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Diagnosing and monitoring of invasive aspergillosis during antifungal therapy by polymerase chain reaction: an experimental study in mice

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Abstract

This study evaluated the value of polymerase chain reaction (PCR) for diagnosing and monitoring of invasive aspergillosis during amphotericin B therapy. PCR, microscopy and culture of tissues samples ($n = 126$) and blood samples ($n = 78$) of experimentally infected mice ($n = 42$) were performed. The PCR results of treated were compared to those of untreated animals; *Aspergillus fumigatus* and *A. terreus* were used in this study. In the amphotericin B treated group the sensitivities of PCR, microscopic examination and culture of the various tissues were 69, 58, and 53%, respectively; the specificities of all examinations were 100%. In the untreated group the sensitivities of PCR, microscopic examination, and culture were 72, 64, and 57%, respectively; the specificities of all examinations were 100%. The 78 blood samples taken from mice under therapy were tested by PCR over a period of 8 days following *Aspergillus* infection. The test sensitivity was 77%, the specificity 46%, the positive predictive value 59%, and the negative predictive value 67%. In the untreated group the sensitivity was 92%, the specificity 46%, the positive predictive value 63%, and the negative predictive value 86%. The results suggest that this PCR method has possible clinical value for improving the diagnosis of invasive *Aspergillus* infection. Monitoring of blood under antifungal therapy is not recommended.

Keywords: Aspergillus; PCR; Aspergillosis; Antifungal treatment

1. Introduction

Invasive aspergillosis (IA) is increasingly recognized in immunocompromised hosts (Denning, 1991). Patients with prolonged and deep granulocytopenia following chemotherapy for hemato-oncologic disorders or allogeneic bone marrow transplant recipients are particularly at risk (Peterson et al., 1998; Anaissie, 1992). The crude mortality rate of IA approaches 100% and results at least partly from difficulties in obtaining a reliable diagnosis at an early stage of the disease, often leading to a fatal delay in adequate therapy (Aisner et al., 1977). No method has proven sufficiently sensitive and specific to allow diagnosis at an earlier stage. Culture detection is often delayed and blood cultures are rarely positive for patients with invasive aspergillosis (Duthie et al., 1995). Thus, the detection of circulating fungal DNA has been advocated as a promising, rapid and more sensitive diagnostic tool for overcoming these drawbacks (Buchheidt et al., 2002; Williams et al., 2000; Einsele et al., 1997, 1998; Bretagne et al., 1995).

Recently, we evaluated the value of twice-weekly screening for circulating fungal DNA using the PCR of whole blood samples (Lass-Flörl et al., 2001). Positive PCR results became negative shortly after commencement of antifungal treatment and did not correlate with the underlying diseases.

The aim of the present study was to evaluate this PCR assay as a tool for monitoring and diagnosing aspergillosis during antifungal therapy. PCR, cultures and microscopic examination of organ specimens and blood of experimentally infected mice were performed. PCR results of amphotericin B-treated mice were compared to PCR results obtained from untreated mice. Since in our hospital *Aspergillus terreus* is the most detectable species of inva-

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sive aspergillosis we performed this study with *A. fumigatus* and *A. terreus*.

2. Materials and methods

2.1. Mice

There were 42 female BALB/c mice, 5-7 weeks old, weighing 20-22 g used throughout the experiment. Mice were housed in groups of 21 and given standardized food and tap water ad lib. Animal experiments were conducted in confirmity with national law and guidelines of the European Communities.

2.2. Infection

An animal model was used as described previously (Dannaoui et al., 2000). There were 28 mice infected with *A. fumigatus* (MIC 1 μg/ml amphotericin B) and 14 with *A. terreus* (MIC 2 μg/ml amphotericin B), (which was kindly provided by E. Dannaoui). The in vitro resistance testing was performed according to a method of Espinel-Ingroff et al. (1995). The inoculum was prepared by culturing these strains on Sabouraud Dextrose Agar (SAB, Merck, Vienna, Austria) for 5 days at 35°C. Conidia were harvested by washing the agar slant with sterile NaCl 0.9% containing Tween 80 0.05%, and the suspension was counted in a hemacytometer. Viability was determined by plating serial 10-fold dilutions prepared in NaCl 0.9% with Tween 80 0.05%. Plates were incubated at 35°C, and the numbers of colony-forming units (CFU) were counted after 24 h and 48 h. On the day of injection, the conidial suspension was adjusted to the required concentration in NaCl 0.9%. A preliminary study was performed to determine the LD_{90} for these strains by testing three inoculum sizes (1-5 $\times$ 10^5, 1-5 $\times$ 10^6, and 1-5 $\times$ 10^7 CFU/mouse). First, each mouse was infected by IV injection of 0.1 ml of conidial suspension into a lateral tail vein. After injection, mice were randomized to the different treatment groups. The 13 noninfected mice served as controls.

2.3. Drugs and therapy

Amphotericin B desoxycholate (Fungizone, Bristol-Meyers Squibb, Vienna, Austria) was given in 5% glucose intraperitoneally (IP). Treatment was begun 3 h after infection and was continued for 10 days. One group was treated with amphotericin B (4.5 mg/kg/day) by once daily injection; control mice were inoculated, but received only glucose 5%. Animals were checked twice daily for illness, distress, suffer, and mortality. The mice were observed for 10 days after the end of treatment; mice surviving on day 20 after infection were sacrificed by cervical dislocation.

2.4. Organ cultures and blood monitoring

Organs (kidneys, brain, and lungs) were removed and homogenized with 3 ml of NaCl 0.9% in a tissue grinder. Homogenates were divided into three identical aliquots for PCR, culture and microscopic examination. Culture technique was performed according to the method of Dannaoui et al. (2000); calcoflour white staining was performed according to the manufacturer's instructions.

For some experiments, sequential blood samplings were taken on day one, three and eight by puncturing the retro-orbital vein-plexus of mice.

2.5. PCR assay

DNA extraction of 1 ml of EDTA-anticoagulated blood was performed using recombinant lyticase (Sigma, Vienna, Austria) and the QIAmp Tissue Kit (Qiagen, Vienna, Austria) as previously described (Einsele et al., 1997). Genes of the 18S rRNA of various fungal pathogens were amplified with PCR using specific primers. Primers (5′-ATT GGA GGG CAA GTC TGG TG and 5′-CCT ATC CCT AGT CGG CAT AG; Roth, Graz, Austria) bind to conserved regions of the fungal 18S small-subunit rRNA. Thirty-four cycles of repeated denaturation (94°C, 30 s), annealing (62°C, 1 min) and extension (72°C, 2 min) were applied. Amplicons were detected by PCR-ELISA system using species-specific digoxin-labeled oligonucleotide (Roth) for *Aspergillus spp.* and antidigoxigenin antibodies conjugated with alkaline phosphatase (Boehringer Mannheim, Vienna, Austria). Negative and positive probes were used routinely as a quality control. Various blood samples contaminated with *A. fumigtus* and/or *A. terreus* served as control to detect PCR inhibitors.

3. Results

For the animal model, a preliminary experiment was performed to determine the LD_{90} of the *A. fumigatus* at day 11 and for the *A. terreus* at day 8. The LD_{90} was estimated at 1-5 $\times$ 10^7 CFU/mouse and this inoculum size was used for the treatment experiment. The presence of disseminated fungal infection was determined by culture and microscopic examination of organs. All infected mice had either positive kidney, brain or lung cultures. A total of 126 samples from 42 mice were analyzed, with the results shown in Table 1.

In the amphotericin B treated group the sensitivities of PCR, microscopic examination and culture of tissue samples were 69, 58, and 53%, respectively; the specificities of all examinations were 100%. In the untreated group the sensitivities of PCR, culture and microscopic examination were 72, 64, and 57%, respectively; the specificities of all examinations were 100%. The calculations are based on at least one positive finding in either lung, kidney, or brain.

The 78 blood samples taken from mice were tested by

Table 1
Distribution of results for the 28 mice tested for *Aspergillus* infection in tissues using PCR, microscopy and culture

PCR results of infected mice						
	Under antifungal therapy			Without antifungal therapy		
A. fumigtus						
Tissues (no. of Animals)	PCR ±	Culture ±	Microscopy ±	PCR ±	Culture ±	Microscopy ±
Lungs $n = 28$	8/6	6/8	4/10	8/6	6/8	6/8
Kidney $n = 28$	12/2	12/2	8/6	14/0	14/0	12/2
Brain $n = 28$	4/10	4/10	4/10	6/8	6/8	4/10
A. terreus						
Tissues (no. of Animals)	PCR ±	Culture ±	Microscopy ±	PCR ±	Culture ±	Microscopy ±
Lungs $n = 14$	4/3	3/4	5/2	5/2	5/2	6/1
Kidney $n = 14$	5/2	3/4	4/3	4/3	4/3	4/3
Brain $n = 14$	5/2	3/4	5/2	6/1	5/2	5/2

PCR over a period of 8 days following *Aspergillus* infection, 21 (72%) samples in the control group, and 12 (41%) in the amphotericin B group were positive. In the untreated group 3 mice were found to be negative by PCR on day 1, 8 on day 3, and 7 on day 8. During amphotericin B therapy 7 mice remained negative on day 1, 11 mice on day 3, and 9 on day 8. Based on at least one positive PCR result the sensitivity was 77%, the specificity 46%, the positive predictive value 59%, and the negative predictive value 67%. In the untreated group the sensitivity was 92%, the specificity 42%, the positive predictive value 63%, and the negative predictive value 83%. Similar data were obtained in the sensitivity to identify *A. fumigatus* or *A. terreus* as shown in Table 2. The blood specimens obtained from uninfected mice showed positivity once in seven cases.

Table 2
Distribution of results of blood monitoring using *Aspergillus* PCR in treated and untreated mice with proven infection

PCR results from blood						
	During antifungal therapy			Without antifungal therapy		
A. fumigatus						
Mice	Day 1	Day 3	Day 8	Day 1	Day 3	Day 8
1	+	−	−	+	+	+
2	+	−	−	+	−	−
3	−	−	−	−	−	+
4	−	+	+	+	−	+
5	−	−	+	+	+	−
6	+	−	−	−	+	+
7	−	−	−	+	−	−
8	+	−	−	+	+	−
A. terreus						
Mice	Day 1	Day 3	Day 8	Day 1	Day 3	Day 8
1	+	−	−	+	+	+
2	+	−	−	+	−	−
3	−	−	−	−	−	−
4	−	+	+	+	−	+
5	−	−	+	+	−	−

4. Discussion

We evaluated the value of *Aspergillus*-specific PCR for diagnosing and monitoring invasive aspergillosis during antifungal therapy in a murine model of systemic aspergillosis.

Organ specimens showed positive PCR results for *Aspergillus* in the glucose (68.2%) and amphotericin B (60.4%) treatment groups. This suggests that amphotericin B treatment does not exhibit an inhibitory effect on PCR when analyzing tissue samples. PCR was the most sensitive method, but not significantly more than microscopic examination in treated and untreated groups. These data are in agreement with the literature showing that microscopy is sensitive and specific for fungal infection, yet with a lack of species-identification (Ascioglu et al., 2002; Denning, 1998). For this reason, the performance of a species-specific PCR is appropriate and clinically useful in diagnosing *Aspergillus* infection in several organs. As shown in our study, there exist no differences in the sensitivity to detect *A. fumigatus* and *A. terreus*. Combining microscopy, culture and PCR may increase the diagnostic outcome, as recently found (Buchheidt et al., 2002; Skladny et al., 2000).

In monitoring the course of fungal disease, PCR of the sequentially blood samples showed inconsistent results. In the amphotericin B group 12 of 39, in the glucose Group 21 of 39 samples revealed positive during the course of the disease. The rate of positive PCR results declined during antifungal treatment and did not correlate with the underlying invasive disease. We observed 66% false-negative results with a negative predictive value of 67%. Similar data were found by Yamakami et al. (1998) when using a nested PCR. Once can imagine that treatment with amphotericin B may in part be responsible for complete clearance of fungi from the blood, whereas complete clearance of fungi from tissue is not provided (Lass-Flörl et al., 2001). The half-life of circulating DNA is short, probably less than 5 min and a positive signal is observed only when the fungal burden is large enough (Rumore et al., 1992). This assumption is supported by the PCR positive results of several tissue samples such as brain, kidneys and lungs. Also, Loeffler et al. (2002) postulate a correlation between high fungus load of tissue and the presence of fungal DNA in the blood. In

their study, *Aspergillus* DNA was detected in only 25% of the blood samples of infected animals. Mice whose blood became PCR positive showed a mean fungus load in their lungs 10 times higher than that in mice whose blood remained PCR negative. However, negative blood samples were also detected in the untreated group of infected mice. Immune response of the host may result in a loss of fungi in blood (Becker et al., 2000). Also, a PCR assay in which circulating DNA is detected in serum can possibly give a better correlation with fungal load and severity of disease and thus gives more consistent results (Bretagne et al., 1998). By contrast, Loeffler et al. (2000) reported that the sensitivity of plasma PCR was lower than that of PCR performed on whole blood samples.

One of the major drawbacks in the application of *Aspergillus* PCR assays is the high rate of transient *Aspergillus* fungaemia without evidence of invasive aspergillosis (Bialek et al., 2002; Lass-Flörl et al., 2001). As shown in our study, it is of major importance to recommend a serial blood sampling, because the value of one positive or negative test may result in a false diagnosis.

5. Conclusion

From the findings in our animal model, we conclude that performance of *Aspergillus* PCR should be recommended in addition to microscopic examination and culture technique for sensitive detection of tissue infection. Serial blood screening is not recommended under antifungal therapy as sensitivity was 77%, specificity 46%, and the negative predictive value 67%. This model is quite different from human infections, further studies are necessary to investigate the in vivo situation.

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