Impact of a Molecular Approach to Improve the Microbiological Diagnosis of Infective Heart Valve Endocarditis

Claudia Breitkopf, MD; Dieter Hammel, MD; Hans H. Scheld, MD; Georg Peters, MD; Karsten Becker, MD

Background—Even today, infective endocarditis (IE) remains a severe and potentially fatal disease demanding sophisticated diagnostic strategies for detection of the causative microorganisms. Despite the use of appropriate laboratory techniques, classic microbiological diagnostics are characterized by a high rate of negative results.

Methods and Results—Broad-range polymerase chain reaction (PCR) targeting bacterial and fungal rDNA followed by direct sequencing was applied to excised heart valves (n=52) collected from 51 patients with suspected infectious endocarditis and from 16 patients without any signs of IE during an 18-month period. The sensitivity, specificity, and the positive and negative predictive values for the bacterial broad-range PCR were 41.2%, 100.0%, 100.0%, and 34.8%, respectively, compared with 7.8%, 93.7%, 80.0%, and 24.2% for culture and 11.8%, 100.0%, 100.0%, and 26.2% for Gram staining. Without exception, database analyses allowed identification up to the (sub)species level comprising streptococcal (n=13), staphylococcal (n=4), enterococcal (n=2), and other signature sequences such as Bartonella quintana and Nocardia paucivorans. Fungal ribosomal sequences were not amplified. All valve tissues of the reference group were negative for both PCR and conventional methods, except one sample that was contaminated by molds.

Conclusions—Culture-independent molecular methods substantially improve the diagnostic outcome of microbiological examination of excised heart valves. Importantly, this was true not only for fastidious, slow-growing, and/or nonculturable microorganisms but also for easy-to-culture pathogens such as streptococci and staphylococci. Both patient management and empiric antibiotic therapy of IE are likely to benefit from improved knowledge of the spectrum of pathogens now causing IE. (Circulation. 2005;111:1415-1421.)

Key Words: diagnosis ■ infection ■ polymerase chain reaction ■ surgery ■ valves

Infective endocarditis (IE) remains a major medical concern because of its associated mortality rate and costs.1 IE is usually diagnosed by clinical, histological, and/or microbiological parameters according to the Duke scheme.2 Although the modified Duke criteria have been validated in several studies, they have several limitations, most important of which is their poor performance in blood culture–negative endocarditis.3–5 Clinical suspicion of IE is often difficult to confirm because blood cultures are negative in 2.5% to 31% of suspected IE cases despite the use of appropriately appropriate laboratory procedures.5–8 Conventional microbiological techniques such as microscopy and (enrichment) cultures may not detect the causative microorganisms in blood because of previous therapy with antimicrobial agents, suboptimal sample collection of specimens, and/or fastidious, slow-growing, or nonculturable microorganisms.5,9 These innate limitations of classic microbiological diagnostics apply also for examination of excised heart valves. To overcome these restrictions, culture-independent molecular techniques based on amplification and subsequent direct sequencing of ribosomal sequences have been applied.10–13 These modern techniques have been evaluated only in a limited number of studies and are currently undergoing optimization, reevaluation, and validation. Thus, further studies are warranted before molecular methods can become part of daily diagnostic procedures.3

See p 1352

Here, we present data on a large number of surgically excised cardiac valves obtained from patients with suspected IE compared with a reference group of patients without any signs of IE. We used a broad-spectrum polymerase chain reaction (PCR) to amplify bacterial and fungal ribosomal sequences, followed by direct sequencing to detect and differentiate the causative agents of IE.

Methods

Specimens

Over an 18-month period, 52 excised heart valves of 51 patients (42 men, 9 women; average age, 52.4 years; range, 17 to 78 years) classified before surgery as having suspected IE (from clinical, microbiological, and/or echocardiographic findings) were analyzed.
in a cross-sectional, observational study in a routine setting at the University of Münster Hospital (Germany). IE was classified according to Duke’s criteria on the basis of gross features and histopathological findings as definite.2-4 IEs classified as rejected, ie, no pathological evidence of IE, were not included in the study. Patients mostly were admitted from other hospitals because of cardiac complications. Sixteen resected heart valves from patients (7 men, 9 women) who did not have any signs of IE were studied as references. Excised native cardiac valves were divided equally for culture and molecular analysis in a laminar flow unit. In addition, material for culture techniques

Culture Techniques

The samples were cultured aerobically with a set consisting of Columbia blood agar (Becton Dickinson), endo agar (Merck), and Kimmig agar (Merck). In addition, cultivation was performed on chocolate agar (Mast) with 5% CO2 and on Schaedler agar (Becton Dickinson) anaerobically. All plates were incubated at 35°C for up to 2 to 3 days. Brain-heart infusion broth was inoculated and incubated at 35°C for up to 10 days. Isolated colonies were identified according to standard procedures.14 Results of blood cultures, if collected before cardiac surgery, were evaluated retrospectively.

DNA Extraction and PCR Amplification

All molecular procedures were carried out according to standard recommendations to avoid contamination events, including room separation and use of the uracil DNA-glycosylase system. DNA was extracted from cardiac tissues with the QIAamp DNA kit (Qiagen). Extracted DNA was stored at −80°C before DNA extraction.

For PCR amplification of bacterial sequences, the primers 27f and 907r(m) were used (Table 1). In addition, panfungal primers ITS4 and ITS5 targeting the ITS-1 and ITS-2 regions of fungal 18S and 28S rDNA were applied.15 PCR amplifications were performed as previously described.16

TABLE 1. Oligonucleotide Primers Used for PCR Amplification and Sequencing of Bacterial 16S rDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Sequence (5’-3’)</th>
<th>Location Within Gene*</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27f</td>
<td>aga gtt tga tcm tgg ctc ag</td>
<td>8–27</td>
<td>Panbacterial</td>
<td>Bacteria</td>
</tr>
<tr>
<td>907(m)</td>
<td>ccc tca att cmt tgg agt tt</td>
<td>907–926</td>
<td>Panbacterial</td>
<td>Bacteria</td>
</tr>
<tr>
<td>357f(m)</td>
<td>act cct acg gga ggc agc ag</td>
<td>338–357</td>
<td>Panbacterial</td>
<td>Bacteria</td>
</tr>
<tr>
<td>785r</td>
<td>gga cta ccm ggg tat cta ac c</td>
<td>785–806</td>
<td>Panbacterial</td>
<td>Bacteria</td>
</tr>
<tr>
<td>127f-Sta</td>
<td>cgg tgt agt aac aac tgg</td>
<td>110–127</td>
<td>Staphylococci</td>
<td>This study</td>
</tr>
<tr>
<td>342f-Sta/Str</td>
<td>ccc tac tgc tgc ctc cgg tag</td>
<td>342–362</td>
<td>Staphylococci, streptococci</td>
<td>48, Modified</td>
</tr>
<tr>
<td>537f-Sta</td>
<td>cgg ata acg ctt gcc acc tac</td>
<td>537–557</td>
<td>Staphylococci</td>
<td>This study</td>
</tr>
<tr>
<td>128f-Str</td>
<td>cgg tgt aac gac cgq tag g</td>
<td>110–128</td>
<td>Streptococci</td>
<td>This study</td>
</tr>
<tr>
<td>540r-Str</td>
<td>cgg aca acg ctc ggg acc</td>
<td>540–557</td>
<td>Streptococci</td>
<td>This study</td>
</tr>
</tbody>
</table>

*According to 16S rDNA sequence of Escherichia coli (Acc. No. V00348).

Results

Heart Valves From Patients With Suspected IE

Fifty-two excised heart valves, including 24 aortic valves, 18 mitral valves, and 6 tricuspid valves, were studied. From one patient, both mitral and aortal valves were obtained. In addition, 4 valve prostheses (mitral, n=2; aortal, n=2) were analyzed.

Results of analyzing heart valves of patients with suspected IE are summarized in Tables 2 and 3. Bacterial broad-spectrum PCR amplification was positive in 22 of 52 specimens (42.3%) (aortic valve, n=11; mitral valve, n=9; tricuspid valve, n=2) obtained from 21 of 51 patients (41.2%).

Fourteen of these 22 specimens tested negative in both conventional methods applied, heart valve culture and Gram staining. Only 4 of 52 heart valves (7.7%) of the 51 patients (7.8%) were positive by culture comprising isolates of Staphylococcus aureus (n=2), Streptococcus equinus [bovis], and Nocardia species. On microscopic examination, Gram-positive cocci were observed in 7 of 52 (13.5%) of the Gram-stained smears; ie, the Gram stain was positive for 6 of 51 patients (11.8%).

All positive heart valve cultures and microscopic results were confirmed by bacterial broad-spectrum PCR and direct sequencing. No discrepancies resulting from misidentification were observed. In one case of suspected IE, both the aortic and mitral valves of the patient were found to be positive by microscopic examination and bacterial broad-spectrum PCR but negative by culture methods. In this particular case, direct sequencing showed 100% homology to S equinus [bovis].

None of the broad-spectrum PCR-negative results were positive by heart valve culture or microscopic examination.

All amplicons of bacterial broad-range PCR were identified by 16S rDNA sequencing up to the (sub)species level comprising predominantly streptococcal sequences (n=13, 59.1%), followed by staphylococcal (n=4) and enterococcal (n=2) sequences (Table 3). Six of the streptococcal sequences showed homology to the 16S rDNA sequence of S equinus [bovis]. Furthermore, ribosomal sequences of Nocardia paucivorans, Bartonella quintana, and Propionibacterium acnes were detected. When primers targeting the ITS-1 sequence

Direct Sequencing

To sequence amplified bacterial 16S rDNA fragments, ~918-bp amplicons of the primers 27f and 907r(m) were reamplified by nested primers 357f(m) and 785r, resulting in amplicons of ~468 bp (Table 1). As standard procedure, nested-PCR amplicons were sequenced with these primers. For sequencing of staphylococcal and streptococcal targets, amplicons of the primers 27f and 907r(m) were also sequenced, and more appropriate oligonucleotide primers were designed and applied as listed in Table 1. Otherwise, direct sequencing was performed as described elsewhere.16 Ribosomal sequences were compared with those available in the NCBI database using Lasergene software suite (DNASTAR Inc).
and ITS-2 regions of fungal 18S and 28S rDNA were applied, no positive results were observed.

Heart Valves From Patients Without Suspected IE

In the reference group comprising patients without any signs of IE (n=16 valves), no positive results were obtained with either bacterial or fungal broad-spectrum PCR (Table 2). Furthermore, Gram stains were completely negative, as were heart valve cultures, except for one specimen infected with *Aspergillus* species. Reexamination of another portion of this heart valve tissue did not reveal any growth, suggesting that the *Aspergillus* infection was the result of contamination.

<table>
<thead>
<tr>
<th>TABLE 2. Results of Testing Heart Valves by Broad-Spectrum PCR Compared With Gram Stain and Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result of Testing Heart Valves of Patients Tested/Total, n</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>With suspected IE</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Without suspected IE</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

* In one case of suspected IE, both the aortic and mitral valves of a patient were found to be positive by Gram stain and PCR but negative by culture methods.
† Possible contamination by molds.

<table>
<thead>
<tr>
<th>TABLE 3. Microbiological Findings of Patients With Suspected Endocarditis Whose Heart Valves Tested Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (Age, Sex)</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>B.S. (67 y, M)</td>
</tr>
<tr>
<td>N.H. (57 y, F)</td>
</tr>
<tr>
<td>N.O. (39 y, M)</td>
</tr>
<tr>
<td>M.M. (33 y, M)</td>
</tr>
<tr>
<td>R.S. (17 y, M)</td>
</tr>
<tr>
<td>H.C. (38 y, M)</td>
</tr>
<tr>
<td>S.M. (56 y, M)</td>
</tr>
<tr>
<td>K.W. (44 y, M)</td>
</tr>
<tr>
<td>V.A. (67 y, M)</td>
</tr>
<tr>
<td>W.M. (39 y, M)</td>
</tr>
<tr>
<td>S.E. (62 y, F)</td>
</tr>
<tr>
<td>M.G. (55 y, F)</td>
</tr>
<tr>
<td>W.E. (74 y, M)</td>
</tr>
<tr>
<td>S.M. (65 y, M)</td>
</tr>
<tr>
<td>S.M. (65 y, M)</td>
</tr>
<tr>
<td>W.J. (44 y, M)</td>
</tr>
<tr>
<td>H.L. (57 y, M)</td>
</tr>
<tr>
<td>S.J. (59 y, M)</td>
</tr>
<tr>
<td>U.S. (49 y, M)</td>
</tr>
<tr>
<td>N.D. (64 y, M)</td>
</tr>
<tr>
<td>R.A. (77 y, F)</td>
</tr>
<tr>
<td>B.H. (62 y, M)</td>
</tr>
</tbody>
</table>

ND indicates no data.
*According to taxonomic rules, *Streptococcus bovis* (later heterotypic synonym) was recently designated as *Streptococcus equinus*.
† The former *Streptococcus bovis* biovar II.2 reclassified as *Streptococcus pasteurianus* was recently reappraised as *S gallolyticus* subspecies *pasteurianus*. 

Breitkopf et al. Molecular Diagnosis of Heart Valve Endocarditis.
Comparison With Previous Blood Culture Results

Results of blood cultures were available for 43 IE patients. Comparison of blood culture results to those obtained by examining the heart valves with PCR/direct sequencing showed agreement in 8 of 22 cases (36.4%) (Table 3). Ten patients with positive blood culture results before surgery (S aureus, n=5; Staphylococcus capitis, n=1; Streptococcus mitis, n=1; S equinus [bovis], n=1; Haemophilus parainfluenzae, n=1; Micrococcus luteus, n=1) tested negative on examination of the heart valves by either molecular or conventional methods.

Of the 30 PCR-negative cases, 14 patients had negative blood cultures, and no blood culture data were available for another 6 patients. These 20 patients all displayed the pathological criteria of IE (Table 4). At least 9 were receiving antibiotics at the time of the blood culture collection.

Sensitivity, Specificity, and Positive and Negative Predictive Values

Definite and possible cases of IE and cases of the reference group without any signs of IE were used to calculate specificity, sensitivity, and positive and negative predictive values. Sensitivity and specificity for the bacterial broad-range PCR were 41.2% and 100.0%, respectively; for culture, sensitivity was 7.8% and specificity was 93.7%. Sensitivity of the Gram stain was 11.8%, and its specificity was 100.0%. The positive and negative predictive values for the bacterial broad-range PCR were 100.0% and 34.8%, respectively; for culture, they were 80.0% and 24.2%; and for Gram stain, they were 100.0% and 26.2%. Thus, with the probable Aspergillus contamination of a heart valve culture excluded, no false-positive results were seen with any method, whereas the molecular approach clearly has the lowest frequency of false-negative results.

Discussion

Although IE is rare, it remains a severe and potentially fatal disease.1,17 Adequate antimicrobial treatment depends on the microbial diagnosis of the causative pathogen. Normally, blood culture is used to identify the pathogenic agent.18 Despite improvements in culture methods in recent years, the conventional microbiological approach often yields false-negative results. Traditionally, it has been assumed in such cases that the IE is due to fastidious, slow-growing, or nonculturable microorganisms.6–8 Prior antibiotic therapy and the host immune response may also cause the microbiological examination to fail despite the use of appropriate laboratory techniques.

In cases of surgical treatment of IE in the setting of negative blood cultures and serology, microbiological examination of excised heart valves is the only way to identify the causative agent. Paradoxically, the improved antibiotic treatment of IE during the past decades may have increased the diagnostic failure rate because the microorganisms present on the excised valve are nonviable. This has limited our knowledge of the current spectrum of organisms causing IE.
Sequencing ribosomal genes is thought to be the most accurate means of identifying microorganisms.\textsuperscript{19} It has recently been suggested that the combination of broad-spectrum PCR amplification and direct sequencing may overcome many of the problems besetting the microbiological diagnosis of IE. Goldenberger et al\textsuperscript{10} first reported the use of broad-spectrum PCR, followed by sequencing, in 18 resected heart valves. Further studies underlined the contribution of PCR to the diagnosis and management of IE; however, these studies were limited by technical issues, by the restricted range of detectable microorganisms, and/or by the taxonomic level of identification.\textsuperscript{12,13,20} Recently, it was suggested that molecular diagnosis of IE should be included in the Duke’s classification scheme.\textsuperscript{21} In the present study, we analyzed a large number of excised heart valves using broad spectrum primers that targeted both bacterial and fungal ribosomal sequences. Amplified PCR products were determined at the (sub)species level by direct sequencing. In the case of staphylococci and streptococci, sequencing of a 429-bp fragment was not discriminative enough to reach the (sub)species level. Therefore, sequences of further fragments of the 16S rDNA were determined with newly designed primer pairs.

Analyzing taxonomic sequence data remains a major challenge in establishing the definitive species classification. Interpretation of rDNA-based data are hampered by deficiencies in public sequence databases such as faulty, truncated, and noncharacterized sequence entries; reiterations; and an often outdated nomenclature. High-quality sequence databases are available for only a few genera, mainly \textit{Mycobacterium}, \textit{Neisseria}, and \textit{Staphylococcus}.\textsuperscript{22–24}

In 13 of 22 cases with negative results from culture of heart valve tissue and blood, the molecular detection and sequencing of bacterial sequences identified the infectious agent. Surprisingly, in many of these cases, the causative organism was not fastidious but one that is generally considered easy to cultivate such as streptococci, enterococci, and staphylococci. This may indicate that many of our patients had been treated with antimicrobial agents over elongated periods before the onset of severe cardiac complications leading to surgery. Furthermore, besides the traditionally recognized fastidious microorganisms, phenotypic variants such as small-colony variants of staphylococci have been described. These variants are increasingly recovered from specimens of antibiotic-refractory, recurrent, and/or persistent infections.\textsuperscript{25–27} Because the generation time for these variants is 6- to 9-fold longer than for metabolically normal staphylococci, recovery and correct identification of small-colony variants in the microbiological laboratory may be difficult. This may result in diagnostic underestimation.\textsuperscript{28}

Less surprisingly, 16S rDNA sequences of \textit{B. quintana}, known to be difficult to recover because of its slow growth rate and highly fastidious nature in vitro, were amplified in one heart valve tissue. \textit{B. quintana} and occasionally other \textit{Bartonella} species have been detected as infectious agents causing IE.\textsuperscript{29–31} \textit{Bartonella} infection must be considered in patients with suspected IE, particularly if regular blood cultures remain sterile despite the absence of antibiotic treatment and if serological assays for other fastidious pathogens such as \textit{Francisella tularensis}, \textit{Coxiella burnetii}, and \textit{Brucella melitensis} biovares are negative.\textsuperscript{32} Because significant cross reactions between \textit{B. quintana} and \textit{Chlamydia/Chlamydophila} antigens have been reported and a high prevalence of chlamydial antibodies is found in the populations, nucleic acid amplification–based methods are needed in such cases of blood culture–negative endocarditis.\textsuperscript{33,34} Recently, Fenollar et al\textsuperscript{35} described patients with PCR-detected mycoplasmal endocarditis for whom blood cultures and cardiac valve cultures were repeatedly sterile. In addition, for \textit{Cardiobacterium hominis}, a member of the HACEK group of bacteria, molecular approaches were shown to be useful.\textsuperscript{36} Thus, there is no doubt that molecular techniques are valuable and necessary to establish a definitive IE diagnosis if fastidious and/or nonculturable microorganisms are involved.\textsuperscript{37,38}

Cases in which examination of the excised heart valve was negative despite the presence of positive blood cultures underline the importance of analyzing the appropriate section of the heart valve. It is currently unclear whether sensitivity can be improved by the analysis of multiple valve fragments. It is also possible, of course, that positive blood culture results may be due to contamination of the sample by skin microorganisms.

In contrast to most former studies, we performed the molecular examinations down to the (sub)species level. As shown by Podglajen et al,\textsuperscript{39} species identification, as opposed to genus identification, may have substantial impact on the management of patients. These authors reported the molecular identification of \textit{Gramlicatella adiacens} in a case of suspected infection with group C streptococci. As a result, prolonged antimicrobial drug treatment was prescribed. Because \textit{S. luedenensis} IE is usually associated with left-sided valvular disruption and life-threatening embolic complications, correct identification to distinguish this aggressive species from other coagulase-negative staphylococci may be of utmost importance.\textsuperscript{40}

Reports on \textit{Candida} endocarditis are becoming more common, particularly in intravenous drug abusers and immunocompromised patients. For this reason, we included fungal ribosomal targets in our diagnostic strategy.\textsuperscript{31,42} \textit{Candida} endocarditis has also been associated with central-line–associated bloodstream infections, especially in patients who have received prolonged courses of intravenous fluids and antibiotics. Although \textit{C. albicans} represents the predominant species, the importance of non–\textit{C. albicans} yeasts has emerged over the last decade.\textsuperscript{43} \textit{C. parapsilosis} is the yeast most commonly isolated from narcotics addicts with fungal IE.\textsuperscript{44} Notably, current routine methods for yeast identification may be insufficient to identify isolates of lipophilic \textit{Malassezia} species, which have been found to be associated to a low but not negligible extent with infection of central venous catheters for parenteral nutrition-bearing lipid emulsions.\textsuperscript{45} Alpert et al\textsuperscript{46} reported \textit{Malassezia furfur} fungemia in infancy, including a patient with evidence of endocarditis. In addition, \textit{Aspergillus} species frequently found in patients after cardiovascular surgery account for a high percentage of fungal IEs.\textsuperscript{43,44} Nevertheless, cultivation of \textit{Aspergillus} and related genera and detection of mold sequences should be done with care because molds are ubiquitous in the environment and the
risk of contamination by airborne spores is high. Even though no fungus was found as a causative pathogen in this study, the increase in risk factors accountable for fungal IE should lead us to include respective targets for molecular detection strategies.

In summary, the diagnosis of IE poses a challenge, particularly in patients who have negative blood culture and/or who were previously treated with antimicrobial agents. Applying molecular detection and identification techniques, especially in the case of negative results of culture and microscopic examination, substantially improves diagnosis not only of fastidious, slow-growing, and/or nonculturable microorganisms but also of pathogens such as streptococci and staphylococci that are generally considered easy to cultivate. In addition to the consequences for patient management, improved knowledge of the real spectrum of pathogens currently involved in IE will improve the basis for empiric antibiotic therapy of IE.47,48

Acknowledgments

We are grateful to Brigitte Schuhen, Martina Schulte, and Barbara Grimmastel for excellent technical assistance and to Christoph von Eiff and Paul Cullen for helpful discussions.

References

Impact of a Molecular Approach to Improve the Microbiological Diagnosis of Infective Heart Valve Endocarditis
Claudia Breitkopf, Dieter Hammel, Hans H. Scheld, Georg Peters and Karsten Becker

Circulation. 2005;111:1415-1421; originally published online March 7, 2005;
doi: 10.1161/01.CIR.0000158481.07569.8D
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/111/11/1415

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/