various other studies done in this context.

The results of the study are in agreement with another study done by Contreras and Slots[23], in which significant correlation was found between *HCMV* and periodontitis, which was significant in deep periodontal pockets compared to shallow pockets. The relationship of herpes virus co-infection with deepening probing depths suggests a positive relationship between herpes virus infection and clinical severity of periodontal disease.

Most of the studies which have been done in past have mainly studied the possible role of *HCMV* and *EBV* in the pathogenesis of periodontal disease, but the present study also attempts to throw some light on the possible role of *HSV-1*. In this study, the detection frequency of *HSV-1* was found to be highest in deep (73.33%) as well as shallow sites (53.33%) followed by *EBV-1, HSV-2* and *HCMV*. The variation found in the detection frequencies of viruses in the present study in contrast to other studies can be attributed to various possibilities. Other possibilities for the varying results can be partly due to patient selection criteria, variations in technique of sample collection and the methodology used for DNA extraction. Also, recent data suggest that viral detection and quantification are useful for identifying the individuals at high risk, but do not consistently predict the clinical outcome. Factors like viral strain, genotype and immune response in association with viral load affect the clinical outcome of the disease.

The most important objective of our study was to compare the clinical parameters (plaque index and gingival index) with the presence of viruses in order to study the relationship between viruses and clinical severity of periodontal disease. When the comparisons were made between viruses and plaque index in both deep and shallow sites, correlation was found to be highly significant in relation to deep sites (in both viral detected and undetected sites) but not in case of shallow sites. These findings are in accordance with the study done by

Saygun et al[18] where the differences in the measurements of plaque index in viral detected and undetected sites were statistically significant.

When the comparisons were made between gingival index and the presence of viruses no statistically significant differences were found in case of deep sites as well as shallow sites except for *HCMV* in case of deep sites. These findings are in accordance with the study done by Kamma et al[16] where *HCMV - EBV-1* co-infection revealed bleeding upon probing, a clinical sign of elevated risk for disease progression.

In a recent study, which was studied on Indian population, a highly statistically significant prevalence of *HSV-1* in subgingival samples from chronic periodontitis patients (76%) and aggressive periodontitis patients (80%) compared to healthy controls (12%) was seen.[24] These findings are in accordance with the present study, where the frequency of *HSV-1* was found to be highest in deep as well as shallow sites.

In another study done by Zeyad T et al, high percentage of chronic periodontitis was detected between 20-39 years old. Nested PCR showed detection of *EBV, CMV* and *HSV* in chronic periodontitis patients. *EBV* and *HSV* showed significant association with chronic periodontitis patients, but there was no association with *CMV.* Also, no statistical association was detected between pocket depth and type of virus detected.[25]

Herpes viral–bacterial interactions in periodontitis:

A periodontal herpes virus infection is typically associated with an increased occurrence of periodontopathic bacteria.[26] A study of adults with gingivitis or periodontitis found statistically significant associations between periodontal *Epstein– Barr virus type 1* or *cytomegalovirus* and the pathogens *P gingivalis, Tannerella forsythia, P intermedia, P nigrescens and Treponema denticola.*[27] Similarly respiratory tract infections, otitis media and other non-oral diseases that were previously thought to be caused solely by bacteria may actually have a combined viral–bacterial etiology.[26] Herpes viruses can exert direct cytopathic effects on fibroblasts, keratinocytes, endothelial cells and inflammatory cells, including polymorphonuclear leukocytes, lymphocytes, macrophages and possibly bone cells.[27] *Epstein–Barr virus* and *Cytomegalovirus* can also infect and alter the functions of monocytes, macrophages and lymphocytes in periodontitis lesions. Perhaps as result of a herpes virus periodontal infection, aggressive periodontitis lesions contain fewer overall viable cells, more T-suppressor lymphocytes and more B-lymphocytes (*Epstein–Barr virus* effect) than chronic periodontitis lesions or healthy periodontal sites.[28] A periodontal herpes virus infection may increase the pathogenicity of the periodontal microbiota. Herpes virus proteins expressed on eukaryotic cell membranes may act as new bacterial binding sites. *Cytomegalovirus* can enhance the adherence of *A. actinomycetemcomitans* to primary periodontal pocket epithelial cells and to HeLa cells.[29] Herpes viruses may induce abnormalities in the adherence, chemotaxis, phagocytic and bactericidal activities of polymorphonuclear leukocytes, which are cells of key importance for the control of periodontopathic bacteria.[26] *Epstein–Barr virus* active infection can also generate anti-neutrophilic antibodies and neutropenia, and polyclonally stimulate the proliferation and differentiation of B-lymphocytes. The pathogenic mechanisms of herpes viruses cooperate in exacerbating disease, and probably for that reason, a periodontal dual infection with *cytomegalovirus* and *Epstein– Barr virus*, or with *cytomegalovirus* and *herpes simplex virus*, tends to occur in severe types of periodontitis.[29] The interaction between herpes viruses and bacteria is probably bidirectional, with bacterial enzymes or other inflammation inducing factors having the potential to activate periodontal herpes viruses.[29] *Epstein– Barr virus* and *cytomegalovirus* infections up-regulate the interleukin-1β and tumor necrosis factor-alpha gene expression of monocytes and macrophages. Increased levels of pro-inflammatory cytokines in periodontal sites are associated with an enhanced risk of periodontal tissue destruction. The herpes virus-associated proinflammatory cytokines and chemokines can hamper the antibacterial host defence, stimulate bone-resorbing osteoclasts, up regulate matrix metalloproteinase and down regulate tissue inhibitors of metalloproteinase, thereby impeding tissue turnover and repair and increasing the risk of periodontal tissue breakdown. Also, periodontitis tends to be of greater severity in carriers of the HLA-DR4 alloantigen, perhaps because *cytomegalovirus* - specific CD8+ T cells can cross-recognize HLA-DR4 molecules and potentially induce autoimmune reactions.[29]

In the present study, the detection frequency of viruses was found to be more in deep sites as compared to shallow sites. Also, the correlation between viruses and clinical parameters was statistically significant in relation to deep sites.

Limitations: The actual viral load could not be detected as qualitative PCR was used rather than quantitative method. Smaller sample size was another drawback of the study.

CONCLUSION

Herpes viruses mainly *HSV-1* and *EBV-1* are frequently detected in chronic periodontitis lesions and viral load may be related to the clinical severity of periodontal disease. Moreover, the relationship between herpes virus infection and clinical periodontal conditions may be valuable in understanding their role in pathogenesis of periodontal disease and open new insights for the better treatment of periodontal disease in future, which may include vaccination against herpes viruses and hence better understanding of pathogenesis of periodontal disease.

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