Detection of cytomegalovirus, Epstein–Barr virus, and Torque Teno virus in subgingival and atheromatous plaques of cardiac patients with chronic periodontitis

Sriraman Priyanka, Gurumoorthy Kaarthikeyan, Jayakumar Doraiswamy Nadathur, Anbarasu Mohanraj, Avinash Kavarthapu

Abstract:
Background: Periodontitis and atherosclerosis represent a chronic inflammatory process. The incidence of periodontitis in cardiac patients with atherosclerosis is a well-established fact. The role of viruses in the etiopathogenesis of both has been proposed. Aim: The aim of the study was to evaluate the prevalence of Torque Teno virus (TTV), Epstein–Barr virus (EBV), and cytomegalovirus (CMV) in cardiac patients with atherosclerosis and coexisting chronic periodontitis (CP). Materials and Methods: Thirty patients (17 males and 13 females) with atherosclerotic plaques and coexisting periodontitis were recruited for this cross-sectional study. Viral DNA was extracted from the subgingival and atheromatous plaque. The presence of CMV, EBV, and TTV in the plaque samples was identified using polymerase chain reaction. The collected data were statistically analyzed for the prevalence of the viruses and Chi-squared test was performed to find out its association with atheroma and CP. Results: The prevalence of CMV, EBV, and TTV in atheromatous plaque was 63.3%, 56.7%, and 46.7%, respectively, as compared to rates of 80%, 63.3%, and 53.3% in subgingival plaque. Results also indicated no significant association of CMV, EBV, and TTV in both samples (P = 0.08, 0.346, and 0.261, respectively). Conclusions: There was no significant association of CMV, EBV, and TTV between subgingival and atheromatous plaque. The prevalence of CMV, EBV, and TTV was high in atheromatous plaque. TTV was isolated from more than 50% of participants in atheromatous plaque, which is a significant finding.

Key words: Atheromatous plaque, cardiac patients, chronic periodontitis cytomegalovirus, Epstein–Barr virus, subgingival plaque, Torque Teno virus

INTRODUCTION
The chronic inflammatory state of periodontal destruction is caused by multiple etiologies and risk factors. The role of Gram-negative anaerobes in periodontal destruction is well established. Recently, the viruses have been identified as playing a major role in the etiopathogenesis of chronic periodontitis (CP). The association of various herpes viruses with several types of periodontal disease has been established by many studies. Among the viruses, cytomegalovirus (CMV), Torque Teno Virus (TTV), and Epstein–Barr virus (EBV) plays a major role.[1-3] The second most common cause of mortality (29%) worldwide after infectious diseases is cardiovascular diseases (CVDs). There are multiple causes for the development of atherosclerosis in cardiovascular and cerebrovascular diseases. The major risk factors for more than 50% of CVD are diabetes, hyperlipidemia, obesity, hypertension, and cigarette smoking accounts for 50%–60% of cases of CVD.[4] There is a continuous debate on the role of these viruses in the etiopathogenesis of atherosclerosis. The presence of EBV and CMV in patients with CVD was proved with well-established studies.[5,6] There are studies that correlate the association between CP and CVD mainly concentrate on the role of bacterial
pathogens such as Porphyromonas gingivalis, Treponema denticola, and Aggregatibacter actinomycetemcomitans. However, there is a lack in the association between periodontitis and CVD with a viral etiology such as CMV, EBV, and TTV. Hence, the aim of the present study was to evaluate the association of CMV, EBV, and TTV in subgingival and atheromatous plaque of cardiac patients with CP.

MATERIALS AND METHODS

Thirty cardiac patients (17 males and 13 females) requiring coronary artery bypass graft (CABG) with a coexisting periodontal disease were screened and selected for the study. The sample size was calculated based on the previous study of Rotundo et al., with a G power of 85%. The Institutional Ethical Committee and Institutional Review Board of the University approved our study (IHEC/SDBDS11PER5). The cases were tested by two periodontists for assessing oral hygiene status, bleeding on probing, periodontal pocket depth, and clinical attachment levels (CAL). One day before surgery, subgingival plaque samples were collected, and the corresponding coronary atheromatous plaques were collected during CABG surgery.

The inclusion criteria were patients belonging to either sex of any age group with a minimum of twenty teeth present, on whom CABG was to be performed, with at least 2–3 sites having clinical attachment loss >5 mm and a periodontal pocket depth >5 mm. These clinical findings were supported by radiographic evidence showing bone loss in the teeth under investigation. Patients with other systemic diseases such as diabetes, immunocompromised state, patients who underwent any periodontal treatment within the past 6 months, and those who were on prolonged antibiotic use were excluded from the study. Periodontal status was assessed by evaluating Muhlemann’s modified sulcular bleeding index, Silness and Loe’s plaque index, probing pocket depth, and CAL. For each participant, 2–3 teeth with pockets >5 mm were selected and isolated for the subgingival plaque collection. Using a clean, sterile curette, subgingival plaque samples were collected after careful removal of the supragingival plaque. Similarly, atheromatous plaque samples were obtained during CABG surgery. Both the samples were transferred to phosphate-buffered saline and stored at −20°C for analysis. The DNA from the virus was extracted using PROTEINASE-K DNA extraction method (Qiagen, USA) for both the subgingival and atheromatous plaque samples.

DNA sample was prepared and PCR was performed. The amplified products were run in 3% agarose gel for 30 min at 50 volts and 100 mA. It was visualized under gel documentation system.

The forward primer sequence of TTV: 5’GCTACGTCACTAAC CACGTG3’ (T 801, sense primer) and the reverse primer 5’CTCGGTGTGTTAACTCAC3’ (T 935, antisense primer) were used for PCR amplification. The PCR amplification was done in a thermocycler (Perkin Elmer Cetus, USA) after the mix was kept at 95°C for 10 min for reverse transcriptase inactivation. The denaturation was carried out for 20 s at 94°C, annealing at 60°C for 20 S, and template extension at 72°C for 30 s. The cycle was repeated 55 times. The PCR amplified products were visualized on a 2% agarose gel with intercalating ethidium bromide dye.

The social sciences version 18 for Windows (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The prevalence and association of CMV, EBV, and TTV in subgingival and atheromatous plaques were calculated using Chi-square test. Statistical significance in all tests was determined at P < 0.05.

RESULTS

The prevalence of CMV, EBV, and TTV in atheromatous plaque and subgingival plaque was 63.3%, 56.7%, and 46.7% and 80%, 63.3%, and 53.3%, respectively [Table 1]. The percentage prevalence of CMV, EBV, and TTV in subgingival and atheromatous plaque samples has been tabulated [Tables 2-4]. While the percentage prevalence of CMV in both subgingival and atheromatous plaque was 40% and 30% respectively. Statistical analysis of the data showed no significant association of CMV, EBV, and TTV in atheromatous and subgingival plaque samples (P = 0.08, 0.346, and 0.261), respectively.

DISCUSSION

Atherosclerosis, the main underlying disease responsible for the cardiovascular and cerebrovascular morbidity, results from multifactorial etiology. Traditional risk factors such as obesity, hyperlipidemia, diabetes mellitus, hypertension, and smoking account for only 50%–60% of cases of cardiovascular

Table 1: Prevalence of cytomegalovirus, Ebstein-Barr virus, and Torque Teno virus in subgingival and atheromatous plaque sample

| Variable | n | Prevalence (%) |
|----------|---|----------------|
| CMV (S)  | 24 | 80             |
| CMV (A)  | 19 | 63.3           |
| EBV (S)  | 19 | 63.3           |
| EBV (A)  | 17 | 56.7           |
| TTV (S)  | 16 | 53.3           |
| TTV (A)  | 14 | 46.7           |
| Both CMV (S) and CMV (A) | 17 | 56.7 |
| Both EBV (S) and EBV (A) | 12 | 40 |
| Both TTV (S) and TTV (A) | 9 | 30 |
| CMV (S), EBV (S), TTV (S) | 6 | 20 |
| CMV (A), EBV (A), TTV (A) | 3 | 10 |

CMV – Cytomegalovirus; EBV – Ebstein-Barr virus; TTV – Torque Teno virus; S – Subgingival; A – Atheromatous; n – number of subjects
disease. The microorganisms initiate atheroma formation which resembles many aspects of chronic inflammation. There are many reviews supporting the venerable hypothesis of an infectious etiology. Contribution of both bacterial and viral agents to atherogenesis has shown in many experimental animal studies. Many such agents such as Chlamydia pneumoniae, CMV can infect blood vessel walls, persist, remain latent, and cause recurrent infection.

A low-grade chronic infection in the body initiates and perpetuates the process of atherosclerosis. As the periodontal pocket harbors multiple pathogens, it could serve as a potential source of microorganisms. A possible association of periodontal pathogens and atherosclerosis has been reported in the literature. These studies found a significant association of P. gingivalis, T. denticola, Campylobacter rectus, and atherosclerosis. Many studies have isolated CMV and EBV from atherogenic plaque. The recent findings suggest a major role for these viruses in the periodontal destruction. An extensive review of literature did not show any study linking the association of the viruses in subgingival and atheromatous plaque and periodontitis. Hence, we framed a hypothesis that the source of the viruses in the atheromatous plaque is from the periodontal pocket. Thus, the aim of our study was to evaluate the association of EBV, CMV, and TTV in subgingival and atheromatous plaque.

From the current study, it was found that the prevalence of CMV and EBV was 63.3% and 56.7%, respectively, in the atheromatous plaque sample. These results correspond to earlier reports wherein a prevalence of CMV in the range of 10%–90% was found in atheromatous plaque. Some authors reported that they were not able to identify CMV in atherosclerotic tissue, but this is in contradiction with our results. However, CMV DNA was detected in both atherosclerotic plaque as well as nonatherosclerotic tissue from the same patient by Xenaki et al.

EBV role in the etiopathogenesis of atherosclerosis and CP is as unsubstantiative as CMV. The prevalence of EBV in atheromatous plaque samples in our study was 56.7%, which agrees with a study by Shi et al. and Horvarth et al. In these studies, the prevalence of EBV was found to be 60%–80%. However, a higher percentage of EBV DNA was detected by a few authors, whereas in case of advanced atheromatous tissue, other authors could not demonstrate EBV DNA.

The recently identified TTV responsible for periodontal destruction belongs to a Circoviridae family (genus Anellovirus), and it was first identified and isolated from the serum with posttransfusion hepatitis patients of the Japanese population, which frequently and ubiquitously infect humans. There are a great genetic variation and lifelong viremia caused as a result of these infections. However, those levels of viral replication might fluctuate among different people, and this might represent a critical marker of the pathogenic part for TTV. There has been a great association found between TTV and many chronic diseases such as bronchiectasis, hepatic failure, and asthma. Since

| Table 2: Percentage prevalence of cytomegalovirus (subgingival) and cytomegalovirus (atheromatous) |
| CMV (S) | Present | Total (n) | Percentage | $\chi^2$ | $P$ |
| Present (17) | - | 6.7 | 19 | 63.3 | 2.907 | 0.08 (NS) |
| Absent (7) | - | 20 | 30 | 100 |
| Total (n) | - | 20 | 30 |

$CMV$ – Cytomegalovirus; $S$ – Subgingival; $A$ – Atheromatous; $NS$ – Not significant; $\chi^2$ – Chi-square value; $P$ – p-value < 0.05; $n$ – number of subjects

| Table 3: Percentage prevalence of Ebstein-Barr virus (subgingival) and Ebstein-Barr virus (atheromatous) |
| EBV (S) | Present | Total (n) | Percentage | $\chi^2$ | $P$ |
| Present (12) | - | 5 | 17 | 56.7 | 0.889 | 0.346 (NS) |
| Absent (7) | - | 20 | 30 | 100 |
| Total (n) | - | 30 | 30 |

$EBV$ – Ebstein–Barr virus; $S$ – Subgingival; $A$ – Atheromatous; $NS$ – Not significant; $\chi^2$ – Chi-square value; $P$ – p-value < 0.05; $n$ – number of subjects

| Table 4: Percentage prevalence of Torque Teno virus (subgingival) and Torque Teno virus (atheromatous) |
| TTV (S) | Present | Total (n) | Percentage | $\chi^2$ | $P$ |
| Present (9) | - | 5 | 14 | 46.7 | 1.265 | 0.346 (NS) |
| Absent (7) | - | 30 | 30 |
| Total (n) | - | 30 | 30 |

$TTV$ – Torque Teno virus; $S$ – Subgingival; $A$ – Atheromatous; $NS$ – Not significant; $\chi^2$ – Chi-square value; $P$ – p-value < 0.05; $n$ – number of subjects
the pathogenesis of atherosclerosis has followed a chronic pattern, we intended to investigate the association of TTV and atherosclerosis.

To the best knowledge of the authors, this is the first study to isolate TTV from atheromatous plaque of 14 participants. The prevalence rate was found to be 46.7%. This showed that TTV was isolated from nearly half of the sample participants, which was a significant finding. The possible pathogenic mechanism of TTV infection includes its ability to twist the immune balance toward the T-helper cell (Th2) response, on replication. The other possible pathogenic mechanisms would be the interference with the activity of nuclear factor (NF)-kappa B by the open reading frame protein ORF2 of TTV. An intracellular signal transcription factor called NF-kappa B is well characterized and is known to play a myriad of roles in inflammation and immunomodulation.

CMV was isolated from both subgingival and atheromatous plaque of 17 participants with a percentage prevalence of 56.7%. Even though the association of CMV in subgingival and atheromatous plaque found to be nonsignificant in our study, but, it is an important finding, since CMV was isolated from both subgingival and atheromatous plaque of more than half of the participants.

Similarly, the association of EBV in subgingival and atheromatous plaque was not statistically significant. However, the virus was isolated from both subgingival and atheromatous plaque sample of 12 participants. There was no significant association of TTV in subgingival and atheromatous plaque. The percentage prevalence of TTV in subgingival and atheromatous plaque was found to be 10%. The results of our study did not confirm the etiopathogenesis but would highlight the plausibility of organisms or a cumulative infectious effect in atherogenesis.

Less sample size and the absence of control groups are considered to be the major limitation of our study. Our study mainly focused on the late-stage atherothrombotic disease, and we are uncertain about the children getting exposed to ubiquitous infections, frequencies of re-exposure, and the age at which these viruses influence atherosclerosis. In addition, we did not describe the roles of status of infection such as past, active, persistent latent, or recurrent infection. These aspects need further investigation.

**CONCLUSION**

Within the limitations of our study, it can be concluded that the prevalence of CMV, EBV, and TTV was high in atheromatous plaque. The role of TTV in the pathogenesis of atheromatous plaque has to be explored. Further studies are required to address the interactions of these viruses in causing infection, possible risk factors, and other determinants of host susceptibility such as gender, genetic, and nutrition using larger sample size.

**Acknowledgement**

We would like to acknowledge Indian council of Medical Research (ICMR) and Madras Medical Mission of granting the permission to do the research.

**Financial support and sponsorship**

This study was supported by the Short Term Studentship grant by Indian Council of Medical Research (ICMR)- STS-20110554.

**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Slots J. Herpesviruses in periodontal diseases. Periodontol 2000 2005;38:33-62.
2. Rotundo R, Maggi F, Nieri M, Muzzi L, Bendinelli M, Prato GP, et al. TT virus infection of periodontal tissues: A controlled clinical and laboratory pilot study. J Periodontol 2004;75:1216-20.
3. Slots J, Saygun I, Sabeti M, Kubar A. Epstein-Barr virus in oral diseases. J Periodontal Res 2006;41:235-44.
4. World Health Organization. The World Health Report. Geneva: World Health Organization; 1997. p. 30-40.
5. Ibrahim AI, Obeid MT, Jouma MJ, Moasis GA, Al-Richane WL, Kindermann I, et al. Detection of herpes simplex virus, cytomegalovirus and Epstein-Barr virus DNA in atherosclerotic plaques and in unaffected bypass grafts. J Clin Virol 2005;32:29-32.
6. Shi Y, Tokunaga O. Herpesvirus (HSV-1, EBV and CMV) infections in atherosclerotic compared with non-atherosclerotic aortic tissue. Pathol Int 2002;52:31-9.
7. Muhlestein JB, Anderson JL. Chronic infection and coronary artery disease. Cardiol Clin 2003;21:333-62.
8. Teles R, Wang CY. Mechanisms involved in the association between periodontal diseases and cardiovascular disease. Oral Dis 2011;17:450-61.
9. Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. J Periodontol 1996;67:1123-37.
10. De Nardin E. The role of inflammatory and immunological mediators in periodontitis and cardiovascular disease. Ann Periodontol 2001;6:30-40.
11. Hendris MG, Salimans MM, van Boven CP, Bruggeman CA. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. Am J Pathol 1990;136:23-8.
12. Stassen FR, Vega-Córdoval X, Vliegen I, Bruggeman CA. Immune activation following cytomegalovirus infection: More important than direct viral effects in cardiovascular disease? J Clin Virol 2006;35:349-53.
13. Daus H, Ozbek C, Saage D, Scheller B, Schieffer H, Pfreundschuh M, et al. Lack of evidence for a pathogenic role of *Chlamydia pneumoniae* and cytomegalovirus infection in coronary atheroma formation. Cardiology 1998;90:83-8.
14. Saetta A, Fanourakis G, Agapitos E, Davaris PS. Atherosclerosis of the carotid artery: Absence of evidence for CMV involvement in atheroma formation. Cardiovasc Pathol 2000;9:181-3.
15. Pinar A, Oç M, Akyön Y, Farsak B, Kocyiirdirim E, Us D, et al. The presence of *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus in human atherosclerosis detected by molecular and serological methods. Mikrobiyol Bul 2004;38:213-22.
16. Xenaki E, Hassoulas I, Apostolakis S, Sourvinos G, Spandidos DA. Detection of cytomegalovirus in atherosclerotic plaques and nonatherosclerotic arteries. Angiology 2009;60:504-8.
17. Kindermann I, Perreten A, Barili F, Kindermann H, Kindermann G, et al. The possible role of human cytomegalovirus (HCMV) in the origin of atherosclerosis. J Clin Virol 2000;20:205-15.
18. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M, et al. A novel DNA virus (TTV) associated with elevated transmitochondial levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 1997;241:92-7.
19. Bendinelli M, Pistrello M, Maggi F, Fornai C, Freer G, Vatteroni ML, et al. Molecular properties, biology, and clinical implications of...
TT virus, a recently identified widespread infectious agent of humans. Clin Microbiol Rev 2001;14:98-113.

20. Pifferi M, Maggi F, Andreoli E, Lanini L, Marco ED, Fornai C, et al. Associations between nasal torquetenovirus load and spirometric indices in children with asthma. J Infect Dis 2005;192:1141-8.

21. Zheng H, Ye L, Fang X, Li B, Wang Y, Xiang X, et al. Torque teno virus (SANBAN Isolate) ORF2 protein suppresses NFκB pathways via interaction with IκB kinases. J Virol 2008;82:593.