Evaluation of PCR in the molecular diagnosis of endocarditis

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Summary Objective. Infective endocarditis (IE) is diagnosed by the Duke criteria, which can be inconclusive particularly when blood cultures are negative. This study investigated the application of polymerase chain reaction (PCR) to identify bacterial DNA in excised valvular tissue, and its role in establishing the diagnosis of IE.

Methods. Ninety-eight patients undergoing valve replacement surgery were studied. Twenty-eight patients were confirmed as definite for endocarditis by the Duke criteria; nine were considered as possible and 61 had no known or previous microbial infection of the endocardium. A broad-range PCR technique was used to amplify prokaryotic 16S rRNA genes present within homogenised heart valve tissue. Subsequent DNA sequencing of the PCR amplicon allowed identification of the infecting microorganism.

Results. PCR results demonstrated the presence of bacterial DNA in the heart valves obtained from 14 out of 20 (70%) definite IE patients with positive blood cultures preoperatively. The causative microorganism for one patient with definite culture negative endocarditis was identified by PCR. Two out of nine (22%) of the valves from possible endocarditis patients also had bacterial DNA present converting them into the definite criteria whereas in the valves of seven out of nine (78%) of these patients no bacterial DNA was detected.

Conclusion. The application of PCR to the explanted valves in patients with possible or confirmed diagnosis can augment the Duke criteria thereby improving post-surgical antimicrobial therapeutic options.

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Introduction

Infective endocarditis (IE) is an infection involving the endothelial lining of the heart, affecting primarily the valves.¹ Diagnosis of IE is based on...
the fulfilment of a number of major and/or minor criteria as set down by the Duke University Medical Centre, principally vegetation on echocardiography and positive blood cultures. Overall, echocardiography alone is diagnostic in 50–94% of cases. Indeed, in some conditions including *Coxiella burnetii* and *Legionella* species IE vegetations are rarely detected by imaging. Up to 31% of suspected IE patients have negative blood cultures. This is frequently due to prior antibiotic therapy or from failure to grow fastidious microorganisms such as *Haemophilus* species, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella* species and *Kingella kingae* (the HACEK group), *Abiotrophia* species, *Bartonella* species and intra-cellular microorganisms including *Chlamydia* species and *C. burnetii*. Slow growing microorganisms including *Legionella* species, *Bartonella* species and *Mycobacterium* species may also be missed in routine clinical microbiology investigations.

Sequence analysis of bacterial 16S rRNA genes using the polymerase chain reaction (PCR) can be performed directly on clinical specimens to establish an aetiological diagnosis. This molecular technique has been shown to be more sensitive than conventional blood culturing techniques for the detection of bacteria. The infecting microorganism was identified in 2.4% of clinical specimens (including tissue biopsies, body fluids and pus samples) where standard bacterial culture had failed. The ability of PCR to detect and identify microorganisms associated with culture negative IE and regardless of prior antibiotic use may allow earlier directed antimicrobial therapy where previously empirical treatment based on clinical judgment alone was used.

In a number of reported case reviews, PCR analysis of excised cardiac tissue or blood cultures has proved to be the only means of identifying the infecting microorganism, particularly with fastidious bacteria. Recent studies have investigated the role of the technique in the routine diagnosis of infection and it has been suggested that the method be included within the Duke criteria. In this current study, PCR amplification of 16S rRNA genes was used to identify prokaryotic DNA present in excised heart valves. To establish the sensitivity and specificity of PCR when applied to explanted valves, unlike previous studies, a large number of control valves were obtained from patients with no evidence of IE. The broad-range eubacterial primers used corresponded to highly conserved regions flanking more variable areas of the nucleotide sequence encoding 16S rRNA. These primers have previously been shown to generate amplicons from a diverse range of eubacteria including staphylococci, streptococci, campylobacters, streptomycetes, mycoplasmas and *Tropheryma* species. We report the use of PCR to amplify a variable region of the 16S rRNA gene and sequence analysis of the amplicon as a means of diagnosing the aetiological agent of IE.

### Patients and methods

#### Heart valve samples

Between August 1999 and December 2001 valves from 98 patients were obtained including 28 with a preoperative diagnosis of definite IE and nine with possible IE as defined by the Duke criteria (M:F ratio of 25:12, mean age of 58 years, age range of 19–91). A further 61 valves were obtained from patients undergoing valve replacement due to degenerative valve disease (M:F ratio of 34:27, mean age of 69 years, age range of 38–82). These valves acted as controls. None of the control patients had a history of IE or of any other infection in the previous 6 months. All valves were native except for one prosthetic valve removed from a patient with definite IE. Samples were aseptically removed from each valve and studied by standard microbiology techniques and the remainder of the valves underwent PCR analysis. Demographic and clinical data, including valvular appearance at the time of surgery, from all the patients were recorded. Ethical Committee approval was obtained for the study.

#### Bacterial culture

A section of heart valve tissue was aseptically transferred to a sterile container containing 15 glass beads (3-mm diameter) and 4 ml of brain-heart infusion broth (Oxoid, UK) and homogenised for 10 s. This disrupted tissue sample was inoculated onto a range of microbiology media, including blood agar (Columbia agar base supplemented with 5% (v/v) defibrinated horse blood, Oxoid) and fungal media (Sabouraud dextrose agar, Oxoid). These plates were incubated for 18 h either in a 5% enriched CO₂ or an anaerobic atmosphere at 37°C. The remaining broth–tissue suspension was incubated for 5 days at 37°C and subsequently sub-cultured at 2 and 5 days onto two blood agar plates which were incubated as previously described. Multiple blood cultures from the majority of patients...
were incubated using a BD Bactec 9240 blood culture system (BD, USA) for a minimum of 14 days. Isolated colonies, from either valve material or blood culture, were identified by standard microbiological procedures.

**Extraction of DNA from tissue samples**

Approximately 100 mg of each valve was investigated. Tissue from the prosthetic valve was obtained by the firm application of a sterile scalpel blade across the valve surface. Each tissue sample was added to 100 µl of PCR grade water containing six sterile DNA-free glass beads (3-mm diameter) and homogenised by vortex mixing for 4 min. The supernatant, containing bacterial DNA template if present, was recovered by centrifugation (11 400 g for 1 min) from the homogenate and used as template for PCR amplification. The DNA extraction, preparation of PCR amplification mixes, amplicon detection, amplicon purification and sequencing of amplicon steps of analysis were all performed in separate laboratories to minimise cross contamination.23

**DNA amplification**

Two microlitres of homogenate supernatant was screened for bacterial DNA with a 25 µl volume PCR reaction using the oligonucleotide primer set DG74: 5’AGG AGG TGA TCC AAC CGC’ and RW01: 5’AAC TGG AGG GTG GGG AT3’.22 These broad-range PCR primers are located at the nucleotide positions 1522-1540 and 1170-1189 within the *Escherichia coli* 16S rRNA genes respectively (Ribosomal DNA Primer Database, http://rrna.uia.ac.be/primers/database.html). A PCR cabinet with UV-light facility and appropriate equipment were used.23 The PCR reaction consisted of 1 × Taq polymerase buffer (containing 1.5 mM MgCl₂) (Qiagen, USA), 0.2 mM each dNTP (Amersham Biosciences, NJ, USA), 25 µM each DG74 and RW01 (Alta Bioscience, Birmingham, UK), 1.25 units HotStar Taq polymerase (Qiagen) and PCR grade water to a final volume 25 µl. The PCR amplification protocol consisted of a Taq activation step of 14 min 30 s at 95 °C followed by 30-cycles of 30 s at 95 °C, 30 s at 60 °C, 60 s at 72 °C (PTC-100 Programmable Thermal Controller, MJ Research, MA, USA). After amplification, the PCR reaction products were electrophoresed through a 2% (w/v) molecular biology grade agarose gel (Flowgen, Leicestershire, UK) in TAE buffer (40 mM Tris, 1 mM EDTA pH 8.0, 0.1% (v/v) glacial acetic acid), pre-stained with ethidium bromide (0.5 µg/ml), at 6 V/cm for 1 h. Amplification was repeated on template DNA from samples which were positive in the initial PCR screen. The scale of the reaction was increased to 8 µl of template in a 100 µl PCR reaction using the same PCR protocol increased to 40-cycles. PCR product was separated by electrophoresis through a 2% (w/v) Sea Plaque agarose gel (Flowgen) in TAE buffer, pre-stained with ethidium bromide (0.5 µg/ml), at 6 V/cm for 1 h and visualised on a trans-illuminator. Purified amplicons (approximately 371 bp based on the *E. coli* genome), recovered from the gels using the QIAEXII DNA extraction kit (Qiagen), were sequenced in a single direction with an Applied Biosystems 377 automated DNA sequencer (Alta Bioscience, Birmingham, UK) using the DG74 or RW01 primers. Subsequently, the nucleic acid sequence database GenBank was searched for amplicon homology by the BlastN algorithm (Workbench, http://workbench.sdsc.edu/). The criteria used for interpretation of the sequences were similar to those employed by Goldenberger;19 the percentage similarity, level of homology shared by the next species of the same genus and the level of homology shared by the next species of a different genus were all taken into consideration.

The DNA extraction/PCR negative control consisted of glass beads and water preparation (no template), whilst whole bacterial cells of *Staphylococcus epidermidis* NCTC 11047, taken from a blood agar plate, provided the PCR positive control.

**Estimation of sensitivity**

Nine prepared 25 µl PCR reactions containing heart valve DNA template, prepared from a tissue sample which had previously been shown to be PCR-negative for bacterial DNA, were inoculated by serial dilution with *S. epidermidis* NCTC 11047 cells, prepared in TSB to an OD₅₅₀ of 1.0 (estimated range of 6.2 × 10⁶ bacteria per PCR reaction to theoretically one bacterium per PCR reaction). Neither enzymic nor chemical extraction of bacterial DNA from the cells of the control strain was attempted prior to the sensitivity assay to reflect the condition of the DNA template prepared from the tissue samples. A negative control containing tissue template alone was incorporated. After PCR, agarose gel electrophoresis was used to establish the lowest number of whole bacterial cells present within an amplification reaction required to generate a band visible by ethidium bromide staining.

**False negatives**

The occurrence of false-negatives due to the presence of inhibitory substances in the valve tissue
extract was investigated by performing PCR amplification on PCR-negative template samples inoculated with bacterial cells. Using a previously prepared culture of *S. epidermidis* NCTC 11047, grown in tryptone soya broth (TSB, Oxoid) and adjusted to an optical density of 1.0 at 550 nm, the PCR-negative samples were ‘spiked’ with $5 \times 10^5$ cfu per 25 $\mu$l PCR reaction and amplification performed by the standard PCR protocol. PCR inhibition was indicated by the failure of PCR to generate an amplicon from template spiked with bacteria.

**Results**

Of the 98 heart valves, 37 were removed from patients diagnosed with IE and 61 from the control group with no infection. Of the 37 IE patients, 19 with definite IE underwent surgery before the end of standard antimicrobial treatment, and are referred to as the active group. Nine definite IE patients underwent surgery after the completion of treatment (time period of 3 months to 7 years after treatment), and are referred to as the resolved group. The remaining nine valvular tissues were obtained from possible IE patients who were receiving either empirical antimicrobial treatment or prophylactic antibiotics at the time of surgery.

The results of blood cultures and PCR, together with patient demographics, for these 37 IE patients are shown in *Table 1*. Of those samples removed from patients within the active group, 12 out of 19 were both blood culture-positive and PCR-positive (PCR results for 10 of these samples matched the blood culture results and two generated PCR amplicons which failed to sequence) (*Table 1*). Four patients were blood culture-positive but PCR-negative (however, DNA sequences confirming the blood culture results for two of these samples were obtained when the number of PCR cycles was increased from 30 to 40). One patient was both blood culture-negative and PCR-negative. Substances within the template of the remaining two tissues inhibited the PCR.

Of the nine samples excised from definite patients in the resolved group, PCR confirmed the blood culture results for two samples (staphylococcal DNA detected 1-year and streptococcal DNA 18-months post-treatment) (*Table 1*). A *Streptococcus* species was also identified by PCR in a sample obtained from a blood culture-negative patient (antibiotic treatment was completed 5 months prior to surgery). Bacterial DNA was not detectable in four of the valves and two other samples were PCR inhibited.

In the possible IE group, two of the nine patients were PCR positive (*Table 1*). For one of these patients, a *Bacillus* species and an *Acinetobacter* species were isolated through valve culture, whilst PCR identified *Bacillus* species DNA. A *Staphylococcus aureus* was identified in the tissue of the second PCR positive sample within this group.

All the tissues from the control patients with non-infective valvular insufficiency were either negative by PCR for bacterial DNA (51 out of 61, 84%) or PCR was inhibited by substances within the template (10 out of 61, 16%).

The amplification sensitivity assay using whole staphylococcal cells (a genus commonly associated with IE) demonstrated that $6.5 \times 10^3$ bacteria were required within a 25 $\mu$l PCR reaction containing tissue extract to generate a detectable PCR amplicon. Based on previous estimates of there being $1 \times 10^9$ bacteria per gram of cardiac vegetation, there would be $2 \times 10^5$ bacteria per 25 $\mu$l PCR reaction if only 10% of the bacterial cells were released from the 100 mg of tissue into the homogenate. For the investigation of false-negatives due to inhibitory substances within the template, each negative sample was spiked with $5 \times 10^4$ bacteria per 25 $\mu$l PCR reaction: a value above the sensitivity threshold but considered achievable within the template produced from the valves of IE patients. Of the PCR negative samples (*n* = 81), which included both infected and non-infected tissues, 67 (83%) generated an amplicon when spiked with bacteria, amplification being inhibited by unknown substances within the template of the remaining 14 (17%) samples. There was no evidence to suggest that false-positive results were being generated in any of the samples.

Samples from all of the 98 valves were also subjected to microbiological culture. *Haemophilus parrophilus* was recovered from the prosthetic valve of a definite IE patient and a *Bacillus* species from a native valve of an IE possible patient, both of which were also detected by PCR. These were the only two isolates from the 98 tissue samples considered to be clinically significant. Isolates considered to be clinically insignificant were recovered from a further seven samples (a culture contamination rate of 7%). These included coagulase-negative staphylococci, propionibacteria, corynebacteria, *Pseudomonas* species, *Stenotrophomonas maltophilia* and a *Candida* species. PCR did not detect any of these isolates in the explanted valves.
Discussion

Bacterial culture of excised valvular material is a useful method for identifying the cause of IE and confirming the diagnosis from blood cultures. As with blood culture however, prior antibiotic therapy reduces the rate of microorganism recovery, whilst fastidious and slow growing microorganisms can be missed. The molecular detection and identification of infecting microorganisms within valvular tissue would avoid these problems and has proved in many instances to be superior to bacterial culture. Overall, the results of PCR-based screening of the samples correlated with those obtained by standard blood cultures. Within the definite active IE group, PCR detected those bacteria, which were also isolated from blood with only a few exceptions and additionally identified one infecting microorganism that failed to grow in blood culture due to prior antibiotic therapy. The five PCR-negative samples taken from patients with active infection were subjected to extended amplification using a 40-cycle protocol. For two of these samples, sequencing of the 40-cycle generated PCR product identified the infecting bacterium; confirming the
blood culture result in one sample whilst identifying a *Staphylococcus* species in the second sample taken from a culture-negative patient. Although this modified PCR screening protocol increased the sensitivity of the assay, false-positives were detected. Therefore, for screening samples, 30-cycles of amplification were performed, whilst 40-cycles were applied only to screen-positive samples to generate a large amount of DNA for sequencing purposes. It was interesting to note the persistence of bacterial DNA within valvular tissue for up to 18 months after the completion of treatment in three samples taken from patients within the resolved definite IE group. Gauduchon made a similar observation. Review of clinical notes discounted both recurrence of infection and treatment failure in these patients. The clinical implication of this finding merits further study.

PCR proved to be particularly useful for patients within the possible IE group. Of these nine patients, PCR provided supplementary evidence for two to be converted into the definite category. Negative tissue PCR, in addition to the absence of pathological tissue evidence at the time of surgery, allowed a further four patients to be retrospectively rejected for bacterial IE. Either the identification of the aetiological microorganism or the rejection of an IE diagnosis can direct post-surgical antibiotic therapy. Of the 61 samples obtained from patients within the control group, 51 were PCR-negative and 10 were PCR inhibited. Juvonen reported the detection of *Chlamydia pneumoniae* within the valvular material of non-rheumatic stenotic aortic valves, tissues analogous to our control patients. In this study, however, and in accordance with the findings of Vainio, there was no evidence to suggest the presence of either *Chlamydia* species or any other prokaryote in these degenerated valve tissues. This indicates that these conditions are unlikely to have a bacterial aetiology.

In this study the sensitivity of the PCR method showed that at least $3.3 \times 10^6$ bacteria per gram of tissue were required to generate sufficient recoverable DNA for sequence analysis: a bacterial count readily achievable within a cardiac vegetation. However, inhibition of the PCR reaction, as also observed by Goldenberger, is a potential restricting factor. Certain substances, including blood, are known to be potent inhibitors of DNA amplification. Initially, various commercial DNA extraction kits were assessed; including those available through Bioline (DNace Spin Tissue mini kit), Qiagen (QIAmp DNA mini kit), and Promega (Wizard Genomic DNA Purification kit). Additionally, physical disruption methods were assessed for their ability to release and/or recover DNA from the tissues. These included lysozyme and lyostaphin treatment, freeze-thaw lysis, glass bead homogenisation and chelex treatment. Of all the methods assessed, simple physical disruption by homogenisation with glass beads without further extraction of DNA was found to be the most effective template preparation method. Qin similarly demonstrated that simple disruption of tissue sufficed in the preparation of template.

Contamination of the clinical sample is another potential limiting factor of the technique. The use of broad-range primers predisposes the assay to the generation of false-positives. However, PCR of control tissues recovered from patients with no clinical history of bacterial infection indicated that in practice there was no evidence of false-positives. Of the 61 control valves seven (11%) had microorganisms isolated, which were considered to be contaminantants. However, these were all either PCR-negative or PCR-inhibited confirming other previous studies that the routine culture of explanted valves for patients without clinical evidence of IE is an unnecessary investigation. Additionally, all positive samples identified medially significant microorganisms that are commonly associated with IE; four *Staphylococcus* species, eight *Streptococcus* species, and one *Haemophilus* species. Seventy-five percent of all IE infections are caused by either *Staphylococcus* or streptococci. The detection of a *Bacillus* species is more unusual. This tissue may have been contaminated, but the sensitivity assay performed on tissue samples suggested that unless there was gross contamination false-positives were unlikely to be detected. Database analysis of a number of the PCR amplicons identified the infecting microorganism to the species level; identifications that correlated with previous blood culture results. However, due to the high level of homology within 16S rRNA sequences of closely related microorganisms, for example between different species of the staphylococci, no attempt was made to record identifications beyond the genus level. If required the employment of specific primers would allow identification to the species level, for example, Millar used additional primers for the *femB* locus to differentiate between *S. aureus* and coagulase-negative staphylococci.

In summary, identification of bacterial DNA present in resected valvular tissue can be considered both as an important and realistic advance in the diagnosis and treatment of IE. We believe that the application of this molecular approach will prove to be most useful in cases where the diagnosis remains unclear, those patients that are currently placed within the Duke possible group, and in those
patients classified as definite for IE but for whom cultures remain negative.

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