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Subgingival Plaque as a preferred Reservoir for EBV-1 in HIV-1 Seropositive Patients: A pilot study.

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ABSTRACT

Introduction - HIV-induced immunosuppression facilitates herpes virus reactivation, but active herpes viruses might also activate latent HIV. There are reports of reactivation of latent HIV virus due to microbial infections. Subgingival region provides a favorable habitat to many microorganisms, including herpes virus group, which in turn plays key role in periodontal breakdown at the infected sites. Objective: To compare the frequency of EBV-1 infection (Epstein - Barr Virus), in peripheral blood, saliva and subgingival plaque from HIV-1 (Human Immunodeficiency Virus) positive patients without HAART (High Activity Antiretroviral Therapy) and its co-relation with clinical parameters. Materials and Methods: 10 HIV-1 seropositive patients (who were not on HAARTtherapy)were enrolled for the study. After informed consent the blood, saliva and subgingival plaque samples were obtained from the subjects. All the participants were subjected to thorough oral examination. The samples were analyzed initially for p24 using polymerase chain reaction (PCR), and then PCR using specific primers for to detect viral DNA of EBV-1. Results: The PCR analysis using specific primer for EBV-1 in Plasma (blood), saliva and subgingival plaque of HIV-1 seropositive patients showed presence of EBV in blood samples in 6/10 patients (60%), saliva 7/10(70%) .The subgingival plaque of these patients showed the maximum frequency of 8/10 (80%). Of interest, in 4 cases EBV was detected only in plaque, and in two cases it was present only in saliva and plaque but not in blood. Conclusion: The results revealed that subgingival plaque can be a preferred ecological niche for the HIV seropositive patients even without any obvious clinical presentation. Hence, a thorough oral prophylaxis can reduce the viral load in the patients thereby improving general oral health

Keywords: subgingival plaque, EBV-1, HIV-1.

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INTRODUCTION

Since the beginning of acquired immunodeficiency disease syndrome (AIDS) epidemic, much has been learnt about HIV (Human Immunodeficiency Virus) related oral mucosal disease. Disease progression and reduction in CD 4 count is correlated directly with the presence of many oral lesions like oral candidiasis, oral hairy leukoplakia, ulcerative periodontal disease and xerostomia [1,2] The other main oral manifestations include HIV associated periodontitis and oral malignancies mainly lymphomas. The etiopathogenesis of a periodontal disease is a complex process which includes multifactorial interactions between microbial, host and environmental factors. The significance of viral infection in medicine and HIV gingivitis and periodontitis in dentistry show the wide range of pathogenic potential of human viruses. [3]

Viral infections facilitate destruction of periodontal tissue by [4, 5]

A. Lytic activity against host periodontal cells by inducing bone resorption and interfere with collagen formation and turnover
B. Immune mediated tissue destruction by producing interleukin 1-□□□tumor necrosis factor□□□□and other cytokines
C. Immune modulation which increases susceptibility of the host to the bacterial attack especially *P.gingivalis, B.forsythus,P.intermedia, P.nigrescene and T. denticola*.[6]

More recently, Herpes viruses especially Human cytomegalovirus (HCMV) and Epstein Barr Virus-1(Epstein - Barr Virus), EBV-2 have been identified in the sub-gingival plaque of patients with advanced periodontitis. As HCMV infects periodontal monocytes and macrophages and T-lymphocytes and EBV infects B-lymphocytes, the presence of these viruses may contribute to increase virulence of pathogenic bacteria causing periodontal damage.[7] The main neoplastic manifestations present in HIV-seropositive patients are Oral Non-Hodgkin's Lymphomas (NHL) and Oral Squamous cell Carcinomas (OSCC), and more commonly reported in western literature i.e., Kaposi's sarcoma. The main causative factor for NHL is EBV and that for OSCC is postulated to be Human Papilloma Virus (HPV).[8]

However it is not known what could be the site of infection of EBV in the oral cavity. We hypothesized that EBV may reside in the subgngival plaques and act as reservoir to precipitate the disease.

The present study was designed to compare the frequency of EBV-1 in the subgingival plaque, saliva and peripheral blood of HIV-1 seropositive patients and to demonstrate whether subgingival plaque harbours EBV-1in the HIV-1 seropositive patients and escape immune attack leadfing to dissemination of the virus in saliva.

MATERIALS AND METHODS

The study designed was an in-vivo observational study which was conducted in Dr. D.Y.Patil Dental college and Hospital, Pune and National Centre for Cell Sciences(NCCS) Pune and Yashwantrao Memorial Hospital ART centre, Pune.The study duration was of around 6 months. The study was approved by the Institutional Review committee for human experiments of DY Patil Medical College and Hospital, Pimpri.

Case selection

10 HIV-1 seropositive patients were included in the study. Complete information was provided to the patient and written consent was taken from them. A thorough examination of the oral cavity was performed for evaluation of oral manifestations. CD4 counts of the patients were also recorded. Inclusion criteria included diagnosed cases of HIV who had more than 20 teeth, and had never taken any anti-retroviral (ART) medication. Any patient who had less than 20 teeth, taken ART or suffering from florid systemic infections was excluded. [9]

Sample Collection

Blood – 3ml of the blood sample was taken in anticoagulant bulbs (Fluoride and EDTA bulb) and was centrifuged. The plasma of the samples thus obtained on sedimentation was removed and stored for further applications at -70° c.

Saliva – The patients were not allowed to brush their teeth or eat 30 mins before sampling to minimize the effect of circadian rhythms. Collection of saliva samples was carried out between 9 to 10.30 am. Patients were advised to rinse mouth with tap water and after 10 minutes saliva samples were collected to avoid sample dilution. The patients were seated in a well-ventilated and well-lit room. The head was kept at 45 degrees flexion with one hand holding onto a 4ml cryoprecipitation vial with a funnel inserted into it, in a calm atmosphere to simulate unstimulated conditions. The saliva was allowed to drip into the funnel held to the lower lip. 2 ml of saliva per patient was obtained. The saliva samples were collected in plain sterile tubes and stored at -70°c till DNA extraction.[10]

Subgingival plaque- supra gingival plaque was gently removed with sterile cotton pellets and air-dried. The subgingival plaque samples were taken from the two deepest periodontal pocket and if the patient didn't have periodontitis then from mesial aspects of lower first molars by a sterile Gracey's curette. The samples were suspended in sterile phosphate buffered saline (0.4 ml) and homogenized by vigorous mixing. [11,12]

Nucleic acid extraction

DNA extraction- plaque, saliva and plasma samples from 4 patients were used to optimize the DNA extraction protocol. Silica-Guanidine thiocyanate (GUSCN) method was used for DNA extraction. 0.4ml of the sample was mixed with 5µl of silica particles in 80µl of lysis buffer (120gm of GuSCN; 100ml of 0.1mM Tris HCL, pH=6.4; 22ml of 0.2M EDTA, pH=8; 2.6g of Triton X100), vortexed for 10 sec and kept at room temperature for 10 minutes. Nucleic acid/ silica combinations were recovered by centrifugation at 12000 rpm for 1 minute, washed twice in buffer (GuSCN-Tris HCL), twice in 70% ethanol and once in acetone. The sample was then dried in a heating block at 56^{0}c for 10 minutes. After centrifugation at 12000g for 2 mins, the supernatant was stored at -70^{0}c [13,14]

RNA extraction- 150 µl each of the twenty plasma sample were used for extraction of RNA using Qiagen Rapid RNA Extraction kit. The quality of the RNA was checked by absorbance at 260/280 using a nanodrop spectrometer. RNA from 20 samples were converted to cDNA using MMLV Reverse Transcriptase.

PCR procedures

The PCR method was used to detect the viral DNA of EBV. The outer and inner oligonucleotide primers that were used were[12]

Outer- F5' AGG GAT GCC TGG ACA CAA GA
R5'TGG TGC TGC TGG TGG CAA
Inner- F5' TCT TGA TAG GCA TCC GCT AGG ATA
R5' ACC GTG GTT CTG GAC TAT TCG GAT C

The target gene was EBNA 2 with an expected base pair size of 497.

Optimal conditions of the PCR reaction mixture and temperature cycling were determined using positive and negative control.

The initial reaction for the outer primer

40 µl of total mixture containing 3 µl of DNA template, 0.8 µl of the pair of outer primers, 1.5mM $MgCl_2$ (10X Buffer A) 4 µl, 0.2mM of each deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP) 0.4 µl of Taq and rest of the volume of double distilled water. The PCR amplification included an initial denaturation step at 95° c for 1 min followed by 30 cycles of denaturation at 94^{0}c for 1 min, prime annealing at 60^{0}c for 1 min, and extension step at 72^{0}c and then a final extension step at 72^{0}c for 1 min.

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The second round of amplification was performed using the inner primers(0.5 µl) with 3 µl of the first PCR product as the template, 0.5 µl of 1mM dNTP's, 1.5mM $MgCl_2$ i.e., 10X Buffer a- 2.5 µl and Taq polymerase. The programme for the second cycle was 35 cycles of a denaturation step 94^0c for 1 min, annealing at 55^0c for 1 min extension at 72^0c for 1 min and a final extension step at 72^0c for 2 min.

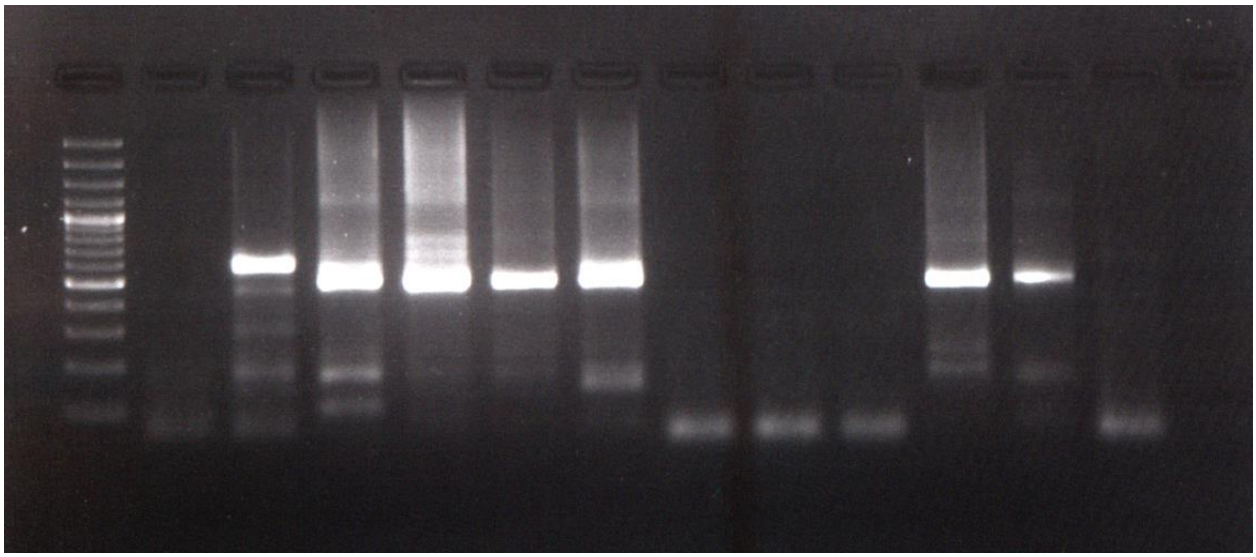
The positive control was cellular DNA extracted from the Raji cell line (EBV-1) and the negative control included normal HIV- negative patients Plasma, Saliva and Subgingival Plaque. To avoid contamination every 2 patient's sample were run with water as negative control. All the samples negative for the EBV virus were tested for GAPDH gene to check the integrity of DNA in the sample.[13] Amplicons were detected by electrophoresis of 20 µl samples in a 2% agarose gel containing 0.5 µl/ml of ethidium bromide. The gels were viewed under UV Transilluminator (Versadoc).

RESULTS

Opportunistic viral infections play a vital role in progression of the disease in the HIV infected individuals. The co-infections with different viruses would require specific treatment when immunosuppression advances. One of the most common virus associated with the opportunistic infections especially concerned with oral lesion like AIDS immunocompromised patient is EBV. [15-18]

Our study had sample size of 10 HIV-1 seropositive patients. The molecular analysis of Plasma (blood), saliva and subgingival plaque of these patients showed presence of EBV blood in 6/10 patients (60%), saliva 7/10 (70%) and subgingival plaque of these patients showed the maximum frequency of 8/10 (80%). Of interest, in 4 cases EBV was detected only in plaque, and in two cases it was present only in saliva and plaque but not in blood.

Figure 1: PCR amplification gel of PLASMA samples.



Lane -1: 100 bp Standard DNA Ladder; Lane -2 to Lane -11: Plasma samples from patient No.1 to patient No.10.; Lane -12: Positive Control(Genomic DNA); Lane -13: Negative Control (Normal HIV-negative Person

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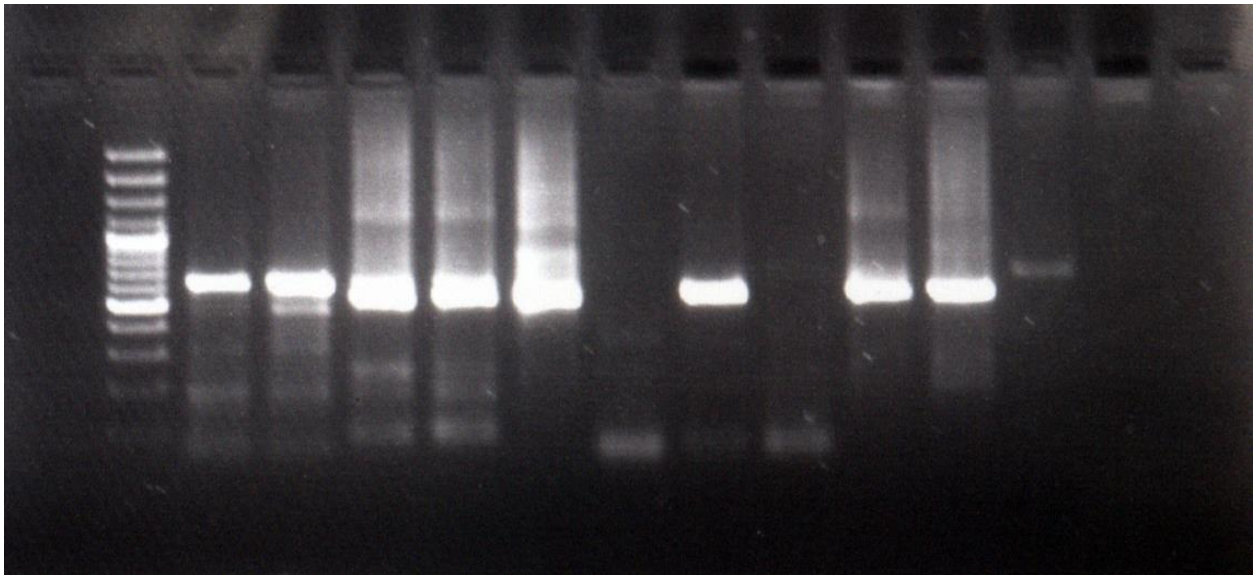
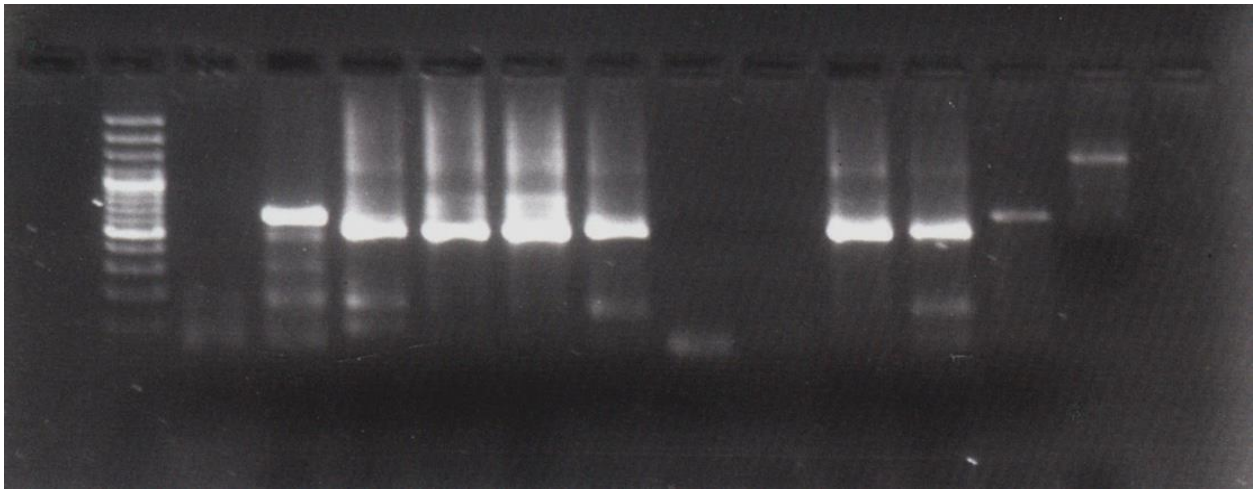
Figure 2: PCR amplification gel of SUBGINGIVAL PLAQUE samples



Lane -1: 100 bp Standard DNA Ladder; Lane -2 to Lane -11: Plaque samples from patient No.1 to patient No.10. ; Lane -12: Positive Control(Genomic DNA); Lane -13: Negative Control (Normal HIV-negative Person)

Figure 3: PCR amplification gel of SALIVA samples.



Lane -1: 100 bp Standard DNA Ladder; Lane -2 to Lane -11: Saliva samples from patient No.1 to patient No.10.; Lane -12: Positive Control(Genomic DNA); Lane -13: Negative Control (Normal HIV-negative Person)

The CD4 count of these patients ranged from 150 to 400cells/□l. Only two patients had the count as low as 75cells/□l, had EBV positivity in all samples.

DISCUSSION

There are many studies which show that EBV is commonly detected the gingival crevicular fluid and plaque in patients showing periodontal breakdown**.** Lawn SD[19] in 2004 reviewed the impact of co-infections the pathogenesis of HIV-1 infections in sub-saharan Africa where cases of AIDS are pandemic. They concluded that the immunogenic implications of having superimposed coinfections are generally results in CD4 count and increased viral replication leading to decreased immune function.

Klemec P et al 2005[20] in their study evaluated the presence of EBV, HHV-6,HHV-8& HCMV in GCF and their clinicopathological correlation. They used restriction endonuclease for specific virus identification in 66 samples of GCF. EBV was found in 29(43.6%) of periodontitis cases whereas they were in healthy volunteers. Thus EBV was seen associated with increased periodontal breakdown.

Similar studies in HIV associated periodontal diseases state that infection from oral pathogens , bacteria and viruses alike, induces a state of chronic inflammation and releases various cytokines. [21]These cytokines are responsible for the periodontal bone loss as well as reactivation of HIV-1 infection thereby causing dual damage and setting a vicious cycle.[22]

Another hypothesis recently proposed by Jakovljevic A et in 2016 states that EBV in the periodontal pocket is instrumental in bone resorption by inducing overproduction of reactive oxygen species , which in turn causes excessive production of receptor activator of nuclear factor kappa B(NF-□b) ligand (RANKL), thereby activating osteoclasts.[23]

The reported prevalence of EBV-1 infection in periodontitis patients range from 17.7% to 70.6%.[23] EBV infection in healthy individuals is between0-100%.[24]There are also studies which report that presence of EBV in saliva could be because of shedding of the virus from periodontitis sites.[25]In the present study also we have been able to show that subgingival plaque is a preferred site for growth of EBV virus . Thus, oral prophylaxis would go a long way in reducing the EBV load in HIV seropositive patients.

CONCLUSION

There could be a focal infection from EBV, in HIV infected patients, without being present in circulating blood. This in association with other studies implies that oral mucosa is a major site of EBV replication and shedding of infectious virions so it acts as a reservoir.[13]

1. The detection of EBV-1 in oral sites of HIV-1 patients would require a combination of saliva and subgingival plaque analysis to avoid false negative results when relying on only one sampling method.
2. The present finding of frequent detection of EBV in subgingival environment of these patients could provide the rationale for further studies evaluating the role of subgingival plaque and periodontal treatment in reducing EBV load in HIV-1 seropositive patients.

A thorough oral prophylaxis, should be added to the standard care regime for HIV-1 seropositive patients, based on the hypothesis that increased viral coinfections could mediate deterioration of oral health.

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