Molecular diagnosis of *Aspergillus* endocarditis after cardiac surgery

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The prevalence of *Aspergillus* endocarditis (AE) is increasing in the hospital population. *Aspergillus* species contribute to approximately 25% of all cases of fungal endocarditis. This study is a descriptive report of the use of nested PCR to detect DNA specific for *Aspergillus* species in serum for the diagnosis of cardiac infections. Open heart surgery was performed on patients and collected samples were examined microscopically and cultured. Ten sera in total from five patients were extracted for *Aspergillus* DNA and nested PCR with *Aspergillus* species primers was carried out. The lowest limit of detection for the PCR assay was 1 c.f.u. (ml serum)⁻¹. The PCR was positive in three patients. Culture of valvular tissue confirmed the growth of *Aspergillus fumigatus* in one patient and *Aspergillus niger* in two patients. In this study we have demonstrated the presence of invasive aspergillosis in patients who had undergone open heart surgery and the usefulness of a molecular assay for the diagnosis of AE.

INTRODUCTION

The prevalence of *Aspergillus* endocarditis (AE) is increasing in the hospital population (Ellis et al., 2001). AE tends to occur in patients who have undergone open heart surgery, although it has also been described as a complication of parenteral nutrition and drug addiction. Most frequently the aortic and mitral valves are the sites of infection. Specific features include large friable vegetations, emboli that obstruct major arteries, particularly those of the brain, that occur in about 80% of cases (Anaissie et al., 2003).

Prosthetic valve endocarditis (PVE), which affects as many as 3.4% of patients in the year following surgery and 1% annually thereafter (Puvimanasinghe et al., 2001), is frequently associated with reoperation and significant mortality. Fungal PVE can occur early (within 60 days of surgery) or late (after 60 days), but an early occurrence is generally not seen in the absence of pre-operative fungal infections. Most fungal endocarditis cases are caused by *Candida* species, although other fungi, including *Aspergillus*, can cause endocarditis. *Aspergillus* species contribute to approximately 25% of all cases (Ellis et al., 2001; Muehrcke et al., 1995). Over half of the cases involving *Aspergillus* infections are identified only at autopsy (Sherman-Weber et al., 2004).

PVE due to *Aspergillus* has been reported on mechanical prostheses in both the mitral and aortic positions (Khan et al., 1968; Stein et al., 1966; Aslam et al., 1970).

Abbreviations: AE, *Aspergillus* endocarditis; PVE, prosthetic valve endocarditis.

Identifying the source, establishing the diagnosis and carrying out the treatment are highly challenging in AE cases, and are often unsuccessful. It also remains a significant complication of prosthetic valve surgery, and is associated with high rates of morbidity and mortality. Like native valve endocarditis, most fungal PVE results in valvular destruction and regurgitation, but large fungal vegetations may also obstruct flow of circulation (Gerritsen et al., 1998; Melgar et al., 1997).

This article describes the use of molecular tests to aid the diagnosis of AE. To the best of our knowledge, this is the first description of the use of nested PCR to detect DNA specific for *Aspergillus* species in serum for the diagnosis of cardiac infection.

METHODS

Five cases of infected endocarditis have been recently diagnosed (within the 12 months preceding June 2008) at the Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, and were carefully investigated. A diagnosis of endocarditis required fulfilment of the Duke criteria and the presence of a vegetation (Durack et al., 1994). All the patients had undergone aortic valve replacement.

To determine the detection limit of the assay for fungal pathogens in the blood, suspensions of serum with *Aspergillus* (*Aspergillus flavus, Aspergillus niger* and *Aspergillus fumigatus*) conidia (1 to 10⁵ conidia ml⁻¹) were diluted, and each solution was cultured for determining the colony count, and for DNA extraction and PCR.

Two blood specimens from each patient where there was a suspicion of fungal infection after heart surgery were examined for *Aspergillus* species in serum for the diagnosis of AE. Open heart surgery was performed on patients and collected samples were examined microscopically and cultured. Ten sera in total from five patients were extracted for *Aspergillus* DNA and nested PCR with *Aspergillus* species primers was carried out. The lowest limit of detection for the PCR assay was 1 c.f.u. (ml serum)⁻¹. The PCR was positive in three patients. Culture of valvular tissue confirmed the growth of *Aspergillus fumigatus* in one patient and *Aspergillus niger* in two patients. In this study we have demonstrated the presence of invasive aspergillosis in patients who had undergone open heart surgery and the usefulness of a molecular assay for the diagnosis of AE.
DNA. *Aspergillus* DNA was extracted from the sera of patients using the QIAamp DNA minikit (Qiagen) in accordance with the manufacturer’s recommendations. PCR was performed as Yamakami et al. (1996) suggested, which is a nested PCR with two sets of primers. It is worth mentioning that this PCR is able to identify all *Aspergillus* species.

After examining the DNA bands of ethidium bromide-stained gels after electrophoresis, one patient received voriconazole immediately, and all patients underwent open heart surgery after 2 days. Infected valve specimens were cultured on Sabouraud dextrose agar (Merck) and all patients underwent open heart surgery after 2 days. Infected material for the diagnosis of AE are invasive. Because specimens must be obtained surgically, and since pathogenic fungi may require culturing times of 2–3 weeks or longer, definitive diagnosis may be delayed, thus impacting patient care. *Aspergillus* may be isolated from the infected valves in post-mortem or biopsy specimens. A positive blood culture is essential to establish an accurate microbiological diagnosis and it is the major criterion in the Duke criteria. However, in patients with *Aspergillus* infection, isolation of the organism from blood cultures is exceedingly rare. Gumbo et al. (2000) reported endocarditis on a native or prosthetic valve or in a mural location characterized by peripheral embolization, and almost always there was a negative result from blood culture. However, Pemán et al. (2007) reported a case of fungal endocarditis in a native mitral valve with the isolation of *A. fumigatus* both in valve vegetation and in blood culture bottles. The patient underwent valve replacement and antifungal treatment with voriconazole and caspofungin, but he died on post-operative day 45 with disseminated aspergillosis confirmed by necropsy. Paradoxically, galactomannan antigen detection in the serum was negative. El-Hamamsy et al. (2004) reported three cases of *Aspergillus* aortic valve endocarditis caused by *A. niger*, *A. fumigatus* and *Aspergillus* spp. Despite attempts at combined medical and surgical therapy, mortality was 100% in their report. Sherman-Weber et al. (2004) reported the major pathogens that caused infective endocarditis in cardiac transplantations were *S. aureus* (four cases) and *A. fumigatus* (three cases). Endocarditis-related mortality was 80%. All patients with AE died. Kotanidou et al. (2004) and McCracken et al. (2003) reported that for their patients with AE all serological tests for *Aspergillus* were negative, and did not help the diagnosis and follow-up.

**RESULTS AND DISCUSSION**

The lower limit of detection of this PCR assay was 1 c.f.u. (ml serum)⁻¹. Nested PCR with *Aspergillus* species primers produced bands on ethidium bromide-stained gels in three patients (Fig. 1). Valvular vegetations were detected by echocardiography and fungal infections were documented in three patients by surgical exploration. Direct microscopic examination of the three samples was positive for fungal growth and on potassium hydroxide smear showed the characteristic pattern of *Aspergillus* (hyphae of 5–10 μm in width, which were branched with numerous septa distributed at regular intervals, consistent with typical *Aspergillus* species morphology). Culture of valvular tissue on Sabouraud dextrose agar confirmed the growth of *A. fumigatus* in one patient and *A. niger* in two patients. Two of the patients with AE that received late antifungal therapy (post-surgery) died and one patient lived. *Staphylococcus aureus* was isolated from the blood and valve replacement from two patients with negative PCR. The demographic and clinical features of the patients are listed in Table 1.

AE, most commonly found in open heart surgery cases, is associated with a high mortality rate in adults and children.

Post-mortem studies indicate that AE is more invasive than *Candida* endocarditis (Feigin et al., 2004). The most common clinical features are fever, major peripheral emboli and a changing heart murmur (Rubinstein & Lang, 1995), as seen in all the presented cases. Non-specific laboratory findings, such as anaemia, leukocytosis, elevated erythrocyte sedimentation rate and elevated C-reactive protein, are often present in patients with suspected PVE. The culture and histopathological samplings from embolic material for the diagnosis of AE are invasive. Because specimens must be obtained surgically, and since pathogenic fungi may require culturing times of 2–3 weeks or longer, definitive diagnosis may be delayed, thus impacting patient care. *Aspergillus* may be isolated from the infected valves in post-mortem or biopsy specimens. A positive blood culture is essential to establish an accurate microbiological diagnosis and it is the major criterion in the Duke criteria. However, in patients with *Aspergillus* infection, isolation of the organism from blood cultures is exceedingly rare. Gumbo et al. (2000) reported endocarditis on a native or prosthetic valve or in a mural location characterized by peripheral embolization, and almost always there was a negative result from blood culture. However, Pemán et al. (2007) reported a case of fungal endocarditis in a native mitral valve with the isolation of *A. fumigatus* both in valve vegetation and in blood culture bottles. The patient underwent valve replacement and antifungal treatment with voriconazole and caspofungin, but he died on post-operative day 45 with disseminated aspergillosis confirmed by necropsy. Paradoxically, galactomannan antigen detection in the serum was negative. El-Hamamsy et al. (2004) reported three cases of *Aspergillus* aortic valve endocarditis caused by *A. niger*, *A. fumigatus* and *Aspergillus* spp. Despite attempts at combined medical and surgical therapy, mortality was 100% in their report. Sherman-Weber et al. (2004) reported the major pathogens that caused infective endocarditis in cardiac transplantations were *S. aureus* (four cases) and *A. fumigatus* (three cases). Endocarditis-related mortality was 80%. All patients with AE died. Kotanidou et al. (2004) and McCracken et al. (2003) reported that for their patients with AE all serological tests for *Aspergillus* were negative, and did not help the diagnosis and follow-up.

The challenge we face today is to find a way to detect evidence of infection with non-invasive and high-accuracy innovative laboratory methods. Molecular identification of fungal infections has typically been presented in other research (Imai et al., 2000; Posteraro et al., 2000; Löffler et al., 1998; Einsele et al., 1997). A PCR assay for the detection of fungal nucleic acids may be the optimal diagnostic approach, because it offers the potential of being more sensitive than current culture-based methods and more applicable to a variety of specimen types (Van Burik et al., 1998; Badiee et al., 2007). A molecular approach to improve the microbiological diagnosis is reported in cardiovascular disease cases by Breitkopf et al. (2005). Also, disseminated aspergillosis in one patient with

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**Fig. 1.** Patient DNA was amplified with specific primers, and amplicons were detected by gel electrophoresis and ethidium bromide staining. 1, 100 bp DNA size marker; 2, negative control; 3, positive control; 4, *A. niger*; 5, *A. niger*; 6, *A. fumigatus*.
endocarditis diagnosed by a RFLP method was also reported (McCracken et al., 2003). In the present study all documented AE cases were positive with nested PCR.

Because of the ubiquitous nature of the organism, establishing a definitive diagnosis of disease caused by Aspergillus is difficult. In order to maximize specificity, we require additionally the presence of valvular vegetation demonstrated by echocardiogram, the pathology of a surgical specimen, or documentation at autopsy. In our study, surgical specimens confirmed the definitive diagnosis by PCR test results.

One patient responded to treatment with an antifungal agent, but unfortunately in two patients, the diagnosis was too late and antifungal therapy was unsuccessful; the patients died. Two patients had negative PCR results and S. aureus was detected in both cultures from the blood and surgical samples. These patients were successfully treated and responded well to antibiotic therapy.

**Conclusion**

We demonstrated in the present study invasive aspergillosis in open heart surgery patients and the usefulness of a molecular assay for the diagnosis of AE. However, the power of such a study is quite limited because of the small number of cases studied.

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**REFERENCES**


**Table 1. Clinical features of patients with infected endocarditis after heart surgery**

<table>
<thead>
<tr>
<th>Age/sex</th>
<th>Time of FIE after surgery (months)</th>
<th>No. of surgeries</th>
<th>Organism</th>
<th>Result of nested PCR</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>42/M</td>
<td>4</td>
<td>2</td>
<td><em>A. fumigatus</em></td>
<td>Positive</td>
<td>None</td>
<td>Dead</td>
</tr>
<tr>
<td>64/M</td>
<td>7</td>
<td>3</td>
<td><em>A. niger</em></td>
<td>Positive</td>
<td>Voriconazole + surgery</td>
<td>Alive</td>
</tr>
<tr>
<td>35/F</td>
<td>8</td>
<td>3</td>
<td><em>A. niger</em></td>
<td>Positive</td>
<td>Amphotericin + surgery</td>
<td>Dead</td>
</tr>
<tr>
<td>31/F</td>
<td>2.5</td>
<td>1</td>
<td><em>S. aureus</em></td>
<td>Negative</td>
<td>Antibacterial + surgery</td>
<td>Alive</td>
</tr>
<tr>
<td>25/M</td>
<td>3.5</td>
<td>1</td>
<td><em>S. aureus</em></td>
<td>Negative</td>
<td>Antibacterial + surgery</td>
<td>Alive</td>
</tr>
</tbody>
</table>

F, Female; M, male; FIE, fungal infected endocarditis.


