

Detection of Herpes Viruses and Quantification of its Load in Periodontitis

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Many studies proved the prevalence of (HSV) Herpes Simplex Viruses and its involvement in occurrence of the periodontal disease and its progression. Recent studies prove effects of HSV viral load on disease severity and rate of progression in periodontitis. This study aimed to compare the prevalence and load of Herpes simplex virus 1 (HSV-1), Epstein-Barr Virus (EBV) and Human Cytomegalovirus (HCMV) in subgingival plaque samples and its effect on severity in chronic periodontitis and aggressive periodontitis. A total of 60 samples from the systematically healthy patients with chronic periodontitis n=30 (mean age, 35 ± 7). And n=30 aggressive periodontitis participated in this study. Clinical periodontal evaluation included the plaque index (PI) (Loe and Silness), bleeding on probing (BOP) (O'Leary), bleeding index, periodontal pocket depth (PPD) and Clinical Attachment Level Measurement. Plaque sample taken from > 6 mm periodontal pockets and from ≥ 3 mm sulcus depth in a quadrant of the same patient using periodontal curettes in chronic periodontitis and plaque samples taken from periodontal pockets in aggressive periodontitis with CAL >6mm. A Taq-man Real-Time Polymerase Chain Reaction assay was used to identify genomic copies of periodontal HSV-1, HCMV and EBV. Data were analyzed by the Wilcoxon-signed ranks and Friedman tests using the SPSS 16 software. Out of 60 samples of subgingival plaque samples taken from the patients with chronic periodontitis and aggressive periodontitis. HSV count was least in the tissue sample with PD < 3 mm to 5mm (P < 0.05) in moderate chronic periodontitis sites. The highest HSV count was in samples with PD > 6 mm (P < 0.05), in severe chronic periodontitis sites and aggressive periodontitis sites. According to the results of this study, quantification of HSV-1, HCMV and EBV observed in this study is high in plaque samples of aggressive periodontitis and severe chronic periodontitis sites compared with moderate chronic periodontitis sites.

Keywords: Periodontitis, hsv viruses, Real-Time PCR.

Apart from periodontopathic bacteria there are numerous infectious agents and its interconnected cellular and humoral host response play an important role in periodontitis. The various infectious agents apart from bacteria like viruses,

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fungi etc may cause the periodontal disease. Majority of studies focus on identification of periodontopathic bacteria, although some recent studies suggest the various viruses like Herpes viruses detection, involvement and in pathogenesis of periodontal disease. (1-3) Herpes viruses are capable of destroying periodontal structural cells and host defense cells thereby reducing its resistance to bacterial colonization. (4)

Many studies have shown the presence of HSV viruses like Herpes simplex-1(HSV-1) Human cytomegalo virus(HCMV) and Epstein –Barr virus(EBV) in periodontal sites .Presence of this virus change the periodontal micro flora favoring a severe pathogenesis^{3,5}.

Many number of studies indicates increased frequency of specific members of the herpes viridae detection, such as EBV-1, HCMV and herpes simplex virus-1 (HSV-1) in various forms of periodontal diseases^{1, 4, 5}.In which EBV-1 and HCMV may reside dormant in periodontal cells even after periodontal treatment⁶ causing destruction of alveolar bone^{7,8} HSV-1 and HCMV synergistically populate porphyromonas gingivalis in periodontitis site⁹.

Although prevalence of hsv viruses in periodontal diseases were detected in these various studies¹⁰, definitive role of virus as a unibody causing periodontal disease Is not yet proved. HSV virus may modify microenvironment by reducing host immunity there by supporting periodontopathic bacteria to colonise in diseased site⁴. Thus the amount of destruction does not depend only on prevalence but also on virus load in diseased site¹⁰.

Objectives

The current study regards to the detection and quantification of herpes viruses in chronic and aggressive periodontitis and correlate with the clinical parameters i.e Clinical attachment loss .there by showing the role of viral load in diseased sites . Subgingival plaque samples were collected from the diseased sites and quantification of HSV viruses is done with Real time PCR(RT-PCR). Study was done in Thai moogambigai dental college in deparment of periodontics.under the ethical clearance from Dr.MGR University And Research Institute.

MATERIALS AND METHODS

The study included n=60 patients (age range, 23 - 65 years) who needed a periodontal pocket elimination surgery. Chronic periodontitis patients(n=30)displayed clinical attachment loss of 4 - 9 mm and gingival inflammation in at least 3 posterior teeth. Aggressive periodontitis patients(n=30 displayed 5-9mm clinical attachment loss in molars and incisors with radiographically

seen vertical bone loss. Inclusion criteria were as follows: The patients did not have any systemic condition such as: diabetes, heart disease, asthma. absence of herpetic infection history and didn't have any history of antibiotic medication within the previous 2 months. None of the patients had previous periodontal surgery. They all had at least 20 teeth. They did not report the history of smoking, pregnancy or lactation. The patients were signed with consent form .

Clinical Procedure

Patients screened with periodontal examinations included plaque index (PI), bleeding on probing (BOP), bleeding index, periodontal pocket depth (PPD) and clinical attachment level measurement, from cemento enamel junction (CEJ) to the deepest probable pocket depth,the measurements with Williams probe (Hu-Friedy, USA) in 6 parts: midfacial, midlingual, mesiofacial, mesiolingual, distofacial, distolingual aspects of each tooth. Sub gingival Plaque samples were taken with curettes from Chronic periodontitis patients in deep CAL >6mm and shallow pockets CAL>3mm in same patients and from aggressive periodontitis in CAL>6mm. The plaque samples were immediately immersed in a transport medium 500µl of Phosphate buffered saline (pH 8.0), transported in ice and stored at -20°C till assay.and given to hi tech diagnostics lab ,Chennai .

Laboratory Procedures

The subgingival plaque samples in Phosphate buffer solution (PBS)were microcentrifuged at 8,000rpm for 5minutes, supernatant discarded and the pellet was resuspended in 100µl of lysis broth (10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, 1.0% Triton X-100, pH 8.0). The lysis broth was further vortexed and kept in water bath at 100°C for 5 minutes, cooled and centrifuged at 4000 rpm for 2 minutes and the supernatant was stored at -20°C .For viruses the last setup of 25 µL Taq Man PCR (including 5 µL of specimen, 12.5 µL Mater Universal mixtures, 5 pmol primer, 4 pmol Taq Man probe) was done. PCR quantification was performed using plasmid dilution (one-copy per mL). The detection process was as follows: 95°C for 10 minutes, followed by 50 cycles of 94°C for 10 seconds, 60°C for 32 seconds, and 72°C for 25 seconds. The reactions are done in a 7500 RT- PCR system tool .

RESULTS

Pairwise comparisons between plaque samples with CAL < 3 mm in CP and plaque samples with CAL > 6 mm in Both CP AND AP mm were checked using the Wilcoxon-signed rank test. HSV-1, EBV and HCMV counts were compared with Friedman test. Differences if were statistically significant, pairwise comparison performed using a Wilcoxon-signed rank test.

The result on the comparison of each virus in plaque samples, cal ≤ 3 mm and cal > 6 mm is demonstrated in the Table 1 and the results of the comparison between hsv-1, EBV and CMV counts in plaque samples are shown in Table 2.

Herpes simplex virus 1

The HSV-1 count in subgingival plaque was more in sites more than CAL > 6 mm and the

load in the subgingival plaque samples with CAL < 3 mm was minimal. statistically significant (P < 0.05) (table 1) in both chronic periodontitis and aggressive periodontitis

Epstein-Barr Virus

The EBV count in subgingival plaque sample was also more than its amount in the SUBGINGIVAL PLAQUE samples with CAL > 6 mm and the EBV load in CAL < 3 mm was the least (Table 1). All the differences were statistically significant (P < 0.05). in both chronic periodontitis and aggressive periodontitis.

Human Cytomegalovirus

The HCMV count was similarly higher in subgingival plaque sample CAL > 6 mm. On the other hand, HCMV count was lower in CAL ≤ 3 mm samples (Table 1). in both chronic and aggressive periodontitis.

Table 1. Based on the Freidman test. P-values less than 0.05 are considered as statistically significant

Groups	Mean ± SD	Median (Mean Rank)	Significant Pairwise Comparison	P Value
HSV-1				0.012
CAL > 6 mm	5632 ± 6934			
CAL ≤ 3 mm	103.34 ± 422.98	0.00 (1.23)	0.00 c PD > 6 mm vs. PD ≤ 3 mm	
EBV				0.00
CAL > 6 mm	3170.24 ± 4877.76	837.00 (2.42)		
CAL ≤ 3 mm	113.22 ± 416.25	0.00 (1.64)	0.00 c PD > 6 mm vs. PD ≤ 3 mm	
HCMV				0.013
PD > 6 mm	623.27 ± 1636.25	0.00 (1.99)	0.00 c PD > 6 mm vs. PD ≤ 3 mm	
PD ≤ 3 mm	119.38 ± 405.67	0.00 (1.80)		

Table 2. Based on the Freidman test. P-values less than 0.05 are considered as statistically significant

Groups	Mean ± SD	Median	P Value
PD > 6 mm			0.001
HSV-1	5632 ± 6934	0.00	
EBV	3170.24 ± 4877.76	0.00	
HCMV	623.27 ± 1636.25	0.00	
PD ≤ 3 mm			0.001
HSV-1	103.34 ± 422.98	0.00	
EBV	113.22 ± 416.25	0.00	
HCMV	119.38 ± 405.67	0.00	

Herpes Simplex -1-1, Human Cytomegalovirus and Epstein-Barr Virus Comparison

In comparison of HSV-1, HCMV and EBV counts, the HSV-1 load was statistically higher in the PLAQUE samples with CAL > 6 mm (P = 0.001). In comparison of HCMV and EBV counts, the difference in PLAQUE samples with CAL < 3 mm was not statistically significant (P > 0.05). On the other hand, EBV count was significantly higher than HCMV in tissue samples with PD > 6 mm (P < 0.05) (Table 2). The frequency of HSV-1 was 28% in PD > 6 mm in CP AND 32% IN CAL > 6MM IN AP and 10.8% in PD d" 3 mm. The frequency of EBV in the same group was 21.6% in PD > 6 mm and 8.1% in PD d" 3 mm. and HCMV was 20.8% in CAL > 6MM and 5.1% in CAL < 3mm similar in both chronic and aggressive periodontitis table 2

DISCUSSION

The Current Study Was Conducted To Detect HSV-1, HCMV And EBV In Subgingival Plaque Samples Of Chronic Periodontitis Patients And Aggressive Periodontitis Using The RT-PCR. We Observed The Highest Amount Of HSV-1 in Samples With CAL > 6 Mm And The Highest Amount Of EBV and HCMV In CAL > 6 Mm Subgingival Plaque Samples Of Patients With Chronic Periodontitis And Aggressive Periodontitis. Many Studies Have Demonstrated The Association Between Herpes Viridae And The Periodontal Disease. Herpes Viruses Like EBV, HCMV, HSV And Human Herpes Virus (HHV 6-8) And Transfusion Transmitted Virus (TTV) (A New One) Causes Periodontal Disease^{10, 13, 14}. Latency and reactivation of viruses other than its cytopathic properties effects periodontitis⁴. cytokine release during its reactivation produces destruction of periodontal tissues⁸. Latency of these viruses may have more damage To The Periodontal Regeneration¹⁰.

EBV and HSV-1 are mostly related to chronic and aggressive periodontitis. probing depth and attachment loss were higher in the presence of these viruses¹². Recent studies show the presence of viruses more in GCF sample collected between periodontitis and gingivitis^{6, 14-17}. Few studies evaluated the frequency of viral presence in saliva and gingival tissues. In the study detection of high frequency of viral load was

reported in the chronic periodontitis in comparison with healthy sites^{6, 10, 13, 15, 18-21}. Present study confirmed the PRESENCE OF HSV -1 EBV and HCMV counts IN Chronic And Aggressive Periodontitis. As Many Studies Like Contreras et al. could not detect any VIRUS particles in healthy periodontal tissue¹. Sample Collection In The Healthy Sites Less Than 3mm Cal Is Avoided . Most Studies Just End In Detection Of The Viruses Our Study Was Presented With The Viral Counts. Of HSV-1 ,EBV, HCMV detected.

Viral prevalence have been reported in different populations. in different studies Saygun in 2002¹⁹, Turkish samples. Imbrono (18) and Bilichodmath¹³ reported that HSV-1 and EBV-1 were more frequently associated with chronic periodontitis and aggressive periodontitis, respectively this study was done in a south indian population Though the percentages varied greatly for EBV: 78.9%¹³, 46.7%, for HSV-1: 100%¹³, 40%¹⁸ and for HCMV: 26.31%¹³, 50%¹⁸. In other studies, the results were similar in presence of the viral contents in samples.

Though PCR Was The Most Preferred Detection Aid In Many Studies¹⁰ or nested PCR^{13, 14, 18, 22} the present study we adopted RT-PCR. Polymerase chain reaction is a gene amplification method, which allows quantification of microorganisms with selective DNA segment manipulation²³. Nested PCR has both advantages (specificity and sensitivity and disadvantages like false positive results due to its contamination susceptibility technique²³. Real-time PCR is a more appropriate quantitative method for detection of viruses. Polymerase chain reaction is a qualitative test that just focuses on the existence of virus, which can be affected by confounding environmental factors. In addition, viruses like EBV and HCMV can remain latent in lymphocytes which are the normal inhabitants of periodontal sulcus and pocket wall; so, mere existence of viruses is not valuable enough²⁴.

Studies Like Saygun R et al.¹⁷ Which Did Not Compare Their Test Group With A Normal Control Group; We Adopted Similar Study Comparing Samples With Cal < 3 Mm In Chronic Periodontitis And Cal > 6 Mm In Both Chronic And Aggressive Periodontitis¹⁷. Yildirim Et Al. Reported Hcmv And Ebv Detection In Kostmann syndrome with RT-PCR in 2006 (25, Saygun *et al.*

classified chronic and aggressive periodontitis just by age range, which does not seem to be scientific¹⁷. OUR STUDY WE attach radiographic aids to vary the bone loss pattern to differentiate chronic and aggressive periodontitis.. The Aggressive Periodontitis Sites >6mm Showed More Viral Load.

We observed 13.5% HCMV and EBV-1 coinfection, 15% HSV-1 AND EBV COINFECTION in some samples CAL d" 3 mm and PD > 6 mm. This result was in line with Jahangirnezhad (10) and Kubar *et al.*,²¹ studies that reported 52% HCMV and EBV-1 coinfection in aggressive periodontitis respectively. Kubar *et al.* also reported a 27% coinfection in chronic periodontitis²⁶. Grenier *et al.*,¹⁴ showed higher copy number of HCMV presence in GCF of deep periodontal pockets which is in agreement with our study on tissue . They also postulated that periodontal therapy can eliminate EBV and HCMV, and reduce HSV in GCF.

Some difficulties of virus DNA extraction from subgingival plaque samples are as follows: 1) It is hard to collect without contamination of blood and saliva , 2) low amount of plaque samples in aggressive periodontitis sites, and 3) absence of viable proteins in plaque samples to detect viral load accurately we suggest a cohort study for an investigation on the cause and effect relationship of the herpes viruses in the periodontal disease.

Current study demonstrated considerable number of viruses present in this sample population. and increase in load causing severity of disease or vice versa which remained a challenge to find.

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