Detection of Diverse Bacterial Signatures in Atherosclerotic Lesions of Patients With Coronary Heart Disease

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Background—Bacterial infection has been discussed as a potential etiologic factor in the pathophysiology of coronary heart disease (CHD). This study analyzes molecular phylogenies to systematically explore the presence, frequency, and diversity of bacteria in atherosclerotic lesions in patients with CHD.

Methods and Results—We investigated 16S rDNA signatures in atherosclerotic tissue obtained through catheter-based atherectomy of 38 patients with CHD, control material from postmortem patients (n/H11005 15), and heart-beating organ donors (n/H11005 11) using clone libraries, denaturating gradient gel analysis, and fluorescence in situ hybridization. Bacterial DNA was found in all CHD patients by conserved PCR but not in control material or in any of the normal/unaffected coronary arteries. Presence of bacteria in atherosclerotic lesions was confirmed by fluorescence in situ hybridization. A high overall bacterial diversity of >50 different species, among them Staphylococcus species, Proteus vulgaris, Klebsiella pneumoniae, and Streptococcus species, was demonstrated in >1500 clones from a combined library and confirmed by denaturating gradient gel analysis. Mean bacterial diversity in atheromas was high, with a score of 12.33±3.81 (range, 5 to 22). A specific PCR detected Chlamydia species in 51.5% of CHD patients.

Conclusions—Detection of a broad variety of molecular signatures in all CHD specimens suggests that diverse bacterial colonization may be more important than a single pathogen. Our observation does not allow us to conclude that bacteria are the causative agent in the etiopathogenesis of CHD. However, bacterial agents could have secondarily colonized atheromatous lesions and could act as an additional factor accelerating disease progression. (Circulation. 2006;113:929-937.)

Key Words: atherosclerosis ■ bacteria ■ coronary disease ■ infection ■ plaque

The pathophysiology of coronary heart disease (CHD) is characterized by degenerative and inflammatory mechanisms. The general importance of elevated C-reactive protein (CRP) as a marker for disease progression has raised interest in the role of bacterial infection as a pathogenic factor. Specific pathogens have been detected in the arterial vessel wall in subsets of the patient population, and an association of disease with serological responses to viral and bacterial pathogens such as cytomegalovirus, herpes simplex virus, Chlamydia pneumoniae, or Helicobacter pylori has been reported. Periodontitis, which would lead to frequent bacteremia from the oral lesions, has been implicated as a risk factor for the development of cardiovascular disease. A recent study by Lehtiniemi et al hypothesized that atheromas might act as mechanical sieves collecting bacteria from the circulation as they observed a diverse colonization of the plaques. However, this small study used only a small number of postmortem specimens in which the origin, time point of colonization, and constitution of the bacterial organisms in the plaques remain unclear.

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The first interventional trials with macrolide antibiotics conducted to specifically eradicate plaque infection by chlamydia after acute coronary syndrome suggested a risk reduc-
such as roxithromycin or azithromycin in the treatment of CHD have recently failed to show a benefit for specific antibiotics. Controlled studies (Weekly Intervention With Zithromax/H11005) showed that CHD were obtained by coronary catheterization and comprehensive detection of the bacteria and an assessment of the presence and the spectrum of bacteria in coronary atherosclerotic lesions on the basis of their molecular phylogeny signatures. In a subanalysis, the results were correlated with demographic (age, gender) and clinical parameters, including serum levels of CRP, age, recurrence of stenotic disease, and cardiovascular risk factors. A systematic detection of the bacteria and an assessment of their diversity was based on different molecular techniques. Atherectomy specimens from 38 patients with CHD were obtained by coronary catheterization and compared with various controls (n=26), including postmortem specimens (n=15) and normal coronary material from heart-beating transplantation donors (n=11). In addition, specific PCR assays were used to assess signals from Chlamydia species, Mycoplasma species, and H pylori in the same material. Selected samples were examined by fluorescence in situ hybridization (FISH) analysis to localize bacteria detected by their molecular signatures.

### Methods

**Patients**

All 38 patients underwent directional coronary atherectomy under sterile conditions from nonostial de novo (n=31) or restenotic (n=7) lesions in native coronary arteries at the Cardiac Catheterization Laboratories of the First Medical Department, University Hospital Schleswig-Holstein, Campus Kiel (Germany). Six of the 38 patients who underwent successful directional coronary atherectomy had acute coronary syndrome; the other 32 patients had stable angina pectoris. Acute coronary syndrome was defined as new-onset or worsening angina that required hospitalization and was associated with alterations of the ST segment, with or without elevation of cardiac markers, including creatinine kinase and troponin measurements. Stable angina was defined as no change in frequency, duration, or intensity of symptoms within 6 weeks before the intervention. A predefined subgroup analysis comprised patients who had an elevated CRP, defined as serum levels >8 mg/dL. The baseline data of the patients included in the study are summarized in Table 1. The acute coronary syndrome, restenosis, and elevated CRP categories describe partially overlapping subgroups. The study was approved by the ethics committee of the medical faculty of the Christian-Albrechts University Kiel. All patients considered for the study gave written informed consent before catheter examination.

### Material for Control Experiments

Blood samples, catheter material (catheter tips, balloons, wires, introducers), swabs, and syringes were collected from 5 patients during heart catheter examination and subjected to the same molecular detection techniques as technical controls. Biological material from 26 control individuals was analyzed; tissue material from 15 postmortem patients (kidney, liver, myocardium, coronary arteries, including 8 splenetic tissue fragments) was obtained from the Institute of Pathology, University Hospital Schleswig-Holstein, Campus Kiel. The 15 postmortem patients suffered from malignant diseases. They were selected because no current infectious disease or secondary events such as cardiovascular complications and restenosis. However, large, randomized, controlled studies (Weekly Intervention With Zithromax Against Atherosclerosis and Related Disorders [WIZARD], Pravastatin or Atorvastatin Evaluation and Infection Therapy [PROVE-IT], Randomised Trial of Roxithromycin in Non-Q-Wave Coronary Syndromes [ROXIS], Azithromycin and Coronary Events Study [ACES]) that were completed recently have failed to show a benefit for specific antibiotics such as roxithromycin or azithromycin in the treatment of CHD.}

### Table 1. Baseline Characteristics of the CHD Patients Investigated in This Study

<table>
<thead>
<tr>
<th></th>
<th>All Patients (n=38)*</th>
<th>Acute Coronary Syndrome (n=31)*</th>
<th>Restenosis (n=7)*</th>
<th>Elevated CRP (n=17)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at intervention, y‡</td>
<td>55.5 (31–83)</td>
<td>65.5 (36–83)</td>
<td>51 (31–63)</td>
<td>54 (36–72)</td>
</tr>
<tr>
<td>Sex, n‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (86.8)</td>
<td>5 (83.3)</td>
<td>7 (100)</td>
<td>15 (88.2)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (13.2)</td>
<td>1 (16.7)</td>
<td>...</td>
<td>2 (11.8)</td>
</tr>
<tr>
<td>Coronary artery, n‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCA</td>
<td>17 (44.7)</td>
<td>5 (83.3)</td>
<td>4 (57.1)</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>LAD</td>
<td>21 (55.3)</td>
<td>1 (16.7)</td>
<td>3 (42.9)</td>
<td>8 (47.1)</td>
</tr>
<tr>
<td>Blood values*§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/dL§</td>
<td>8.6±10.5 (&lt;3.2–51.1)</td>
<td>13.3±20.2 (&lt;3.2–51.1)</td>
<td>5.8±9.6 (8.4–25.5)</td>
<td>16.8±10.1 (8.4–51.1)</td>
</tr>
<tr>
<td>Leukocytes (per nL), n</td>
<td>8.1±2.0 (5.2–14.6)</td>
<td>7.9±0.9 (6.7–9.4)</td>
<td>5.8±2.9 (6.2–14.6)</td>
<td>9.3±2.1 (6.5–14.6)</td>
</tr>
<tr>
<td>Cardiovascular risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>19 (50)</td>
<td>1 (16.7)</td>
<td>3 (42.9)</td>
<td>8 (47.1)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>20 (52.6)</td>
<td>2 (33.3)</td>
<td>4 (57.1)</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5 (13.2)</td>
<td>1 (16.7)</td>
<td>1 (14.3)</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Smoking</td>
<td>23 (60.5)</td>
<td>2 (33.3)</td>
<td>6 (85.7)</td>
<td>12 (70.6)</td>
</tr>
</tbody>
</table>

RCA indicates right coronary artery; LAD, left artery descending artery. Acute coronary syndrome, restenosis, and elevated CRP describe partially overlapping subgroups as detailed in Methods.

*Including patients for FISH analysis.
†Expressed as median age (range).
‡Expressed as number of patients (frequencies are indicated in parentheses).
§Values <8 mg/dL were considered normal (detection limit, 3.2 mg/dL).
||Information on leukocytes was available for 35 of the 38 patients; information on CRP was available for 37 of the 38 patients.
the last 3 months before death. Permission for postmortem examination and research use of tissues was obtained from the next of kin.

Native coronary arteries from explanted hearts were obtained from 11 heart-beating tissue donors (7 men, 4 women; median age, 52 years; range, 34 to 64 years; left anterior descending artery, n = 5; right coronary artery, n = 6) from the Kiel transplantation program. The explanted hearts were discarded from implantation for morphological or technical reasons. None of these patients had an active infectious disease (CRP levels not elevated), recent or current treatment with antibiotics, or atherosclerosis of any coronary artery in macroscopic examination. The main inclusion criteria for the study were no external trauma, no infection, no use of antibiotics, and age of between 30 and 70 years. The cause of death was cerebral hypoxemic state after arrhythmia resulting from idiopathic dilated cardiomyopathy, n = 3; and cerebrovascular event, n = 2; severe intracerebral hemorrhage, n = 3; and cerebral hypoxic state after arrhythmia resulting from idiopathic dilated cardiomyopathy, n = 1. None of these patients had clinical or macroscopic signs of CHD.

Experimental Design

Because the PCR-based techniques used require the full use of the entire specimen obtained by arterectomy, a hierarchical study design was used. Thirty-three samples were extracted for analysis by arterectomy, a hierarchical study design following the protocol of Heuer et al. As negative controls, gel slices containing cell and tissue fragments from surrounding regions of the bands were investigated. Isolation of DNA fragments from destained gel slices was performed as described by Schwieger and Tebbe. The recovered DNA was reamplified with primers U968-GC and L1401. For characteristics of microorganisms, the protocol of Heuer et al.26 was followed.

TABLE 2. Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Species</th>
<th>Dir</th>
<th>Position</th>
<th>L, bp</th>
<th>T, °C</th>
<th>C, n</th>
<th>Sequence (5′-3′)</th>
<th>Gene</th>
<th>Ref.</th>
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<tr>
<td>U968-GC</td>
<td>Eubacteria</td>
<td>f</td>
<td>968–84*</td>
<td>400</td>
<td>53</td>
<td>30/10</td>
<td>CGCCCCGCGGCGGCCGGCGCGGC-ACGGGGGAGAAGAAGGCTACAC</td>
<td>16S</td>
<td>20</td>
</tr>
<tr>
<td>L1401</td>
<td></td>
<td>r</td>
<td>1378–1401*</td>
<td>150</td>
<td>55</td>
<td>15/10</td>
<td>CGGTGGTACAGGCGGAGAAGCG</td>
<td>16S</td>
<td>27</td>
</tr>
<tr>
<td>COM1</td>
<td>Eubacteria</td>
<td>f</td>
<td>519–36*</td>
<td>370</td>
<td>50</td>
<td>25/10</td>
<td>CAGCGGCGCCGTAATAC</td>
<td>16S</td>
<td>18</td>
</tr>
<tr>
<td>COM2</td>
<td></td>
<td>r</td>
<td>907–26*</td>
<td>250</td>
<td>50</td>
<td>15/10</td>
<td>CGGTCAATCTCTTGAAGTT</td>
<td>16S</td>
<td>18</td>
</tr>
<tr>
<td>M13(21)</td>
<td>Vector</td>
<td>f</td>
<td>389–404†</td>
<td>300</td>
<td>55</td>
<td>30/10</td>
<td>TGGAAAGACGCGGAGT</td>
<td>16S</td>
<td>27</td>
</tr>
<tr>
<td>M13(24)</td>
<td></td>
<td>r</td>
<td>205–21$</td>
<td>300</td>
<td>55</td>
<td>30/10</td>
<td>AACACGGTACGAGCT</td>
<td>16S</td>
<td>27</td>
</tr>
<tr>
<td>GADPH_F2</td>
<td>Human DNA</td>
<td>f</td>
<td>...</td>
<td>300</td>
<td>62</td>
<td>40</td>
<td>ACCCAACTCTCCACCTT</td>
<td>16S</td>
<td>27</td>
</tr>
<tr>
<td>GADPH_R2</td>
<td></td>
<td>r</td>
<td></td>
<td>300</td>
<td>62</td>
<td>40</td>
<td>CTGTTTGCTGAGCAATATTG</td>
<td>16S</td>
<td>27</td>
</tr>
<tr>
<td>Chlam_F</td>
<td>Chlamydia species</td>
<td>f</td>
<td>40–55*</td>
<td>250</td>
<td>51</td>
<td>45</td>
<td>CGGGGTGAATACGATGC</td>
<td>16S</td>
<td>24</td>
</tr>
<tr>
<td>Chlam_R</td>
<td></td>
<td>r</td>
<td>310–24*</td>
<td>250</td>
<td>51</td>
<td>45</td>
<td>TCACTCCAGGTGTTGG</td>
<td>16S</td>
<td>24</td>
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<tr>
<td>H_pyl_F</td>
<td>H pylori</td>
<td>f</td>
<td>629–46*</td>
<td>80</td>
<td>48</td>
<td>35</td>
<td>AACGTGATTTGAAACTAC</td>
<td>16S</td>
<td>22</td>
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<tr>
<td>H_pyl_R</td>
<td></td>
<td>r</td>
<td>721–39*</td>
<td>80</td>
<td>48</td>
<td>35</td>
<td>TCGCTCTGCAATGTA</td>
<td>16S</td>
<td>22</td>
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<tr>
<td>NUC1</td>
<td>S aureus</td>
<td>f</td>
<td>...</td>
<td>270</td>
<td>55</td>
<td>35</td>
<td>GCGATTGATGATGATACGTT</td>
<td>Nuc</td>
<td>25</td>
</tr>
<tr>
<td>NUC2</td>
<td></td>
<td>r</td>
<td></td>
<td>270</td>
<td>55</td>
<td>35</td>
<td>AGCCGACCGTTCGAGAATAAAGC</td>
<td>Nuc</td>
<td>25</td>
</tr>
<tr>
<td>TP5_F</td>
<td>Eubacteria</td>
<td>f</td>
<td>906–26*</td>
<td>490</td>
<td>56</td>
<td>35</td>
<td>AAACCTAAATGATTGACGCG</td>
<td>16S</td>
<td>18</td>
</tr>
<tr>
<td>Enter1416</td>
<td>Enterobacteriaceae</td>
<td>f</td>
<td>1416–23*</td>
<td>1416</td>
<td>55</td>
<td>30</td>
<td>CGTTTGCAACGCACTTC</td>
<td>16S</td>
<td>27</td>
</tr>
</tbody>
</table>

Dir indicates direction; L, length of fragment; T, annealing temperature; C, number of PCR cycles; Ref, reference; f, forward; r, reverse. Chlam represents Chlamydia; and Ecoli represents Escherichia coli.

$ denotes a GC-rich sequence (GC clamp) was attached to the 5′ end to prevent complete melting of double-strand PCR products during separation.

The 5′ terminal of the primer was 5′-3′ reconditioning PCR.

Universal and Specific Primers

The established PCR primer pairs U968-GC/L1401 and COM1/COM2 were used.19–21 The oligonucleotides target the V6 through V9 and the V4 and V5 hypervariable regions of the 16S rRNA gene, respectively. Oligonucleotides for the detection of H pylori were designed with the PRIMER-DESIGN tool of the ARB software.22 To detect Enterobacteriaceae, the eubacterial primer TP5 was combined with a primer specific for Enterobacteriaceae (Enter1432, modified from Sghir et al23). The primer sequences for the detection of Chlamydia species and Staphylococcus aureus were obtained from the literature.24,25 Characteristics of primers are listed in Table 2. Detection of Mycoplasma species was conducted according to the instructions of the manufacturer of the PCR assay (VeneroM Mycoplasma Detection Kit, Minerva Biologs).

DGGE Analysis

Samples from 33 patients were investigated by DGGE analysis as previously described.21,26 A 2-step reconditioning PCR approach was used to reduce formation of heteroduplexes and PCR artifacts, following the protocol of Thompson et al and Acinas et al.27,28 Briefly, a first PCR with 30 cycles was performed in the presence of excess PCR reagents to avoid effects of limited amplification. A second PCR step using only 10 cycles (reconditioning PCR) was then performed after 1:10 dilution of PCR products. Identification of the operational taxonomic units (OTUs) underlying the bands followed the protocol of Heuer et al.26 As negative controls, gel slices from surrounding regions of the bands were investigated. Isolation of DNA fragments from destained gel slices was performed as described by Schwieger and Tebbe.26 The recovered DNA was reamplified with primers U968-GC and L1401. For characteristics of primers, see Table 2. The PCR products were cloned into competent Escherichia coli cells with the PCR 2.1 TOPO TA Cloning Kit for solid catheter materials (swabs, introducers, wires, catheter tips, balloons) were washed in demineralized water for 30 minutes to dissolve cell and tissue fragments.

The treatment of atherectomy particles, control material, and DNA extraction DNA from blood and tissue specimens was extracted following a protocol adapted for the characteristics of microorganisms. The
sequencing (Invitrogen) according to the manufacturer’s instructions. Sequencing of the inserts was performed as described. More than 250 clones (n=254) were sequenced and analyzed. Image analysis was performed as described previously.

Clone Libraries
More than 1500 clones were sequenced from a library that was generated from pooled PCR products of 10 atherectomy samples. An ~400-bp fragment of the 16S rDNA containing the V4/5 variable region was amplified and cloned into E. coli cells as described. A 2-step reconditioning PCR approach (25/10 cycles) was used as described above. Cloning and sequencing of inserts were performed as described above.

FISH and Oligonucleotide Probes
Atherectomy specimens from 5 patients with CHD were fixed in 500 µL freshly prepared 4% buffered formalin and embedded in Shandon Histoplast Paraffin (Thermo Electron Corp) according to routine procedures. Cross sections (2 µm) were cut and placed on coated microscope slides (Superfrost±/Plus). Sections were hybridized as described previously with the following 16S/23S rRNA-targeted oligonucleotide probes: an equimolar mixture of 5 bacteria-directed probes—EUB 338, EUB 785, EUB 927, EUB 1055, and EUB 1088, referred to as EUB mix—to detect all bacteria.

Data and Sequence Analyses
Most of the data did not follow a normal distribution. An explorative analysis was carried out to investigate relationships between diversity (assessed as number of bands) and demographic (age, gender) or clinical (indication for examination: restenosis versus myocardial syndrome, elevated versus normal levels of CRP, chlamydia infection, cardiovascular risk factors) characteristics. Because the number of bands was asymmetrically distributed in some of the subgroups, data are represented by median values with quartiles and illustrated via box-and-whisker plots. Statistical tests of differences in location parameters were performed by 2-sided Wilcoxon rank-sum tests for independent samples at a 5% level of significance. The statistical analysis was conducted with SPSS (version 10.0). Alignment and assembly of partial sequences were performed with the Sequencher software package (Gene Codes Corp). OTUs were identified by Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information) analysis using search results of at least 97% similarity. The sequences were examined for chimera with the Chimera Check tool of the Ribosomal Data Projects of the Center for Microbial Ecology, Michigan State University. For the taxonomic classification of sequences that could not be assigned to known bacterial species in the BLAST analysis, comparative sequence analysis using the ARB software package was used.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Detection of Bacterial DNA in Atherosclerotic Plaques
Total amounts of 60 to 610 ng (mean, 135.6 ng) of genomic DNA were extracted from coronary atherectomy specimens. Bacterial DNA was found in all 38 CHD patient samples by either PCR using the primer pairs U968-GC/L1401 and COM1/COM2 (n=33) or FISH analysis (n=5). The presence of bacterial rRNA could be demonstrated by FISH analysis in selected patients using a mix of 16S/23S rRNA-targeted oligonucleotide probes. Figure 1 shows single bacteria (Figure 1A and 1D) and groups of bacteria (Figure 1B and 1C) in the cross sections of atherosclerotic plaques.

Overall Bacterial Diversity of the Clone Library
Clone libraries are currently the most accurate molecular method to represent the microbial composition of a complex habitat. A library was generated from pooled PCR products. For this experiment, the 10 patient samples with the highest bacterial diversity in the DGGE examination described below were used. More than 1500 clones were sequenced (mean length of sequences, 338.9±69). Chimeric sequences and incomplete inserts were discarded, resulting in 903 sequences that were used for the final analysis. More than 50 different clones were detected. They represented bacteria involved in skin infections such as Staphylococcus species, respiratory infections such as Klebsiella pneumoniae or Proteus vulgaris, or oral bacteria such as different Streptococcus species. Table 3 shows the molecular OTUs as a representation for the variety...
of species involved. Assignment to known bacterial species used BLAST analysis. Most interestingly, >100 clones of those shown in Table 3 (uncultured bacteria) did not correspond to presently known bacterial species. Table 4 shows the taxonomic relationships that were assigned by comparative phylogenetic sequence analysis.

### Formal Assessment of Bacterial Diversity in Coronary Atherosclerotic Lesions

To explore the diversity and distribution of bacterial DNA in atherosclerotic plaques, DGGE analysis was performed in the samples of 33 patients. A representative gel demonstrating the variety of the different interindividual banding patterns is shown in Figure 2A. DNA from selected bands was reamplified and sequenced. The spectrum of bacteria identified through DGGE and band-matching analysis was concordant with that found in the clone libraries, except for 1 additional *Bacillus* species (Figure 2B) that was detected only through DGGE. Additional controls included amplification of species identified by DGGE through independent direct PCR (eg, *S. aureus*, *Enterobacteriaceae*, data not shown).

A total of 40 different band classes were calculated. The frequency of occurrence for each band ranges from 3% (only 1 patient) to 93.9% (31 of 33 patients). The diversity of bands varies from 5 (patient 8) to 22 (patient 16). The mean diversity of bands was 12.33% (range: 5–18), whereas the Shannon-Wiener diversity index of bands was 3.81. Diversity was calculated for each band according to its frequency of occurrence for each band ranges from 3% (only 1 patient) to 93.9% (31 of 33 patients). The diversity of bands varies from 5 (patient 8) to 22 (patient 16). The mean diversity of bands was 12.33% (range: 5–18), whereas the Shannon-Wiener diversity index of bands was 3.81. Diversity was calculated for each band according to its frequency of occurrence for each band ranges from 3% (only 1 patient) to 93.9% (31 of 33 patients). The diversity of bands varies from 5 (patient 8) to 22 (patient 16). The mean diversity of bands was 12.33% (range: 5–18), whereas the Shannon-Wiener diversity index of bands was 3.81. Diversity was calculated for each band according to its frequency of occurrence for each band ranges from 3% (only 1 patient) to 93.9% (31 of 33 patients). The diversity of bands varies from 5 (patient 8) to 22 (patient 16). The mean diversity of bands was 12.33% (range: 5–18), whereas the Shannon-Wiener diversity index of bands was 3.81.

#### Table 3

<table>
<thead>
<tr>
<th>Closest Relative (NCBI BLAST)</th>
<th>Frequency</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em> species</td>
<td>218</td>
<td>24.94</td>
<td></td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>164</td>
<td>18.76</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>99</td>
<td>11.33</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>66</td>
<td>7.55</td>
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<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>37</td>
<td>4.23</td>
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<tr>
<td><em>Pseudomonas diminuta</em></td>
<td>33</td>
<td>3.78</td>
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<tr>
<td><em>Streptococcus parasanguis</em></td>
<td>22</td>
<td>2.52</td>
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<tr>
<td><em>Enterobacteriaceae</em> bacterium*</td>
<td>18</td>
<td>2.06</td>
<td></td>
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<tr>
<td><em>Streptococcus bacterium</em></td>
<td>16</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td><em>Brucella</em> bacterium*</td>
<td>12</td>
<td>1.37</td>
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<td><em>Acetobacteraceae</em> bacterium*</td>
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<td><em>Pseudomonas</em> species</td>
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<td><em>Sphingobacterium</em> species</td>
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<td><em>Enterobacter</em> dissolvens</td>
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<tr>
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<tr>
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<tr>
<td><em>Ochrobactrum</em> species</td>
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<td></td>
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<tr>
<td><em>Pantoea</em> agglomerans</td>
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<td>0.34</td>
<td></td>
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<tr>
<td><em>Streptococcus</em> anginosus</td>
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<td>0.34</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> constellatus</td>
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<td>0.23</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> cristaure</td>
<td>2</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> sinensis</td>
<td>2</td>
<td>0.23</td>
<td></td>
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<tr>
<td><em>Staphylococcus</em> haemolyticus</td>
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<td></td>
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<tr>
<td><em>Staphylococcus</em> hominis</td>
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<td>0.23</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus</em> pasteurii</td>
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<td>0.23</td>
<td></td>
</tr>
<tr>
<td><em>Burkholderiaceae</em> bacterium</td>
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<td>0.23</td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia</em> mallei</td>
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<td></td>
</tr>
<tr>
<td><em>Citrobacter</em> freundii</td>
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<td>0.11</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter</em> cloacae</td>
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<td>0.11</td>
<td></td>
</tr>
<tr>
<td><em>Nocardia</em> species</td>
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<td>0.11</td>
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<td><em>Ralstonia</em> species</td>
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<td>0.11</td>
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<td>0.11</td>
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<td>Uncultured bacteria</td>
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<td>12.47</td>
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</tbody>
</table>

*OTUs were identified by NCBI BLAST analysis using search results of at least 97% similarity (NCBI BLAST, available at http://www.ncbi.nlm.nih.gov/). Nomenclature according to Bergey's *Manual of Determinative Bacteriology* (Holt, 1994).*

#### Table 4

<table>
<thead>
<tr>
<th>No.</th>
<th>Phylum†</th>
<th>Class‡</th>
<th>Order‡</th>
<th>Percent of Clones</th>
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<td>Enterobacterales</td>
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<td>Rhizobiales</td>
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<td>Caulobacterales</td>
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<tr>
<td>4†</td>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>5</td>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Bacillales</td>
<td>5.0</td>
</tr>
<tr>
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<td>Proteobacteria</td>
<td>γ-Proteobacteria</td>
<td>Xanthomonadales</td>
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<tr>
<td>7</td>
<td>Proteobacteria</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>5.0</td>
</tr>
<tr>
<td>8</td>
<td>Proteobacteria</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>5.0</td>
</tr>
<tr>
<td>9</td>
<td>Proteobacteria</td>
<td>α-Proteobacteria</td>
<td>Sphingomonadales</td>
<td>2.5</td>
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<tr>
<td>10</td>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Bacillales</td>
<td>2.5</td>
</tr>
<tr>
<td>11</td>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Lactobacillales</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*Sequences of unidentified clones were analyzed with the ARB software package using the Parsimony interactive algorithm including local optimization modus; comparative sequence analysis was performed without specific filtering. †Nomenclature according to Bergey's *Manual of Determinative Bacteriology* (Holt, 1994). ‡Further taxonomic classification was not possible.*
Control Individuals and Technical Controls
Primer pairs U968-GC/L1401 and COM1/COM2 were used for universal bacterial DNA amplification. As a technical control, blood samples, catheter material (catheter tips, balloons, wires, introducers), swabs, and syringes were collected from 5 patients during heart catheter examination. Direct universal PCRs as described above gave negative results. Control biological samples were obtained postmortem.

Figure 2. A, Original DGGE profiles of 8 patients with CHD (patients 1 through 8). All 33 patients were examined by DGGE analysis with similar results. The distribution of bands demonstrates the interindividual variability of banding patterns. Selected bands were excised from the gels, reamplified, cloned, and sequenced as described above. The OTUs were identified with NCBI BLAST comparative analysis using results of ≥97% sequence identity. B, Species that could be assigned to bacterial species. The spectrum of species identified by DGGE analysis and subsequent taxonomic classification is concordant with that of the clone library, except for one additional Bacillus species (band VI). Nomenclature according to Bergey’s Manual of Determinative Bacteriology.43

Figure 3. Explorative analysis carried out to investigate relationships between diversity (assessed as number of bands) and demographic (age) or clinical (indication for examination: restenosis vs myocardial syndrome, elevated vs normal levels of CRP, Chlamydia infection) characteristics. None of the results reached statistical significance. Indicated are the medians/interquartile ranges (IQR). A, Bacterial diversity in relation to patient age. Diversity tends to higher values in younger patients (16/6.75 vs 14/3.5 in patients >55 years of age; P=0.488). B, Diversity is higher in the group of CRP-positive patients (16/7 vs 13.5/3.25; P=0.255). C, Diversity shows only slight differences between patients referred for acute coronary syndrome and patients with stable angina pectoris (16/5.75 vs 13/3.5; P=0.1). D, The subgroup analysis for the Chlamydia group showed higher bacterial diversity in those patients who tested positive for Chlamydia (13.5/4 vs 16/5.5; P=0.053).
(n=15, including splenetic tissue from 8 individuals) and from nontransplanted hearts from the organ donor program (n=11, healthy coronaries). No bacterial DNA was found, including the macrophage-rich spleen tissue. Five additional PCR cycles were used in the controls to exclude low-level bacterial signals, also. The efficacy of nucleic acid extraction and the presence of DNA in the control samples were verified with a GADPH-PCR that is routinely performed to detect DNA contamination during RNA extraction that was conducted according to an established protocol. In addition, a 16S-based clone library was constructed, and 100 clones were sequenced.

**Specific Detection of Chlamydia Species, Mycoplasma Species, and H pylori**

Specific PCR assays were applied to determine known bacterial species that have previously been implicated in the pathophysiology of CHD. *Chlamydia* species were detected in 17 of the 33 atherectomy samples (51.5%). Further characterization by sequencing revealed most of them as *C pneumoniae* and *C trachomatis*. *Mycoplasma* species and *H pylori* could not be detected.

**Discussion**

We observed frequent colonization of atherosclerotic lesions in CHD. The high overall diversity of bacterial DNA in coronary lesions supports the hypothesis of multiple bacterial colonizations of the arterial lesions. These findings underscore the infection hypothesis in the pathophysiology of coronary atherosclerosis. However, several explanations are contradicting according to an established protocol. In addition, a 16S-based clone library was constructed, and 100 clones were sequenced.

Specific Detection of *Chlamydia* Species, *Mycoplasma* Species, and *H pylori*

Specific PCR assays were applied to determine known bacterial species that have previously been implicated in the pathophysiology of CHD. *Chlamydia* species were detected in 17 of the 33 atherectomy samples (51.5%). Further characterization by sequencing revealed most of them as *C pneumoniae* and *C trachomatis*. *Mycoplasma* species and *H pylori* could not be detected.

In our study, we detected only a nonsignificant tendency for an association of CRP levels with the degree of bacterial diversity. This could be due to a lack of power through the limited numbers of patients in the subgroup analysis. On the other hand, the extent of infected lesions (and not the degree of diversity in the bacterial flora within the lesions) could be the main driver for the height of the CRP levels.

The high rate of positive *C pneumoniae* signals corresponds with previous studies. The range of *C pneumoniae*–positive patients varies extremely in the literature. Chiu and colleagues found *C pneumoniae* in 54 of 76 endarterectomy specimens (71%) by immunostaining, whereas Maass et al could detect *C pneumoniae* in only 11 of 70 atheromas (16%) by direct cultivation and in 21 (30%) by PCR. In one of the first studies, Shor et al demonstrated *C pneumoniae* in coronary fatty streaks in 5 of 7 autopsy patients (71%). The differences in detection of *C pneumoniae* in view of constantly high seropositivity rates for this infectious agent could also point to an important role of individual innate immunity mechanisms involved in the elimination of pathogenic bacteria.

The broad bacterial spectrum in CHD included a host of species that are commonly found on human barrier organs like skin or the oral cavity. Interestingly, animal models with experimentally induced periodontitis, which leads to frequent Gram-negative bacteremia, had more extensive accumulations of lipids in the aorta compared with animals without periodontitis, suggesting that the inflammatory burden provided by the defective oral mucosal barrier directly contributes to atherosclerosis. Several of the species detected in human coronaries in this study are part of the commensal human flora including *Staphylococcus* species, *Streptococcus* species, *K pneumoniae*, *P vulgaris*, or *Burkholderia* species but might exhibit pathogenic characteristics under certain conditions. For example, the group of nonfermentative Gram-negative bacilli (including *Pseudomonas* species), especially *Pseudomonas aeruginosa*, is involved in respiratory tract infections and bacteremia in immunocompromised patients, patients with cystic fibrosis, children, or elderly people. The spectrum of bacteria in CHD lesions also comprises true pathogens such as *K pneumoniae*, a facultative member of the gastrointestinal microflora.

There are several possible explanations for the presence of the highly diverse bacterial DNA signatures in atherosclerotic coronary lesions. Bacterial translocation from the gastrointestinal tract, especially the oral cavity, is common. The incidence of bacteremia after dental procedures such as tooth extraction, endodontic treatment, periodontal surgery, and root scaling is well documented. Transient bacteremia is also common after surgical procedures, endoscopy, manipulation of infected tissue, or local infections. Infections of mucosal organs but also physiological processes such as defecation can lead to transient detection of bacterial material in the circulation. The correlation of *C pneumoniae* infection and detection rates in atherosclerotic plaques of a subgroup of CHD patients supports this hypothesis. Up to 60% of the population have serological evidence of previous infection, and individuals are probably infected several times throughout their lives or harbor chronic infection. Recent publications showed that even rare bacteria might be present in the blood if the mucosal barrier is defective, as demonstrated for *Mycobacterium paratuberculosis* in patients with Crohn’s disease.

Most significantly, the high overall bacterial diversity in coronary artery plaques, in combination with large interindividual differences, suggests that CHD is no monoinfectious disease caused by a single, specific pathogen. Our results are compatible with the hypothesis that infectious agents could be secondary factors that lead to the progression of lesions in the vessel wall through secondary
colonization of atheromatous material. However, the etiological role of microbiota in the pathophysiology of CHD remains to be defined. 

Acknowledgments

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Disclosures

Dr Schreiber has been a consultant to Applied Biosystems. The other authors report no conflicts.

References

CLINICAL PERSPECTIVE

Coronary heart disease (CHD) is one of the major health problems in the western industrialized world. CHD is widely recognized as an inflammatory disease. The general importance of elevated C-reactive protein (CRP) as a marker for disease progression has raised interest in the role of inflammation, specifically bacterial infection, as a pathogenic factor. Microbiota such as *Chlamydia pneumoniae* have been described in coronary atherosclerotic plaques in subsets of patients, but results were inconsistent. This study reports a systematic phylogenetic analysis of molecular signatures of bacteria in a series of patients with CHD. A whole spectrum of bacteria was found through PCR-based techniques and confirmed by direct morphological localization of bacteria with fluorescence in situ hybridization (FISH). Coronary plaques excised by atherectomy contained a wide variety of bacterial species. Interindividual variation was prominent. Control tissues were free of bacterial signatures. These findings suggest the possibility that recurrent microbial challenge may be a major risk contributor to atherosclerotic lesions in the vessel wall in CHD.
Detection of Diverse Bacterial Signatures in Atherosclerotic Lesions of Patients With Coronary Heart Disease

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