DETECTION OF MULTIPLE MICROBIAL DNA IN ATHEROMATOUS PLAQUES BY POLYMERASE CHAIN REACTION

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ABSTRACT
The present study was aimed at evaluating the relationship between the presence of microbial infections and cardiovascular disease by testing the atheromatous plaque and non-atheromatous tissue samples. We analyzed prospectively endarterectomy specimens from 25 consecutive patients (Group I) and left internal mammary artery pieces from 40 subjects who underwent coronary artery bypass grafting (Group II). Polymerase chain reaction (PCR) was performed to look for the DNA of Chlamydia pneumoniae, Helicobacter pylori, Cytomegalovirus (CMV) and Herpes simplex viruses (HSV) 1 and 2. Four (16%) specimens from Group I patients and thirteen (32.5%) specimens from Group II subjects were negative for all the microbes studied (p<0.01). The incidence of infection with C. pneumoniae (64%), H. pylori (44%) and HSV 1 (52%) were significantly high in Group I patients in comparison to those of Group II. There was not much difference in the incidence of CMV and HSV 2 infections among the 2 groups. The occurrence of multiple infections was also significantly higher in group I subjects with 64% showing concurrent microbial infections with 3 or 4 organisms studied. C. pneumoniae always coexisted with other microbe/s. The outcome of our study strongly supports the hypothesis that microbial infections play an important role in atherosclerosis either by initiating or accelerating the process. They do not appear to be bystanders. The high incidence of HSV1 and H. pylori need to be investigated further.

KEY WORDS: Atherosclerosis, Polymerase chain reaction, Risk Factors, C. pneumonia, H. pylori, HSV, CMV

INTRODUCTION
Atherosclerosis is the major underlying disease leading to most cases of myocardial infarction and cerebrovascular accidents. Atherosclerotic heart disease is the leading cause of mortality and morbidity all over the world and ranks only second to infectious and parasitic diseases
. It has been projected that atherosclerotic cardiovascular disease (CVD) will be the leading cause of death and disability in the world by the year 2020
. It is well accepted that atherosclerotic CVD is multifactorial in etiology and pathogenesis. However, the traditional risk factors such as smoking, obesity, hypertension, hyperlipidemia, diabetes mellitus and genetic factors account for only 40-50% of patients with myocardial infarction
. This has prompted the investigators to look for newer risk factors and one of the putative risk factors is the role of infectious agents, which has evinced a lot of interest in recent years. They may play a direct role in these patients of atherosclerosis or they may intensify the effect of other risk factors
. A number of microbes have been investigated, but most of the research work is centered on C. pneumoniae
, H. pylori
, CMV
, HSV
 and periodontal pathogens
. The association of coronary artery disease (CAD) and C. pneumoniae is firmly established but the links with other infectious agents is more controversial. However, new data on this topic are being published at a rapid pace and the potential for novel therapeutic management of CAD is enormous, if infection is proved to be the cause or accelerators of atherosclerosis. In spite of the enthusiasm and interest generated by the concept, very few investigators have looked for the presence of multiple organisms in the same affected tissue. Keeping this in mind, the present study was undertaken to detect the DNA of C. pneumoniae, H. pylori, CMV and HSV type 1 & 2 by PCR in atheromatous and non-atheromatous tissues.

MATERIALS & METHODS
Study subjects: the present study was conducted at Heart Foundation, KLEs Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum from July-2007 to March-2009. A total of 65 patients, all men who were randomly chosen were included in the study and were
categorized into two groups. Group I comprised of 25 patients who underwent atherectomy and were in an age range of 37-71 years (mean 55.6 ±11.67). Group II was made up of 40 patients who were subjected to CABG: they were in an age range of 38-74 years (mean 57.9 ± 7.71). The study was approved by the hospital institutional review and ethics committee. Informed consent was obtained from each patient. A thorough clinical examination was conducted for all the patients and detailed history regarding the presence of various risk factors such as hypertension, hyperlipidemia, diabetes mellitus and smoking were recorded. Body Mass Index (BMI) was calculated for each subject and recorded. Internationally accepted guidelines were followed for defining risk factors21.

Specimen: Atheromatous plaques following coronary endarterectomy were collected from patients in group I and pieces of left internal mammary artery (LIMA) used for grafting were picked up from patients in group II. The atheromatous tissue / LIMA pieces that were collected during surgery were immediately transferred to the laboratory. Each tissue was divided into three portions. One part was used for DNA extraction, another portion was sent for histopathological evaluation in buffered formalin. Remaining tissue was stored frozen at -80°C for further use. Other relevant investigations including fasting and post-prandial glucose, glycosylated hemoglobin and lipid profile were also carried out immediately after admission.

DNA extraction: The specimen was cut into small bits, finely minced and the DNA was extracted by modified proteinase K method22. Briefly, the minced arterial tissue was repeatedly washed in Tris- EDTA buffer and the deposit was subjected to lysis with lysis buffer I (Tris-Hcl 10mM, EDTA 1mM, Triton X- 100 0.45%) for five minutes followed by lysis buffer II (Tris-Hcl 50mM, Kcl 50mM, MgCl2 2.5mM, Tween -20 0.45%, Nonidet P-40 0.45%) with freshly added proteinase K solution (500ug / ml). The suspension was kept in a water bath at 65°C for 2 hrs followed by boiling for 10 minutes. After cooling, the sample was centrifuged and the supernatant was stored at -20°C till amplified by PCR.

PCR amplification and analysis: Amplification of C. pneumoniae DNA was performed using primers that amplified a 437 bp fragment of the gene23. PCR for H. pylori was carried out by using primers that targeted Cag A gene24 and amplified a 394 bp fragment. Reactions for HSV1, HSV2 and CMV were done with the aid of primers that amplified 147 bp, 227 bp and 256 bp respectively25.

All the PCR reactions were carried out in individual tubes and the guidelines optimized by the original workers were strictly followed. After amplification, the products were subjected to agarose gel electrophoresis and the captured images were analyzed for the presence or absence of specific bands.

STATISTICAL ANALYSIS:
Differences between groups were tested by $\chi^2$ for categorical variables. Logistic regression analysis and coregression analysis were performed for adjusted age, sex, cardiovascular risk factors (smoking, hyperlipidemia, arterial hypertension, diabetes mellitus and BMI) in a multivariate model. Confidence intervals at the 95% level were calculated for the odds ratios. A value of $p \leq 0.05$ was considered significant.

RESULTS
The tissues collected from all the patients included in the study were subjected to histopathological examination for evidence of atheroma. None of the specimens from group II were atheromatous where as all 25 specimens from group I showed clear evidence of atheroma in the tissues. Risk Factors: The distribution pattern of conventional risk factors diabetes mellitus, hypertension, hyperlipidemia, smoking and abnormal BMI in subjects of both groups is shown in Table 1. The combination pattern and the presence of multiple risk factors in patients of groups I and II are shown in Table 2. The findings depicted in these tables show that there is not much difference in the distribution pattern of risk factors in the patients studied. On the other hand, it is evident that the occurrence of multiple risk factors is slightly higher in patients from group I and 5 patients from group II had no detectable risk factors. These differences were statistically significant ($p = 0.032$).

When comparison was made between the occurrence of number of microbial infections with the presence of risk factors, the association was seen to be significant (Correlation Coefficient $p=0.012$, Spearman’s rank correlation $p=0.010$).

Infections: Four (16%) specimens from patients with atheroma in group I and thirteen (32.5%) LIMA specimens from patients of group II did not show any evidence of infection with the microbes included in the study. This difference was significant with a $P$ value of <0.001.

When the incidence of individual infections in patients from both groups was compared, it could be seen that the infection rates for C. pneumoniae, H. pylori and HSV 1 were significantly much in group I patients (figure1). On the other hand, infection with CMV and HSV 2 did not show much difference between the two groups. In fact HSV 2 infection rates were slightly higher in group II subjects compared to those of group I. A look at the distribution pattern of these microbial infections revealed that 64% of patients in group I had

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**References:**
1. Dixit Mahadev Damodar et al. IRJP 2 (5) 2011 214-219
2. [Cited Reference 1]
3. [Cited Reference 2]
concurrent microbial infections with either 3 or 4 organisms studied. In contrast only 10% of patients from group II were infected with 3 organisms and simultaneous infection with 4 organisms was not seen at all. None of the subjects in both the groups had concurrent infections with all the five organisms tested (Table 3).

Univariate analysis of the risk factors in various microbial infections showed that CMV (p=0.098) and HSV1 (p=0.035) infections were more prevalent in older subjects. Similarly CMV (p=0.118) and H. pylori (p=0.058) were more frequently seen in patients with abnormal BMI. A significant association was seen between smoking and HSV1 (p=0.019) and C. pneumoniae (p=0.001). Chlamydia pneumoniae infections were also more prevalent in subjects with Diabetes Mellitus (p=0.004). But the levels of significance were reduced after adjustment for variables by multivariate analysis (Table 4).

**DISCUSSION**

Atherosclerosis is an inflammatory process that selectively affects arteries and is highly prevalent in both women and men. It develops as a result of sustained injury to the arterial wall and several culprits have been identified. One of them is the chronic infective state produced by several microorganisms. The mechanisms by which the association may be explained include increased coagulation, endothelial dysfunction, plaque instability and increased progression of atherosclerosis. Organisms that have been implicated include C. pneumoniae, H. pylori, periodontopathogens, Herpes viruses, influenza viruses, parovirus, enteroviruses, and hepatitis viruses.

In the present study, we have included C. pneumoniae, H. pylori, CMV and HSV 1 and 2 because all of them are intracellular pathogens and known to cause low grade, persistent infections. A number of techniques have been used to investigate the presence of these microbes either in the blood or affected blood vessels. Serological tests to detect various classes of specific antibodies (IgG, IgM, and IgA) have generally proved to be inconclusive. Hence, tests such as PCR, Immunocytochemistry (ICC) and Electron microscopy (EM) are commonly used to look for the evidence of infection produced by these organisms. Detection of the microbe in atheromatous plaques with its absence in normal arteries has been a most popular method of establishing association with atherosclerosis and the disease resulting from subsequent complications. Most studies investigating the relationship between infection and atherosclerosis related disease concentrate on the effect of single, individual pathogen. To our knowledge there are very few studies who have examined the effect of multiple pathogen burden on atherosclerosis, that too by using a technique such as PCR. In this context, the results of the present study have shown some interesting features.

In our study, the incidence of C. pneumoniae was highest (64%) in atheromatous tissues. This was followed by HSV 1 and H. pylori which were detected in 52% and 44% of the specimens respectively. These three microbes were found at relatively lower concentrations in LIMA specimens and the differences were statistically significant. In contrast, CMV and HSV 2 infections were found almost with similar incidence in specimens from patients of both groups. The strongest evidence for an association between infectious agents and cardiovascular disease is for C. pneumoniae and this has been confirmed by several studies. Our findings also are in accordance with these observations. We found the evidence for infection with C. pneumoniae in LIMA specimens with no evidence of atheroma to be higher (22.5%) compared to the projected incidence in normal vessels. This could be related to the age factor and the presence of risk factors such as smoking in these subjects. Another interesting observation made by us in the present study was that C. pneumoniae infection never existed alone and always co-existed with a combination of other microbes, but most commonly with CMV and HSV. Similar findings have been reported by other workers.

Several studies that have included HSV in their panel have detected these viruses at low rates both in atheromatous and normal vessels. But our findings differ from these observations: we have detected HSV 1 at a high frequency in atheromatous specimens. The incidence of HSV 2 and CMV do not differ much in diseased and normal arteries and their presence appears to be non-specific with no predilection for diseased vessels. Moreover, CMV is implicated in accelerating atherosclerosis following heart transplantation and post-coronary atherectomy restenosis.

The present study revealed the presence of H. pylori infection in a significant number of atheromas (44%) compared to LIMA specimens (12.5%). The findings of various workers regarding the association of H. pylori with atherosclerosis have been contradictory. It has been suggested that these discordant findings may be due to the genetic heterogeneity of the organism and the association may be restricted to Cag-A bearing, pathogenic strains of H. pylori. Several studies conducted recently show a definite role for cag A positive H. pylori strains in atherosclerosis and the findings assume significance for India where the incidence of H. pylori in adults is quite high.
Recently, it has been postulated that if infection does play a role in pathogenesis, then multiple pathogens are causally involved and that CAD risk relates to the aggregate pathogen load or pathogen burden. The results of our study also support this hypothesis and it was seen that the incidence of multiple infections was much higher in patients with plaque when compared to those without plaque. Similar observations have been made by other workers. 

When we compared the occurrence of infections with the presence of traditional risk factors, it could be seen that most of the infections occurred independent of the risk factors and there was no correlation. Only exceptions were association of smoking and diabetes mellitus with C. pneumoniae and that of obesity with H. pylori. They also became non-significant after appropriate adjustment for variables. 

In conclusion, our study has shown that the incidence of C. pneumoniae, H. pylori and HSV 1 is significantly higher in atheromatous individuals and multiple infections with several organisms also are common in these patients. Except for certain associations, most of the infections occurred independent of the traditional risk factors. These observations strongly support the hypothesis that multiple infections play an important role in atherosclerosis by either initiating or accelerating the process. They do not appear to be innocent bystanders. The role of H. pylori and HSV 1 in atherosclerosis has to be defined by additional studies because these organisms may play an important role in developing countries such as India where the incidence of infection with them is quite high, compared to western countries.

REFERENCES

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26. Epstein SE. The multiple mechanisms by which infections may contribute to atherosclerosis development and course. Circulation Res 2002; 90: 2-4

### TABLE 1: SHOWING DISTRIBUTION OF RISK FACTORS IN DIFFERENT GROUPS

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>16 (64%)</td>
<td>27 (67.5%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>10 (40%)</td>
<td>16 (40%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>19 (76%)</td>
<td>18 (45%)</td>
</tr>
<tr>
<td>Obesity</td>
<td>16 (64%)</td>
<td>16 (40%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>16 (64%)</td>
<td>06 (15%)</td>
</tr>
</tbody>
</table>

### TABLE 2: SHOWING COMBINATION PATTERN AND OCCURRENCE OF MULTIPLE RISK FACTORS IN DIFFERENT GROUPS

<table>
<thead>
<tr>
<th>No of Risk factors</th>
<th>Group I</th>
<th>Group II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>5 (12.5%)</td>
<td>05</td>
</tr>
<tr>
<td>1</td>
<td>3(12%)</td>
<td>7(17.5%)</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>5 (20%)</td>
<td>17(42.5%)</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>7(28%)</td>
<td>6 (15%)</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>7 (28%)</td>
<td>4 (10%)</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>3 (12%)</td>
<td>1 (2.5%)</td>
<td>04</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>40</td>
<td>65</td>
</tr>
</tbody>
</table>

### TABLE 3: SHOWING INCIDENCE OF MULTIPLE INFECTIONS IN GROUPS I AND II

<table>
<thead>
<tr>
<th>No of Infections</th>
<th>Group I</th>
<th>Group II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4 (16%)</td>
<td>13 (32.5%)</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td>5 (20%)</td>
<td>11 (27.5%)</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>12 (30%)</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>10 (40%)</td>
<td>4 (10%)</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>6 (24%)</td>
<td>0</td>
<td>06</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>40</td>
<td>65</td>
</tr>
</tbody>
</table>
TABLE 4: SHOWING UNIVARIATE AND MULTIVARIATE ANALYSIS OF DIFFERENT RISK FACTORS IN MICROBIAL INFECTIONS.

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted OR</td>
<td>P-Value</td>
</tr>
<tr>
<td></td>
<td>CMV</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.051</td>
<td>0.098</td>
</tr>
<tr>
<td>BMI</td>
<td>2.230</td>
<td>0.118</td>
</tr>
<tr>
<td>Age</td>
<td>4.269</td>
<td>0.019</td>
</tr>
<tr>
<td>Smoking</td>
<td>3.190</td>
<td>0.058</td>
</tr>
<tr>
<td>DM</td>
<td>5.412</td>
<td>0.004</td>
</tr>
<tr>
<td>Smoking</td>
<td>7.071</td>
<td>0.001</td>
</tr>
</tbody>
</table>

FIGURE 1: GRAPH SHOWING COMPARISON OF DIFFERENT INFECTIONS IN GROUPS I AND II

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