10.5005/jp-journals-10024-1781

ORIGINAL RESEARCH

Prevalence of Herpesvirus and Correlation with Clinical Parameters in Indian Subjects with Chronic Periodontitis

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ABSTRACT

Objective: The identification of new uncultured species and viruses supports the possibility of combination of the herpesvirus-bacterial periodontal infection for periodontitis. The paucity of data and studies with larger sample size in Indian subjects provides an unclear picture of the presence of the herpesvirus in this population.

Materials and methods: This was a cross-sectional study consisting of 100 each in the healthy group and chronic periodontitis (CP) group. The subgingival plaque was collected and polymerase chain reaction was performed post deoxyribonucleic acid (DNA) extraction by using specific primers for human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV). The data were analyzed using Fisher's exact test, Mann-Whitney U test and Spearman's coefficient correlation.

Results: Human cytomegalovirus and EBV viruses were significantly higher in the CP group as compare to the healthy group. A higher percentage of those with CMV positive had EBV also positive (28.3%) compared to only 9.1% of CMV negative being EBV positive in the CP group. When both the healthy and CP group in total was compared, there was a significant correlation with all clinical parameters.

Conclusion: Both the viruses dominated in disease as compared to health were similar to the earlier findings. The CP group had higher pocket depth and clinical attachment loss in the virus positive subjects. These findings could suggest that virus serves as a prelude to the disease and the combination of the two viruses could play a role in the pathogenesis.

Keywords: Chronic periodontitis, Epstien-Barr virus, Human cytomegalovirus, Polymerase chain reaction, Subgingival plaque.

How to cite this article: Joshi VM, Bhat KG, Katti SS, Kugaji MS, Ingalgi PS. Prevalence of Herpesvirus and Correlation with Clinical Parameters in Indian Subjects with Chronic Periodontitis. J Contemp Dent Pract 2015;16(11):915-920.

Source of support: Nil

Conflict of interest: None

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INTRODUCTION

The occurrence of periodontitis has been associated with multiple factors of that the bacteria and the host immune response were customarily considered the most important. However, with the identification of new uncultured species and viruses the new concept supports the possibility of a combination of the herpesvirus-bacterial periodontal infection for periodontitis.[1,2]

There has been recent evidence of the various different types of viruses in the human oral cavity.[3] The herpesviruses, especially Epstein-Barr virus (EBV) and the human cytomegalovirus (HCMV) have been detected at destructive periodontitis and sites with active periodontal disease.[4] The higher prevalence of these viruses in combination with the anaerobic bacteria and the host immune factors may aggravate the progress of the disease.[5] There has been conflicting evidence found in a few studies from around the globe which suggest that the EBV and the HCMV are present at fewer periodontal sites than considered otherwise.[6-12]

Because of the paucity of data and studies with larger sample size in Indian subjects, the exact picture of the presence of the herpesvirus in this population is still unclear. This study was undertaken to determine

the prevalence of the EBV and HCMV in subjects with healthy periodontium and chronic periodontitis patients and also to determine if any relation exists between the occurrence of a virus and periodontal status.

MATERIALS AND METHODS

This was a cross-sectional study consisting of 200 subjects (healthy subjects healthy group n = 100 and chronic periodontitis (CP) subjects–CP group n = 100) from Belgaum, Karnataka, India. The subjects included in the study were periodontally healthy with the absence of sites with pocket depth of > 3 mm in the healthy group. In the chronic periodontitis group the subjects were selected according to the classification laid down by the American Association of Periodontology, International Workshop for Classification of Periodontal Diseases.[13] Subjects with history of smoking, undergoing orthodontic therapy, have had antibiotic therapy or professional cleaning within the previous 3 months, need for antibiotic coverage before dental treatment, currently on immunosuppressant medications, bisphosphonates or steroids, or having diabetes. The selected subjects were with at least 20 natural, non-carious teeth, with at least three posterior teeth (premolars and molars) in two quadrants (maxillary and mandibular right or left sections) without interproximal restorations (fillings that extend to the sides of a tooth), ≥5 mm probing pocket depths (PD) and clinical attachment loss (CAL) ≥ 3 mm at the test sites (indicative of periodontal pockets). All subjects signed an informed consent after the details of the study were explained. The ethical committee of the Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belgaum, reviewed and approved the study.

CLINICAL PROCEDURE AND SAMPLE COLLECTION

The clinical parameters (PD, CAL), plaque and gingival indices (PI, GI) were recorded using a UNC-15 probe. For the collection of the subgingival plaque, the selected area was air dried and supragingival plaque removed with sterile cotton rolls. Using a universal curette subgingival dental plaque was collected from the most apical portion. Plaque from a total of six sites was harvested, pooled and transferred into a vial containing reduced transport fluid medium for each patient. Sites for sampling were randomly selected from both the posterior and anterior teeth.

Polymerase Chain Reaction Assay

The collected plaque samples were then used for deoxyribonucleic acid (DNA) extraction. The modified proteinase K method was used for DNA extraction. The method is mentioned briefly here. Samples are vortexed and washed in Tris-EDTA buffer, pH 7.5. Lysis buffer I and II are added, followed by Proteinase K (10 mg/ml) (Chromous Biotech, Bengaluru, Karnataka, India) and incubated at 600 C for 2 hours then kept in boiling water bath for 10 minute to inactivate the enzyme. The sample was then centrifuged and the supernatant containing the DNA was elected in a separate tube and stored at –200°C till further processing.[14]

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed by using specific primers for the 16S rRNA gene of HCMV and EBV virus. The following primers were used:

Human cytomegalovirus primer sequences 5′-ACG TGT TAC TGG CGG AGT CG -3′ as forward and 5′-TTG AGT GTG GCC AGA CTG AG -3′ as reverse Epstein-Barr virus primer sequences 5′-AGC ACT GGC CAG CTC ATA TC -3′ as forward 5′-TTG ACG TCA TGC CAA GGC AA -3′ as reverse[15] Polymerase chain reaction amplification was done in a veriti thermal cycler (Applied Biosystems, Grand Island, NY, USA) in a 20 µl reaction mixture (Qiagen, Duesseldorf, Germany) containing: dNTP mix-10 mM each, 10× PCR buffer containing 15 mM of $MgCl_2$, Taq DNA polymerase 1.5 units/reaction, DNA templates ≤ 1µg/reaction. A primer concentration of 2.5 pico mole was used. The following thermal cycle conditions were applied; initial denaturation at 95°C for 5 minutes followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds, and a final step of 72°C for 5 minutes.[15]

Polymerase chain reaction products were detected by agarose gel electrophoresis of amplified samples on 2% agarose. A 100bp DNA ladder was loaded onto the gel simultaneously with the samples. The gel was stained with 0.5 µg/ml of ethidium bromide for 30 minutes. The gel was visualized and the results were recorded by Gel doc system. (Major Science, Saratoga, CA, USA). The molecular size of 368 base pairs for HCMV and 326 base pairs for EBV was detected by comparing the band position with the 100bp DNA ladder.

STATISTICAL ANALYSIS

The results from the current study were analyzed taking into account the group-by-group presence or absence of HCMV and EBV viruses. The detection of HCMV and EBV viruses in healthy and CP group was compared using Fisher's exact test. The clinical parameters were averaged across the subjects in each group and the difference among the virus positive and virus negative was compared using Mann-Whitney U test. The Spearman's rank correlation coefficient was used to compare the clinical parameters with the presence of viruses. The difference of $p < 0.05$ among the groups was

considered as statistically significant. All calculations were done using the Statistical Package for the Social Sciences (SPSS) software package, version 11.0.

RESULTS

In this study, a higher percentage of CMV positive cases were diseased (67.4%) when compared to CMV negative cases (44.8%). Also, higher disease percentage was observed in EBV positive cases (77.8%) when compared to EBV negative cases (45.7%) and the difference between the groups was statistically significant ($p < 0.011$, $p < 0.003$ respectively). The prevalence of HCMV was 31 and 15% and that of EBV was 21 and 6% in chronic periodontitis and healthy group respectively (Table 1).

There was a significant relationship between CMV and EBV prevalence. A higher percentage of those with CMV positive had EBV also positive (28.3%) compared to only 9.1% of CMV negative being EBV positive. This relationship was evident in the diseased group when analyzed separately. However, in healthy individuals there was no significant relationship between EBV and CMV prevalence (Table 2).

The comparison of the clinical parameters in the virus positive and virus negative subjects showed a significant higher CAL in HCMV and EBV-positive individuals in the healthy group ($p \leq 0.01$). The CP group showed a significantly higher pocket depth in HCMV positive subjects ($p = 0.019$) as seen in Table 3. Although, EBV positive cases in CP group had higher levels with all clinical parameters the difference was not statistically significant when compared to the negative case (Table 4). When both the healthy and CP group in total was compared, there was a significant correlation with all clinical parameters as shown in Tables 5 and 6.

The linear regression analysis showed none of the factor influencing the clinical parameters when adjusted for age, sex and herpesvirus (CMV, EBV) when analysed for both groups.

Table 1: Cross tabulation showing relationship between the CMV and EBV positive status and the proportion of CP group and healthy group

		Group			
Measure		*CP group (n = 100)*	*Healthy group (n = 100)*	*Total*	*Fisher's exact test*
Presence of CMV (n = 100)		69	85	154	p < 0.011, S
	Negative	44.8%	55.2%	100.0%	
		31	15	46	
	Positive	67.4%	32.6%	100.0%	
		100	100	200	
	Total	50.0%	50.0%	100.0%	
Presence of EBV (n = 100)		79	94	173	p < 0.003, S
	Negative	45.7%	54.3%	100.0%	
		21	6	27	
	Positive	77.8%	22.2%	100.0%	
		100	100	200	
	Total	50.0%	50.0%	100.0%	

p < 0.05; S: Significant

Table 2: Relationship between the CMV and EBV in all cases, CP group and healthy group

		EBV			*Fisher's exact test*
Measure		*Absent*	*Present*	*Total*	
CMV in all cases (n = 200)		140	14	154	p < 0.002, S
	Absent	90.9%	9.1%	100.0%	
		33	13	46	
	Present	71.7%	28.3%	100.0%	
		173	27	200	
	Total	86.5%	13.5%	100.0%	
CMV in diseased (n = 100)		60	9	69	p < 0.007, S
	Absent	87.0%	13.0%	100.0%	
		19	12	31	
	Present	61.3%	38.7%	100.0%	
		79	21	100	
	Total	79.0%	21.0%	100.0%	

p < 0.05; S: Significant

Table 3: Comparison of periodontal parameters between the CMV negative and positive cases compared by Mann-Whitney U test in periodontally healthy and patients

		Healthy group				*CP group*			
	CMV	*N*	*Mean*	*Std. Dev.*	*p-value*	*N*	*Mean*	*Std. Dev.*	*p-value*
GI	CMV Negative	85	0.48	0.19	0.565, NS	69	2.52	0.21	0.168, NS
	CMV Positive	15	0.53	0.19		31	2.57	0.20	
PI	CMV Negative	85	0.46	0.16	0.710, NS	69	2.50	0.20	0.580, NS
	CMV Positive	15	0.45	0.22		31	2.52	0.20	
PD	CMV Negative	85	2.45	0.47	0.854, NS	69	5.83	0.51	0.019, S
	CMV Positive	15	2.42	0.61		31	6.11	0.57	
CAL	CMV Negative	85	2.47	0.36	0.011, S	69	5.38	0.37	0.104, NS
	CMV Positive	15	2.69	0.26		31	5.56	0.58	

p < 0.05; NS: Nonsignificant; S: Significant

Table 4: Comparison of periodontal parameters between the EBV negative and positive cases compare by Mann-Whitney U test in periodontally healthy and CP group

		Healthy individuals				*CP group*			
	EBV	*N*	*Mean*	*Std. Dev.*	*p-value*	*N*	*Mean*	*Std. Dev.*	*p-value*
GI	EBV Negative	94	0.48	0.19	0.242, NS	79	2.52	0.21	0.375, NS
	EBV Positive	6	0.59	0.20		21	2.57	0.21	
PI	EBV Negative	94	0.46	0.17	0.152, NS	79	2.50	0.21	0.421, NS
	EBV Positive	6	0.36	0.14		21	2.52	0.15	
PD	EBV Negative	94	2.45	0.50	0.850, NS	79	5.90	0.53	0.714, NS
	EBV Positive	6	2.40	0.49		21	5.98	0.57	
CAL	EBV Negative	94	2.49	0.35	0.004, S	79	5.41	0.37	0.299, NS
	EBV Positive	6	2.83	0.18		21	5.54	0.66	

$p < 0.05$; NS: Nonsignificant; S: Significant

Table 5: Comparison of periodontal parameters between the CMV negative and positive cases compare by Mann-Whitney U test

	CMV	*N*	*Mean*	*Std. Dev.*	*p-value and significance*
GI	CMV Negative	154	1.39	1.03	0.002, S
	CMV Positive	46	1.90	0.99	
PI	CMV Negative	154	1.37	1.03	0.015, S
	CMV Positive	46	1.84	1.00	
PD	CMV Negative	154	3.97	1.76	0.004, S
	CMV Positive	46	4.81	1.76	
CAL	CMV Negative	154	3.78	1.49	0.001, S
	CMV Positive	46	4.62	1.45	
AA culture	CMV Negative	154	6.29	15.08	0.023, S
	CMV Positive	46	11.80	18.04	

$p < 0.05$; S: Significant

Table 6: Comparison of periodontal parameters between the EBV negative and positive cases

	EBV	*N*	*Mean*	*Std. Dev.*	*p-value and significance*
GI	EBV Negative	173	1.41	1.04	0.001, S
	EBV Positive	27	2.13	0.86	
PI	EBV Negative	173	1.39	1.03	0.002, S
	EBV Positive	27	2.04	0.93	
PD	EBV Negative	173	4.00	1.77	0.002, S
	EBV Positive	27	5.17	1.60	
CAL	EBV Negative	173	3.82	1.50	< 0.001, S
	EBV Positive	27	4.94	1.29	
AA culture	EBV Negative	173	5.97	14.46	0.002, S
	EBV Positive	27	17.70	20.96	

$p < 0.05$; S: Significant

DISCUSSION

The etiology for periodontitis has been known to be multifactorial, but primarily is the interplay among the host immune response, and a wide range of bacteria along with the viruses.[3] The herpesvirus related destructive periodontitis has been proposed to be a complex involvement of host factors, the multiple bacteria, environmental factors and herpesviruses.[2]

The current cross-sectional study of 200 subjects that included 100 each of periodontally healthy subjects and CP patients. The subgingival plaque samples were subjected to PCR for highly specific conserved regions of 16s rDNA of HCMV and EBV.

The occurrence of the virus could depend on the periodontal disease status, geographic location, ethnicity and herpesvirus state of latency or reactivation.[2,16] The prevalence of the herpesvirus is shown to be varying in studies conducted all around the globe. The data from the healthy group in our study was similar to the findings of Dani et al,[17] who found 20% of HCMV and 7% EBV prevalence in Indian subjects. In a few studies, EBV is detected in a healthy oral cavity which is similar to our findings, but with no HCMV detection.[7,18,19] Although few studies showed similar detection rates of HCMV as in our study, there has been a wide and varied report with high levels of viruses to none in certain population with healthy periodontium.[7,9,17,18,20-23]

In CP, there have been varied reports with some studies finding very high levels of EBV and HCMV.[10,18,20,21,23] Our results showed significantly higher levels of both the HCMV and the EBV virus in CP as compared to the healthy periodontium but still was lower than the median range as quoted by Slots 2010.[2] In his study, we noted that a co-infection of HCMV and EBV existed in CP group and this relationship was significant. In the CP group 28.3% of the HCMV positive sites also had EBV positive and this association was in line with previous reports which identified this close relationship. A study done by Contreras et al in American patients showed that EBV and CMV were detected in 79% and 17% respectively.[22] In Greece, the prevalence of EBV and CMV was 44 and 56% in CP.[24] Chalabi et al showed the prevalence of EBV-1 and CMV in Iran patients was 78.3 and 59% respectively.[18] Similarly, Kubar et al found

EBV and HCMV at 56% for both in periodontitis in their study.[25] In our study the prevalence of EBV and HCMV were 21 and 31% respectively in CP patients.

The occurrence of herpesvirus is known to vary with different geographical locations, ethnicity, type of periodontal lesion being studied and the viral identification technique employed.[2,7] The current study found the occurrence of both the viruses similar to the previous reports by Sharma et al[9] and Dani et al,[17] however, Bilichodmath et al[10] found higher levels of EBV in chronic periodontitis patients. The findings of the present study were at lower levels as compared to some studies done in other countries.[18,20-23] This could also be because the detection levels may depend on the virus being in the active or latent stage. The primary infection of the host with herpesvirus is followed by a state of latency and reactivation. In an inactive state, the viruses are known to reside in the cell for a very long-time without been identified by the host immune system. It is seen that an individual with herpesvirus infection may not reveal herpesvirus in all periodontal sites or if inactive state.[26] The other possibility being the samples could be from sites which are inactive or the virus being in the latent state, or the plaque collected may not be from the sites which were virus-rich in periodontium.[7]

When the clinical parameters between the virus positive and negative sites were compared, it showed a significantly higher CAL in the healthy group for both viruses and significantly deeper pockets in the CP group with HCMV only. In the current study, pocket depth was deeper in EBV positive sites as compared the negative sites, the difference was not significant. Although the findings of present study were similar to the finding of Sharma et al,[9] in this study there was no correlation between the viruses and clinical parameters when adjusted for age and gender (Univariate analysis). They did not see any CAL in healthy subjects which could depend on the different subject inclusion criteria. Saygun et al[4] found a significant difference in the pocket depth and the CAL of detected and undetected sites for both HCMV and EBV. Similarly, Ling et al[8] found higher levels of both HCMV and EBV in diseased sites, but only showed a significantly higher level of EBV in deeper pockets. The variation in the results may be because of discrepancies in the sample size, the study design with differences in inclusion criteria or it is a site-specific study design and ethnic differences in the study subjects.[8] In the current study, when the clinical parameters of both the group in total were compared with the occurrence of the HCMV and EBV virus, all the clinical parameters were at significantly higher levels in both the virus positive sites as compared to the virus negative sites. These findings may suggest that virus serves as a prelude to the disease and the combination of the two viruses could play a role in the pathogenesis.[7,8]

Further, studies could be planned which will include a larger sample size with techniques and measures that will determine the disease activity at the individual site and provide insight into the role of the herpesvirus. We are conducting a study on the relationship between these viruses and some periodontal bacteria.

CONCLUSION

The findings of this study of 200 Indian subjects suggest that HCMV and EBV are detected from subgingival plaque samples of both healthy oral cavity and in CP. The clinical parameters were significantly increased in HCMV and EBV positive sites as compared to virus negative sites when considered in total. The role of these viruses will be well defined from further studies that shall evaluate the presence of key periodontal pathogens from the same subgingival samples.

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