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Molecular identification of pathogens from excised heart valves of infective endocarditis cases

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Abstract

Infective endocarditis is a life-threatening disease and identification of the pathogenic organisms is crucial for better prognosis. However, in some conditions, such as previous antibiotic usage or infection with fastidious organisms, obtaining positive cultures results is difficult. In addition to conventional culture methods, a molecular approach has been developed over several decades, such as broad-range 16S rRNA gene PCR and sequencing using excised tissues, to detect the pathogens. The current consensus is that this method is useful for the diagnosis and management of infective endocarditis patients and it has been suggested that these molecular results should be included as a new Deke criterion. Although molecular technique cannot replace culture methods and should be interpreted carefully, broad-range 16S rRNA/sequencing has great value in complementing conventional methods. Tissue samples from infective endocarditis cases should be examined by this method for better management of the patients.

Keywords

Infective endocarditic, broad-range 16S rRNA gene PCR/sequencing, pathogenic organisms, excised heart valves, blood culture

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1. Introduction

Infective endocarditis (IE) is a life-threatening disease, in which heart function deteriorates because of the destruction of the heart valve. This manifests as thrombosis in several organs, including the brain, spleen, and kidneys. The pathogenesis of IE occurs through infection of the heart valve and subsequent dissemination of the lesion. Early diagnosis and prompt treatment with antibiotics are crucial for good prognosis. Diagnosis of IE worldwide is based on the modified Duke criteria. The criteria are pathological and clinical, with the latter including major and minor criteria (Li *et al.*, 2000). The pathological criterion requires confirming the infection by culture or histological analysis of the infected regions. The major clinical criteria are positive blood culture results and evidence of endocardial involvement. In most IE cases, blood and tissue culture are useful to identify the pathogenic organisms. However, some conditions, such as previous antibiotic usage or infection with fastidious causative organisms, preclude clinicians from obtaining positive culture results. By amplifying and sequencing bacterial DNA from blood or tissue samples, it is now possible to identify pathogens even in these conditions (Goldenberger *et al.*, 1997). It has been suggested that these molecular results should be included as a new Duke criterion (Millar *et al.*, 2001). In this review, we discuss the utility of polymerase chain reaction (PCR)-based identification of organisms, particularly broad-range 16S rRNA gene PCR/sequencing, in the management of IE cases.

2. Pathogenic organisms of IE

IE is caused by bacteremia, through several avenues of suspected bacterial entrance, such as the oral cavity, intestines, and skin. Once the bacteria invade the blood stream, they colonize the heart valves and proliferate there. Common pathogenic organisms are gram-positive bacteria, such as viridans streptococci, *Staphylococcus aureus*, coagulase-negative staphylococci, and *Enterococcus* spp. (Vogkou *et al.*, 2016). Others include gram-negative bacteria, fastidious organisms, and fungi. *Streptococcus* spp. and *Staphylococcus* spp. account for 70-90% of all IE cases (Mylonakis *et al.*, 2001, Fukuchi *et al.*,

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2014, Takayama *et al.*, 2010). Gram-negative bacilli, which include enterobacteriaceae, *Pseudomonas* ssp., and the HACEK group (*Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp.), cause 2-8% of cases (Mylonakis *et al.*, 2001, Fukuchi *et al.*, 2014, Takayama *et al.*, 2010). These bacteria can be reliably isolated using conventional automated blood culture systems (Benett *et al.*). However, 5-20% of all cases have negative blood culture results (Mylonakis *et al.*, 2001, Fukuchi *et al.*, 2014, Takayama *et al.*, 2010, 9-12 Werner *et al.*, 2003, Nakatani *et al.*, 2013, Hoen *et al.*, 2002, Tornos *et al.*, 2005), and in half of these cases, this is due to infection with fastidious pathogens, such as intracellular, slow-growing, and nutritionally deficient bacteria (Brouqui *et al.*, 2006).

Coxiella burnetii and *Tropheryma whipplei* are intracellular pathogens; the isolation of which require cell culture systems and experienced laboratory staff. *C. burnetii*, the etiological agent of Q fever, causes 5% of all cases of endocarditis (Brouqui *et al.*, 2006), and accounts for 48% of culture negative endocarditis cases in France (Houpikian *et al.*, 2005). When Geiβdöfer et al. analyzed cardiac valve tissues from 255 culture negative IE patients by PCR of the bacterial 16S rRNA gene, 6% of the cases were *T. whipplei*-induced IE (Geiβdörfer *et al.*, 2012). *Bartonella* spp. and *Brucella* spp. are slow-growing bacteria; isolation takes a month for the former and more than 10 days for the latter. *Bartonella* spp. are facultative intracellular bacteria that cause various clinical syndromes. For example, *B. henselae* causes cat scratch disease and meningoencephalitis, and *B. quintana* causes trench fever and lymphadenopathy. The prevalence of *Bartonella* endocarditis is reported to be 1-3% of IE cases in Europe, and 10-15% in African countries (Edouard *et al.*, 2015). *Brucella* spp. are also facultative intracellular bacteria, which cause brucellosis. *Brucella* spp. cause only 1-4% of all cases of IE, but their prevalence depends on geographic area (Fernandez-Guerrero *et al.*, 1993). *Abiotrophia defectiva, Granulicatella adiacens*, and *Granulicatella elegans* are nutritionally deficient bacteria termed

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nutritionally variant streptococci (NVS). IE caused by NVS has a very poor prognosis, and a higher mortality rate than that caused by viridans streptococcal or enterococcal endocarditis (Brouqui *et al.*, 2001). These bacteria can be detected using a conventional blood culture system, but subculture requires special conditions, such as supplementation of blood agar with pyridoxal hydrochloride or cysteine. NVS is currently reported to account for 5-6% of microbial endocarditis cases (Ruoff *et al.*, 2001); however, owing to its unique culture requirements and the number of endocarditis cases with negative blood culture results, this figure may be underestimated (Brouqui *et al.*, 2001). Identification of these fastidious bacteria requires techniques other than conventional culture methods.

3. Broad-range 16S rRNA gene PCR and direct sequencing

The sequence of the small-subunit rRNA gene varies across phylogenetic lines, and contains segments that are conserved at the species, genus, and kingdom levels. A molecular approach has been developed over several decades involving the use of broad-range DNA probes to detect and amplify bacterial DNA, enabling sequencing of the genes encoding 16S rRNA (Edwards *et al.*, 1989, Chen *et al.*, 1989. Wilson *et al.*, 1990). In 1989, Edwards reported the first analysis of direct sequencing of amplified DNA fragments without subcloning, and demonstrated the usefulness of their universal primers in the amplification and almost complete sequencing of the 16S rRNA gene of *Mycobacterium kansasii*. The procedure required much less time (3-4 days) than that required for traditional molecular techniques using cloning (3-6 months) (Edwards *et al.*, 1989). This technique provided an attractive alternative when conventional culture methods failed to identify a microorganism, as in the initial molecular characterization of *B. henselae* from an angiomatosis lesion (Relman *et al.*, 1990).

The diagnostic value of broad range 16S rRNA gene PCR has been investigated in patients with endocarditis. In 1995, Jalava et al. conducted PCR with broad-range bacterial primers and DNA sequencing to identify *B. quintana* in a case of culture-negative IE (Jalava *et al.*, 1995). In

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this case, the blood, resected valve, and vegetation were all culture-negative. However, PCR amplification of the vegetation yielded a nucleotide sequence identical to *B. quintana* (Jalava *et al.*, 1995). Whipple's disease is a systemic infectious disease characterized by fever, weight loss, lymphadenopathy, diarrhea, and occasionally cardiac manifestations, such as myocarditis and endocarditis. The pathogen responsible, *T. whipplei*, was first identified by amplification of the 16S rRNA gene and sequencing from duodenal samples and lymph nodes of the patient (Wilson *et al.*, 1991, Relman *et al.*, 1992). Later, Raoult et al. succeeded in isolating the organism from the mitral valve of a patient with endocarditis using a human fibroblast cell line (Raoult *et al.*, 2000). Nevertheless, *T. whipplei* is difficult to cultivate using conventional bacterial culture techniques, and diagnosis of Whipple's endocarditis requires molecular analysis. *C. burnetii* is a strict intracellular pathogen, which requires a cell culture system for isolation (Mühlemann *et al.*, 1995). PCR is a useful tool to detect *C. burnetii* from blood or infected heart valve with either broad-range 16S rRNA primers (Houpikian *et al.*, 2005) or species-specific primers (Harris *et al.*, 2014).

Species-specific PCR primers can be used if a pathogenic organism is suspected based on the clinical characteristics of the patient, or in combination with primers for several commonly isolated IE pathogens. Recent analysis has demonstrated that specific real-time PCR (RT-PCR) is much more sensitive than conventional broad-range 16S rRNA gene PCR. This was indicated by the study conducted by Morel et al., in which both broad-range PCR and specific RT-PCR were performed using blood or valvular tissues from 123 diagnosed endocarditis cases. Of the 123 cases, 76 were established by RT-PCR only (61.7%), whereas only two cases were established by 16S PCR only (Morel *et al.*, 2015). Nevertheless, broad-range 16S rRNA gene PCR allows us to identify bacteria for which specific primers are not available, such as emerging, rare, or unexpected pathogens.

As mentioned previously, cases with negative blood culture results constitute a considerable proportion of all the IE cases,

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and PCR analysis can be helpful to detect pathogenic organisms in these cases. Houpikian et al. investigated samples from 348 patients suspected of having blood culture-negative endocarditis (BCNE) to identify pathogens. They performed serology tests, shell vial blood cultures, and, when available, analyzed resected valves by culture, microscopic examination, and PCR amplification (Houpikian *et al.*, 2005). They used species-specific primers to detect *C. burnetii* DNA, genus-specific primers for *Bartonella* DNA, and universal 16S rRNA gene primers when no evidence of any causative agent was available. Of the 167 cases diagnosed as *C. burnetii* endocarditis by serology test, 41 (24.5%) were also PCR-positive, and of the 99 cases having *Bartonella* endocarditis, 47 (47.5%) were specifically PCR-positive for *Bartonella*. In *Bartonella* cases, PCR was useful to identify the organism at the species level. In addition, the universal PCR primers detected two cases of *T. whipplei* and one case of *A. elegans*. Among 58 patients previously treated with antibiotics, PCR of the 16S rRNA gene from valve tissue was performed in 27 cases, and detected four cases of gram-positive cocci (*Streptococcus gallolyticus* and *Streptococcus mutans*; both commonly isolated from IE cases). In their analysis, valvular specimens from 151 cases were analyzed, and pathogenic organisms were identified in 96 (63.5%) by PCR with specific or universal primers. They concluded that PCR analysis of resected valves was of great value for the diagnosis of IE caused by rare or fastidious microorganisms, or when blood cultures have been sterilized by previous administration of antibiotics. The improvement of these identifications has contributed to a significant improvement in patient management (Houpikian *et al.*, 2005). This group subsequently reported analysis of another 819 cases of suspected BCNE using a comprehensive strategy, incorporating serological, molecular, and histopathological assays (Fournier *et al.*, 2010). Of the 227 patients from whom valves were available, 16S rRNA gene PCR/sequencing allowed the detection of pathogens in 150 (66.1%), and provided a diagnosis for 109 patients with negative serological results. Thus, they suggested that broad-range PCR might be preferred as the first-line test for valvular specimens,

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although sensitivity was poor when the method was applied to blood (Fournier *et al.*, 2010).

Broad-range 16S rRNA gene PCR can also be applicable to blood culture-positive endocarditis (BCPE) cases, especially those in which the blood culture result is positive only once, is contradictory to clinical features, or the species of the isolated organism is unclear. In these cases, broad-range PCR can provide confirmation of pathogen identity. Moreover, the technique could lead to reclassification of organisms previously obtained by culture methods, such as in *Aerococcus urinae* and *Streptococcus tigurinus* IE cases we have reported (Miyazato *et al.*, 2011, Miyazato *et al.*, 2014). The former was initially misidentified as *Streptococcus acidominimus* because of the limited discriminative power of conventional culture methods, and the latter is a newly described species in the *Streptococcus mitis* group. Automatic identification systems, such as Vitek 2 and MALDI-TOF MAS, incorrectly identified *S. tigurinus* as *S. mitis* or *S. oralis* at the time, owing to gaps in their databases. Broad-range 16S rRNA gene PCR/sequencing can augment culture methods and identify rare or emerging pathogens.

4. Evaluation of broad-range 16S rRNA gene PCR and sequencing for management of IE

Among IE cases, 25-50% of the patients undergo valve surgery (Tornos *et al.*, 2005). There have been several reports evaluating the routine molecular diagnosis of IE using resected valves (Goldenberger *et al.*, 1997, Harris *et al.*, 2014, Miyazato *et al.*, 2012, Gauduchon *et al.*, 2003, Voldstedlund *et al.*, 2008, Rovery *et al.*, 2005, Marin *et al.*, 2007). In these reports, 16S rRNA gene PCR/sequencing results were compared to those of blood and valve cultures, and the utility of the molecular technique was discussed. Miller et al. reviewed previous reports and found that the broad-range PCR assay has a wide range of performance in patients with definite IE with a sensitivity range of 41.2-100%, specificity range of 61.5-100%, positive predictive value range of 79-100%, and negative predictive value range of 34.4-100%. They suggested that this variability might be attributed to differences in primers, or differences in

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diagnostic criteria (Miller *et al.*, 2016). Heart valve culture had a less optimal range of pathogen recovery (11.2-32.3%) compared to either blood culture or broad-range 16S rRNA gene PCR/sequencing on heart valve tissue (Miller *et al.*, 2016). Overall, these reports recommended the application of molecular techniques for the microbiological diagnosis of BCNE. In addition, in BCPE, 16S rRNA gene PCR/sequencing could clarify the diagnosis in cases where culture results were positive only once, or were contradictory or insufficient for species identification (Gauduchon *et al.*, 2003). In both blood culture-negative and -positive cases, the molecular method has impacted the diagnosis and subsequent management of patients. Miller et al. described in their report that analysis of heart valve tissue by broad-range 16S rDNA PCR/sequencing contributed to the microbiologic diagnosis of 31% of their patients, and changed the antibiotics administered to 13% of them (Miller *et al.*, 2016). Marsch et al. analyzed valve samples in 46 patients with culture-negative IE or inconsistent microbiological diagnosis by PCR, and the results led them to change the antibiotic regimens of 7 of their 46 patients (15.2%) (Marsch *et al.*, 2015). Gauduchon et al. reported that the 16S rRNA gene PCR assay contributed to the diagnosis and the management of IE in six of 29 (20%) cases; three cases were diagnosed by PCR alone, and three cases were correctly diagnosed by PCR, allowing better patient management (Gauduchon *et al.*, 2003). In our previous work, identification of pathological organisms affected patient treatment in four of 19 IE cases (21.0%) (Miyazato *et al.*, 2012). Taken together, broad-range 16S rRNA gene PCR/sequencing results contributed to better antibiotic use and IE management in 10-20% of the IE patients in these reports.

5. Future perspectives

The current consensus among researchers is that broad-range 16S rRNA gene PCR/sequencing of resected valves is useful for the diagnosis and subsequent management of IE. Several researchers have suggested that the results of broad-range 16S rRNA gene PCR/sequencing should be included in the Duke criteria as a major criterion because of its usefulness for detecting pathological

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agents in IE. The British Society for Antimicrobial Chemotherapy recommend in their guidelines that excised heart valves from cases of culture-negative IE should be referred for broad-range PCR and sequencing, and that positive broad-range bacterial PCR results can be reliably used to identify the cause of endocarditis (Gould *et al.*, 2012). The European Society of Cardiology (ESC) only mentions specific PCR using blood or surgical material for *Brucella* spp., *Coxiella* spp., *Bartonella* spp., *T. whipple*, *Mycoplasm* spp., and *Legionella* spp. following serology tests (Habib *et al.*, 2015). Conversely, the American Heart Association (AHA) does not mention any molecular diagnostic methods in their statement (Baddour *et al.*, 2015). The modified Duke pathological criterion requires proof of microorganisms, demonstrated by culture or histological examination of vegetation or an abscess (Li *et al.*, 2000). However, heart valve culture has a much lower pathogen recovery rate (11.2-32.3%) compared to broad-range 16S rRNA gene PCR on heart valve tissue (41.2-100%) (Miller *et al.*, 2016). These results indicate that broad-range PCR is superior to valve culture in detecting pathological agents, although antibiotic susceptibility of pathogens cannot be investigated by PCR. Thus, it is recommended that, if possible, samples of excised heart valve from IE cases should be referred for broad-range bacterial PCR and sequencing. PCR-based methods using heart valve tissue are of great value for diagnosing pathological agents in BCNE and even BCPE, by complementing incomplete information about the epidemiology and spectrum of causative agents of IE (Moter *et al.*, 2010). In cases where *T. whipplei*, *Coxiella* spp., or *Brucella* spp. is clinically suspected as a pathogen, specific PCR targeting these organisms should be considered first. When applied to blood, 16S rRNA gene PCR exhibited poor sensitivity, although dedicated real-time PCR assays targeting small DNA fragments and using fluorescent probes exhibited high sensitivity for *Bartonella* spp., *C. burnetii*, and *T. whipplei* (Fournier *et al.*, 2010). Therefore, a real-time PCR assay targeting these bacteria could be useful with blood from patients for whom valve specimens are not available. Recently, a commercially available real-time PCR assay,

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LightCycler® SeptiFast, has been tested for diagnosis of IE (Cambau *et al.*, 2017. Leli *et al.*, 2014, Menacacci *et al.*, 2012). This system covers most common bacterial and fungal pathogens of IE, but does not cover fastidious or intracellular microorganisms. Further molecular methods and commercial assays will need to be developed to account for the spectrum of causative pathogens of IE. There are also drawbacks of molecular analysis. It requires specific techniques and laboratory equipment, and there is currently no standardized method of 16S rRNA gene PCR/sequencing for IE cases. Clinical samples tested may contain factors that inhibit the PCR reaction, causing false-negative results; contamination may occur, causing false-positive results; and the quality of sequences in gene databases may be insufficient to identify the amplicons. Moreover, PCR-based methods cannot differentiate between viable and dead microorganisms. Therefore, PCR cannot replace conserved culture techniques, and results should be interpreted carefully and assessed in the context of clinical findings and culture results. However, broad-range 16S rRNA gene PCR/sequencing has great value in complementing culture methods in the identification of pathological agents of IE, particularly fastidious, intracellular, or emerging microorganisms, and in the condition of previous antibiotic administration. Although the molecular method may not be available in most laboratories, tissue samples could be investigated in certain reference laboratories using this technique. Samples of excised heart valve from IE cases, if possible, should be examined by broad-range bacterial PCR and sequencing for better management of IE patients.

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