The role of nucleic acid amplification techniques (NAATs) in the diagnosis of infective endocarditis

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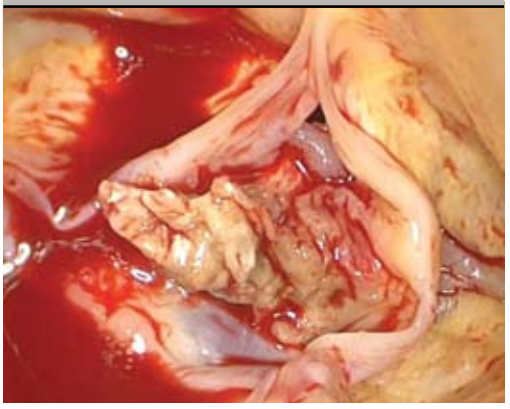
Infective endocarditis (IE) causes high rates of morbidity and mortality. Clinical management is problematic if there are uncertainties over the identity, viability or antibiotic susceptibility of the causative organism. Between 10% and 30% of IE blood cultures are negative, usually a result of prior antimicrobial therapy, but also occurring when causative micro-organisms are non-cultivable or fastidious. While evidence-based guidelines exist for treatment of IE caused by defined agents, clinicians are often faced with the dilemma of IE of unproven aetiology. Duration of empirical therapy is usually titrated against overall clinical response and non-specific laboratory markers of inflammation, but these may bear little relation to ongoing microbial activity in the heart valve. There is an increasing need for more specific, sensitive and rapid tests for the identification of causative organisms. Nucleic acid amplification technologies (NAATs) show promise for rapid detection of pathogen nucleic acid in blood or tissue. This review discusses the developments in this field, and the potential for the application of NAATs to improve aetiological identification in IE.

Introduction

Untreated infective endocarditis (IE) is fatal; even with appropriate treatment, IE is associated with high rates of morbidity and mortality worldwide.[1] The annual incidence of IE over the past two decades has remained relatively constant, ranging between 1.7 and 6.2 cases/100,000 population. Neither advances in healthcare nor revisions made to the current diagnostic criteria have substantially altered this.[1-3] The current definition for IE now incorporates infections of prosthetic heart valves (both bioprosthetic and mechanical), implanted devices (such as pacemakers or ventricular assist devices) and cardiac endothelial surfaces.[4] **Figure 1** illustrates a large vegetation on the aortic valve from a patient with IE.

Figure 1. A large vegetation on the aortic valve from a patient with infective endocarditis



The variability in both the clinical manifestations and the course of IE reflect, in part, the heterogeneity of causative micro-organisms; this makes accurate diagnosis by clinical means alone problematic.[5,6] Currently, the likelihood of IE is based on a score derived from a combination of clinical, microbiological and echocardiographic evidence.[7-10] Laboratory diagnosis consists of culture of the infectious organism from the blood and/or heart valve material. However, the sensitivity of this scoring system is significantly compromised when IE is caused by fastidious or non-cultivable organisms or when patients have received previous antibiotic therapy such that positive cultures cannot be obtained. Novel diagnostic tests, such as nucleic acid amplification technologies (NAATs), that can

Table 1. Microbiology and typical acquisition patterns of infective endocarditis[1,2,4,11,15,21,23,35-37]

Organism	Approximate proportion of cases (%)	Acquisition pattern (CA/HCA)	Valve type (NVE/ PVE)
Gram-positive bacteria			
Streptococcus spp - viridans group *Streptococci* - NVS - Streptococcus pneumoniae - ß-haemolytic *Streptococci*	50–60 35–45 2–3 1–3 5–10	CA	NVE; late PVE
Gemella spp	Rare	CA	NVE; late PVE
Enterococcus spp	5–18	CA	NVE; late PVE
Staphylococcus spp - *Staphylococcus aureus* - CoNS - *Staphylococcus lugdunensis*	30–50 25–45 10 Rare	 CA HCA HCA CA	 NVE; late PVE NVE; PVE (early, late) Early PVE NVE
Corynebacterium spp	Rare	HCA CA	NVE and PVE NVE
Gram-negative bacteria			
HACEK - *Haemophilus* spp - *Aggregatibacter actinomycetemcomitans* - *Cardiobacterium hominis* - *Eikenella corrodens* - *Kingella kingae*	2–5	CA	NVE
Pseudomonas spp	Rare	CA HCA	NVE (in IVDU) early PVE
Non-cultivable/fastidious organisms		CA	NVE unless stated
Coxiella burnetii	3–5		
Bartonella spp	3		
Brucella spp	1–4		
Chlamydia spp	Rare		
Legionella spp	Rare		Rarely early PVE
Anaerobic bacteria	Rare	CA	NVE
Fungi			
Candida spp, *Aspergillus* spp	2–4	HCA CA	NVE, early PVE NVE
Polymicrobial	Rare	CA	NVE in IVDU

Key: CA = community acquired; CoNS = coagulase-negative *Staphylococci*; HCA = healthcare acquisition; IVDU = intravenous drug use; NVE = native valve endocarditis; NVS = nutritionally variant *Streptococci* (*Abiotrophia* spp, *Granulicatella* spp); PVE = prosthetic valve endocarditis

identify the presence of infective organisms irrespective of the culture constraints will not only improve sensitivity but may reduce empiric treatment by permitting targeted antibiotic therapy. To be clinically useful any such technique must be able to identify the causative micro-organism from blood or tissue rapidly, have appropriately high positive- and negative-predictive values, and demonstrate good reproducibility between laboratories.

This review discusses the developments and application of NAATs for rapid and accurate detection of the infectious aetiology of IE.

Epidemiology and microbiology of IE

IE affects both native and prosthetic (bioprosthetic and mechanical) valves, and may develop through community- or healthcare-associated acquisition (CA-IE and HCA-IE, respectively). Worldwide, CA-IE of the native valve is by far the most common form of IE with rheumatic valve disease remaining the major risk factor;[4] in resource-rich countries, however, profound changes in the epidemiology and aetiology of IE have been seen in recent years. There are many reasons for such shifts, but the principles for IE risk remain essentially unaltered: increased opportunity for microbial entry to the blood circulation, the presence of abnormal endocardial surfaces or flow patterns, and diminished host immune capacity.[3,4,11] In developed countries with expanding elderly populations, degenerative valve lesions and congenital defects have far outstripped chronic rheumatic valve disease as major underlying risk factors, being present in up to 50% of IE patients over the age of 60 years.[5]

Although native valve endocarditis remains mostly CA-IE, the incidence of HCA-IE is steadily increasing[12] as medical interventions allow greater opportunities for microbial access to the bloodstream via prolonged or repeated intravascular access or cannulation. Cardiothoracic surgical advances in developed healthcare settings have provided the emergence of new risk groups, including patients with prosthetic valves, intravascular devices or endovascular repairs. The flora associated with HCA-IE, predominantly skin-dwelling staphylococcal species, is fundamentally different to that of CA-IE. Such differences therefore

demand different treatment.[13,14] IE associated with intravenous drug use (IVDU) is in many ways unique – left- and right-sided valves are affected in approximately equal proportions,[14] and the variety of microbial flora reflects the different opportunities for contamination at different stages of the process.[15,16] *Staphylococcus aureus* is the predominant organism of IVDU-related IE as it colonises skin flexures; IE caused by *Pseudomonas* species and other motile Gram-negative bacteria have been attributed to the use of contaminated water to clean needles,[17] and dissolving heroin in lemon juice predisposes to candidaemia and *Candida* IE.[18]

Prosthetic valve endocarditis occurs at a rate of 3–6/1,000 patient-years, accounting for an estimated 1–5% of all IE cases in resource-rich settings. Early prosthetic valve endocarditis occurs within 60 days of valve surgery[11] and is typically HCA-IE, with *Staph. aureus*, *Staph. epidermidis* and other coagulase-negative staphylococci the most common pathogens. By contrast, the microbiology of late prosthetic valve endocarditis resembles that of native valve CA-IE.[19] The typical microbiology of IE and its patterns of acquisition are summarised in **table 1**.

Current diagnostic methods used in IE

The recognised epidemiological characteristics, described above, are only associations, and, while they may guide empiric therapy, they are not sufficiently reliable to allow antimicrobial prescribing in individual cases with certainty. For this, microbiological identification of the causative organism is needed; this will also inform the decisions on duration of therapy and even whether surgical intervention should be anticipated.[20]

However, direct microbiological culture from blood is not without pitfalls. The protean manifestations of IE that in part reflect the variety of aetiological agents, mean that patients frequently present to primary care with non-specific symptoms. Bacteraemia associated with IE is usually continuous but low grade, averaging at 1–10 organisms

Table 2. Advantages and disadvantages of existing infective endocarditis diagnostic criteria[10,21,36,38-43]

Duke criterion	Advantages	Disadvantages
Direct culture from valve material	• Good specificity: accurate reflection of microbiology at site • Isolates viable organisms permitting antibiotic sensitivity testing	• Poor sensitivity: no growth if infection due to non-cultivable fastidious organisms; or prior antibiotic therapy • Slow process: ≥24 hours to grow organism • Enrichment processes may be susceptible to contamination
Histology/microscopy	• Presence of lesions/organisms identifies active endocarditis: direct observation of evidence of inflammatory response	• Poor sensitivity for certain microorganisms (e.g. *Bartonella* spp) • Antibiotic therapy alters morphology and affects Gram staining • Accurate speciation not possible • No antibiotic susceptibility information derived
Blood culture (B/C)	• Sequential sampling increases specificity • Detects viable organisms permitting antibiotic sensitivity testing	• Contamination rates of up to 30% of all positive B/Cs • Slow process: incubated ≤5 days, ≥24 hours to grow organism, further ≤24 hours to determine antibiotic sensitivity • Negative B/C may lead to misclassification of patients
Serology	• Identifies fastidious organisms (e.g. *Bartonella* spp, *C. burnetii, Chlamydia* spp, *Brucella* spp and *Legionella* spp)	• Antibody cross reactivity between *Bartonella* spp and *C. burnetii* or *Chlamydia* spp reduces sensitivity • Slow process: often requires 'convalescent' samples after 2–3 weeks to confirm rising antibody titres
Echocardiogram	• Rapid, direct evidence of abscess formation, vegetations, valvular regurgitation or new partial dehiscence of prosthetic valve • TTE: high specificity, poor sensitivity • TOE: Oesophageal: high specificity, improved sensitivity especially for smaller lesions or prosthetic valve vegetations	• Infrequent detection via imaging of vegetations caused by fastidious organisms (e.g. *C. burnetii* and *Legionella* spp) • Absence of cardiac murmur in early infective endocarditis may not trigger request for echocardiography • Inconclusive echocardiograms can lead to misclassification of patients
Biomarkers – C-reactive protein (CRP), procalcitonin	• Easy to perform • May be used to monitor treatment response • Possibility for defining organism – or process-specific proteomic profiles	• Elevated markers of systemic bacterial infections do not identify the organism or necessarily reflect microbiology at site of lesion

Key: TOE = transoesophageal echocardiography; TTE = transthoracic echocardiography

per ml of blood;[21,22] blind trials of antibiotic therapy may, therefore, suppress microbial activity at the valve sufficiently to reduce the sensitivity of blood cultures taken at the time of hospital presentation.[2,20-23] Indeed, up to 30% of all IE cases have negative blood cultures.[24] A minor proportion of these cases are due to the presence of micro-organisms that are either non-cultivable, fastidious in their nutritional requirements in culture, or are extremely slow-growing on conventional media. The modified Duke criteria try to compensate for these issues, but the scoring system remains flawed **(table 2)**.

Rationale for use of NAATs in IE

The advent of molecular technology, the "diagnostic tool for the new millennium",[25] has turned opportunities for rapid organism identification and even detection of certain drug susceptibility patterns from concept into a working reality. The rapid exponential generation of billions of copies of target DNA template from a single original sequence accounts for the potential for NAATs to be sensitive, specific and timely. One of the greatest assets of molecular tests is their potential to be adapted according to purpose. Amplification tests may be designed to be so specific as to detect only a single species, strain, or even resistance-inducing mutation; alternatively, the use of commonly shared genetic sequences as amplification targets allows detection of much broader categories of organisms. For example, the 16S rDNA gene codes for the RNA component of the 30S sub-unit of the prokaryotic ribosome. As it has both highly conserved and variable regions, pan-bacterial primers can be developed to target the conserved regions that immediately adjoin variable regions.

Table 3. Advantages and disadvantages of using molecular-based techniques targeting the 16S rDNA gene to diagnose infective endocarditis[3,22,26,28,31,44-53]

	Advantages	Disadvantages
Sample handling/ collection	• May be able to identify micro-organisms from formalin-fixed or paraffin-embedded histology specimens	• DNA extraction difficult from histological specimens • Requires sterile, DNA-free sampling equipment/containers • Appropriate storage to avoid nucleic acid degradation
Sensitivity/specificity	• Potential for designing highly specific and sensitive assays capable to detect to single organism numbers	• High sensitivity broad-range targets assays like 16S rDNA increase the risk of amplifying contaminants or 'clinically irrelevant organisms'
16S rDNA gene	• Can identify non-cultivable, fastidious or cell dependent organisms	• Limited to discriminate between recently diverged species compared with well-resolved species • Copy variants make sequence analysis problematic when using multi-copy targets
Contamination/inhibition	• Specific gene targets reduce risk of detection of contaminants • Inclusion of inhibition controls and the use of real-time PCR can measure inhibition	• May amplify contaminating non-viable DNA sequences present in sampling equipment and/or reagents • Potential for generating false-positive results • Limited applicability in polymicrobial infections • PCR inhibition can cause false-negative results
Antibiotic susceptibility	• Potential to target specific antibiotic resistance genes	• Cannot detect expressed antibiotic resistance phenotype • Unable to detect antibiotic resistance mechanisms that are not confined to single gene mutations
Laboratory infrastructure	• Contamination risks can be reduced by structural planning	• Separate pre- and post-PCR rooms require large space allocation
Requirements/costs	• Early organism identification diagnosis may reduce costs by allowing focused antimicrobial therapy, reducing empirical treatment and hospital stays • Real-time PCR reduces post-PCR analysis equipment • High through-put reduces run costs	• Requires specific equipment, reagents and controls • Run costs increased with low through-put
Time	• Rapid diagnostic technique • Amenable to automation • High through-put runs offer rapid turnarounds	• Low through-put runs may increase turnaround times
Training of personnel	• Many common laboratory training issues relevant to both routine clinical microbiology laboratory and molecular diagnostics – health and safety, minimisation of contamination risks • Adherence to diagnostic protocols standard in clinical laboratories	• Molecular techniques not part of standard training for all clinical laboratory staff • Optimisation and validation of assay system essential but may be difficult
Interpretation of results/ clinical relevance	• Easy to identify organisms by matching derived sequences with database of known isolates • Copy number quantification may help determine relevance of identified organism • Potential for assessment of organism viability by RNA detection • Sequential blood sampling for quantification may allow improved assessment of therapeutic response	• Identification match dependent upon quality of matching database – usually less strong with rarer organisms, or those with greater inherent genetic variability • Greater assay sensitivity may increase false-positive rate • Essential to interpret molecular results in clinical context as many typical contaminant organisms also have potential to be causative • DNA detection does not indicate viability • 16S rDNA provides organism identification, but not antibiotic susceptibility profile

Key: PCR = polymerase chain reaction

Consequently, a single set of primers can be used to amplify the DNA from an enormous range of different bacteria. Subsequent sequencing of this amplified DNA can identify the variable region and thereby the bacterium.[26-28] As the 16S rDNA gene sequence is universal throughout all phyla of bacteria it is, therefore, ideally suited for the diagnostics of IE, where there is such immense diversity of possible causative organisms including those that are fastidious or non-cultivable, or even no longer viable.[3,29] The majority of NAATs applied to IE have used polymerase chain reaction (PCR) to amplify and subsequently sequence the 16S rDNA gene. To date, this technique has been used most successfully on excised valve tissue, and at present cannot replace microbiological culture of blood. **Table 3** outlines the potential advantages and disadvantages of using molecular techniques to diagnose IE, with specific regard to targeting the 16S rDNA gene.

In fungal endocarditis, the 18S rDNA gene – the equivalent of the bacterial 16S rDNA gene – is more problematic as a diagnostic target since 18S rDNA gene sequences are highly conserved and demonstrate insufficient variability to differentiate between many fungal species. Instead, alternative targets such as short non-coding ribosomal internal transcribed spacer (ITS) regions are increasingly being used. These regions are located between conserved genes encoding for 18S, 5.8S and 28S rDNA, and are highly variable in both length and sequence, and, thus, more efficient for discriminating species than 18S sequences.[30]

Real-time PCR

More recently, 16S real-time or quantitative PCR (qPCR) has been applied to bacterial IE.[2,31] There are a number of advantages of qPCR over conventional PCR: not only is it a more sensitive technique, but it is also more rapid as it eliminates the need for post amplification steps such as gel electrophoresis of PCR products.[11] Importantly, qPCR can also measure the amount of inhibition from clinical samples and, hence, evaluate the effectiveness of the nucleic acid extraction method. For this, internal extraction and amplification controls are added to the sample before each of these steps.[32]

Previous studies have recommended that molecular-based techniques are included as a major criterion in the Duke criteria.[22,31,33] Indeed, several studies have now demonstrated PCR positivity on valves in patients classified as possible or definitive IE, even when blood culture was negative.[3] Results from these studies are undoubtedly promising, with the sensitivity of PCR from valve material ranging from 41.2% to 96%, compared with direct culture rates of 7.8–24.3%. Information of all published trials using 16S rDNA PCR in IE is available in a supplementary appendix (available online at www.bjcardio.co.uk).

Conclusions

It is important to remember that whatever the method of organism identification in IE, whether culture isolation or molecular nucleic acid detection, the result must still be open to careful interpretation. With an ever-increasing list of organisms that have been associated with both native and prosthetic valve IE, this assessment becomes more complex. Attribution of causation must always be weighed against the possibility of contamination. Appropriate measures to prevent contamination are as critically important in microbiological culture as in the molecular laboratory.

Molecular tests undoubtedly advance the diagnosis of IE; however, a much greater understanding of the variables that influence the sensitivities and specificities of the molecular methods needs to be defined. Recent calls to introduce basic standards in molecular diagnostic test protocols,[34] in conjunction with the use of common targets, would allow for a much more accurate comparison between studies. With advances in molecular technology, NAATs now provide far more sensitive and rapid methods to detect the micro-organisms that cause IE. Comparative results from recent studies using NAATs indicate the clear superiority of valve material PCR over conventional valve culture. PCR can provide a positive identification where one or more of the definitive Duke criteria have been inconclusive. Development of consensus guidelines is needed to overcome the difficulties that the lack of standardisation of targets and protocols present to enabling valid comparisons between studies. With recent advances in nucleic acid quantification, NAATs technology may provide a tool to help answer some of the outstanding challenges that remain for IE diagnostics: accurate treatment response monitoring and reliable outcome prediction

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Conflict of interest

None declared.

Editors' note

A supplementary appendix containing information on all published trials using 16S rDNA PCR in IE is available online at www.bjcardio.co.uk

Key messages

- Infective endocarditis (IE) causes high rates of morbidity and mortality
- Clinical management is problematic if there are uncertainties over the identity, viability or antibiotic susceptibility of the causative organism
- Conventional diagnostic microbiological techniques fail when patients have received prior antibiotic therapy, or when the causative organism is fastidious or non-cultivable
- Nucleic acid amplification techniques (NAATs) now represent a much more sensitive and rapid method for detection of the micro-organisms that cause IE compared with culture alone

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APPENDIX: Comparison of the use of 16S rDNA polymerase chain reaction (PCR) and gene sequencing with microbiological techniques to diagnose infectious endocarditis (IE)

Reference	Number of patients/valves/blood cultures	16S PCR sensitivity/specificity
1	Valve material: 18 IE patients (n=10 IE of aortic valve, n=3 IE of mitral valve and n=2 prosthetic valve IE)	Valve material: 15/18 positive
2	Valve material: 30 IE patients (n=9 definite IE†, n=21 controls*) Blood cultures: 47 patients (≥3 sets) (n=18 definite IE, n=3 possible IE, n=26 rejected†) (n=5 controls*)	Valve material: 9/29 positive Blood culture: 10/25 positive
3	Valve material/blood culture: n=4 patients definite IE, n=4 patients possible IE† (B/Cs from 7, valve extract from 1)	All 16S rDNA positive (*Staphylococci*), MRSA n=1, MSSA n=3, CoNS n=4
4	Valve material: 2 IE patients (culture negative) plus B/C's	16S PCR V3 primer 100%
5	Whole blood: 51 patients with suspected bacteraemia	Sensitivity 86.7%, specificity 86.9%, PPV 76.5%, NPV 94.1%
6	Valve material: 49 IE patients (n=22 definite IE, n=13 possible IE, n=14 rejected†)	Sensitivity 82.6%, specificity 100%, PPV 100%, NPV 76.5%
7	Valve material: 52 patients (n=38 suspected IE†, n=14 controls*)	21/28 PCR matched blood culture result
8	Valve material: 15 IE patients (n=12 possible IE, n=3 definite IE†, total n=17 samples); n=13 controls*	14/15 positive (93%) 13/13 controls negative (100%)
9	Valve material: 46 IE patients (n=36 definite IE, n=10 possible IE†); n=25 controls*	24/30 positive
10	Valve material: 8 patients	2/8 positive for organism identified by culture from previous IE
11	Valve material: 98 patients (n=28 definite IE, n=9 possible IE†, n=61 controls*)	PCR: Active group: 12/19 positive, Resolved group: 3/9 positive
12	Valve material: 51 patients suspected IE† (n=52 samples); n=16 controls*	Sensitivity 41.2%, specificity 100%, PPV 100%, NPV 34.8%
13	Valve material: 238 patients (total n=245 samples; n=127 from IE patients – n=98 definite IE, n=29 possible IE†, n=118 controls*) plus B/Cs	Sensitivity 61%, specificity 100%, PPV 100%, NPV 74%
14	Valve material: 147 IE patients† (n=156 valves), B/Cs 3 sets/patient	Group 1: 76/126 positive (60%) Group 2: 11/30 positive (37%)
15	Valve material: 56 IE patients (n=36 definite IE, n=2 possible IE, n=18 rejected IE†) plus B/Cs	Sensitivity 65.8%, specificity 100%, PPV 100%, NPV 58%
16	Valve material: 35 definite IE patients† (n=48 samples); 120 controls* (n=129 samples)	Sensitivity 96%, specificity 95.3%, NPV 98.4% and PPV 88.5%
17	Valve material: n=71 valves from IE patients (42 IE episodes†); n=1,030 valves from controls* (total valves n=1,101)	Valve tissue 34/37 positive
18	Valve material: 74 patients (n=57 definite IE, n=7 possible IE, n=10 rejected†); n=16 controls*	Sensitivity 72%, specificity 100%

Key: B/Cs = blood cultures; PPV = positive predictive value; NPV = negative predictive value; MRSA = methicillin resistant *Staph. aureus*; MSSA = methicillin-sensitive *Staph. aureus*; CoNS = coagulase-negative *Staphylococci*

*Valves replaced due to non-infective indication

†Classified according to the Dukes criteria

Two sets of 16S primers were used in this study: one targeting V3 region and one targeting V4-V7 regions;[4]

Eight patients undergoing valve replacements following a previous episode of IE;[10]

Of the 28 definite IE, 19 had surgery before end of antibiotic treatment – active group, 9 had surgery after antibiotics – resolved group;[11]

Group 1: 126 patients receiving antibiotics at the time of surgery; Group 2: 30 patients with a history of IE who had completed antibiotic therapy before surgery;[14]

PCR performed on all excised valves in first 9 months of study, PCR only performed on valves with a positive culture or on clinical suspicion of IE in remaining 27 months of study[17]

Appendix References

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