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Original Article

Microbiology Section

Association of Herpes Viruses with Mild, Moderate and Severe Chronic Periodontitis

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ABSTRACT

Introduction: Periodontal disease is an inflammatory condition of the supporting tissues of the teeth. It is a multi-factorial and multi-etiological infectious disease process. Recent evidences shows that human herpes viruses could be putative pathogens. The aim of the present study was to evaluate the association of Herpes viruses especially Herpes simplex viruses (HSV-1 and 2), Epstein-Barr virus (EBV) and Human cytomegalovirus (HCMV) in patients with chronic periodontitis.

Materials and Methods: A total of 75 patients with periodontitis were included in the study (25 each with mild, moderate and severe periodontitis) with ethical approval and informed consent. Sub gingival plaque sample was collected and subjected to extraction of DNA and further analysis with multiplex Polymerase chain reaction for the presence of herpes viral DNA.

The collected data was entered in the excel sheet format. It was subjected to statistical analysis using SPSS software. The Chi-Square statistical tests was applied and p-value<0.05 was taken as significant.

Results: The overall association of HSV-1, HSV-2, EBV and CMV was 28%, 32%, 30.66% and 37.33% respectively in the present study from the cases of chronic periodontitis.

Conclusion: Epstein Barr viruses were detected from all types of cases of chronic periodontitis in the present study. Though, EBV was not significantly associated with periodontitis; they were significantly increased in severe periodontitis. Herpes viruses were significantly associated with periodontal disease, more so with severe periodontal disease. They could thus be playing a role in increasing the severity of the disease. Therapeutic and prophylactic intervention planned against these viruses could decrease the tooth loss associated with this disease.

Keywords: Interactions, Multiplex PCR, Periodontal disease

INTRODUCTION

Periodontal disease is an inflammatory condition of the supporting tissues of the teeth. It is a multi-factorial and multi-aetiological infectious disease process [1,2]. The clinical presentation is a result of interaction between microbes and the host immune response. The microbe triggers the inflammatory reactions and this result in the loss of connective tissue attachment and alveolar bone around the teeth.

The primary aetiological factor in periodontitis is the bacterial plaque which accumulates due to poor oral hygiene [3]. The microbes involved in periodontal disease are a complex community of microorganisms, many of which are still difficult to isolate and culture in the laboratory. The most important hurdle in identifying association of pathogens with the disease condition is that the pathogens involved are present in disease but occasionally are also present in the healthy periodontium though in low numbers. The chronic nature of periodontal disease has made the search for the infecting pathogens more difficult. This is due to fact that the disease progresses at different rates with alternating episodes of rapid tissue destruction and period of remission. To date bacteria are the main aetiological factors in the periodontal diseases especially the Gram negative species are more commonly reported [4].

Recent evidences shows that human herpes viruses and yeast could be putative pathogens also [5,6]. Various studies have shown that human herpes viruses, especially Herpes simplex virus (HSV-1&2), Human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) play a role in the pathogenesis of periodontal diseases [7-9]. It is often felt that the development of periodontal disease depends upon cooperative interactions between herpes viruses, specific pathogenic bacteria and destructive inflammatory mediators [10,11].

Viruses could be playing a role by increasing the severity of the periodontal disease. The herpes viruses encourage inflammatory and non-inflammatory cells to release cytokines and chemokines which may impair the local periodontal immune defense, resulting in the establishment of more virulent resident periodontopathic bacteria. Thus, together they may cause more severe forms of the disease [12,13].

The aim of the present study was to evaluate the association of Herpes viruses especially Herpes simplex viruses (HSV-1 and 2), Epstein-Barr virus (EBV) and Human cytomegalovirus (HCMV) in patients with chronic periodontitis.

MATERIALS AND METHODS

The present study was undertaken in Sinhgad Dental College and Hospital Pune, India. Ethical committee approval and informed consent from participating patients were taken during the study period from January 2012 to June 2012. A total of 75 patients with periodontitis were included in the study (25 each with mild, moderate and severe periodontitis).

The inclusion criteria: No history of smoking, No antiviral drugs in the previous six months and absence of systemic disease such as diabetes and cardio vascular diseases. The patients were included as per clinical attachment loss based upon the classification given below.

The classification of mild, moderate and severe chronic periodontitis was done as previously described [14]:

Mild : 1 to 2 mm of clinical attachment loss

Moderate : 3 to 4 mm of clinical attachment loss

Severe : > 5 mm of clinical attachment loss

Exclusion criteria: The patient who had a history of smoking, antibiotics in recent and had any systemic illness such as diabetes, cardiovascular disease.

Sample collection: The sub gingival plaque sample was collected with the help of curette from the affected site from each category of mild, moderate or severe chronic periodontitis. It was then transferred to the tube containing Tris-EDTA (TE) buffered saline (Hi Media India). It was further processed for DNA extraction and multiplex polymerase chain reaction. The procedure used for DNA extraction is given below.

DNA Extraction [15]: The sample was vortexed to dislodge the plaque into the TE buffer medium (HiMedia) and then centrifuged at 5,000 rpm for 5 minutes and the supernatant discarded. Five hundred microliter fresh TE buffer was added and centrifuged for 3-4 minutes. The procedure was repeated for 2-3 times using fresh buffer each time. Supernatant was discarded. Fifty micro liter Lysis buffer was then added and vortexed for 5 minutes. Fifty microliter of Lysis buffer II (HiMedia) and 10 microliter Proteinase–K (100ug/ml) (Chromous Biotech India) was added and the mixture was vortexed vigorously. The tubes were then kept in water bath for 2 hours and then kept in boiling water bath for 10 minutes to deactivate the enzyme. Finally all extracted samples were stored in -20^{0}C

Multiplex PCR: PCR Master Mix was prepared containing dNTPs: 10mM, PCR Taq polymerase buffer: 10X Taq DNA polymerase: 1.5 U/reaction) (Chromous Biotech India)

The primers used in the study were as follows [16]:

HSV-1:

Forward: 5'-CGTACCTGCGGCTCGTGAAGT-3'

Reverse: 5'-AGCAGGGTGCTCGTGTATGGGC-3'

HSV-2:

Forward: 5'-TGGTATCGCATGGGAGACAAT-3'
Reverse: 5'-CTCCGTCCAGTCGTTTATCTTG-3'

CMV:

Forward: 5'-ACGTGTTACTGGCGGAGTCG-3'
Reverse: 5'-TTGAGTGTGGCCAGACTGAG-3'

EBV:

Forward: 5'-AGCACTGGCCAGCTCATATC-3'
Reverse: 5'-TTGACGTCATGCCAAGGCAA-3'
(Bioserve India Pvt. Ltd India)

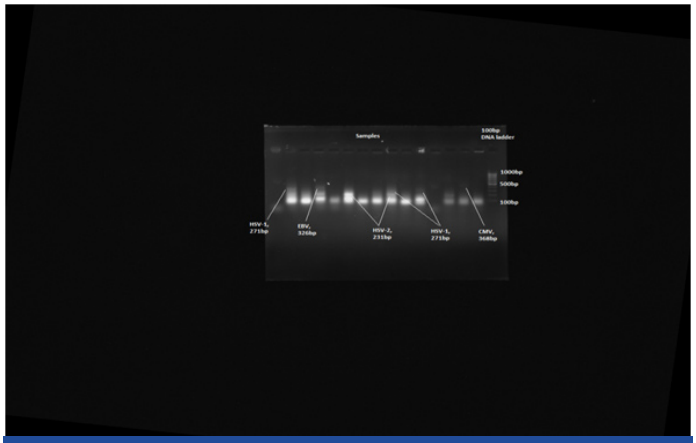
Oligonucleotide primers were prepared at a concentration of 2.5pm each in DEPC Water. A premix was prepared and aliquots were made. The premix contained the following components in a final volume of 50 µl/ aliquot.

A 25 µl PCR Master Mix + 1 µl of forward and reverse oligonucleotide primers of each herpes viruses (HSV-1, HSV-2, CMV and EBV) and DEPC water adjusted to the final volume of 50 µl. A 3µl of sample DNA was added to each tube and subjected to Multiplex PCR (MPCR).

Multiplex PCR: MPCR conditions were as follows: Initial denaturation (95^{0}C for 5min), 45 cycles of denaturation (95^{0}C for 30sec) annealing (54^{0}C for 30 seconds) and extension (72^{0}C for 30 seconds). Final extension was given at 72^{0}C for 5 minutes. Samples were kept at 4^{0}C and then the PCR product was detected by gel electrophoresis.

Detection of amplified products: This was done by electrophoresis on 2% agarose gel containing 1x TAE. 20 µl of each amplified product mixed with 5 µl of bromophenol blue (HiMedia) was loaded onto the gel, one product in each lane along with 100bp DNA ladder.

Electrophoresis was performed at 25V for 2 hour. The gel was visualized under UV light illuminator after staining with Ethidium bromide. A 100bp DNA ladder was run simultaneously with the test samples in agarose gel electrophoresis for locating the accurate amplicon size of positive test samples. Known control samples along with test samples were also run. The amplicon size of known samples of HSV-1, HSV-2, C.M.V and EBV were detected as 271, 231, 368 and 326 bp respectively [Table/Fig-1].



[Table/Fig-1]: Multiplex Polymerase Chain Reaction (MPCR) showing the positive bands for HSV-1, HSV-2, CMV and EBV
(HSV1- Herpes Simplex Virus type 1 (271 bp), HSV2- Herpes Simplex Virus type 2 (231bp), CMV- Cytomegalovirus (368bp), EBV- Epstein Barr Virus (326bp), (Lane number 1 to 15 samples and lane number 16 DNA ladder)

STATISTICAL ANALYSIS

The collected data was entered in the excel sheet format. It was subjected to statistical analysis using SPSS software. The Chi-Square statistical tests was applied and p-value <0.05 was taken as significant.

RESULTS

Total 75 patients (47 Males and 28 females) with clinically confirmed chronic periodontitis (mild, moderate and severe) were recruited in the study. Mean age in the mild, moderate and severe cases of chronic periodontitis was 43.16, 40.76 and 43.16 respectively. The prevalence of herpes viruses in the various grades of Periodontitis is shown in [Table/Fig-2]. The present study showed the detection of Herpes viruses in all grades of chronic periodontitis. The prevalence of herpes viruses in severe chronic periodontitis was high as compared to mild and moderate chronic periodontitis except EBV. The present study shows the strong association of Herpes viruses and the severity of the chronic periodontitis. There was no difference found in the occurrence of herpes viruses and age and the sex of the patient. HSV-1, HSV-2 and CMV were found to be significantly associated with periodontitis. HSV1 and HSV-2 were more commonly associated with severe periodontitis. This was not seen with EBV infection.

	HSV-1		HSV-2		CMV		EBV	
	No	%	No	%	No	%	No	%
Mild (n=25)	1	(4)	3	(12)	4	(16)	11	(44)
Moderate (n=25)	7	(28)	6	(24)	7	(28)	8	(32)
Severe (n=25)	13	(52)	14	(56)	12	(48)	10	(40)
Total (n=75)	21	(28)	23	(32)	23	(30.66)	29	(37.33)
p - value	0.001*		0.003*		0.046*		0.477#	

[Table/Fig-2]: Shows the presence of Viral DNA in Chronic periodontitis (HSV-1 Herpes simplex virus-1, Herpes simplex virus-2, CMV- Cytomegalovirus, EBV- Epstein Barr virus)
*Statistically significant # Non significant

DISCUSSION

Many microbial agents have been implicated in the causation of chronic periodontitis including viruses. Association of herpes viruses with periodontitis in various studies has ranged from 0 to 100% [17-19]. In the present study Herpes viruses as a group were detected from 81.33 % of cases of chronic periodontitis.

Co-infection with viruses can increase the complexity of the clinical picture and various studies have reported that Herpes viruses are associated with cases of chronic periodontitis. The overall association of HSV-1, HSV-2, EBV and CMV was 28%, 32%, 30.66% and 37.33% respectively in the present study from the cases of chronic periodontitis. These results are similar to various other studies. The prevalence of 26%, 31% and 21% of HSV-1 was detected from chronic periodontitis cases in the studies carried out by Nishiyama et al., [20], Ling et al., [21] and Contreras et al., [19] respectively. While 28%, 38% and 37% EBV was detected in the studies carried out by Dawson et al., [4], Wu et al., [22] and Wu et al., [23] in their studies and 35% & 34% CMV was detected in the studies carried out by Grenier et al., [24] and Tantivanich et al., [25] respectively.

Severe chronic periodontitis was more commonly associated with the herpes viruses HSV1 and HSV2. Similar observations were made by Contreras et al., in their study [5]. Herpes simplex virus-1 was detected in 52% and herpes simplex virus-2 was detected in 56% of patients with severe chronic periodontitis which is higher than that reported by Imbronito et al., [12], Greiner et al., [24] and Grande et al., [26]

Cytomegalo viruses were detected from 48% of the severe cases of chronic periodontitis in the present study which is similar to the studies carried out by Imbronito et al., [12] (50%) and Ling et al., [21] (52%). However, the studies carried out by Bilchodmath et al., [18] (26%), Sunde et al., [10] (12%) and Grenier et al., [24] (35%) showed a low occurrence of CMV from cases of chronic periodontitis. This difference in detection of herpes viruses from cases of chronic periodontitis might be due to the different geographical distribution, ethnicity of the population studied and other risk factors [27]. Epstein Barr viruses were detected from all types of cases of chronic periodontitis in the present study. The results are correlating with studies carried out by Imbronito et al., [12]. Though, EBV was not significantly associated with periodontitis; they were significantly increased in severe periodontitis. The definitive association of Herpes viruses with periodontal disease indicates that they probably play a role in the aetio-pathogenesis of the disease. Herpes viruses have the ability to multiply in gingival tissue. They may have a direct cytopathic effect on fibroblasts, keratinocytes, endothelial cells and inflammatory cells such as polymorphonuclear cells, lymphocyte, macrophages and bone cells [28,29].

Herpes viruses may also mediate damage to the host immune response and thus play a role in the pathogenesis of periodontal diseases. EBV and HCMV can infect monocytes, macrophages and lymphocytes and can also alter their functions [30-33]. Another mechanism by which they may act is by promoting sub gingival attachment and colonization of periodontopathic bacteria by providing receptors on their surfaces [34,35].

The concept of herpes viruses playing roles in severe periodontitis may have significant therapeutic implications. A novel way to prevent and treat periodontitis may focus on controlling disease initiating herpes viruses. This could be achieved by taking measures to remove plaques and to reduce the herpes virus load form the periodontal sites. Antiviral treatment could assist in this. Some studies have reported the use of antivirals to treat the refractory periodontitis [36].

Vaccines against herpes viruses could play a role in the prevention and progression of periodontitis. Thus, studies on the role of herpes viruses, their interaction with bacteria and host inflammatory mechanisms in periodontitis could result in development of novel ways to prevent and cure the disease.

LIMITATION OF THE STUDY

The present study could detect the herpes viruses qualitatively and not quantitatively.

CONCLUSION

Herpes viruses were significantly associated with periodontal disease, more so with severe periodontal disease. They could thus be playing a role in increasing the severity of the disease. Therapeutic and prophylactic intervention planned against these viruses could decrease the tooth loss associated with this disease.

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