23S rDNA real-time polymerase chain reaction of heart valves: a decisive tool in the diagnosis of infective endocarditis

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Aims
A new diagnostic strategy to improve the detection of pathogens in heart valves (HVs) from patients with infective endocarditis (IE) was evaluated.

Methods and results
Three hundred and fifty seven HVs surgically removed from 326 patients with proven IE or suspicious intra-operative findings, examined by 16S rDNA polymerase chain reaction (PCR) and culture were retrospectively analysed according to the predictive value of various PCR methods. Patients were classified into four categories: active IE, IE with ambiguous infective status, healed IE, and valve diseases but no IE. Retained samples of 200 HVs were analysed by real-time PCR targeting bacterial 23S rDNA, fungal 28S rDNA, and mycoplasmal tuf gene. 16S rDNA PCR revealed 80.6% sensitivity, 100% specificity, 100% positive predictive value, and 71% negative predictive value (NPV), compared with cultivation with 33.4, 96.6, 95.5, and 40.9%, respectively. The use of real-time PCR increased diagnostic sensitivity to 96.4%, and NPV to 92.5%. Bacterial load, C-reactive protein, and white blood cell counts (WBCs) decreased during antibiotic treatment. Bacterial load showed no correlation to C-reactive protein or WBCs, whereas C-reactive protein and WBCs were significantly correlated.

Conclusion
23S rDNA real-time PCR of surgically removed HVs improves the diagnosis of IE. Polymerase chain reaction analysis of explanted HVs allow the optimization of the antimicrobial therapy, especially in patients with culture-negative IE.

Keywords
Infective endocarditis • Real-time polymerase chain reaction • Conventional polymerase chain reaction • C-reactive protein • White blood cell count

Introduction
According to current guidelines, the diagnosis infective endocarditis (IE) is established if microbiological infection of the endocardium is demonstrated. The standard procedure includes clinical and echocardiographic findings plus identification of the causative pathogen.1 In unclear cases, diagnosis may be based on Duke criteria.2 Identification of the causative microorganism is crucial, not only for proper diagnosis, but also for appropriate antibiotic treatment and management decision, e.g. surgery during active IE. Blood cultures are accepted as the gold standard for pathogen identification and quantitative susceptibility testing. However, in up to 31% of suspected IE cases,3,4 blood cultures remain negative due to previous antibiotic treatment,5 slow-growing, fastidious, or difficult-to-culture organisms,6 or small amounts of microorganisms per volume. Patients with culture-negative endocarditis (CNE), often associated with more complicated courses,7 may benefit from identification of pathogens by polymerase chain reaction (PCR) techniques.1

As surgically removed infected heart valves (HVs) usually contain large numbers of pathogens, a PCR analysis is supposed to be much more effective.8–16 Cultures of surgically removed
specimens often suffer the same limitations as blood cultures. Providing potential advantages, molecular genetic screening methods have been used in studies including small numbers of patients only and limited controls to confirm the validity of PCR results. The sensitivity of PCR diagnostics is influenced by false-negative results due to amplification inhibitors in PCR assays, contamination, and owing to standardization of PCR. Broad-range real-time PCR amplification has been established as standard in clinical laboratories because of enhanced assay sensitivity and a short diagnostic time delay. Real-time PCR allows for the calculation of the bacterial load by quantification of gene copies. However, real-time PCR has rarely been used for pathogen detection in HVs, due to environmental contamination of 16S rDNA real-time PCR assays.

Traditional inflammatory markers, such as white blood cell count (WBC) and C-reactive protein, as well as new parameters, e.g. lipopolysaccharide-binding protein (LBP), are currently used to monitor the progression of infection and the effect of therapy. In these patients, the first five criteria were negative, with the relapse (within one month or more) after a full course of antimicrobial treatment.

Healed IE was defined as proven IE without echocardiographic, histopathological, surgical findings, and/or inflammatory treatment for less than 14 days and/or revealed inconclusive results. The sensitivity of PCR diagnostics is influenced by false-negative results due to amplification inhibitors in PCR assays, contamination, and owing to standardization of PCR. Broad-range real-time PCR amplification has been established as standard in clinical laboratories because of enhanced assay sensitivity and a short diagnostic time delay. Real-time PCR allows for the calculation of the bacterial load by quantification of gene copies. However, real-time PCR has rarely been used for pathogen detection in HVs, due to environmental contamination of 16S rDNA real-time PCR assays.

In the present study, we analyse the sensitivity of the standard broad-range 16S rDNA PCR in comparison to a novel 23S rDNA real-time PCR assay in HVs surgically removed for suspected, proven active, or healed IE.

Methods

Patients

From September 2001 to December 2007, 269 surgically removed HVs from 241 patients with proven IE, and 88 surgically removed HVs from 85 patients presenting pre- or intra-operatively with a valve morphology suspicious for IE but without any evidence of IE according to the modified Duke criteria (no positive blood cultures and not more than one minor criterion) were routinely examined by standard microbiological cultivation methods and 16S rDNA PCR. One hundred and eighty-five patients with proven IE had two major Duke criteria (endocardial involvement and positive blood cultures). Fifty-six patients had evidence of endocardial involvement and had at least three minor Duke criteria (fever, predisposing HV disease, arterial emboli, or glomerulonephritis, but negative blood cultures). Clinical records were reviewed with regard to the following criteria: (i) echocardiographic demonstration of vegetations, (ii) blood culture results within six weeks prior to surgery, (iii) increased inflammatory parameters [C-reactive protein (>0.5 mg/dL), WBC (>11,000/μL)], (iv) positive findings (e.g. vegetations, abscesses) during cardiac surgery, (v) positive histopathological findings (leucocyte infiltration, bacteria), and (vi) duration of antibiotic therapy. These parameters were used for disease staging: (i) active IE, (ii) IE with ambiguous infective status, (iii) healed IE, and (iv) valve disease, no IE. Patients classified as having ambiguous infective status had received antibiotic treatment for less than 14 days and/or revealed inconclusive echocardiographic, histopathological, surgical findings, and/or inflammatory parameters. Healed IE was defined as proven IE without relapse (within one month or more) after a full course of antimicrobial therapy. In these patients, the first five criteria were negative, with the exception of a moderately elevated C-reactive protein level. Patients classified as having valve diseases but no IE had HV analyses on a routine basis because of an immunocompromised situation (patients on dialysis or patients with diabetes) or due to morphological alterations of the valve suspicious for IE to the surgeon. Preoperatively, six of these patients revealed suspicious echocardiographic findings and four also had increased C-reactive protein levels eventually due to previous urinary tract infection, pneumonia, or continuous dialysis. None of these patients had clinical signs for alternative explanations, e.g. collagen vascular disease. Demographic data are shown in Table 1.

Cultivation and 16S rDNA polymerase chain reaction analysis of heart valves

Heart valve tissues were aseptically disrupted in a sterile mortar and cultivated in tryptone soya broth and brain heart infusion bouillon (bioMérieux, Nürtingen, Germany) at 37°C under aerobic and anaerobic conditions for a maximum of 21 days, as well as on Columbia sheep blood agar and chocolate agar at 37°C for 5 days, 5% CO2. All isolates were identified by morphology and the biochemical identifying methods ID 32C and VITEK 2 ID-YST (bioMérieux). Total DNA was extracted from the second part of the valve using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was eluted from the QIAamp column with 50 μL sterile distilled water. Broad-range bacterial 16S rDNA PCR followed by sequencing analysis was performed as described previously.

Real-time polymerase chain reaction analysis

Broad-range real-time PCR assays of retained samples of DNA extracts (n = 200) were performed for the fungal 28S rDNA gene and the mycoplasmal tuf gene on the Rotor-Gene 3000 system (Corbett Life Sciences, Sydney, Australia) as described previously. For real-time PCR detection of bacterial DNA, a previously described

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<thead>
<tr>
<th>Table 1 Patient characteristics</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>Age [years, median (SD), range]</td>
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<tr>
<td>Male (n = 235)</td>
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<tr>
<td>Female (n = 91)</td>
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<td>Affected valves (n)</td>
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<td>Type of valve (n)</td>
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<td>Aortic valve</td>
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<td>Mitral valve</td>
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<td>Pulmonary valve</td>
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<tr>
<td>Tricuspid valve</td>
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<tr>
<td>Other specimen</td>
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<td>Classification of infective status before surgery</td>
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<td>Active IE, (HV PCR positive)</td>
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<td>Active IE, (HV PCR negative)</td>
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<td>IE with ambiguous infective status</td>
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<td>Healed IE</td>
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<td>HVD but no IE</td>
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assay developed for the amplification of the 23S rRNA gene was adapted for the detection of the 23S rDNA gene. For all assays, the validity of PCR results was confirmed using exogenous internal controls. We modified the internal control of the 23S rDNA assay to avoid competitive co-amplification of the β2-microglobulin gene using a 278 bp fragment of the bacteriophage lambda (position 2214–2471, accession number J02459). The internal MS2 control of the 28S rDNA assay was adapted for the mycoplasmal tuf assay. Cut-off values were routinely set to 0.01 relative fluorescence units.

The analytical sensitivity of the 23S rDNA real-time PCR assay was determined by a 10-fold dilution of the DNA extract from a Staphylococcus aureus infected HV with negative sample material. External plasmid standards were used to determine the concentration of bacterial DNA. Plasmid standards of the complete 23S rDNA of S. aureus strain ATCC25923 from position 1490 (16S–23S internal transcribed spacer, accession number J01859) to position 2763 (23S rDNA, accession number J01859) were established. The samples were analysed in triplicate in three independent PCR assays.

The bacterial concentration was determined by relative quantification using the ratio of the β2-microglobulin and 23S rDNA C_{T} values. Quantitative real-time PCR was performed with the following modification: amplification was accomplished in two separate PCR assays to avoid competitive inhibition due to high concentrations of human β2-microglobulin copies.

### Statistical analysis

All calculations for statistical analysis were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Values are given as mean including standard deviation (± SD). Testing for Gaussian distribution utilized the Kolmogorov–Smirnov test. We used Pearson and Spearman correlation coefficients to assess correlations between two variables (two-sided testing). P-values < 0.05 were considered significant.

### Results

**Routine analysis of heart valve samples by culture and 16S rDNA polymerase chain reaction**

Microorganisms were identified in 160 (45%) out of 357 surgically removed HVs. Application of 16S rDNA PCR detected bacterial DNA in 149 of these 160 HVs (93%). HV cultures were positive in 64 HVs (40%), and both were positive in 53 HVs. In 96 of these 160 HVs, bacterial DNA was detected by PCR, whereas HV cultures remained negative. Cultures of valve specimens were positive in 15 out of 160 HVs, while no bacterial DNA was detected by PCR. These 160 HVs came from 141 patients in whom IE had been proved according to the modified Duke criteria pre-operatively. Blood cultures were positive in 109 of these patients. Figure 1 demonstrates the results of PCR and HV cultures with respect to the causative organism and the duration of pre-operative antibiotic treatment. All pathogens were identified to species level, but are given as genus in Figure 1.

PCR facilitated the detection of Streptococcus spp. with an almost equal frequency independent of the duration of antibiotic treatment. Heart valve cultures detected Streptococcus spp. primarily within the first 10 days of antibiotic treatment. Staphylococcus spp. had a maximum recovery rate within 20 (PCR) and 10 days (HV culture) after initiation of antibiotic treatment, respectively. The detection of Enterococcus spp. and other pathogens revealed no periodic differences between the two methods dependent on the duration of antibiotic treatment. In 10 out of 15 HVs removed from 14 patients with active but CNE, PCR identified the causative pathogen as Streptococcus spp. (n = 5), Enterococcus spp. (n = 2), Bartonella spp. (n = 2), and Tropheryma whipplei (n = 1). In addition, in 23 of 26 HVs surgically removed from 25 patients with active IE but without positive blood culture results prior to surgery, the analysis of HV tissue by 16S rDNA PCR resulted in the initial pathogen identification [Streptococcus spp. (n = 12), Enterococcus spp. (n = 3), Staphylococcus spp. (n = 4), A. haemolyticus (n = 4)].

The 16S rDNA PCR results were coincident with those from HV cultures, with the exception of five patients. In these patients, PCR results corresponded with pathogens previously isolated from blood culture. In 23 HVs (21 patients), pathogens identified by PCR and from blood cultures were inconsistent. Sequencing analysis exposed minor differences regarding species identification in 13 patients: species identifications differed in seven patients, whereas in six patients, causative microorganisms had been identified only to genus, not to species level from previously drawn blood cultures, and identification was first proved by sequencing analysis of HV tissue. In eight patients, sequencing analysis obtained inconsistent results already on the level of genus identification. For example, Pasteurella multocida or coagulase-negative Staphylococci were identified from blood culture, whereas Actinobacillus actinomycetemcomitans or Streptococcus pneumoniae were identified from HV tissue.

No infective agent was identified by 16S rDNA PCR in 208 (58%) of the 357 removed valves. This high frequency of negative 16S rDNA PCR represents the routine setting where PCR testing was performed irrespective of clinical or infectious patient parameters. Analysis of the medical reports revealed that 88 HVs were removed from patients with non-infective valve disease, 47 from patients with healed IE, and only 36 from patients with active IE, and 37 from patients with an ambiguous infective status. After excluding 135 HVs removed from patients who had either a non-inflammatory heart disease or a healed IE, identification of an infective agent by 16S rDNA PCR still remained negative in 73 (33%) of 222 HVs.

The comparison of the 16S rDNA PCR detection rate for prosthetic HVs and native HVs in patients with active IE or an ambiguous infective status revealed a considerable lower frequency for prosthetic valves (52%) compared with native valves (69%), whereas the positive findings in HV culture were nearly equivalent for both groups (prosthetic valves: 31%, native valves: 29%).

**Comparison of bacterial polymerase chain reaction assays**

The comparison of real-time 23S rDNA PCR results with a routine analysis of HV samples by 16S rDNA, and HV cultures are given in Table 2. No positive results were obtained by 16S rDNA PCR in patients classified as no IE or healed IE. Heart valve cultures were positive for six HVs; however, the second culture with oppositional culture conditions did not reveal growth, indicating
possible contamination. Regarding all patients with active IE at the
time of surgery, positive 16S rDNA PCR results were obtained in
149 of 185 HVs (80.5%), whereas the cultures of HV tissue were
positive in only 63 of 185 HVs (34.0%). In addition to the standard
16S rDNA PCR, remaining samples of retained DNA extracts (\(n = 200\)) were examined and quantified by real-time PCR targeting the
bacterial 23S rDNA, the fungal 28S rDNA, and the mycoplasmal
tuf gene. Head-to-head comparison of 16S rDNA and 23S
rDNA real-time PCR is given in Table 2. 23S rDNA real-time
PCR revealed positive PCR results in 107 of 111 specimens
(96.4%) from patients with active IE. 16S rDNA PCR results
were negative in 16 of these HVs. Only 23S rDNA real-time
PCR detected bacterial DNA in 11 HVs of patients whose infective
status was considered ambiguous and in five HVs of patients with
clinically healed IE since 2–10 months. 28S rDNA real-time
PCR confirmed fungal DNA in two valves, concordant to HV culture
results. Mycoplasma spp. was not found in any of the examined
HVs. No PCR inhibition or contamination of PCR reagents or
sample material was detected using real-time PCR assays. The effi-
cacy of 23S rDNA real-time PCR was lower in explanted prosthe-
sic HVs (72% positive 23S rDNA findings in patients with active IE
or ambiguous infective status) than in native valves (94% positive
results).

### Sensitivity, specificity, positive, and negative predictive values

The diagnostic sensitivities, specificities, positive predictive value
(PPV), and negative predictive value (NPV) for the 16S rDNA assay, the 23S rDNA assay, HV culture, WBC, and C-reactive
protein are given in Table 2. PCR assays detect IE pathogens in
80.5% (16S rDNA), and 96.4% (23S rDNA), both with a specificity
and a PPV of 100.0%, while 16S rDNA had an NPV of 71.0%
compared with 92.5% (23S rDNA). In comparison, HV cultures
had a diagnostic sensitivity of 34.1%, a specificity of 96.6%, a PPV
of 95.5%, and a 40.9% NPV. The inflammatory parameters C-reactive protein and WBC had diagnostic sensitivities of 96.4
(C-reactive protein) and 39.3% (WBC), specificities of 47.5
(C-reactive protein) and 97.4% (WBC), PPV of 80.6 (C-reactive
protein) and 97.3% (WBC), and NPV of 84.4 (C-reactive
protein) and 40.0% (WBC).

### Quantification of bacterial DNA in heart
valve samples and correlation with
inflammatory markers

The analytical sensitivity of the 23S rDNA real-time PCR assay was
determined to 35–100 copies/PCR reaction. In comparison, the
16S rDNA PCR assay revealed a 10–100-fold lower sensitivity.
Bacterial DNA was quantified in 116 HVs of 104 patients with
an active, an ambiguous infective status, or healed IE. In the case
of two or more analysed HVs per patient, the bacterial load was
averaged. The mean concentration of the bacterial load, C-reactive
protein, and WBC decreased parallel to the duration of antibiotic
treatment (Figure 2). The mean concentrations of bacterial DNA
were 1.28 (± 0.45) RU (relative units) within the first 10 days,
1.08 (± 0.33) RU between days 11 and 20, 1.08 (± 0.25) RU
between days 21 and 30, and 1.00 (± 0.28) RU after more than
40 days of antibiotic treatment. Comparatively high concentrations
of bacterial DNA were observed between days 31 and 40 [1.64
(± 0.43) RU] of antibiotic treatment. In contrast, the mean
values of C-reactive protein decrease continuously during anti-
biotic treatment [0–10 days: 10.20 (± 9.10) mg/dL, 11–20 days:
6.93 (± 5.80) mg/dL, 21–30 days: 6.30 (± 4.17) mg/dL, 31–40
days: 5.02 (± 5.26) mg/dL]. Interestingly, persistently high
C-reactive protein values of 5.75 (± 9.35) mg/dL were observed.
| Table 2 | Results of polymerase chain reaction, cultures of heart valves, and inflammatory parameters before valvular surgery |
|-----------------|--------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | 16S rDNA  | 16S rDNA retained samples | 23S rDNA  | 23S rDNA retained samples | HV culture | C-reactive protein | WBC |
|                | Tested/total, n (%) | Tested/total, n (%) | Tested/total, n (%) | Tested/total, n (%) | Tested/total, n (%) | Mean value (± SD) | Tested/total, n (%) | Mean value (± SD) |
| Active IE      |             |                         |             |                         |             |                 |                 |
| Positive       | 149/185 (80.5) | 91/111 (82.0) | 107/111 (96.4) | 63/185 (34.0) | 175/182 (96.2) | 8.63 (7.33) | 72/183 (39.3) | 16.07 (5.09) |
| Negative       | 36/185 (19.5) | 20/111 (18.0) | 4/111 (3.6) | 122/185 (66.0) | 7/182 (3.8) | 0.26 (0.11) | 111/183 (60.7) | 7.61 (1.88) |
| Ambiguous infectious status |             |                         |             |                         |             |                 |                 |
| Positive       | 0/37 (0.0) | 0/19 (0.0) | 11/19 (57.9) | 2/37 (5.4) | 30/34 (88.2) | 5.10 (5.09) | 6/33 (18.2) | 26.80 (22.91) |
| Negative       | 37/37 (100.0) | 19/19 (100.0) | 8/19 (42.1) | 35/37 (94.6) | 4/34 (11.8) | 0.19 (0.17) | 27/33 (81.8) | 6.90 (2.06) |
| Healed IE      |             |                         |             |                         |             |                 |                 |
| Positive       | 0/47 (0.0) | 0/21 (0.0) | 5/21 (23.8) | 1/47 (2.1) | 24/43 (55.8) | 2.38 (2.73) | 4/47 (8.5) | 13.15 (1.05) |
| Negative       | 47/47 (100.0) | 21/21 (100.0) | 16/21 (76.2) | 46/47 (97.9) | 19/43 (44.2) | 0.28 (0.14) | 43/47 (91.5) | 6.60 (1.59) |
| HVD but no IE  |             |                         |             |                         |             |                 |                 |
| Positive       | 0/88 (0.0) | 0/49 (0.0) | 0/49 (0.0) | 3/88 (3.4) | 42/80 (52.5) | 3.76 (4.82) | 2/76 (2.6) | 13.20 (0.00) |
| Negative       | 88/88 (100.0) | 49/49 (100.0) | 49/49 (100.0) | 85/88 (96.6) | 38/80 (47.5) | 0.27 (0.16) | 74/76 (97.4) | 7.16 (1.89) |

*Possible contamination.
*Both were positive for *Candida parapsilosis*, original pathogens were *Staphylococcus aureus*. 
even later than 40 days of antibiotic treatment. In 19 PCR-positive patients and in 13 PCR-negative patients, the C-reactive protein values were below the upper normal value (0.5 mg/dL) or close to it (0.5 ± 0.5 mg/dL). White blood cell count values decreased continuously during antibiotic treatment \([0–10 \text{ days: } 11 670 \pm 579/\mu L, \ 11–20 \text{ days: } 10 540 \pm 560/\mu L, \ 21–30 \text{ days: } 10 280 \pm 630/\mu L, \ 31–40 \text{ days: } 8 060 \pm 263/\mu L, \ >40 \text{ days: } 7 600 \pm 264/\mu L]\). Analysis of bacterial load vs. C-reactive protein \((r = 0.09, P = 0.38)\) and bacterial load vs. WBC \((r = 0.10, P = 0.31)\) revealed no correlation. A strong correlation,
however, was found between C-reactive protein and WBC ($r = 0.5$, $P < 0.0001$).

**Discussion**

The causative microorganism is unknown in up to 50% of patients undergoing HV surgery for IE. The importance of PCR techniques has been evaluated in many studies. However, study results are limited by technical issues, the number of HVs analysed, a restricted range of detectable microorganisms, the taxonomic level of bacterial identification, consideration of antibiotic therapy, and limited controls to confirm the validity of PCR results.

In the present study, we demonstrate for the first time that the ultrasensitive 23S rDNA real-time PCR is superior to the conventional standard 16S rDNA PCR for the detection of the infective agent in surgically removed HVs from IE patients.

**Polymerase chain reaction techniques**

16S rDNA PCR facilitates the identification of causative pathogens, as shown for *T. whipplei* or *Bartonella spp.*, but 16S rDNA real-time PCR is often subject to environmental contamination. Applying 16S rDNA conventional PCR, approximately 20% of HVs removed during active IE remain false-negative. In our study, the causative pathogen could not be detected in 33% of infected HVs. It is known that quantitative real-time PCR applications achieve higher diagnostic sensitivities, but for the detection of pathogens in HVs, 16S rDNA real-time PCR has been used successfully in only one study.

For the first time, we utilized 23S rDNA as a target for real-time amplification combined with the application of internal controls to confirm the validity of PCR results. This technique allowed increasing the sensitivity to detect IE pathogens from 80.5 to 96.4% (compared with 16S rDNA conventional PCR). Both PCR techniques revealed specificities and a PPV of 100%, but with 23S rDNA, a higher NPV (92.5 vs. 71.0%) was obtained. The advantage of 23S rDNA over 16S rDNA as a target for real-time PCR is essentially due to the fact that contaminations are only seen when using 16S rDNA. In patients with an ambiguous infectious status, and in patients with active IE but negative conventional PCR results, real-time PCR improved the diagnostic sensitivity of PCR by more than 50%. Unfortunately, the PCR product of 23S rDNA real-time PCR is short, not allowing the identification of the pathogen to species level, which, however, can easily be done with 16S rDNA conventional PCR.

For the subsequent examination of HVs, we suggest routine detection of pathogens by 23S rDNA real-time PCR followed by 16S rDNA conventional PCR to identify the pathogen to species level when appropriate. The algorithm shown in Figure 3 represents a pathology-based approach to verify clinically diagnosed or suspected IE. Future investigations will address the extension of the amplification region of the 23S rDNA to facilitate species identification by sequencing analysis directly after 23S rDNA real-time PCR amplification.

PCR techniques have become a standard application to overcome inherent limitations of culture methods, facilitating a rapid diagnosis of infectious diseases. In the present study, PCR applications were focused on the detection of microorganisms in HVs. This restricts its potential role in routine clinical practice to patients with active IE requiring HV replacement. We also performed molecular genetic whole blood testing using the 23S rDNA PCR assay in 33 patients with IE (unpublished data), but the detection of bacterial pathogens in whole blood by PCR often failed due to the low amount of bacteria. We demonstrated that the PCR analysis of HVs is more successful, probably due to the higher concentration of pathogens. The main incremental value of PCR analysis in HVs is the detection and identification of microorganisms especially in patients without previous pathogen detection to allow an optimized antimicrobial treatment. In nine patients with CNE and in 22 patients without positive blood culture results prior to surgery, the identification of the causative pathogen using 16S rDNA PCR had a major impact on antibiotic therapy. Subsequently, 27 of these 31 patients had a favourable outcome with a rapid resolution of inflammation. Only four patients developed relevant post-operative complications (e.g. heart failure treated with intra-aortic balloon counterpulsation and catecholamines, long-term mechanical ventilation, acute renal failure). Application of 23S rDNA real-time PCR confirmed the presence of bacteria in two further HVs of patients with CNE.

Sequencing analysis of HVs revealed inconsistent results compared with blood cultures in eight patients in whom antibiotic treatment was changed accordingly after species had been identified by PCR. The antibiotic therapy was not altered in those 13 patients with different species identification between HV PCR and blood culture results because the initially established antibiotic resistogram reflected the effective antibiotic susceptibilities of the underlying causative pathogens. In these cases, 16S rDNA PCR may offer an improved species identification and differentiation between closely related species, which are not achieved by biochemical identifying methods (e.g. differentiation between *Streptococcus mitis* and *Streptococcus oralis* applying ID 32C and VITEK 2 ID-YST).

Infective endocarditis due to fungal or mycoplasmal pathogens is rare. Most epidemiological data were derived from small case series or case reports. The importance of *Candida* endocarditis, particularly in intravenous drug abusers and immunocompromised patients, is growing. For this reason, we included rapid real-time PCR screening for fungal and mycoplasmal spp. in our diagnostic strategy, and detected two valves infected by *Candida* spp.

To optimize the PCR results of HVs we learned from cases of active IE with negative PCR results, even utilizing the ultrasensitive 23S rDNA real-time technique, that it is important to examine an appropriate section of the HV as bacterial colonization is definitely not uniform. For example, transmission electron microscopy of tissue infected with *Bartonella henselae* revealed areas with tight bacterial colonization and non-infected areas. The partitioning of the surgically removed HV into several pieces for histology, culture, and PCR examinations has to be done carefully to avoid sampling errors.

**Influence of antibiotic treatment on identification of causative pathogens**

Blood cultures and tissue cultures of HVs from patients with active IE are hampered by antibiotic treatment. In our study, tissue cultures from infected HVs provided positive results if antibiotics had been given for less than 11 days prior to surgery. Positive tissue culture results decreased significantly if antibiotic therapy lasted longer. Polymerase chain reaction identified bacterial DNA mainly within the first 20 days of antibiotic treatment, but even after longer treatment
periods, a significant number of positive results were found. The highly sensitive real-time PCR assay successfully detected bacterial DNA after more than 40 days of effective antibiotic treatment. Furthermore, the persistence of bacterial DNA was documented in valves from patients with healed IE. The infectious potential of the microorganism, the degree of tissue infiltration, and the effect of antibiotics all influence the retention of pathogens.1,29

**Bacterial load in explanted heart valves**

To the best of our knowledge, bacterial load in explanted infected HVs was measured in this study for the first time. Parallel to the duration of antibiotic therapy, bacterial load, as well as C-reactive protein and WBC, decreased. High bacterial loads after 31–40 days and high values for C-reactive protein after more than 40 days of antibiotic treatment indicated inadequate antibiotic treatment. In this series, the lack of correlation among bacterial load and C-reactive protein or WBC might be explained by long-term persistence of bacterial DNA and the sampling error by partitioning of the HV.

**Pathogen viability**

The presence of bacterial DNA does not necessarily indicate the presence of viable bacteria. To distinguish between acute disease and DNA persistence, a careful simultaneous consideration of clinical symptoms, laboratory, and microbiological findings is mandatory. Long-term persistence of bacterial DNA may be explained by: (i) a recurrent infection; (ii) the initial infection that was not resolved completely due to deep valve penetration, or was not curable even by adequate antibiotic treatment,1,29 and bacteria remained viable but non-growable; (iii) PCR-detected DNA from killed bacteria. We observed long-term persistence, particularly in cases with *Streptococcus* spp. None of the patients classified as healed had any clinical evidence of infection at the time of surgery; thus, the assumption of DNA persistence in the debris of healed cardiac vegetations seems to be obvious. However, the implication of long-term bacterial persistence for the patient is unclear. Presence of viable, but not yet growable, bacteria may predispose for recurrences. Future applications should address the usage of additional reverse transcription real-time PCR assays based on the amplification of mRNA targets, such as *hsp60* or elongation factorTU,30,31 to provide additional information about pathogen viability.

In conclusion, examination of explanted HVs by PCR, especially in patients with CNE, allows identification of the causative pathogen and improves stratification of antimicrobial therapy. Positive culture results have the advantage to allow antimicrobial sensitivity
testing. If blood and/or HV culture results are negative or unreliable, positive PCR results from explanted HVs offer important information. For the examination of HVs, we suggest the following diagnostic strategy: (i) ultrasensitive screening for bacterial DNA using real-time PCR in parallel to HV cultivation in cases of suspected or definite active IE, (ii) in case of positive results, identification of the causative microorganism by 16S rDNA PCR followed by sequencing analysis, (iii) in case of negative results, additional examination by real-time PCR for the detection of not yet detected bacterial pathogens, including fungal or mycoplasmal pathogens. The detection of bacterial DNA in HVs does not imply an active valve infection as so far PCR cannot differentiate viable from non-viable microorganisms. The state of infection has to be classified by consideration of clinical, laboratory, and microbiological findings of the patient.

Study limitations
The retrospective character of this study hampered the application of additional diagnostic approaches (e.g. repeated collection of blood cultures, molecular genetic screening of whole blood), but our results showed that 23S rDNA real-time PCR analysis of explanted HVs is helpful to detect causative microorganism with a very high sensitivity. Although our cohort included only a small number of patients with ambiguous infective status or healed IE, the PCR results revealed the known general limitation of PCR analysis to distinguish between viable pathogens and persisting DNA. Therefore, the infective status of a patient cannot be determined or monitored by PCR analysis.

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References