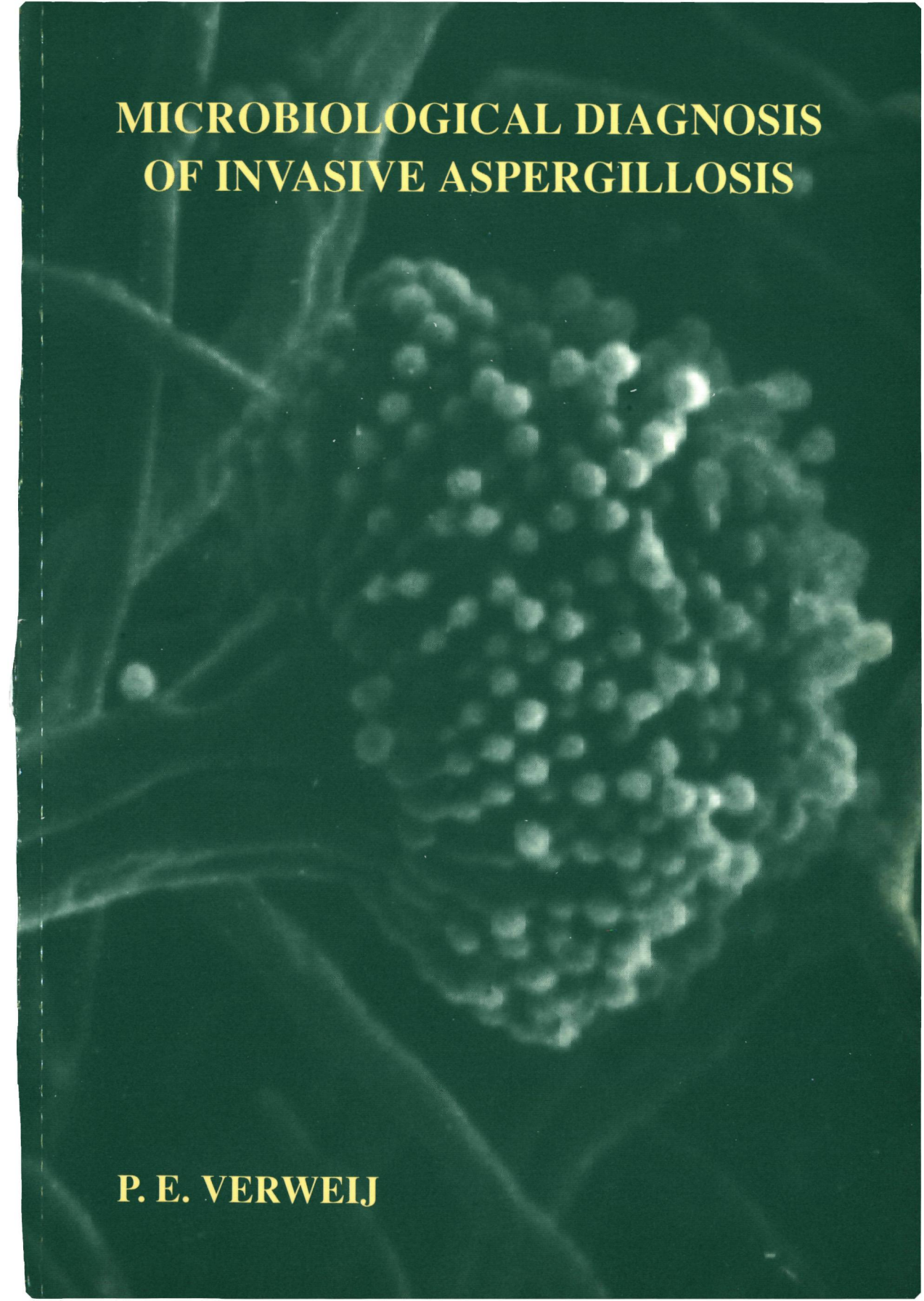
MICROBIOLOGICAL DIAGNOSIS OF INVASIVE ASPERGILLOSIS



P. E. VERWEIJ

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CIP data, Koninklijke Bibliotheek, Den Haag
ISBN 90-5651020-7

1996
Drukkerij: Ponsen & Looijen, Wageningen

MICROBIOLOGICAL DIAGNOSIS OF INVASIVE ASPERGILLOSIS

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen,

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Katholieke Universiteit Nijmegen,
volgens besluit van het College der Decanen
in het openbaar te verdedigen op
donderdag 1 februari 1996,
des namiddags te 1.30 uur precies

door

Paul Eduard Verweij

geboren op 31 augustus 1962
te 's Gravenhage.

PROMOTOR: Prof. Dr. J.A.A. Hoogkamp-Korstanje

CO-PROMOTORES: Dr. J.F.G.M. Meis
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The studies presented in this thesis were performed at the Department of Medical Microbiology, University Hospital Nijmegen, Nijmegen, The Netherlands in collaboration with the Department of Hematology, University Hospital Nijmegen, Nijmegen, The Netherlands and Unité de Mycologie, Institut Pasteur, Paris, France.

The publication of this thesis was financially supported by Janssen-Cilag bv, The Liposome Company inc., Pfizer bv, Sanofi Diagnostics Pasteur bv, Vestar Benelux bv, and Zeneca Farma. This is gratefully acknowledged.

Cover illustration: Scanning electron microscopic picture of *Aspergillus fumigatus*, H.A.L. van der Lee.

Aan mijn ouders

Aan Liesbeth, Lukas, en .....

CONTENTS

The genus Aspergillus *was first described by Micheli in the year 1725. He named the fungus* Aspergillus *because of the resemblence of the conidiophore and conidia to the Aspergillum, which was used to sprinkle holy water (aspergere). This figure shows an Aspergillum dated between 1709 and 1746 (reprinted with permission of the Rijksmuseum Catherijneconvent, Utrecht, The Netherlands).*

GENERAL INTRODUCTION

(Adapted and translated from: Verweij PE, de Pauw BE, Hoogkamp-Korstanje JAA, Kullberg BJ, Meis JFGM. Invasieve aspergillose: epidemiologie, diagnose en therapie. Ned Tijdschr Geneeskd 1994;138:752-7).

Aspergillus is a saprophytic fungus which is ubiquitous throughout the world. Although more than one hundred *Aspergillus* species are known, only 16 have been documented as etiologic agent in human disease[68]. *Aspergillus* species can cause several clinical pathological conditions in humans, but the scope of this thesis includes only the invasive pulmonary infections. The use of immunosuppressive treatment over the past 30 years has led to an increase in the incidence of opportunistic infections with *Aspergillus* species, and it is now the second most common systemic mycosis in immunocompromised patients[14]. Patients at high risk of invasive aspergillosis include patients with AIDS, those who are granulocytopenic as a result of cytotoxic therapy, patients receiving prolonged courses of high-dose corticosteroids and recipients of organ transplants.

The sources of *Aspergillus* conidia include contaminated ventilatory and airconditioning systems, potted plants and, in particular, construction work carried out in or near hospitals[8]. Invasive aspergillosis is considered a nosocomial infection, the route of entry usually being the inhalation of airborne *Aspergillus* conidia which may primarily cause a pulmonary infection. In immunocompromised patients germination of *Aspergillus* conidia and tissue invasion cannot be prevented due to the elimination of normal defense mechanisms[132]. The etiological agents of invasive aspergillosis include *Aspergillus fumigatus* and *A. flavus*. Although, approximately 90% of invasive *Aspergillus* infections concern invasive pulmonary disease, dissemination to other organs such as the brain, liver and kidneys may occur.

Incidence data of invasive aspergillosis in high risk patient groups are based on autopsy findings because of the difficulty in establishing an antemortem diagnosis. Although the overall incidence of invasive aspergillosis, even in those predisposed, is variable and often unpredictable, an increase of the number of invasive *Aspergillus* infections has been reported[14]. More intensive and effective cytotoxic treatment regimens resulting in enhanced mucosal damage and prolonged neutropenia and thrombocytopenia may acount for the rise of the incidence. At the University Hospital Nijmegen fungal infections were the established cause of death at autopsy in 36% of patients with hematological malignancies during the period 1987-1992, of which 41% was caused by *Aspergillus* species.

The prognosis of patients with suspected invasive aspergillosis is very poor and depends

on a number variables, such as remission of underlying disease, the certainty of the diagnosis, the early administration of antifungal treatment[1] and recovery of the granulocytes (Table 1).

***Table 1.** Mortality of invasive aspergillosis in treated and untreated patients and the certainty of diagnosis[33,92,125,133,141].*

Aspergillus Infection	Mortality (%)	
	Untreated	Treated
Proven[a]	90	60-90
Probable[b]	70	30-40
Possible[c]	30	5

[a]Histopathological evidence for tissue invasion in tissue sections and/or positive biopsy culture.
[b]Persistent fever despite the administration of broadspectrum antimicrobial agents, pulmonary infiltrates on the chest X-ray and, *Aspergillus* species cultured from sputum or BAL fluid samples.
[c]Persistent fever despite broadspectrum antibiotics and pulmonary infiltrates on the chest X-ray, but culture for *Aspergillus* species negative.

The main difficulty in the management of invasive aspergillosis remains the diagnosis of the infection at an early stage of disease. The clinical presentation of invasive aspergillosis is often non-specific and typical pulmonary signs such as cough and dyspnoea may be absent in the early stages of infection. Fever is frequently found, but may be masked by the use of corticosteroids. Plain chest X-ray films lack specificity and sensitivity in neutropenic patients, and typical infiltrates, such as the air-crescent sign, often develop during advanced disease or after recovery of the granulocytes[61]. A definitive etiological diagnosis of invasive aspergillosis can be established only by the demonstration of mycelial elements in tissue and by recovering *Aspergillus* species in culture. However, the presence of severe thrombocytopenia often precludes the use of invasive diagnostic methods to obtain affected tissue samples, and therefore fiber-optic bronchoscopy is

commonly used to obtain a specimen for diagnosis[51]. The conventional microbiological techniques which are used to diagnose invasive aspergillosis include culture of sputum or bronchoalveolar lavage (BAL) fluid samples. *Aspergillus* species may be isolated from these specimens by culture on Sabouraud medium, incubated at 42°C. However, the sensitivity of culture from sputum samples and BAL fluid samples is low, 25%[81] and 40-58%[51,81,104,146] respectively. Although a positive culture result is highly indicative of *Aspergillus* infection in neutropenic patients[146], the interpretation of a positive *Aspergillus* culture result remains difficult because conidia may contaminate the specimen.

The detection of specific anti-*Aspergillus* antibodies in serum has demonstrated poor sensitivity in immunocompromised patients[145] and therefore tests have been developed which detect *Aspergillus* antigens, especially the polysaccharide galactomannan[114]. Several serological tests have been developed which allowed detection of *Aspergillus* antigens in serum, urine and BAL fluid, but the sensitivity of these tests is too low to early diagnose invasive aspergillosis.

The aim of this thesis is to develop and evaluate techniques which may allow the microbiological diagnosis of invasive aspergillosis at an early stage of infection.

Chapter 1

DNA-BASED DETECTION OF *ASPERGILLUS*

PYLOGENETIC RELATIONSHIPS OF FIVE SPECIES OF *ASPERGILLUS* AND RELATED TAXA AS DEDUCED BY COMPARISON OF SEQUENCES OF SMALL SUBUNIT RIBOSOMAL RNA

P.E. Verweij, J.F.G.M. Meis, P. Van Den Hurk, J. Zoll, R.A. Samson, and W.J.G. Melchers

Journal of Medical and Veterinary Mycology 1995;33:185-90.
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Summary

The nucleotide sequences of the genes encoding the 18S rRNA of *Aspergillus flavus*, *A. nidulans*, *A. terreus* and *A. niger* were elucidated and aligned to the sequences of *A. fumigatus*. In addition, the 18S rRNA sequences of the V4 - V9 region of morphologically similar filamentous fungi, e.g. *Penicillium chrysogenum*, *P. marneffei* and *Paecilomyces variotii*, were elucidated. Phylogenetic analysis and comparison showed a very close intergeneric relationship of the genus *Aspergillus* to species of the genera *Paecilomyces* and *Penicillium*. However, the sequenced *Aspergillus* species also showed a very close relationship to *Eurotium rubrum* and *Monascus purpureus*. Phylogenetic analysis of fungal 18S rRNA sequences divided the genera *Aspergillus*, *Penicillium*, and *Paecilomyces* into two coherent clusters and showed a close intergeneric relationship which is in keeping with the existing morphological and taxonomic classification.

Introduction

The genus *Aspergillus* is ubiquitous within the environment and is found throughout the world. Although clinical entities caused by *Aspergillus* species show great variety, the upper and lower respiratory tract are the main focus of primary infection. In immunocompetent hosts, allergic reactions to inhaled *Aspergillus* conidia and saprophytic colonization of pre-existing cavities in the lung are the syndromes most frequently encountered[68]. Invasive pulmonary aspergillosis occurs in immunosuppressed patients and is the second most frequent cause of opportunistic fungal infection[14]. The pathogenesis involves inhalation of conidia followed by localized or disseminated disease. More than 90% of the patients have either received corticosteroid therapy[43], had prolonged neutropenia[41] or are receiving cytotoxic chemotherapy[144]. Although more than 180 *Aspergillus* species, with about 70 named teleomorphs have been listed[95,106,107], only 16 species have been documented as etiologic agents of human disease[68]. *A. fumigatus* is the most common cause of both invasive and non-invasive aspergillosis worldwide[14]. To examine the origin of pathogenicity, it is important to determine the evolutionary relationships of

the *Aspergillus* species to each other, as well as similar fungi.

The most important pathogenic species of *Aspergillus*, including *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*, are classified in an artificial system (Fungi Imperfecti), because they are not known to produce an ascomycetous state[107], although *A. fumigatus* is associated with species of the ascomycete *Neosartorya*, and *A. nidulans* is known to produce a teleomorph in the genus *Emericella*. Although the conidial structures of aspergilli are fairly uniform, the morphological features of the teleomorph, when present, are very diverse. The diversity of the cleistothecia and the absence of ascocarps in many species makes studies of evolution and phylogenetic relationships difficult. Therefore, molecular data obtained from ribosomal nucleotide sequences are increasingly used to elucidate fungal phylogeny[38]. These molecules are well suited as taxonomic tools because they are universally present and their function in protein synthesis is highly conserved both in pro- and eukaryotes[86].

In addition, knowledge about the sequence variability exhibited by these fungi is of importance, because DNA amplification techniques using *Aspergillus* specific primers and oligonucleotide probes are increasingly implemented for the diagnosis of invasive aspergillosis[78,111,119] and epidemiological analysis[122].

Here we describe the phylogenetic relationships of five pathogenic *Aspergillus* species based on 18S rRNA sequence analysis and attempt to relate these results to the presently known taxonomic situation. Two species of *Penicillium*, *P. marneffei* and *P. chrysogenum* and the morphologically related species *Paecilomyces variotii* were also included in the comparison because high levels of homology with *Aspergillus* have been reported[78,111].

Materials and Methods

Organisms

A. flavus (CBS108.30; source: ex *Pseudococcus*), *A. nidulans* (CBS100.20; source: ex man), *A. terreus* (CBS106.25; source: unknown), *A. niger* (CBS102.12; source: unknown, type of *A. niger* mutant altipes), and *P. marneffei* (CBS 385.89; source: ex man) were obtained from the Centraalbureau voor Schimmelcultures (CBS, Baarn, The

Netherlands). *P. chrysogenum* (AZN 1126; source: ex man) and *Paecilomyces variotii* (AZN 731; source: ex man) were isolated at our hospital. All fungi were grown in culture on Sabouraud agar at 28°C for 4 days. Mycelial growth was peeled off from the agar surface using sterile forceps, and suspended in 600 μl distilled water. This suspension was added to 1 ml of 0.5 mm zirconium beads which had been washed and autoclaved in 0.2% sodium dodecyl sulphate in 1 M sodium bicarbonate. Complete cell destruction was achieved by high frequency shaking at 6000 rev. min^{-1} for 160 sec in a MiniBeadbeater system (Tecnolab., Alkmaar, The Netherlands). Next, the mixture was centrifuged for 15 min at 1500 *g*. DNA was purified successively by phenol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol extraction[74] followed by precipitation with ethanol overnight at -20°C. DNA was finally dissolved in 100 μl distilled water.

PCR amplification

The complete 18S rDNA was amplified by PCR using general eukaryotic 5' and 3' 18S rDNA primers 5'-CCTGGTTGATCCTGCCAGTA-3' and 5'- GCTTGATCCTTCTGCA-GGTT-3' respectively[27]. PCR was performed in a 100 μl reaction mixture containing 10 mM Tris-HCL (pH 9.0 at 25°C), 10 mM KCl, 1.4 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.1% Triton X-100, 50 pmol each of two primers, 0.2 U superTaq DNA polymerase (HT Biotechnology, Cambridge, UK) and 0.5 μg DNA of each *Aspergillus*, *Penicillium* or *Paecilomyces* isolate which had been denatured at 94°C for 5 min. Next, 30 cycles of amplification were performed by denaturating for 1 min at 94°C, annealing the primer for 1 min at 42°C and allowing elongation for 3 min at 72°C. When successful, amplification generated a product of approximately 1800 basepairs. Each product was analyzed by electrophoresis in 1.5% agarose gels. The amplification products required for sequencing were purified from low-melting agarose (LKB) using the Prep-a-Gene system (BioRad Laboratories, Veenendaal, the Netherlands). DNA sequencing was performed by the dideoxy nucleotide method[108] using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Gouda, the Netherlands) according to the manufacturer's instructions. The nucleotide sequence was determined in both directions. Sequencing reactions were analysed on an Applied Biosystems 373A DNA sequencer. Primers selected from

several conserved regions of the 18S RNA served as sequencing primers (Table 1). From *A. flavus*, *A. nidulans*, *A. terreus* and *A. niger* the complete nucleotide sequence of the 18S rRNA was determined, and from *P. chrysogenum*, *P. marneffei*, and *Paecilomyces variotii* the nucleotide sequence of the V4 - V9 region.

Table 1. *List of primers used to determine the sequence of 18S rDNA of* A. flavus, A. terreus, A. nidulans, A. niger, Penicillium marneffei, P. chrysogenum, *and* Paecilomyces variotii.

Primer	Orientation	Sequence (5'-3')	Position
P56.1	5'-3'	CCTGGTTGATCCTGCCAGTA	2-22
P35.1	5'-3'	GAAACTGCGAATGGCTCATT	83-102
P36.1	5'-3'	ATTCCGGAGAAGGAGCCTGA	376-395
P55.4	5'-3'	GGTGCCAGCAGCCGCGGTAA	539-578
P55.7	5'-3'	GAGTGTTCAAAGCAGGCCTT	761-780
P55.11	5'-3'	AGGTGAAATTCTTAGATTTG	899-918
P81.1	5'-3'	AAGTTTTTGGGTTCTGGGGG	1092-1112
P55.13	5'-3'	GATTTGTCTGCTTAATTGCG	1301-1320
P55.15	5'-3'	CGCGCGCTACACTGACAGGG	1461-1480
P55.17	5'-3'	TACTACCGATTGAATGGCTC	1640-1659
P35.2	3'-5'	GGTTTTTTATCTAATAAATA	201-220
P35.3	3'-5'	AATCGAACCCTAATTCTCCG	359-378
P36.2	3'-5'	GCAACAACTTTAATATACGC	593-612
P55.5	3'-5'	AAGGCCTGCTTTGAACACTC	761-780
P55.6	3'-5'	TTTCGCAGTAGTTAGTCTTC	921-940
P55.8	3'-5'	CAAAAACTTTGATTTCTCGT	1081-1100
P55.9	3'-5'	AGACAAATCACTCCACCAAC	1290-1309
P55.10	3'-5'	GGCATCACAGACCTGTTATT	1421-1440
P81.2	3'-5'	TACAAAGGGCAGGGACGTAA	1615-1634
P56.2	3'-5'	GCTTGATCCTTCTGCAGGTT	1784-1803

Phylogenetic analysis

The 18S rRNA sequences were aligned with other eukaryotic sequences available on the EMBL database. A subset of this alignment, including the presently sequenced fungi and 23 other fungal 18S rRNA sequences (EMBL database), was selected for inference of phylogenetic trees. Sequence analysis and comparisons were made using PHYLIP version 3.4 (the default of the program)[35]. Neighbour-joining[105] and Fitch-Margoliash distance trees were inferred using NEIGHBOR and FITCH, respectively. The confidence limits in the trees were estimated by using the bootstrap method (SEQBOOT, 100 replicates)[36].

Nucleotide sequence accession numbers

The 18S rRNA sequences of the *Aspergillus* species reported here have appeared in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers X78537 (*A. flavus*), X78538 (*A. niger*), X78539 (*A. nidulans*), X78540 (*A. terreus*).

Results

Figure 1 shows the sequence alignment data of the 18S rRNA from the different *Aspergillus* species. The sequence of *A. fumigatus*, which was published earlier by Barns et al.[11], was used as a template. A very extensive homology was found between *P. chrysogenum*, *P. marneffei*, *Paecilomyces variotii*, *A. fumigatus*, and the presently sequenced *Aspergillus* species: *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* for the V4 - V9 region of the 18S rRNA gene. A representative collection of levels of evolutionary distance estimates is presented in Table 2. The regions V4 - V9 of the 18S rRNA, consisting of approximately 1200 basepairs, were used to draw up a phylogenetic tree. The Fitch-Margolish distance tree is shown in Figure 2. Use of neighbour-joining methods gave trees topologically identical to that shown in Figure 2 in all major respects. The statistical reliability of this tree was examined by using the bootstrap method[36], which estimates the uncertanty in a measurement due to a finite sample size (Figure 2). The fungi sequenced in this study were divided into two clusters of organisms with a high intersequence similarity. *Penicillium marneffei* and *P. chrysogenum* were found to be very closely related to *Paecilomyces variotii*. The four sequenced *Aspergillus* species were also highly related to each other and to *A. fumigatus*. Comparison with the 18S rRNA sequences of 23 other eukaryotes revealed a very close relationship between *Aspergillus* species and two other ascomycetous species, e.g. *Eurotium rubrum* and *Monascus purpureus*.

Discussion

The introduction of molecular criteria in fungal phylogeny has contributed to understanding the evolution and phylogenetic relations of fungi. Branching patterns derived from

Table 2. Levels of evolutionary distances[a] between small-subunit rRNA sequences of five Aspergillus *species,* Penicillium marneffei, P. chrysogenum, Paecilomyces variotii, *and representitives of related taxa.*

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *Aspergillus terreus*	0.008	0.022	0.006	0.012	0.006	0.020	0.024	0.032	0.038	0.037	0.075	0.134	0.118	0.105	0.104	0.103
2. *A. niger*		0.020	0.010	0.011	0.009	0.019	0.021	0.028	0.039	0.036	0.074	0.130	0.113	0.100	0.099	0.098
3. *A. nidulans*			0.019	0.020	0.019	0.026	0.033	0.041	0.048	0.044	0.084	0.140	0.120	0.108	0.106	0.105
4. *Eurotium rubrum*				0.008	0.005	0.017	0.022	0.028	0.039	0.036	0.072	0.133	0.112	0.101	0.100	0.099
5. *A. flavus*					0.008	0.018	0.025	0.031	0.039	0.037	0.073	0.137	0.115	0.104	0.103	0.102
6. *A. fumigatus*						0.015	0.021	0.028	0.037	0.034	0.071	0.130	0.114	0.101	0.100	0.099
7. *Monascus purpureus*							0.028	0.032	0.045	0.036	0.072	0.131	0.106	0.091	0.092	0.091
8. *Paecilomyces variotii*								0.015	0.027	0.041	0.079	0.140	0.118	0.105	0.107	0.106
9. *Penicillium chrysogenum*									0.027	0.050	0.090	0.143	0.130	0.119	0.119	0.118
10. *P. marneffei*										0.051	0.089	0.148	0.128	0.119	0.116	0.115
11. *Blastomyces dermatitidis*											0.063	0.114	0.097	0.089	0.085	0.084
12. *Aureobasdium pullulans*												0.114	0.075	0.059	0.059	0.058
13. *Candida albicans*													0.137	0.122	0.124	0.123
14. *Neurospora crassa*														0.071	0.070	0.069
15. *Ophiostoma ulmi*															0.014	0.014
16. *O. stenoceras*																0.001
17. *Sporothrix schenkii*																

[a]The values represent the average numbers of substitutions per nucleotide according to Kimura[35].

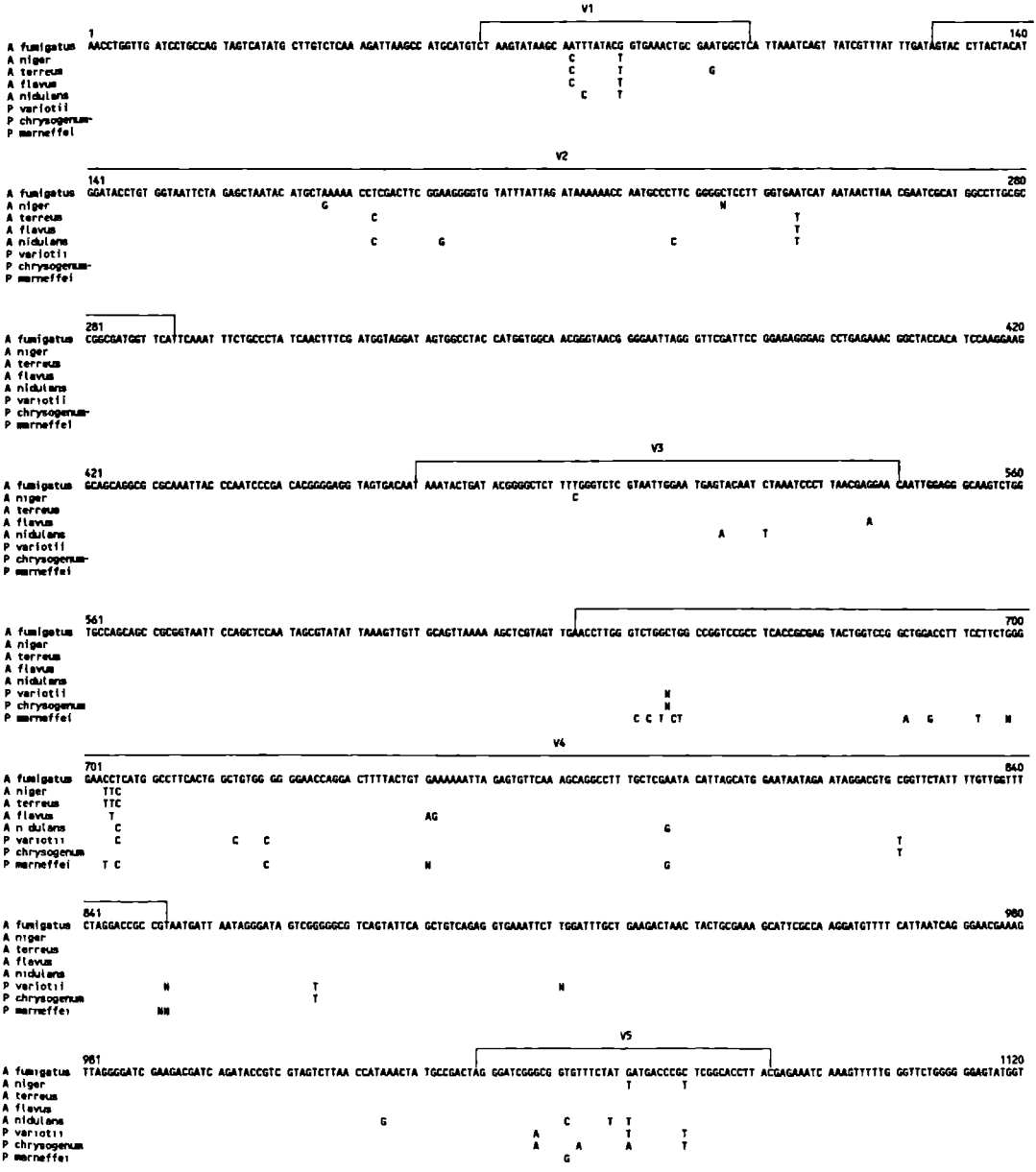
V1
V2
V3
V4
V5
A fumigatus
A niger
A terreus
A flavus
A nidulans
P variotii
P chrysogenum
P marneffei

Figure 1. Sequence alignment of 18S rRNA of Aspergillus fumigatus, A. niger, A. terreus, A. flavus, A. nidulans, Paecilomyces variotii, Penicillium chrysogenum, *and* P.marneffei. *Nucleotide residues in the sequences of the isolates are indicated by "." for corresponding residues; mutated residues are indicated by their corresponding letter, and N indicates uncertain residues; regions that were not sequenced are indicated by "-". The variable regions V1 - V9 are indicated above the sequences.*

18S rRNA[12] and orotidine 5'-monophosphate decarboxylase protein sequence analysis[94] show that the ascomycetes initially split from the zygomycetes and basidiomycetes. The first branch in the ascomycete line seperates the budding yeasts from the filamentous ascomycetes. In the present study a phylogenetic trees was constructed for the 18S rRNA by neighbour-joining and Fitch-Margoliash analyses and was found to be similar to those reported by other investigators[34]. The sequenced species of the genera *Aspergillus*, *Penicillium* and *Paecilomyces* were found to be closely related to each other and two coherent clusters were formed. High levels of intersequence similarities between morphological similar species of *Aspergillus* and *Penicillium* was also demonstrated by Min-Wei Chen et al[23]. Our analysis, based on the fungal 18S rRNA sequence, confirms these findings and also suggests a close relationship of these taxa to *Paecilomyces variotii*. *Paecilomyces variotii* was found to be more closely related to *Penicillium* than to *Aspergillus*. The conidiogenous structures of *Paecilomyces variotii* are similar to

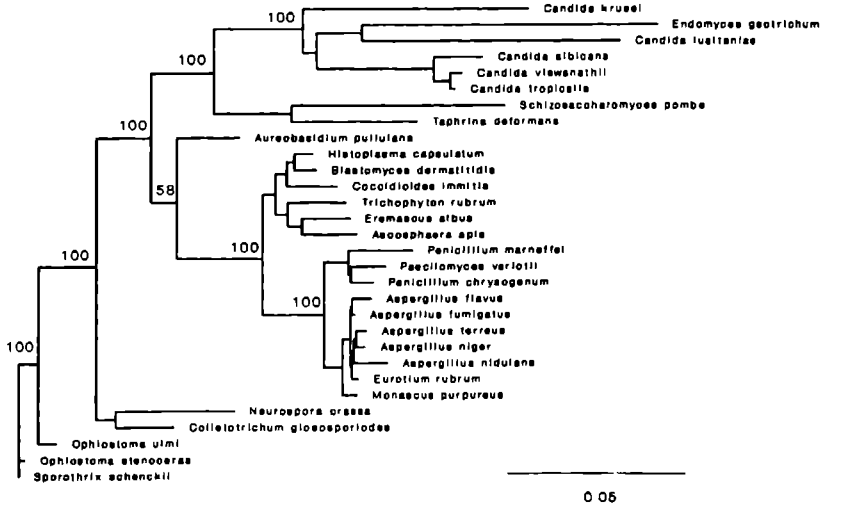

Figure 2. *Fitch-Margoliash distance tree based upon analysis of the regions V4 - V9 of the 18S rRNA, consisting of approximately 1200 basepairs, showing intrageneric phylogenetic relationships of five* Aspergillus *species and the position of these species with respect to* Penicillium, Paecilomyces *and 22 other eukaryotes. Bootstrap values of major branches were displayed on the tree. Scale bar = 0.05 substitution per position.*

Penicillium species, differing by the shape of the philides and the branching pattern. It is therefore not surprising that this species clusters closely with the *Penicillium* species examined. The latter belong to two different subgenera of *Penicillium*, *P. marneffei* to subgenus *Biverticillium* and *P. chrysogenum* to subgenus *Penicillium*. Accordingly, *P. marneffei* is more distantly related to *P. chrysogenum*.

The intrageneric relationship of the *Aspergillus* species which were examined showed very high levels of intersequence similarities. *Aspergillus fumigatus* and *A. flavus* were found to be closely related to each other and *A. niger* to *A. terreus*. The close relationship between *A. fumigatus* and *A. flavus* is interesting because these are opportunistic pathogens most often encountered in invasive human disease. The highest divergence among the *Aspergillus* species in the present study was found between the cluster *A. flavus* and *A. fumigatus*, and *A. nidulans*. These results differ from observations based on restriction endonuclease cleavage patterns of mitochondral DNA[59]. In their study, the phylogenetic distance between *A. nidulans* and *A. flavus* was less than the distances between *A. flavus* and *A. niger*. However, 5S rRNA sequence analysis of *A. niger* and *A. flavus* also showed very high levels of similarities[55]. Since these two species are also morphologically closely related they are placed in the subgenus *Circumdati* and no teleomorph are known[106].

Phylogenetic analysis showed that the two ascomycetous species *E. rubrum* and *M. purpureus* are also closely related to *Aspergillus* species. Although *Eurotium* has an *Aspergillus* anamorph, it is clearly different from the species examined. *Monascus* has a quite different *Basipetospora* anamorph and with respect to the morphology and taxonomy of these species, a close relation to *Aspergillus* is difficult to understand. Since the interpretation of the phylogenetic relationship of *Eurotium* and *Monascus* to *Aspergillus* is unclear, sequence data and phylogenetic analysis of more species within these genera are needed to gain more insight in the nature of this relationship.

With exception of the fungi *E. rubrum* and *M. purpureus*, phylogenetic analysis of fungal 18S rRNA sequences divided the genera *Aspergillus*, *Penicillium* and *Paecilomyces* into two coherent clusters and showed a close intergeneric relationship which is in keeping with the existing morphological classification.

POLYMERASE CHAIN REACTION AS A DIAGNOSTIC TOOL FOR INVASIVE ASPERGILLOSIS: EVALUATION IN BRONCHOALVEOLAR LAVAGE FLUID FROM LOW RISK PATIENTS

P.E. Verweij, J.F.G.M. Meis, P. van den Hurk, B.E. De Pauw,
J.A.A. Hoogkamp-Korstanje, and W.J.G. Melchers

Serodiagnosis and Immunotherapy in Infectious Disease 1994;6:203-8.
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Summary

An *Aspergillus* genus-specific PCR, which has been developed for the diagnosis of invasive aspergillosis (IA) in high risk patients[78], was evaluated in 72 bronchoalveolar lavage (BAL) samples obtained from 70 non-neutropenic, low risk patients in order to establish the rate of detectable colonization with *Aspergillus* species of the respiratory tract. A positive amplification was found in 11 out of 72 samples (15%). Risk factors for colonization, e.g. corticosteroid therapy and cigarette smoking, were present in 64% of the evaluable cases, and a significantly higher rate of colonization was found when risk factors were present ($p < 0.05$). The low rate of detectable colonization justifies further evaluation of this PCR assay in the diagnosis of IA.

Introduction

The genus *Aspergillus* is a saprophytic fungus which can cause severe pulmonary conditions in humans, including allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA). Life threatening invasive infections by *Aspergillus* species increasingly occur in immunocompromised patients. Particulary at risk are leukemic patients with cytotoxic-induced neutropenia and transplant recipients receiving high-dose corticosteroid therapy. The diagnosis of IA in an early stage is of great importance, since early treatment with amphotericin B has been shown to reduce the mortality rate[1]. However, conventional diagnostic methods, e.g. culture of sputum[81] or bronchoalveolar lavage (BAL) fluid[51] and serology[6], lack sensitivity and definite diagnosis is often made only at autopsy. In our hospital the diagnostic yield of cultures obtained from patients suspected for IA and proven at autopsy was 39%, but in half of these cases culture became positive only within 7 days before death[126]. Therefore, sensitive and reliable assays for the detection of IA in an early stage are needed. DNA-based methods for the detection of *Aspergillus* species have been developed and several studies have shown that PCR could detect *Aspergillus* DNA in BAL fluid samples of high risk patients with a very high sensitivity and specificity and the PCR may therefore be a promising diagnostic tool

for the diagnosis of IA[78,97,111,119]. However, since *Aspergillus* is also known to colonize the respiratory tract, the interpretation of a positive amplification can be difficult. The prevalence of detectable colonization of the respiratory tract with *Aspergillus* species is the most important issue that must be addressed in the clinical evaluation of the PCR.
In the present study, we have evaluated a PCR assay by determining the rate of *Aspergillus* species in BAL fluid obtained from non-neutropenic patients at low risk for IA and have compared this with the results obtained by culture. In addition, we have reviewed the patient records for risk factors for colonization of the respiratory tract with *Aspergillus* species.

Materials and Methods

Clinical samples

BAL fluid specimens (5-10 ml) from hospilatized patients, which were sent to the laboratory for microbiological analysis, were assigned a number and stored at -80 °C until use. BAL fluid samples from the Departments of Hematology and Oncology were excluded from the study. All samples were analysed without knowledge of the patients' medical history. After thawing, the BAL fluid samples were centrifuged at 2,500 rpm for 15 min, and the supernatant was discarded leaving a residue of 0.5 ml in which the pellet was resuspended. One hundred μl of this suspension was added to 1 ml of 0.5-mm-diameter zirconium beads which had been washed and autoclaved in 0.2% sodium dodecyl sulphate (SDS) in 1 M sodium bicarbonate. Complete cell destruction was achieved by high frequency shaking at 6000 vibrations per min for 160 sec in a Mini-Beadbeater system (Tecnolab Int., Alkmaar, The Netherlands)[78]. Next, the mixture was centrifuged for 15 sec at 11 000 rpm. Nucleic acids were purified by a guanidinium thiocyanate containing lysis buffer and silica particles, as descibed by Boom et al.[17] and were finally dissolved in 100 μl of distilled water. The DNA content of each sample was determined by spectrophotometry (GeneQuant RNA/DNA Calculator, Pharmacia, Cambridge, UK).

Polymerase chain reaction

All DNA samples were tested in two dilutions containing 0.1 μg and 0.5 μg of total DNA, for the presence of *Aspergillus* species by a hot-start PCR as described previously[78]. Briefly, PCR was performed in a final reaction volume of 100 μl containing 75 mM Tris-HCl (pH 9.0 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 0.33 mM (each) deoxynucleoside triphosphates (dNTPs), 50 pmol of each of the two *Aspergillus* genus-specific primers (*Asp1*, 5'-CGGCCCTTAAATAGCCCGGTC-3'; and *Asp-2*, 5'-ACCCCCCTGAGCCAGTCCG-3'), 0.2 U of Taq DNA polymerase (Thermoperfect plus DNA polymerase, Integro, Zaandam, The Netherlands), and 0.1 μg or 0.5 μg of DNA from each BAL fluid sample which had been pre-denatured at 94 °C for 5 min. Next, 40 cycles of amplification were performed by denaturating for 1 min at 94°C, annealing the primer for 1 min at 62°C and allowing elongation for 1 min at 72°C. Internal control of DNA amplification of each sample was performed with the ß-globin primers PCO3/ PCO4, which amplify a 326-bp fragment of the ß-globin gene[103].

Analysis of amplified DNA

Aliquots of 25 μl samples were analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide[74]. For Southern blotting, the agarose gel was depurinated in 0.25 N HCl, denaturated in 0.5 N NaOH-1.5 M NaCl, and transferred to a nylon membrane (Hybond; Amersham Int., Bucks, England) by diffusion blotting in 0.5 N NaOH-1.5 M NaCl. DNA was covalently bound to the membrane by baking at 80 °C for 2 h. Hybridization was performed with an internal *Aspergillus*-specific oligonucleotide probe (*Asp-p*, 5'-ATGGAAGTGCGCGGCAATAAC-3'), which was non-radioactively labelled with Digoxigenin-11-dUTP (DIG oligonucleotide 3'-end labeling kit, Boerhinger GmbH, Mannheim, Germany) according to the manufacturers instructions. The hybridization signal was detected on an X-ray film using a chemiluminescent substrate (Lumigen™ PPD, Boehringer GmbH, Mannheim, Germany). All positive samples were confirmed to be *Aspergillus* specific by *Sty*I restriction endonuclease digestion[78].

For prevention of contamination, the recommendations of Kwok and Higuchi[67] were followed and each technical step was kept strictly isolated. Furthermore, a limited number

of five BAL fluid samples were analysed at a time.

Culture results and patient records

After the PCR analysis of the BAL fluid samples was completed, the results were compared with bacteriological, virological, parasitological, and mycological culture results. In addition, the patient records were reviewed for the following features: age, sex, leucocyte count, underlying disease, corticosteroid therapy, and cigarette smoking.

Data analysis

The Statistical Program for the Social Sciences (SPSS) was used for data-analysis. Chi-square tests were used to determine statistical significance

Results

A total of 72 BAL fluid samples obtained from 70 patients, 50 men and 20 women, were tested. The mean age of the patients was 52 years (range 4 - 88). Patient records were available from 64 patients (91%), and the main underlying diseases are listed in Table 1. The patients were non-neutropenic and at low risk for IA, but 41 patients (64%) had risk factors for colonization of the respiratory tract with *Aspergillus* species (Table 1). The macroscopic aspect of the BAL fluid samples varied from clear to purulent and the mean concentration of DNA which was isolated from the BAL fluid specimens was 79 ng/μl (range 30 - 478 ng/μl). Amplification with *Aspergillus* genus-specific primers and hybridization with an *Aspergillus* specific oligonucleotide probe was found positive in 11 out of 72 (15%) of the BAL fluid samples (Table 1). Ten positive samples were obtained from patients with risk factors for colonization, and one sample was obtained from a patient without presence of the analyzed risk factors. However, the latter patient had a chronic granulomatous disease which is an additional risk factor for colonization and infection with *Aspergillus*. Statistical analysis showed that corticosteroid therapy and cigarette smoking were significantly associated with a positive PCR amplification ($p < 0.05$).

***Table 1.** Underlying diseases and risk factors for colonization with* Aspergillus *species in 70 patients at low risk for IA, and results of* Aspergillus *genus-specific PCR.*

Underlying disease	Number of patients	Analysed risk factors			
		absent	cortico-steroids	smoking	both
Absent	4	1		3[1]	
Transplantation					
kidney	5		3[1]		2
bone marrow	1	1			
Pulmonary conditions					
COPD	2	1		1	
malignancies	4			3	1
TBC	4	1	1	2	
RA / SLE	5		3		2
HIV / AIDS	3			2[1]	1
Ulcerative colitis	2		2		
NIDDM	3	3			
Malignancies	10	6	1	3[1]	
Other	21	11[1]	2[2]	6[2]	2[2]
Unknown	6				
Total	70				

[1]/[2]=number of patients with a positive *Aspergillus* genus-specific PCR amplification; COPD = chronic obstructive pulmonary disease; TBC = tuberculosis; RA = rheumatoid arthritis; SLE = systemic lupus erythemathodus; HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome; NIDDM = non-insulin dependant diabetes mellitus.

The culture of the BAL fluid samples yielded many different microorganisms (Table 2). Filamentous fungi were cultured from two BAL fluid specimens. In one case *A. fumigatus* was cultured, together with *Pseudomonas aeruginosa* and cytomegalovirus, from a patient with agammaglobulinemia, and PCR was also positive. *Paecilomyces* species were

Table 2. Diagnostic yield of 72 BAL fluid specimens and results of Aspergillus *genus-specific PCR.*

Culture	Number of BAL specimens	PCR result (positive/negative)
negative	14	1 / 13
Bacteria[a]		
alone	24	3 / 21
+ *Candida* spp	13	2 / 11
+ virus	3	0 / 3
+ *Pneumocystis carinii*	3	0 / 3
+ virus + *Aspergillus fumigatus*	1	1 / 0
+ virus + *Candida* spp	3	0 / 3
+ virus + *P. carinii*	1	1 / 0
+ virus + *Candida* spp + *P. carinii*	1	0 / 1
+ *Candida* spp + *Paecilomyces* spp	1	0 / 1
Yeasts[b]		
alone	4	3 / 1
+ *P. carinii*	1	0 / 1
Viruses[c]		
alone	2	0 / 2
+ *P. carinii*	1	0 / 1
Total	72	11 / 61

[a]Gram-positive and Gram-negative bacteria, *Mycobacterium tuberculosis*, *Mycobacterium species*, and *Legionella pneumophila*; [b]*Candida albicans*, *Torulopsis glabrata*, *C. tropicalis* and *C. lusitaniae*; [c]Herpes simplex, Cytomegalovirus, Influenza A and Rhinovirus.

cultured, together with *Enterobacter cloacae* and *Candida albicans*, from a sample obtained from a patient with a tumor of the hypopharynx, and in this case the PCR was negative. Amplification with ß-globin primers was performed in order to determine the

quality of the isolated DNA and was found to be positive for all cases. An example of the PCR results is shown in Figure 1. In some cases nonspecific amplification products were visible on agarose gel (Figure 1A, lanes 3 - 5), but no hybridization was found with an *Aspergillus* specific oligonucleotide probe (Figure 1B).

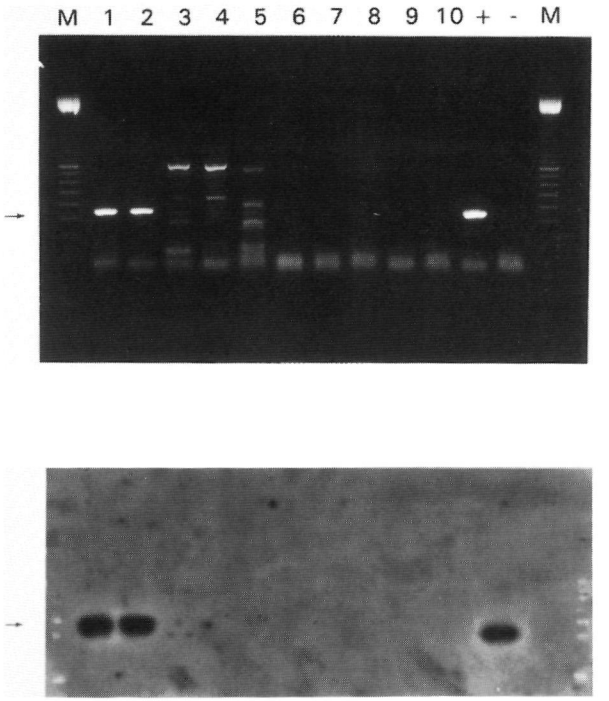

Figure 1. *PCR analysis of patients at low risk for invasive aspergillosis. Nucleic acids were extracted from BAL fluid samples, subjected to PCR amplification, and analyzed by agarose gel electrophoresis (1A) and Southern blot hybridization (1B). Lanes: M, size marker (HinfI-digested pBR322); 1 and 2, BAL fluid sample with positive amplification in both dilutions containing 0.1 μg and 0.5 μg of DNA respectively; 3 and 4, BAL fluid sample with non-specific amplification in both dilutions; 5 and 6, BAL fluid sample with non-specific amplification in the lowest dilution (0.1 μg); 7 and 8, negative BAL fluid sample; 9 and 10, negative BAL fluid sample;* +, A.fumigatus *DNA; -, distilled water. Arrows indicate PCR fragment of 363-bp.*

Discussion

The sensitivity of the PCR for the detection of *Aspergillus* DNA in BAL fluid samples is very high, and the DNA content equivalent to two *Aspergillus* genomes can be detected[19,78,97,111,119]. Since colonization of the respiratory tract with *Aspergillus* species is common, a positive amplification in the case of colonization may be a serious drawback for the use of the PCR to diagnose IA.

Three research groups have thus far reported the use of PCR for the detection of *Aspergillus* DNA in BAL fluid samples[78,97,111,119]. These studies indicated that PCR detection was more sensitive than conventional diagnostic methods and therefore might be a valuable adjunct to current laboratory methods to diagnose IA. Two research groups have evaluated the PCR assay for a small number of patients at low risk for IA[78,111,119]. Spreadbury et al.[111] have evaluated their PCR assay, which detected only *A. fumigatus*, in BAL fluid samples of seven patients at low risk for IA and found two samples positive. In another study[119], the same investigators found five positive samples obtained from 28 non-immunosuppressed patients, and we previously found no positive samples in 14 non-risk patients[78]. In the present study, review of the patient records showed that all patients were non-neutropenic, but that several patients could have been at high risk for IA. Patients with kidney - or bone-marrow transplantion or patients with AIDS are at high risk for IA at certain stages of therapy or disease. A high risk for IA is present in the second and third month after kidney transplantation[138], during the first four months after bone-marrow transplantation[96], and in patients with advanced AIDS and low levels of CD4+ lymphocytes[30]. None of the patients with these underlying diseases met these criteria and were therefore regarded to be at low risk for IA. The prevalence of detectable colonization of the respiratory tract with *Aspergillus* species was found to be low: 11 of the 72 samples (15%) were PCR positive, which is similar to the prevalence found by others[119]. The low prevalence of respiratory colonization with *Aspergillus* must be noted when one takes into account that humans are continuously exposed to *Aspergillus* conidia, the conidia are small and after inhalation reach the alveoli of the lungs, and *Aspergillus* species grow readily at body temperature. A low prevalence of colonization suggests that after inhalati-

on, the *Aspergillus* conidia are effectively removed from the lungs. Animal studies have shown that bronchoalveolar macrophages play a central role in the host defense against *A. fumigatus*, and that after intranasal inoculation *A. fumigatus* conidia are effectively cleared from the lungs[132].

We have also evaluated well known risk factors for colonization of the respiratory tract with *Aspergillus* species. Corticosteroids have been shown to diminish severely the ability of bronchoalveolar macrophages to kill *A. fumigatus* conidia in mice[132]. Furthermore, the growth rate of *Aspergillus* species in vitro is greatly enhanced by corticosteroids[84]. These factors could predispose patients treated with corticosteroids to colonization of the respiratory tract with *Aspergillus* species. Smoking of tabacco or marihuana is also regarded to be a risk factor for colonization, because *Aspergillus* species have been cultured from these substances[37,50]. *Aspergillus* species are frequently isolated from respiratory secretions obtained from smokers[146], and also elevated precipitins against *Aspergillus* have been found in sera of smokers[63]. All but one of the positive *Aspergillus* PCR amplifications were found in samples obtained from patients with risk factors, and this confirms the predisposition of these patients for colonization of the respiratory tract with *Aspergillus* species. However, despite the high prevalence of risk factors in the patient group which was studied, the prevalence of colonization was found to be low. Therefore additional factors such as underlying disease, duration of corticosteroid therapy or dosage must play an important role.

The amount of DNA isolated from the BAL fluid samples, of course, represents mainly human DNA. The impact of an excess of background human DNA on the sensitivity of the PCR is not clear, but comparitive studies in respiratory secretions have shown no influence of excess of background human DNA on the sensitivity[120]. Since the presence of inhibitory factors in the sample could generate false-negative PCR results[120], the BAL fluid samples were analysed in two dilutions. In most cases, in which a positive amplification was found, only the lowest dilution was positive, indicating that inhibition did not play an important role, which was also demonstrated by the positive amplification with ß-globin primers.

Although the specificity of the *Aspergillus* genus-specific PCR assay is high, specific

amplification was found to occur also with *Penicillium marneffei* and *Paecilomyces variotii*[78]. These moulds could be distinguished from *Aspergillus* species by Southern blot analysis with an *Aspergillus* specific oligonucleotide probe and restriction enzyme digestion of the amplification product by *StyI*[78]. Indeed, although culture of the presently tested BAL fluid specimens yielded many different microorganisms (Table 2), no specific amplification of DNA from other microorganisms than *Aspergillus* species was observed, including a non-speciated *Paecilomyces*. In a number of samples in which no *Aspergillus* DNA was detected, non-specific amplification was observed despite the use of hot-start PCR (Figure 1A, lanes 3 - 5). Some of these amplification products were of similar size to the 363-bp fragment of the *Aspergillus* amplification, which makes direct visual examination of the agarose-gel sometimes difficult. Southern blot analysis, however, showed clearly only hybridization with the *Aspergillus* positive samples (Figure 1B). We believe that the excess of background human DNA could account for non-specific amplification because, in our experience, BAL fluid obtained from neutropenic patients rarely exhibits this phenomenon. This is supported by the fact that the total cell count in BAL fluid obtained from neutropenic patients is significantly lower than compared to non-neutropenic patients[26].

The PCR is a sensitive and specific assay and the use of this technique could contribute to the diagnosis of IA in an early stage of the infection. The results of the present study indicate that the prevalence of colonization of the respiratory tract with *Aspergillus* species in non-neutropenic patients is low, even in patients with risk factors. However, a positive amplification could represent colonization and therefore the PCR results should be interpreted in the whole clinical context of the patient. Although the use of the PCR for the diagnosis of IA is promising, the diagnostic value can only be assessed in prospective studies, and a prospective evaluation of the PCR assay is now being performed at our institute.

GENERAL PRIMER-MEDIATED PCR FOR DETECTION OF *ASPERGILLUS* SPECIES

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Journal of Clinical Microbiology 1994;32:1710-7.
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Summary

A polymerase chain reaction (PCR) assay was developed for the diagnosis of invasive aspergillosis in immunocompromised patients. For this purpose, the complete nucleotide sequences of the genes encoding the 18S rRNA from *Aspergillus nidulans*, *A. terreus*, *A. niger*, and *A. flavus* were elucidated and aligned to the sequences of *A. fumigatus* and other clinically relevant prokaryotic and eukaryotic microorganisms. Genus specific sequences could be identified in the V7 to V9 region of the 18S rRNA. Using hot-start PCR, Southern-blot hybridization and restriction enzyme analysis, *Aspergillus* specific and -sensitive determination was achieved.

Five of six immunosuppressed mice experimentally infected with *A. fumigatus* developed infection, and rRNA could be detected in each case, even in the livers in the absence of positive cultures. *Aspergillus* species were detected by PCR in four neutropenic patients with proven aspergillosis although *Aspergillus* had been isolated from only one bronchoalveolar lavage (BAL) fluid sample. *Aspergillus* species were detected by PCR in two more patients suspected of having infection. Positive PCR signals were obtained from the BAL samples of three of eight neutropenic patients who had developed pulmonary infiltrates, but none were obtained from the samples of 14 nonimmunosuppressed patients. These results indicate the potential value of PCR to detect *Aspergillus* species in BAL samples and, therefore, to identify neutropenic patients at risk for invasive aspergillosis.

Introduction

The genus *Aspergillus* is an ubiquitous saprophytic soil fungus which colonizes the respiratory tract in humans[24] and is responsible for opportunistic infection in immunocompromised patients[100,118,134]. Indeed, invasive aspergillosis (IA) is responsible for up to 41% of the deaths of patients with acute leukemia, and despite the severity and high mortality attributable to this mycosis, there has been little progress in accurately diagnosing infection ante mortem[14,79]. While isolation of the fungus from sputum and bronchoalveolar lavage (BAL) fluid is indicative of infection, both specimens lack sufficient specificity and

sensitivity[51,81]. Although the invasive techniques necessary to obtain a biopsy are usually precluded because of profound thrombocytopenia, the histological demonstration of fungal elements in tissue is mandatory for proven infection[24]. Serology has little to offer as an alternative since the immune response is desultory and, hitherto, the detection of antigen has met with little success. Since successful treatment depends upon intervening before fungal proliferation becomes overwhelming[1,42] and since there is, as yet, no rapid reliable means of diagnosis, therapy is begun empirically, often on the most permissive basis such as persistent unexplained fever despite broad-spectrum antibacterial therapy, and continued until after the recovery of granulocytes.

Recent studies have described PCR methods for detecting *A. fumigatus* and *A. flavus* in clinical material from immunosuppressed patients[97,111,119]. However, other species can be involved[24], and so it seemed important to us to ascertain that a patient had infection with any *Aspergillus* organism. We therefore elucidated the complete sequences of 18S rRNA of several species of *Aspergillus* and related fungi and aligned them with sequences from other sources. In this way, it was possible to develop a sensitive and specific detection assay for the genus *Aspergillus* which could be validated in experimentally infected mice as well as in BAL fluid from patients who were at risk of pulmonary infection.

Materials and Methods

Organisms and growth conditions

Aspergillus fumigatus (CBS113.26), *A. flavus* (CBS 108.30), *A. nidulans* (CBS100.20), *A. terreus* (CBS106.25), and *A. niger* (CBS102.12) were obtained from the Centraalbureau voor Schimmelcultures (CBS, Baarn, The Netherlands). In addition, a panel of micro-organisms was chosen to represent molds related to *Aspergillus* organisms, including two *Penicillium* strains, *Penicillium marneffei* (AZN 747) and *P. chrysogenum* (AZN 1126), *Pseudoallescheria boydii* (AZN 409), a *Fusarium* strain (AZN 441), *Paecilomyces variotii* (AZN 731), and *Rhizopus oryzae* (AZN 593); the yeasts *Candida albicans* (ATTC 90028), *C. tropicalis* (AZN 393), *C. krusei* (AZN 416), *Torulopsis (Candida) glabrata* (ATCC 90030), and *Cryptococcus neoformans* (ATCC 90112); as well

as the bacteria, *Streptococcus sanguis*, *S. mitis*, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), and *Enterobacter cloacae* (ATCC 13047). Clinical isolates were identified with standard methods and were assigned a University Hospital Nijmegen storage number (AZN-number).

Sequence analysis of the 18S rRNA genes

Specific primers of *Aspergillus* organisms included the 18S rRNA sequence of *A. fumigatus* described by Barns and associates[11] and the 18S rRNA from *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus* which were sequenced by us.

Mycelial growth was peeled off from the agar surface with sterile forceps, and suspended in 600 μl of distilled water which had been pretreated with diethyl pyrocarbonate (DEPC). This suspension was added to 1 ml of 0.5-mm-diameter zirconium beads which had been washed and autoclaved in 0.2% sodium dodecyl sulphate in 1M sodium bicarbonate. Complete cell destruction was achieved by high-frequency shaking at 6000 vibrations per min for 160 sec in a Mini-Beadbeater system (Tecnolab Int., Alkmaar, The Netherlands). Next, the mixture was centrifuged for 15 min at 2,500 rpm (Eppendorf centrifuge). Nucleic acids were purified by successively phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol extraction[74] followed by precipitation with ethanol overnight at -20°C and were finally dissolved in 100 μl of DEPC-treated distilled water which contained 0.4 U of human placental RNase inhibitor per μl (RNasin, Promega, Leiden, the Netherlands).

The complete 18S rDNA was amplified by PCR using the general eukaryotic 5' and 3' 18S rRNA primers 5'-CCTGGTTGATCCTGCCAGTA-3' and 5'-GCTTGATCCTTCTGCAGGTT-3', respectively[27]. PCR was performed in a 100 μl reaction mixture containing 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 1.4 mM $MgCl_2$, 0.2 mM of (each) deoxynucleoside triphosphates (dNTPs), 0.1% Triton X-100, 50 pmol of each of the two primers, 0.2 U superTaq DNA polymerase (HT Biotechnology, Cambridge, England), and 0.5 μg of DNA from each *Aspergillus* isolate which had been denatured at 94°C for 5 min. Next, 30 cycles of amplification were performed by denaturating for 1

min at 94°C, annealing the primer for 1 min at 42°C, and allowing elongation for 3 min at 72°C. When successful, amplification generated a product of approximately 1800 basepairs. Each product was analyzed by electrophoresis in 1.5% agarose gels. The amplification products required for sequencing were purified from low-melting-point agarose (LKB) by the Prep-a-Gene system (BioRad Laboratories). DNA sequencing was performed by the dideoxy method[108]. Annealing of a primer to the template was performed by heat denaturing double-stranded PCR products in the presence of a primer and then immediately freezing them in a CO_2-cooled ethanol bath. Primers selected from several conserved regions of 18S RNA served as sequencing primers. The resultant sequences were aligned to those of *A. fumigatus* and the other organisms detailed earlier by a sequence analysis software package (Genetic Computer Group at the University of Wisconsin) implemented on a VAX computer, and *Aspergillus* genus-specific sequences were selected and tested for their specificity. Initially, it appeared impossible to distinguish *Aspergillus* organisms from the *Penicillium* and *Paecilomyces* strains. Consequently, the 18S rRNA genes of these fungi were also sequenced. Subsequent alignment resulted in two primers for the *Aspergillus* genus-specific PCR assay which yielded a 363 bp fragment for positive amplification; *Asp1*, 5'-CGGCCCTTAAATAGCCCGGTC-3', located in the V7 region; and *Asp2*, 5'-ACCCCCCTGAG-CCAGTCCG-3', located in the V9 region of the 18S rRNA (see Figure 1).

Experimentally infected mice

Ten CD-1 Swiss mice (Central Animal Laboratory, Nijmegen, the Netherlands) were immunosuppressed with 150 mg cyclophosphamide (Endoxan-Asta) per kg of body weight given subcutaneously three days before and on the day of infection and protected from bacterial infection by daily intraperitoneal injections of 40 mg of imipenem-cilastatin (Merck, Sharpe & Dohme, Haarlem, the Netherlands) per kg. Six mice were infected intranasally with approximately 10^6 conidia of *A. fumigatus* under general anaesthesia with 0.1 ml of 4.5% chloral hydrate per 10 g of body weight administered intraperitoneally while the other four served as controls. Treatment with imipenem was continued until four days after infection when the mice were killed and their lungs and livers were

removed. The tissues were cut and divided into three portions. The first was used to inoculate Sabouraud glucose agar which was then incubated at 42°C for 5 days. A second portion was homogenized with disposable pellet pestles and tubes (Kontes, Vineland, NJ) in 600 μl of DEPC-treated distilled water, after which nucleic acids were obtained as described above. The remaining tissue was fixed in buffered formalin (4%), embedded in paraffin, and examined for the presence of fungus after *p*-aminosalicylic acid staining.

Clinical samples

BAL fluid samples were tested retrospectively by the PCR. Eighteen samples of BAL fluid had been obtained from 14 neutropenic patients who had developed a variety of pulmonary infiltrates during neutropenia induced by treatment for hematological malignancy (Table I). Aspergillosis was proven in four cases and suspected in two cases. As controls 14 BAL fluid samples were obtained from patients who were unlikely to be at any risk for IA. The BAL fluids were centrifuged at 2,500 rpm in an Eppendorf centrifuge for 15 minutes, and the supernatant was discarded. The residue was resuspended in a total volume of 600 μl of DEPC-treated water, and nucleic acids were isolated as described above.

Aspergillus *culture*

A pellet of BAL fluid was microscopically examined after fluorescent staining (Fungiqual: CIBA-GEIGY, Basel, Switzerland), plated on Sabouraud glucose (2%) agar, and cultured for 5 days at 28°C and 42°C. Histopathological evidence of IA was obtained from autopsy material.

Reverse transcription and hot-start PCR

rRNA was transcribed into cDNA at 37°C for 60 min in 20 μl of reaction mixture of 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 10 mM dithiotreitol, 0.2 mM dNTPs (Boehringer GmbH, Mannheim, Germany), 50 pmol of the downstream primer *Asp2* (see above), 5U of avian myeloblastosis virus reverse transcriptase (Promega) and 1 μg of nucleic acid (determined on a GeneQuant apparatus from Bio-Rad). One waxgem (Ampli-

Wax; Perkin-Elmer Cetus, Leiden, the Netherlands) was added to the sample for PCR analysis, heated to 80°C to allow the wax to melt and to cover the surface, and then returned to room temperature to allow the wax to solidify. This prevents the nucleic acids from melting into the PCR mixture and allows the amplification reaction to start at a high temperature, thereby preventing premature annealing and extension of the primer. Eighty microliters of the following PCR mixture was then deposited on the surface of the wax: 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 1.0 mM $MgCl_2$, 0.2 mM (each) dNTPs, 0.1% Triton X-100, 80 pmol of the upstream primer *Asp1* (see above), 40 pmol of the downstream primer *Asp2*, and 0.2 U of SuperTaq DNA polymerase (HT Biotechnology Ltd.). RNA-cDNA hybrids were denatured at 94°C for 5 min and then underwent 40 cycles consisting of denaturation at 94°C, primer annealing at 62°C, and elongation 72°C, each lasting 1 min. The recommendations of Kwok and Higuchi[67] were followed and each technical step was kept strictly isolated to prevent contamination.

Analysis of amplified DNA

Aliquots of 25 μl samples were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide[74]. Before Southern blotting, the agarose gel was depurinated in 0.25 N HCl, denatured in 0.5 N NaOH-1.5 M NaCl, and transferred to a nylon membrane (Hybond; Amersham Int., Bucks, England) by diffusion blotting in 0.5 N NaOH-1.5 M NaCl. DNA was covalently bound to the membrane by baking at 80°C for 2 h. Membranes were then prehybridized in 6 x SSC (1 x SCC consists of 15 mM sodium citrate and 150 mM sodium chloride)-5 x Denhardt solution (1 x Denhardt solution consists of 0.02% polyvinylpyrollidone, 0.02% Ficoll, and 0.02% bovine serum albumin)-0.1% SDS-250 μg of denatured sonicated herring sperm DNA per ml at 42°C for 2 h. Hybridization was achieved over 16 h at 42°C in 6 x SSC-1x Denhardt solution-0.1% SDS-100 μg of herring sperm DNA per ml-10^6 cpm of a ^{32}P-5'-end-labelled *Aspergillus*-specific oligonucleotide probe (*Asp-p*, 5'-ATGGAAGTGCGCGGCAATAAC-3') per ml. The blots were washed twice at 42°C in 2 x SSC-0.1% SDS and once in 0.5 x SSC-0.1% SDS at 55°C, each for 30 min. Kodak Royal X-Omat films were exposed to the blots for 4 to 8 h between intensifying screens (Dupont) at -80°C to allow autoradiography.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers X78537 (*A. flavus*), X78538 (*A. niger*), X78539 (*A. nidulans*), and X78540 (*A. terreus*).

Results

Selection of an Aspergillus *genus-specific primer set and probe*

The sequence alignment data of the V7 to V9 regions of the rRNA from the different *Aspergillus* species showed extensive homology with those of *P. marneffei*, *P. chrysogenum*, and *Paecilomyces variotii* (Figure 1) but not with those of the other microorganisms. One primer which was fully homologous to all *Aspergillus* species but had a single mismatch to *P. chrysogenum*, *P. marneffei*, and *Paecilomyces variotii* (Figure 1) was selected from the variable region V7. The other primer selected from the variable region V9 was not absolutely conserved for all *Aspergillus* species and displayed one or two mismatches and dispersed variability. *P. marneffei* contained only one mismatch while *Paecilomyces variotii* was completely homologous (Figure 1). *Penicillium chrysogenum* contained four mismatches making it unsuitable for hot-start PCR with high-temperature annealing.

The probe finally selected comprised part of the conserved region between the variable regions V7 and V8 and was homologous among the *Aspergillus* isolates and *Paecilomyces variotii* but not *P. marneffei*, in which the sequence contained two mismatches. A single base difference in the variable region V8 between the *Aspergillus* species and the other molds yielded an endonuclease *Sty*I site, thereby permitting endonuclease restriction analysis of the amplification products to distinguish the *Aspergillus* species from *Paecilomyces variotii* (Figure 1). Thus, by using a PCR assay that combines amplification, hybridization and endonuclease restriction analysis, it should be possible to specifically identify *Aspergillus* species.

Specificity of the Aspergillus *genus-specific PCR assay*

Hot-start PCR was performed to exclude nonspecific primer annealing. At an annealing temperature of 62°C, specific amplification was observed with all the *Aspergillus* species and also with *P. marneffei* and *Paecilomyces variotii* but with none of the other microorganisms, including *P. chrysogenum* (Figure 2). Southern blot analysis with the internal oligonucleotide probe showed clear hybridization with all the *Aspergillus* species and *Paecilomyces variotii* but not with *P. marneffei* (Figure 2). To distinghuish *Aspergillus* species from *Paecilomyces variotii*, 25 μl of each amplication reaction was digested with *Sty*I (Figure 3) and the amplification product of 363 bp found in each *Aspergillus* species was digested in two fragments of 160 bp and 203 bp, whereas the PCR product of *Paecilomyces variotii* remained intact.

Sensitivity of the Aspergillus *genus-specific PCR*

Serial 10-fold dilutions of purified *A. fumigatus* nucleic acids isolated from the same sample were tested by PCR. Without prior transcription of the rRNA, a sensitivity of 1 pg of nucleic acid was obtained (on the basis of the DNA content), as detected by gel electrophoresis (Figure 4A). However, when the rRNA was first transcribed into cDNA, 10 fg of nucleic acids was detected on gel (Figure 4B). Southern blot analysis with the *Aspergillus* oligonucleotide probe increased the sensitivity another 10-fold.

Experimentally infected mice

A. fumigatus was cultured from multiple foci in the lungs of all six infected mice (mice 1 to 6, Table II) while the PCR was positive in only five animals (Figure 5). Hyphae were also found in the livers, although *A. fumigatus* was not recovered by culture. PCR on liver tissue was positive in each case, although the amplification signal was less intense than that obtained from the corresponding lungs (Figure 5). In mouse 2, there was no histological evidence for invasive aspergillosis in either the lung or liver, and the culture and PCR both proved negative. There was neither histological nor cultural evidence of *A. fumigatus* in any of the lungs and livers from the healthy, immunosuppressed control mice (mice 7 to 10, Table II), and the PCR was negative in every case.

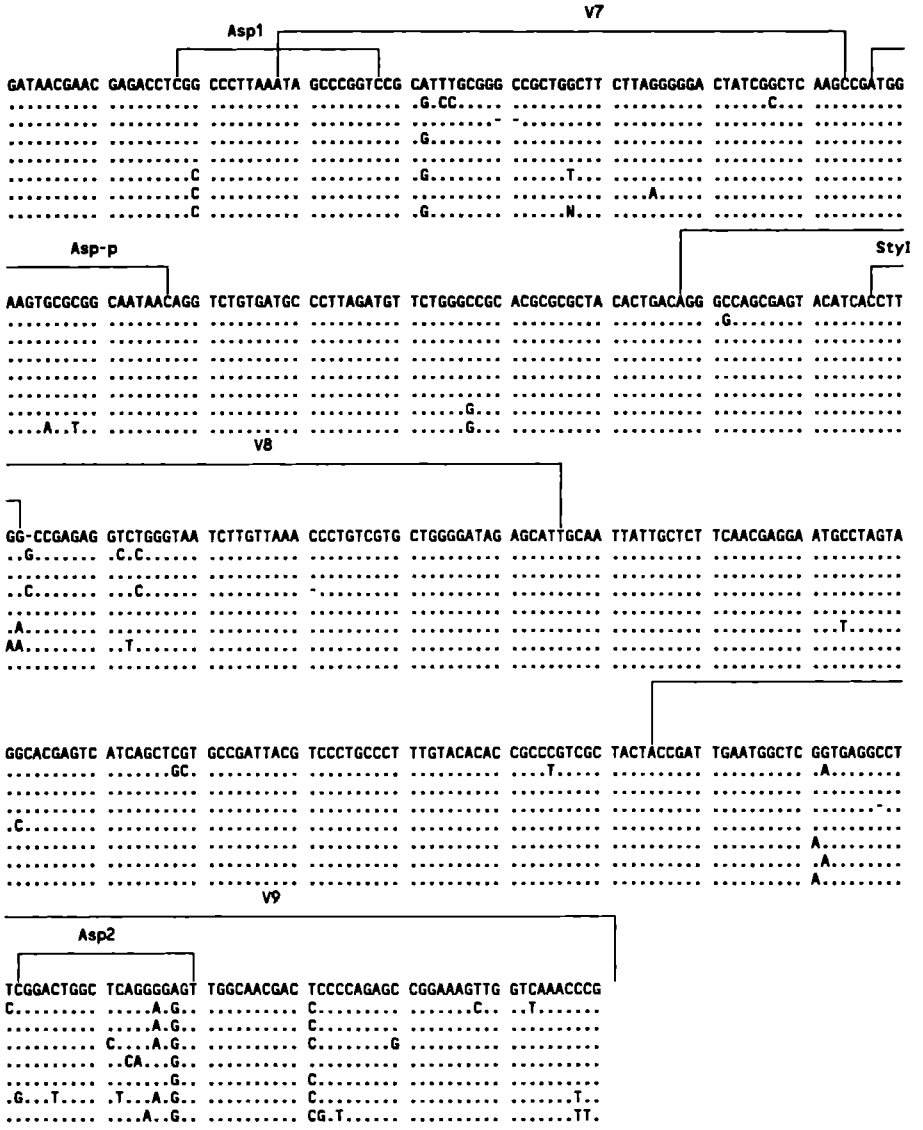

Figure 1. *Sequence alignment and primer selection for* Aspergillus *genus-specific PCR assay. 1,* A. fumigatus; *2,* A. nidulans; *3,* A. niger; *4,* A. flavus; *5,* A. terreus; *6,* Paecilomyces variotii; *7,* Penicillium chrysogenum; *8,* P. marneffei. *Asp1, Asp2, and Asp-p represent the regions of the* Aspergillus *primers and probe. Above the sequences, the variable regions V7 to V9 are indicated. StyI, the StyI endonuclease restriction site CCT(T/A)GG.*

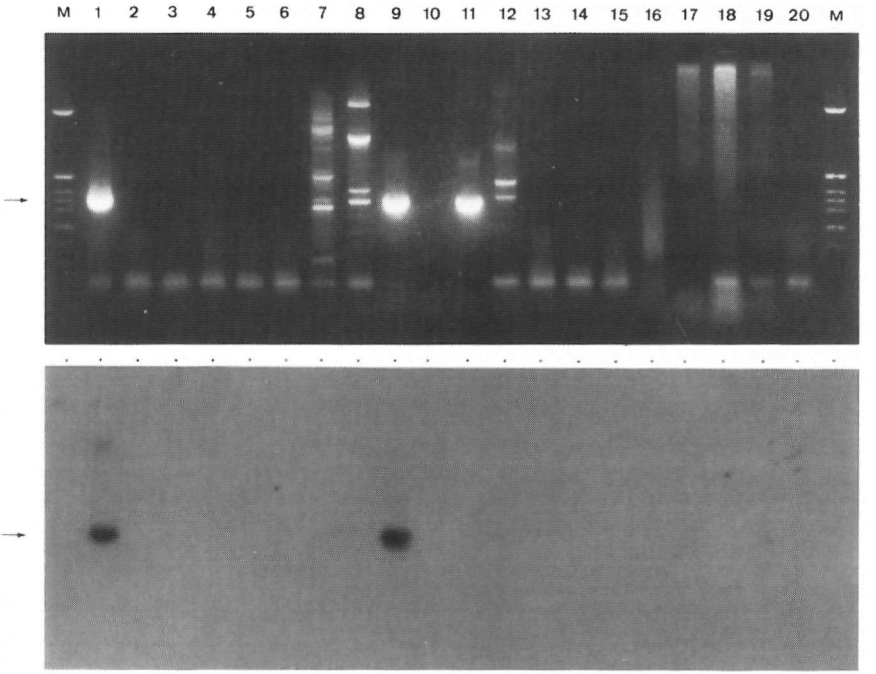

Figure 2. *Specificity of the* Aspergillus *genus-specific PCR. PCR with the* Aspergillus *genus-specific primer set was performed on a number of different fungal and bacterial species. The reactions were analysed by agarose gel electrophoresis (top) and Southern blot hybridization with a* τ-^{32}*P-labeled oligonucleotide probe (Asp-p)(bottom). Lanes: M, size marker (HinfI-digested pBR322); 1,* A. fumigatus; *2,* Candida albicans; *3,* C. tropicalis; *4,* C. krusei; *5,* Candida (Torulopsis) glabrata; *6,* Pseudoallescheria boydii; *7,* Fusarium species; *8,* Enterobacter cloacae; *9,* Paecilomyces variotii; *10,* Penicillium chrysogenum; *11,* P. marneffei; *12,* Cryptococcus neoformans; *13, a* Rhizopus *strain*; *14,* Streptococcus sanguis; *15,* S. mitis; *16,* Staphylococcus aureus; *17,* Pseudomonas aeruginosa; *18,* Escherichia coli; *19,* Klebsiella pneumoniae; *20, distilled water. Arrows indicate PCR fragment of 363 bp.*

Table 1. Characteristics of hematological patients at risk for invasive aspergillosis

Pat.	Sex/age	Underlying disease and/or condition[a]	*Aspergillus* infection	BAL culture	Other diagnostic procedure(s)	PCR	Granulocyte count (10^9/liter)	Antifungal i.v therapy[b] (Total dose)	Outcome
1	♂, 38	CML,BMT	Proven	*Aspergillus* species	Invasive aspergillosis at autopsy	+	<0.1	Ampho B (660 mg)	Deceased
2	♀, 51	MDS	Proven	*Corynebacterium jeikeium*	Disseminated aspergillosis in cerebrum and lungs; *Aspergillus* antigen in serum positive	+	<0.1	Ampho B (1,006 mg), then itra (2,000 mg)	Deceased
3	♀, 42	ALL,BMT	Proven	Negative	*Aspergillus* hyphae in open-lung biopsy	+	0.1	Ampho B (196 mg) then itra (800 mg)	Survived
4	♀, 33	AML	Proven	*Candida albicans*	None	+	<0.1	Ampho B (496 mg)	Survived
5	♀, 55	AML	Probable	Herpes simplex virus		+	<0 1	Ampho B (286 mg)	Survived
6	♂, 45	NHL	Probable	Negative		+	<0.1	Liposomal ampho B (12,600 mg)	Survived
7	♀, 61	AML	Possible	*Candida albicans*		+	<0.1	Flu (6,000 mg) and ampho B (1,206 mg)	Deceased
8	♂, 44	BMT, GVHD	Possible	*Penicillium* species *Candida albicans*, Influenza virus type A		+	5.0	Ampho B (550 mg) then itra (146,000 mg)	Survived
9	♂, 22	ALL	Unlikely	Negative		+	<0.1	Ampho B (463 mg) then itra	Survived
10	♀, 55	CML	None	*Pseudomonas aeruginosa*		-	2.0	—	Survived
11	♂, 41	AML	None	*Candida albicans*		-	<0.1	Ampho B (366 mg)	Survived
12	♀, 60	AML	None	Herpes simplex virus		-	<0.1	Ampho B (196 mg)	Survived
13	♀, 32	ALL,BMT	None	Negative		-	<0.1	—	Survived
14	♀, 60	NHL	None	Negative		-	<0.1	Ampho B (70 mg)	Deceased

[a]CML, chronic myeloid leukemia; AML, acute myelogenous leukemia; ALL, acute nonlymphocytic leukemia; BMT, bone marrow transplant; NHL, non-Hodgkin lymphoma, MDS, myelodysplastic syndrome; GVHD, graft versus host disease.
[b]i v., intravenous, ampho B, amphotericin B, itra, itraconazole, flu, fluconazole.

Clinical samples

Positive amplification was obtained from nine patients, four of whom had proven case and two had suspected cases of aspergillosis. An unspeciated *Aspergillus* species had been isolated from one BAL fluid sample from which a strong amplification signal was also obtained (patient1; Figure 6). This patient was one of two who succumbed to IA. The other patient (patient 2) died of disseminated aspergillosis, and only *Corynebacterium jeikeium* had been isolated from BAL fluid, although serum samples had been repeatedly

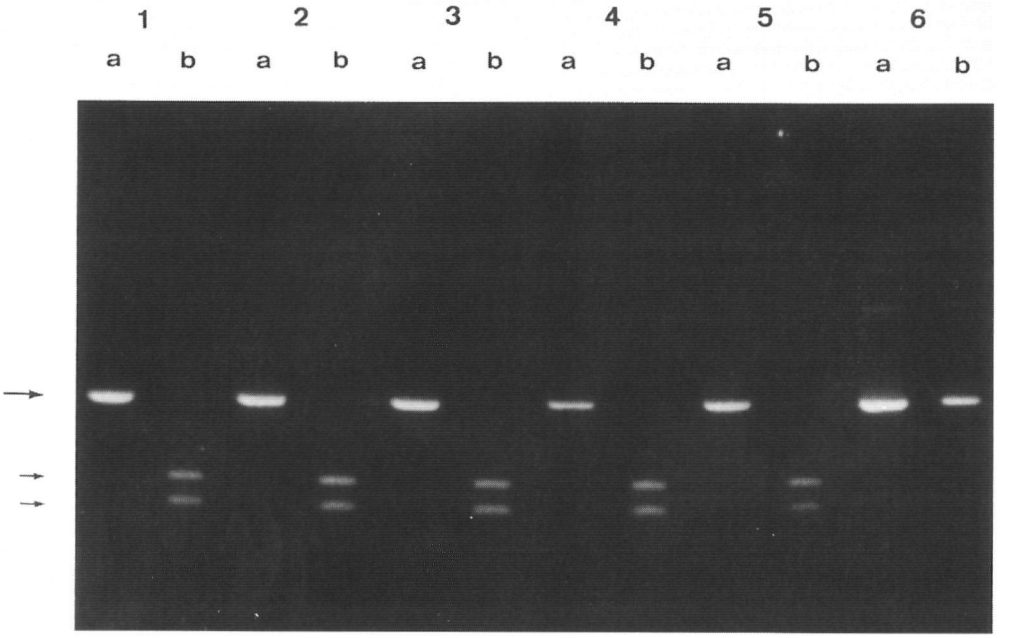

Figure 3. *Analysis of* Aspergillus *genus-specific PCR fragments. A sample of the PCR mixture was digested with StyI. The restriction endonuclease reactions were analysed by agarose gel electrophoresis. Lanes: 1,* A. fumigatus; *2,* A. flavus; *3,* A. terreus; *4,* A. nidulans; *5,* A. niger; *6,* Paecilomyces variotii; *a, PCR control fragment of 363 bp (also indicated by large arrow); b, PCR fragments digested with StyI (also indicated by small arrows at 160 and 203 bp, respectively).*

positive for an *Aspergillus* antigen (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). Hyphae consistent with *Aspergillus* species were seen in lung biopsy material obtained from patient 3, who had undergone open-lung surgery to exclude aspergillosis. Culture of the biopsy remained negative, but PCR was positive for *Aspergillus* species (Figure 6, lanes 4). Three months later, this patient underwent BAL, having developed a pulmonary infiltrate during another episode of neutropenia. Cultures of BAL fluid were again negative, but PCR remained positive (Figure 6, lanes 5). The last patient with proven aspergillosis (patient 4) had initially developed a pulmonary infiltrate eight months earlier during remission induction therapy. *Candida albicans* was isolated from the BAL fluid sample, although *A. fumigatus* had been isolated from oral cultures. Then, during a second course of chemotherapy, a cavity developed in the right upper lobe consistent with lesions if IA but culture of the BAL fluid was again negative. Both BAL fluid samples were found positive by PCR for *Aspergillus* species. Patient 5 survived a pulmonary infiltrate suspected to be due to aspergillosis. Herpes simplex virus was isolated from the BAL fluid, and only 286 mg of amphotericin B was given as therapy when neutrophils recovered. BAL fluid from patient 6 was negative in culture, and he survived after being treated with 4 mg of liposomal amphotericin B (Ambisome; Vestar Europe) per kg for 36 days. *C. albicans* was both seen microscopically and isolated from BAL of patient 7, who ultimately died of her underlying disease. However, no autopsy was performed to exclude IA. She had been treated with more than 1 g of amphotericin B and had also received fluconazole (400 mg/day) for 15 days. Patient 8 was suffering from severe graft versus host disease after an allogeneic bone marrow transplant. Two BAL fluid samples were available for PCR analysis. The first BAL fluid sample had been obtained 20 months after transplant when the patient had developed an infiltrate in the right lower lobe. An unspeciated *Penicillium* isolate and *C. albicans* were cultured. The patient received a short course of amphotericin B and was given 400 mg itraconazole per day as a prophylaxis during the next 8 months. A second BAL fluid sample was obtained when no infiltrates were apparent on a chest X-ray. This time, a *Penicillium* species together with Influenza virus type A was isolated. However, both BAL fluid samples were positive by PCR (Figure 6, lanes 2 and 3).

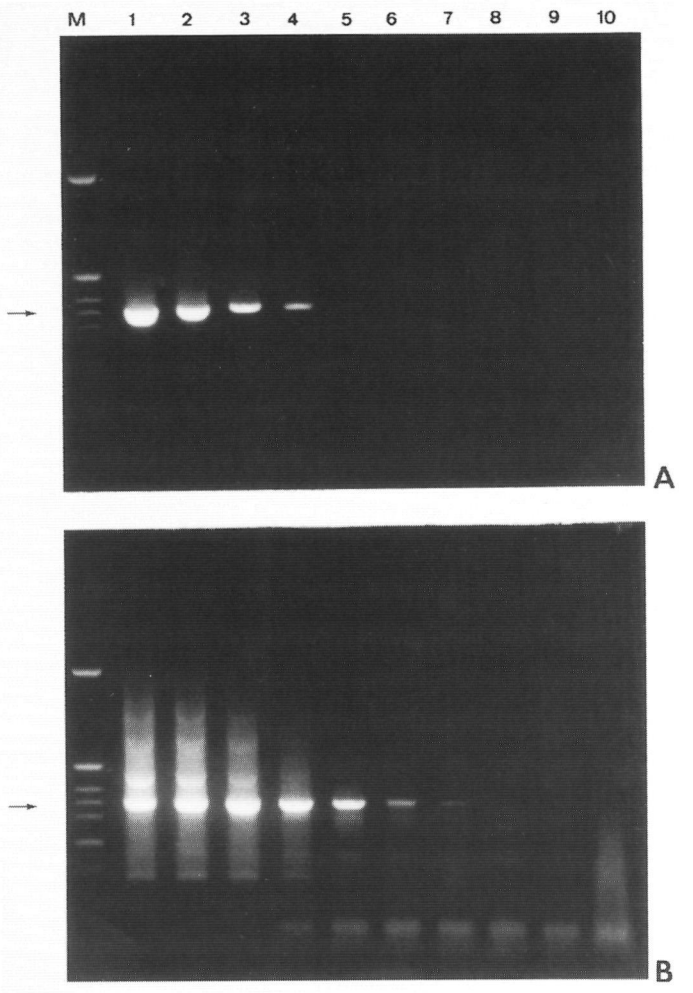

Figure 4. *Sensitivity of the detection of diluted purified nucleic acids isolated from* A. fumigatus *by PCR. The nucleic acid extract either was used directly in the PCR (DNA PCR)(A) or was first transcribed into cDNA by reverse transcription prior to the PCR (RNA/DNA PCR)(B). Lanes: M, size marker (HinfI-digested pBR322); 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, 10 fg; 8, 1 fg; 10, 100 ag; 11, 10 ag. Arrows indicate PCR fragment of 363 bp.*

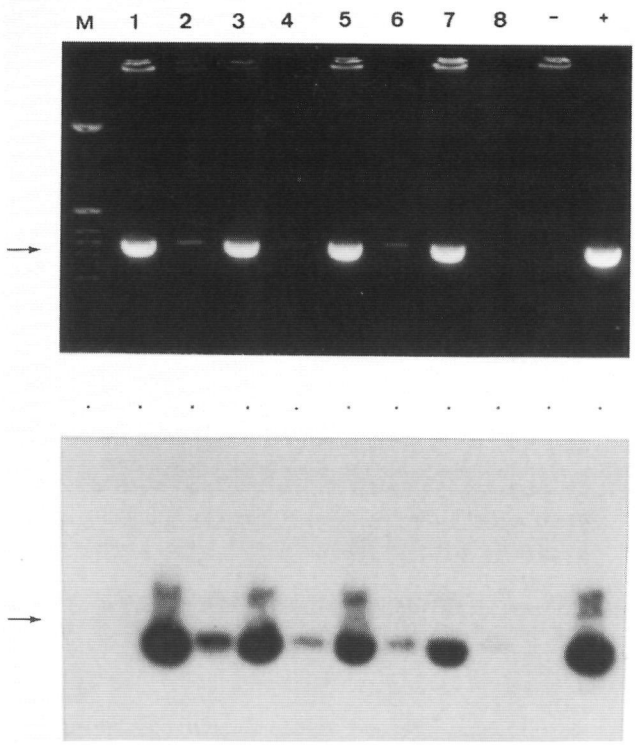

Figure 5. *PCR analysis of experimentally infected mice. Nucleic acids were extracted from the lungs and livers, subjected to PCR amplification, and analysed by agarose gel electrophoresis (top) and Southern blot hybridization (bottom). Lanes: M, size marker (HinfI-digested pBR322); 1, lung of mouse 1; 2, liver of mouse 1; 3, lung of mouse 2; 4, liver of mouse 2; 5, lung of mouse 3; 6, liver of mouse 3; 7, lung of mouse 4; 8, liver of mouse 4, -, lung of uninfected mouse; +,* A. fumigatus *DNA. Arrows indicate PCR fragment of 363 bp.*

The last patient (patient 9) from whom the BAL fluid sample was PCR positive had developed bilateral pulmonary infiltrates which resolved following neutrophil recovery while completing treatment for acute nonlymphocytic leukemia. All positive samples were confirmed to be *Aspergillus* specific by StyI restriction endonuclease digestion. Samples from the five remaining patients were negative by PCR as were those obtained from 14 nonimmunosuppressed patients.

Table 2. Detection of A. fumigatus *in organs of experimentally infected mice.*

Mouse	Clinical sample	Detection by		
		Culture	Histology	PCR
1	Lung	+	+	+
	Liver	-	+	+
2	Lung	+	-	-
	Liver	-	-	-
3	Lung	+	+	+
	Liver	-	+	+
4	Lung	+	+	+
	Liver	-	+	+
5	Lung	+	+	+
	Liver	-	+	+
6	Lung	+	+	+
	Liver	-	+	+
7	Lung	-	-	-
	Liver	-	-	-
8	Lung	-	-	-
	Liver	-	-	-
9	Lung	-	-	-
	Liver	-	-	-
10	Lung	-	-	-
	Liver	-	-	-

Discussion

IA is one of the major clinical concerns in immunocompromised patients because it is difficult to detect, even in the case of extensive systemic infections and, treatment appears to be successful only when started in an early stage of disease[42]. While prevention of infection remains the ultimate goal, identifying those individuals at greatest risk by a rapid diagnosis would provide an alternative since these are most likely to benefit from early treatment. Recently, several investigators reported the use of PCR to detect *A. fumigatus* and *A. flavus* in high-risk patients[97,111,119]. However, infection due to other species is not uncommon[54,134], and so it seems important to detect all clinically relevant

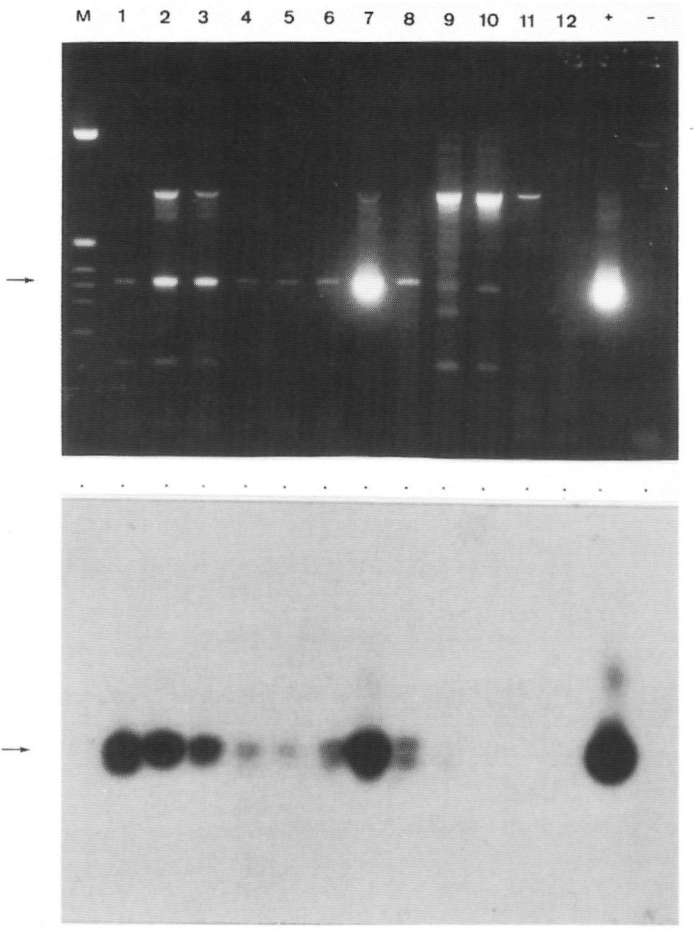

Figure 6. *PCR analysis of patients at risk for invasive aspergillosis. Nucleic acids were extracted from BAL fluid samples, subjected to PCR amplification, and analysed by agarose gel electrophoresis (top) and Southern blot hybridization (bottom). Lanes: M, size marker (HinfI-digested pBR322); 1, Patient 7; 2, Patient 8 BAL fluid sample A; 3, Patient 8 BAL fluid sample B; 4, Patient 3 BAL fluid sample A; 5, Patient 3 BAL fluid sample B; 6, Patient 2; 7, Patient 1; 8, Patient 4; 9 through 12, BAL fluid samples from control patients;* +, A. fumigatus *DNA;* -, *distilled water. Arrows indicate PCR fragment of 363 bp.*

Aspergillus species by developing a genus-specific PCR assay such as we describe. rRNA has been used as the target for the development of a species- or genus-specific PCR assays for several different microorganisms, such as mycobacteria and mycoplasmas[16,124]. Since only the 18S rRNA of *A. fumigatus* had been previously sequenced[11], we elucidated the complete 18S rRNA sequences of the other opportunistic pathogens *A. flavus*, *A. terreus*, *A. nidulans*, and *A. niger*. Alignment of the sequences revealed the impossibility of selecting *Aspergillus* species-specific primers for PCR. However, several domains, mainly in the variable regions V7 to V9, which appeared to be *Aspergillus* genus specific were identified. A suitable-looking primer pair was therefore selected from this region, but strong cross-hybridization was observed with *Paecilomyces variotii*, *P. marneffei*, and *P. chrysogenum*. Spreadbury and colleagues[111] also found reactions suggesting a close homology between *Penicillium* and *Aspergillus* spp. based on the 26S rRNA sequence. Subsequent sequencing of the rRNAs of these fungi showed a close homology to exist among the different *Aspergillus* species. A primer pair from the V7 and V9 region was selected (Figure 1), and it was possible to differentiate *Aspergillus* species from these three closely related genera by combining hot-start PCR with high annealing temperature, hybridization with a specific oligonucleotide probe, and restriction endonuclease digestion with *Sty*I. However, it can not be excluded that other *Penicillium* or *Paecilomyces* species or, in fact, other related genera that might be contaminants can be reactive in the assay. On the other hand, no specific cross-hybridization was observed with any other prokaryotic or eukaryotic microorganism. Without prior transcription of the rRNA, 1 pg of nucleic acids was detected on gel. Sensitivity was increased to 10 fg when the rRNA was first transcribed into cDNA, and it was extended 10-fold by Southern blot hybridization. However, RNA amplification did not lead to the identification of more positive clinical samples than were found by rDNA amplification (data not shown), indicating that sufficient sensitivity was obtained without this additional step. Moreover, this provides sufficient sensitivity for clinical samples since a single genome of *A. nidulans* is estimated to contain approximately 50 fg of chromosomal DNA, based on the karyotype of *A. nidulans* which is equivalent to about 20 fungal elements[19].

The potential value of the PCR was demonstrated in a mouse model where the PCR was positive in five of the six infected immunocompromised mice which had developed IA following intranasal inoculation with *A. fumigatus*. In contrast to the lung specimens, the liver specimens revealed positive PCR signals and histological demonstration of hyphae but negative cultures. A possible explanation could be the absence of conidia during the growth of *A. fumigatus* in liver tissue. Air-containing lung tissue possibly induces better sporulation of growing hyphae. None of the control immunosuppressed mice had evidence of infection, and each PCR was negative.

In a clinical setting, a single positive culture of *Aspergillus* organisms from BAL fluid might be indicative for IA[81,134] but only multiple samples yield adequate sensitivity[146]. The fungus was isolated from only one of the four patients with IA, although PCR was positive in each case. In fact, BAL fluids from nine patients were positive for *Aspergillus* species directly on gel, though infection was either proven or likely in only six cases. Although it is possible that the other three patients with a positive PCR test result might have had incipient aspergillosis, bronchial colonization cannot be excluded[24]. Additional Southern blot hybridization did not alter the number of patients with positive results, suggesting that no more than nine of the 14 patients (64%) who developed pulmonary infiltrates during immunosuppression were actually at risk of aspergillosis. Although our tertiary care 950-bed University Hospital admits several immunocompromised patients, the study of diseases of low prevalence, like IA, will inevitably be limited by the small number of patients. The only definitive diagnosis of proven IA is by histopathological examination of tissues. In most instances, this is only possible postmortem. Antemortem diagnosis is, in our hospital, only possible on BAL fluid and, in most cases, not by open-lung biopsy because of the associated thrombocytopenia. Therefore, most cases will be probable or possible. There is always a possibility of false positivity with this approach. However, PCR results should be interpreted in the whole clinical context of the patient (e.g., a neutropenic or otherwise immunocompromised patient, treatment with a broad-spectrum antibiotics, persisting fever, and new infiltrates on chest X ray). We have begun to study a larger group of patients to prospectively determine the value of PCR for the rapid and early diagnosis of IA. Autopsy surveys have shown that IA developed in around

45% of patients who died of hematological malignancy[79,124]. However, current treatment modalities are not without risk, and even though the practice of early empiric therapy is fairly widespread[53,92], a better determination of the group of patients at risk remains a desirable goal. This requires a test with maximum sensitivity, and so it seems appropriate to consider any immunosuppressed patient with a BAL fluid sample yielding a positive (Southern blot) PCR result as being at risk of IA. We recently used interepeat PCR fingerprinting to demonstrate genotypic heterogenicity among *A. fumigatus* isolates originating from different patients and different anatomical locations[122]. This assay by which different isolates can be genotypically characterized can be used in the study of the epidemiology of IA.

In conclusion, the PCR technique we have developed appears suitable to study a large group of patients and to identify those at risk of IA. One of the main limitations now is that the method itself cannot yet be easily performed in every microbiological laboratory, since trained personel and experience with PCR are necessary. However, once validated, a method such as ours could have a major impact in reducing the morbidity and mortality of patients undergoing treatment for cancer and hematological malignancy by allowing the early institution of therapy on a more selective basis than is possible at present.

Acknowledgements

We are grateful to J. Peter Donnelly for critical comments on the manuscript, Irena Nováková for clinically assessing each of the patients, Jo Curfs for animal infection and histology, and Ton Rijs for mycological assistance.

Chapter 2

DETECTION OF *ASPERGILLUS* ANTIGEN

CLINICAL EVALUATION AND REPRODUCIBILITY OF THE PASTOREX *ASPERGILLUS* ANTIGEN LATEX AGGLUTINATION TEST FOR DIAGNOSING INVASIVE ASPERGILLOSIS

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Journal of Clinical Pathology 1995;48:474-6.
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Summary

The performance of Pastorex *Aspergillus* antigen latex agglutination test for the detection of galactomannan in sera of patients at risk for invasive aspergillosis was evaluated, and the impact of storage on the reproducibility of the antigen titer was tested. During a one year period, 392 serum samples were obtained from 46 patients at risk for invasive aspergillosis and tested for the presence of galactomannan using an *Aspergillus* latex agglutination test (Pastorex). Twenty three positive serum samples which had been stored at -20°C for 2 - 16 months were retrospectively retested. Furthermore, two positive serum samples were stored at -20°C and -70°C and prospectively tested at three month intervals for a period of 15 months. The Pastorex *Aspergillus* test was positive in eight patients with microbiological, radiological or histological evidence for invasive aspergillosis, but was negative in the initial serum sample of five of these patients. In two patients with histological evidence for invasive aspergillosis no positive reaction was found in six samples. Six of 13 (45%) serum samples which had been stored at -20°C for six months had lost reactivity, while one of 10 (10%) samples had lost reactivity when stored up to six months. Two serum samples which had been stored at -20°C and -70°C and prospectively retested at three month intervals for 15 months, maintained stable antigen titers. The Pastorex *Aspergillus* test is too insensitive to diagnose IA in an early stage, but may contribute to the diagnosis when cultures remain negative and serial samples are obtained. To maintain a good reproducibility, serum samples should be stored at -70°C when the period of storage exceeds six months.

Introduction

Invasive aspergillosis (IA) is a serious opportunistic infection with a high mortality rate in patients with compromised host defences. Since early treatment of IA is associated with a reduced mortality rate[1], reliable diagnostic tests capable of detecting IA in an early stage are of great importance. Early diagnosis is, however, extremely difficult. Therefore tests have been developed to detect *Aspergillus* antigens in body fluids[6]. Several serological

tests to detect antigenemia have been used, including an enzyme linked immunoassay and a radioimmunoassay using purified galactomannan[102,117,139]. Monoclonal antibodies raised against galactomannan[114] are the basis of a rapid commercial latex agglutination test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) on serum for detection of circulating antigen. This test, which has been applied in many hospitals, yielded sensitivities up to 95% for patients with proven invasive aspergillosis[31,44], but has been reported to be of limited value because results became negative when testing was repeated after storage[57,137]. Here, we report our experience with the Pastorex *Aspergillus* test in patients at risk for IA, and the results of a study we conducted to determine the impact of the duration of storage and repeated freezing and thawing on the antigen titer.

Materials and Methods

During a one year period, 392 serum samples were obtained from 46 patients at risk for IA. At least two serum samples per patient were collected and processed within 24 hours of collection, and frozen at -20°C until retesting. Testing of all samples was performed by one technician according to the manufacturer's instructions. Titration of positive samples was performed by successive twofold dilutions (1:2 to 1:32) in glycine dilution buffer. To determine the impact of storage on the antigen titer, all positive serum samples were retested retrospectively.

In addition, a prospective study was performed with two positive serum samples obtained from one patient with a histologically proven disseminated infection with *A. flavus*. The impact of repeated freezing and thawing was studied by storing 6 ml of each serum sample at -20°C and -70°C, respectively, for a total period of 15 months. At three month intervals the samples were thawed and the agglutination reaction was performed. Positive agglutination reactions were titrated. The impact of storage on the antigen titer was studied by dividing the remainder of the two serum samples into 20 Eppendorf tubes (300 μl). Ten tubes were stored at -20°C and 10 at -70°C for a total period of 15 months. At three month intervals two tubes stored at -20°C and two stored at -70°C were thawed. The first of each pair of samples was used to perform the agglutination reaction. When

positive, the second sample was used for titration.
The Mann-Whitney test was used to determine statistical significance.

Results

A total of 392 serum samples was tested from 46 patients suspected of IA. Seventy serum samples were collected from eight patients with microbiological, radiological or histological evidence of IA (Table 1). Of these, 24 samples were found to be positive (titers ranged from 1:1 to 1:8). In all cases the Pastorex *Aspergillus* test results were interpreted in the whole clinical context of the patient. Antifungal therapy was not given in two cases (Table 1, patient Nos 2 and 4). In five cases the antigen test was negative at the time antifungal therapy was started, but became positive during treatment (Table 1, patient Nos 1,3,5,7 and 8). In one case (Table 1, patient No 6) antifungal therapy was initiated when the Pastorex *Aspergillus* test was found positive after five successive samples had been negative. Culture of a bronchoalveolar lavage performed the same day yielded *A. fumigatus*. Two patients (acute lymphoblastic leukemia and myelodysplastic syndrome) with histologic evidence of IA showed no positive reaction in six samples.

***Table 2.** Effect of storage on* Aspergillus *antigen titer. A: Storage at -20°C and -70°C. Serum samples were thawed before every agglutination test. B: Storage at -20°C and -70°C. Serum samples were stored untill antigen titer was determined. Serum 1 (S1) and 2 (S2) were taken 3 and 1 day, respectively, before death due to invasive aspergillosis.*

	Storage			
	A		B	
Time between tests (months)	S1 (-20°C)	S2 (-70°C)	S1 (-20°C)	S2 (-70°C)
0	1:8	1:32	1:8	1:32
3	1:8	1:16	1:8	1:16
6	1:8	1:16	1:8	1:16
9	1:8	1:16	1:8	1:16
12	1:8	1:16	1:8	1:16
15	1:8	1:16	1:8	1:16

Table 1. Clinical evaluation and reproducibility of Pastorex Aspergillus *latex-agglutination test in patients at risk for invasive aspergillosis.*

Pat. No	Age/sex	Underlying disease[a]	Postmortem diagnosis	No of samples tested/ No of positive samples	Titer	No of samples retested/ No of positive samples	Titer	Time between first/ second test (months)
1.	16/F	NHL, BMT	*Aspergillus* in bronchi, not invasive	6/1	1:4	1/0	neg	16
2.	58/M	Hepatocellular carcinoma	Mycotic necrotising bronchopneumonia	3/2	1:4 1:4	2/2	1:2 1:2	15
3.	36/F	AML	ND	1/1	1:8	1/1	1:4	14
4.	45/F	Kahler, BMT	Survived	18/2	1:8 1:4	1/1	1:4 ND	11
5.	30/M	AML	Cavitating bronchopneumonia invasive in lung tissue	22/8	1:4 1:1 1:4 1:1 1:4 1:1 1:4 1:1	8/3	1:1 1:1 1:1 neg neg neg neg neg	9
6.	44/F	AML, BMT	Mycotic necrotising bronchopneumonia	8/3	1:2 1:2 1:2	3/3	1:2 1:2 1:2	6
7.	53/M	CML, BMT	ND	9/4	1:1 1:1 1:2 1:1	4/3	1:1 1:1 1:1 neg	2
8.	37/M	AIDS	ND	3/3	1:1 1:1 1:4	3/3	1:1 1:1 1:2	2

[a]NHL, non-Hodgkin lymphoma; BMT, bone marrow transplantation; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; AIDS, acquired immunodeficiency syndrome; ND, not done.

We found only one false positive agglutination in a sample which arrived in the laboratory more than 48 hours after collection. Eight other samples from this patient with chronic granulomatous disease, processed within six hours of collection, were negative. A necropsy performed on this patient, who died of cardiac failure, revealed no evidence of IA.

Twenty-three positive samples, which had been stored at -20°C for 2 - 16 months, were available for retesting. The results of retesting are shown in Table 1. Nine out of 10 (90%) positive serum samples, which had been stored up to six months at -20°C, showed a positive agglutination reaction when retested retrospectively, while only seven of 13 (54%) samples were found positive when stored at -20°C for longer than six months (Table 1; $p = 0.067$).

The effect of storage at -20°C and -70°C and of repetitive freezing and thawing of serum on the galactomannan antigen titer is shown in Table 2.

Discussion

Since serial antigen determination has been shown to be of great importance[139], we obtained as many serum samples as possible from patients at risk for IA. Several investigators have reported the use of a number of methods to detect circulating *Aspergillus* antigen in body fluids[6,64,102,117,139]. Although antigen detection appears to be highly specific, the sensitivity of the tests used until now has been quite low. The Pastorex *Aspergillus* test is the only antigen test which is commercially avaiable, but its use to diagnose IA in an early stage is controversial. Several investigators have reported a high sensitivity of this test, up to 95%[31,44], and found that the Pastorex test allowed diagnosis of IA to be made earlier in 68% of cases when compared to conventional methods[44]. However, other studies have shown that the Pastorex *Aspergillus* test had a low sensitivity[22] and a poor positive predictive value[7,44] in necropsy proven cases of IA. Our results also indicate that the Pastorex *Aspergillus* test is too insensitive to diagnose IA in an early stage, but may contribute in the diagnosis if cultures remain negative and serial serum samples are obtained.

Poor reproducibility of the Pastorex *Aspergillus* test has been reported[57,137], which can make the interpretation of positive agglutination results difficult. Warnock et al.[137] retested 10 positive serum samples which had been stored for three days to 10 months at -30°C, and found that all samples had become negative, and Knight et al.[57] retested 32 positive samples with a different batch and found loss of reactivity in 14 samples. However, these reactions could have been false positive as the manufacturer's instructions were not followed[115]. In the present study, serum samples which had been stored at -20°C for more than six months tended to lose reactivity. Although, because of the limited number of positive samples available, the difference was not statistically significant in comparison to serum samples stored for less than six months, it shows a trend towards loss of reactivity when serum samples are stored at -20°C for long periods of time. Also, the titer found at retesting tended to be lower when the period of storage became longer.

The serum samples which were stored at -20°C and -70°C and prospectively retested at three month intervals were all found to be positive with stable antigen titers. Repeated freezing and thawing of these serum samples (Table 2, A) did not appear to have an effect on the reproducibility. However, to maintain a good reproducibility in the Pastorex *Aspergillus* test, we recommend that serum samples should be stored at -70°C when the period of storage exceeds six months.

In the absence of better commercial antigen detection, the Pastorex *Aspergillus* test remains useful although a better test needs to be developed. Other techniques, including DNA based assays, may contribute in the diagnosis of IA in an early stage. Several investigators have reported the use of the polymerase chain reaction (PCR) for the detection of *Aspergillus* DNA in BAL fluid[78,97,111,119]. The PCR showed a very high sensitivity and specificity in patients at high risk for IA. However, the value of the PCR in the diagnosis of this condition remains to be established and prospective evaluations are now being performed. The diagnosis of IA is difficult and demands a multidisciplinary approach. The implementation and combination of novel or improved methods, for example PCR and antigen detection, to diagnose IA in an early stage or in guiding treatment may improve the clinical outcome of this disease.

SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY COMPARED WITH PASTOREX LATEX AGGLUTINATION TEST FOR DIAGNOSING INVASIVE ASPERGILLOSIS IN IMMUNOCOMPROMISED PATIENTS

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Journal of Clinical Microbiology 1995;33:1912-4.
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Summary

The performance of a direct sandwich enzyme-linked immunosorbent assay (ELISA) for detecting *Aspergillus* galactomannan was compared with that of the Pastorex *Aspergillus* antigen latex agglutination (LA) test by using 532 serum samples from 61 patients at risk for invasive aspergillosis. The ELISA gave positive results earlier in the course of infection than did the LA test. A sensitivity of 70% and a specificity of 86%, were obtained for the LA test and corresponding values of 90 and 84% were obtained for the ELISA when a series of serum samples was employed.

Introduction

Aspergillus fumigatus is a ubiquitous fungus capable of causing life-threatening opportunistic infections in immunocompromised patients[13]. Inhalation of airborne *Aspergillus* conidia and germination of spores in the alveoli primarily results in a pulmonary infection, although dissemination to other organs may occur. The successful diagnosis of invasive aspergillosis (IA) is frustrated by the difficulty in obtaining specimens which demonstrate the organism directly. Serologic techniques have been used in an attempt to establish diagnosis in early stages of infection but have proven to be unsuccessful in immunocompromised patients because of the patients' impaired ability to produce a humoral response. The detection of circulating *Aspergillus* antigens in body fluids, e.g. serum and urine, is promising, but despite the development of several methods for such detection[6,64], none has gained widespread acceptance. Recently, a direct sandwich enzyme-linked immunosorbent assay (ELISA) has been developed; this assay employs the rat monoclonal antibody EB-A2 to detect galactomannan (GM), a cell wall component of *Aspergillus* spp.[114,116]. The EB-A2 antibody is also used in the commercially available Pastorex *Aspergillus* latex agglutination (LA) test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France)[114], but the detection limit can be lowered 10-fold by employing the antibody as a captor and detector[116], which may allow IA to be diagnosed earlier. We investigated this possibilitiy by testing the ELISA with a series of 532 serum samples

which had been previously analysed by LA[128] to attempt to diagnose IA in 61 immunocompromised patients at high risk for the disease.

Materials and Methods

A total of 532 serum samples from 61 patients were collected during chemotherapy-induced neutropenia, and were stored at -80°C. At least two serum samples were available from each patient. Before testing, all serum samples were treated as descibed previously for *Candida* mannan[99] to dissociate immune complexes. The LA tests were performed by the same technician exactly as described by the manufacturer. For the ELISA, the supernatants of the serum samples were obtained by centrifugating samples at 15,000 x *g* for 10 min. The ELISA was performed as described previously[116]. Briefly, 50 μl of the treated sample and 50 μl of horseradish peroxydase-conjugated EB-A2 were placed in the wells of a microtiter plate coated with monoclonal antibody EB-A2, and the plates were incubated at 37 °C for 90 min. After the plates were washed, 100 μl of buffer containing *ortho*-phenylenediamine dihydrochloride was added to the wells, and the plates were incubated for 30 min. The reaction was stopped with 50 μl 4M sulphuric acid and the optical density was read at 492 nm. The optical density of antigen negative serum spiked with 1 ng of GM per ml provided the cut-off value[116], and a positive and negative control were included with each test.

Results

The results for the 532 serum samples tested by LA and ELISA are shown in Table 1. A total of 325 serum samples were obtained from 33 patients who had a low likelihood of IA, i.e., patients for whom there was no clinical, microbiological, histological or radiological evidence of infection. Three samples were positive in both tests, two samples (1.5%) were positive in the LA test alone, and 22 samples (7.7%) were positive only in the ELISA. There was clinical evidence for IA in the other 28 patients, who had all developed pulmonary infiltrates and had been febrile (temperatures of > 38°C) for three

Table 1. The performance of the sandwich ELISA and the Pastorex LA test for diagnosis of IA

Test		Patient groups					
		Low index of suspicion for IA[a]		IA suspected but unlikely[b]		High index of suspicion for IA[c]	
		N° of patients	N° of positive samples/ N° of samples analyzed	N° of patients	N° of positive samples/ N° of samples analyzed	N° of patients	N° of positive samples/ N° of samples analyzed
LA	+	4	(5/35)	3	(3/27)	7	(16/52)
LA	-	29	(0/290)	15	(0/93)	3	(0/35)
ELISA[d]	+	10	(25/88)	5	(10/41)	9	(58/85)
ELISA	-	23	(0/237)	13	(0/79)	1	(0/2)
ELISA[e]	+	5	(20/52)	3	(8/33)	9	(58/85)
ELISA	-	28	(5/273)	15	(2/87)	1	(0/2)

[a]Patients in this group showed no clinical, microbiological, histological, or radiological evidence of infection. n=33.
[b]Patients in this group showed fever and pulmonary infiltrates, but culture results and courses of infection suggested that IA was unlikely. n=18.
[c]Patients in this group had histopathological, radiological, or microbiological evidence of IA. n=10.
[d]A patient was considered positive when one or more serum samples was positive by ELISA
[e]A patient was considered positive when two or more serum consecutive samples were positive by ELISA.

days despite broadspectrum antimicrobial therapy. For 18 of these patients, the course of infection and the results of cultures suggested that IA was unlikely. With the 120 serum samples from these 18 patients, positive results were obtained for three samples (2.5%) in both tests and 10 samples (8.3%) were positive by ELISA (Table 1). The clinical characteristics of the remaining 10 patients with possible IA (patients showing clinical and radiological evidence for IA but having negative culture results) or proven IA (patients showing histopathological evidence of tissue invasion at autopsy and having positive *Aspergillus* culture results) are shown in Table 2, together with the results of the analyses of 87 serum samples from these patients. GM was not detected by either test in any of the serum from patient 1, who had a proven infection. Very low levels of GM in serum, due to limited fungal invasion of the pulmonary blood vessels or the release of low levels of GM by the fungus into the body fluids, may have contributed to the false-negative result for this patient. For the entire patient population, the sensitivity and specificity of the LA test were 70 and 86%, respectively, and the sensitivity and specificity of the ELISA were 90 and 71%, respectively. When a true-positive result for the ELISA was defined as two consecutive positive serum samples, the sensitivity was similar (90%) but the specificity increased to 84%.

Discussion

Diagnosing IA at an early stage of infection is difficult. The culture of *Aspergillus* species from the sputa or bronchoalveolar lavage fluids of patients at high risk for IA is highly indicative of infection, but the diagnostic reliability of culture is low[81]. Therefore, more sensitive methods, such as PCR[78,97,111,119] and *Aspergillus* antigen detection[7,31,44,47,57,64,75,114,128,137], are under investigation. The detection of GM by the Pastorex *Aspergillus* LA test has been evaluated at several institutes[7,31,44,47,57,75,128,137], and the test showed sensitivies of up to 95% with serum samples from patients with a high index of suspicion for IA[31,44]. The LA test was also found to yield positive results earlier than conventional tests for 68% of patients with proven IA[44]. However, these observations were not confirmed by others[7,47,57,75,128,137], and a sensitivity of only 38% has been reported[75]. In our study, the

Table 2. Clinical characteristics and serum analyses of 10 neutropenic patients with proven or possible IA

Pat. No	Sex[a]/Age (yr)	Underlying condition(s)[b]	*Aspergillus* infection[c]	No. of serum samples tested	No. of positive by LA	No. positive by ELISA	Time (days) between fever and positive test by[d]: LA	ELISA
1.	F/33	AML	Proven, bronchopneumonia	2	0	0	-	-
2.	M/30	AML	Proven, cavitating bronchopneumonia	19	5	17	+10	+7
3.	F/44	AML, BMT	Proven, bronchopneumonia	5	3	5	+12	+9
4.	F/60	Kindney transplant	Proven, disseminated	2	1	2	+14	+9
5.	F/51	MDS, BMT	Proven, disseminated	12	1	7	+10	+7
6.	M/58	Hepatocellular carcinoma	Proven, bronchopneumonia	2	2	2	+4	+4
7.	M/53	CML, BMT	Possible	9	1	8	+16	+13
8.	F/50	CML, BMT	Possible	15	0	6	-	-5
9.	M/38	CML, BMT	Possible	18	0	8	-	-4
10.	M/37	AIDS	Possible	3	3	3	+90	+90
Total				87	16	58		

[a]F,female; M, male.

[b]AML, acute myeloid leukemia; BMT, bone marrow transplantation; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia. All patients died.

[c]"Proven" refers to patients with histopathological evidence of tissue invasion and positive *Aspergillus* cultures. "Possible" indicates that cultures from sputa or bronchoalveolar lavage fluids were negative for *Aspergillus* species but clinical and radiological evidence for IA was present.

[d]Values are numbers of days elapsed between the first day of fever (temperature of > 38.5°C) and a positive result by either test. Patient 9 developed severe graft-versus-host disease (III) and was treated with high doses of corticosteroids. With patient 10, a fever of unknown origin was present for three months, but three days before the first serum sample was obtained, respiratory failure developed and infiltrates were demonstrated on the chest roentgenogram.

LA test yielded positive results only during advanced stages of infection in most patients with suspected IA and did not attribute to an earlier diagnosis[128]. The present study showed that the ELISA detected GM in serum up to five days earlier than the LA test did. Although both LA and ELISA failed to detect one proven infection, the ELISA detected GM in two additional patients for whom the LA test continued to yield negative results. Moreover, GM was detected by ELISA in more serum samples by ELISA than by LA. This suggests that monitoring sequential serum samples from high-risk patients during neutropenia may allow the diagnosis of IA to be made at an earlier stage of infection.

The increased sensitivity of the ELISA, however, was associated with an increase in false-positive results. A rate of 8% false positives by ELISA has been found by others[116]. Therefore, in order to identify a genuine elevation of the GM level in serum, positive ELISA results should be found in at least two consecutive serum samples.

Our results suggest that antigen detection at regular intervals by the sandwich ELISA may allow the early diagnosis of IA in immunocompromised patients. Twice-weekly collection and testing of serum samples from a patient during the period of neutropenia should be sufficient to detect an increase in the GM level in serum early in the course of infection. In the case of a positive ELISA result, the collection and testing of serum samples should be continued in order to exclude the possibility of a false-positive result. Furthermore, confirmation of suspected IA should be obtained by chest roentgenogram, computer tomography, or bronchoalveolar lavage. Prospective studies following these guidelines are needed to establish the clinical value of this new ELISA.

Acknowledgements

We thank Marc Tabouret, Sanofi Diagnostics Pasteur, for the collaboration and Dr. J. Peter Donnelly, University Hospital Nijmegen, for discussion and revision of the manuscript.

COMPARISON OF ANTIGEN DETECTION AND PCR ASSAY USING BRONCHOALVEOLAR LAVAGE FLUID FOR DIAGNOSING INVASIVE PULMONARY ASPERGILLOSIS IN PATIENTS RECEIVING TREATMENT FOR HEMATOLOGICAL MALIGNANCIES

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J Clin Microbiol 1995;33:3150-3.
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Summary

The performance of a sandwich enzyme-linked immunosorbent assay (ELISA) which detects *Aspergillus* galactomannan (GM) was evaluated in bronchoalveolar lavage (BAL) fluid samples from 19 patients who were treated for hematological malignancies and who were suspected of having invasive pulmonary aspergillosis (IPA). All patients had fever and pulmonary infiltrates on the chest roentgenogram on the day that the BAL fluid was obtained. The ELISA results were compared with the results of culture and *Aspergillus* genus-specific PCR analysis of BAL fluid samples. ELISA was also performed with serum samples. *Aspergillus* species were detected by PCR or ELISA with BAL fluid samples from five of seven patients who had radiological evidence of IPA. Serum ELISA results were positive for all patients with ELISA-positive BAL fluid, and for four patients the serum ELISA was positive before the BAL fluid was obtained. PCR and ELISA were positive for 2 and 1 of 10 BAL fluid samples, respectively, obtained from patients without radiological evidence of IPA, and 5 and 2 of 35 BAL fluid samples, respectively, obtained from nonneutropenic patients. This preliminary investigation suggests that GM may be detected by ELISA in BAL fluid samples from patients at risk of IPA, but that monitoring of serum GM levels may allow for the earlier diagnosis of IPA. However, further evaluation in prospective studies is required.

Introduction

Invasive pulmonary aspergillosis (IPA) is a major threat to leukemic patients with cytotoxic therapy-induced neutropenia and transplant recipients receiving high-dose corticosteroid therapy. Since the diagnosis of IPA in an early stage is seldom possible and the mortality rate is very high[14], successful treatment is directly related to early diagnosis[1]. Several methods for obtaining an early and rapid diagnosis of IPA are now under study. *Aspergillus* DNA can be detected in bronchoalveolar lavage (BAL) fluid by PCR[78,97,119,127], but the numbers of patients and controls tested have been too limited to establish the diagnostic value of this technique. An alternative approach is to detect

circulating *Aspergillus* antigen in body fluids, e.g., serum or urine, by a number of in-house methods[64,101,102]. The *Aspergillus* antigen test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) is commercially available and uses the rat monoclonal antibody EB-A2 to detect galactomannan (GM), a polysaccharidic antigen of *Aspergillus* species[69]. This antigen test has been evaluated in several institutes[7,44,47,128,137], but it has been found to lack sufficient sensitivity[44,47,128,137]. A recently developed sandwich enzyme-linked immunosorbent assay (ELISA) allowed the detection of low levels of circulating *Aspergillus* GM in sera from patients at high risk of IPA, but the increase in sensitivity was also associated with false-positive results in up to 8% of the serum samples[116,129]. Since IPA is predominantly a pulmonary infection in immunocompromised patients, and *Aspergillus fumigatus* is known to release GM into the culture medium when it is grown in vitro[69], detectable antigen levels may be present in BAL fluid samples from patients suspected of having IPA and therefore may be of use for the diagnosis of IPA.
We therefore investigated the use of the sandwich ELISA to detect GM with BAL fluid samples which had been collected from patients at high risk and from patients at low risk of IPA. The results were compared with those obtained by culture and *Aspergillus* genus-specific PCR amplification and were related to the clinical outcome.

Materials and Methods

Patients

During a 12 month period 19 consecutive BAL fluid samples had been collected from patients who were neutropenic as a result of cytotoxic therapy or from whom circulating granulocytes were recovered. In each patient a bronchoscopy was performed because fever (temperature, > 38.3°C) persisted despite treatment with broad-spectrum antibacterial agents and pulmonary infiltrates developed on the chest roentgenogram. The BAL fluid sample was obtained by wedging a flexible bronchoscope in the area of maximal roentgenographic involvement, after which 150 ml of sterile 0.9% saline was instilled and approximately 100 ml was recovered by suctioning. The medical, histopathological, microbiological, and radiological records of all patients were reviewed for evidence of

IPA or other respiratory disease. Since a definitive diagnosis of IPA is very difficult to establish in this patient group, the patients were allocated to three categories by review of the appearance of the pulmonary infiltrates on the chest roentgenogram by two physicians who were blinded to the medical histories of the patients. The patients were allocated to one of the following groups: A, probable IPA (n = 7), with the chest roentgenogram showing focal nonanatomical infiltrates or any cavitating lesion; C, unlikely IPA (n=10), with the chest roentgenogram showing diffuse infiltrates characterized by nodular, reticular, or reticulonodular lesions which were distributed throughout both lung fields or anatomical focal infiltrates[73]; and B, possible IPA (n=2), which included patients with infiltrates on the chest roentgenogram who could not be classified into group A or C. Of course, the reliability of these definitions is limited because the roentgenographic pattern is not always specific for the infectious etiology of the infiltrates. Furthermore, 35 BAL fluid samples were collected from nonneutropenic patients who were at low risk for IPA.

Sample collection

BAL fluid samples were routinely examined for the presence of bacteria, viruses, fungi, and parasites. A total of 5 to 10 ml of each sample was stored at -80°C until analysis by ELISA and PCR. The microbiological records were reviewed for serum samples which had been collected from each patient between 6 weeks before and 2 weeks after the BAL fluid sample was obtained. These samples had also been stored at -80°C until testing by ELISA was performed.

Aspergillus *Culture*

Routine culture for fungi was performed after centrifugation of the BAL fluid sample at 1,500 x *g* for 10 min. The pellet was plated onto Sabouraud glucose (2%) agar and was cultured for 5 days at 28°C and 48°C. *Aspergillus* species were identified by one technician (A.J.M.M.R.) by their cultural characteristics and the morphologies of their conidiophores and conidia.

PCR amplification

After thawing, the BAL fluid samples were centrifuged at 1,500 x *g* for 10 min. The supernatant was separated from the pellet, leaving a residue of 0.5 ml in which the pellet was resuspended. PCR analysis was performed as descibed previously[78,127]. Briefly, high frequency shaking was performed to achieve complete cell destruction; this was followed by purification of nucleic acids by guanidium thiocyanate-containing lysis buffer and silica particles. A hot-start PCR was performed using two *Aspergillus* genus-specific primers. This PCR assay can detect a wide range of the *Aspergillus* species involved in human disease specifically when it is combined with Southern blot hybridization and restriction enzyme analysis[78].

ELISA

The sandwich ELISA used to detect *Aspergillus* GM in serum samples and BAL fluid supernatants was performed exactly as described previously[116]. Saline 0.9% and culture- and PCR-negative BAL fluid supernatants from patients at low risk of IPA containing a range of dilutions of GM (range: 0.25 to 10 ng/ml) and a negative control BAL fluid were included in each test. All serum and BAL fluid samples were tested in duplicate by one technician who was unaware of the clinical data. The microtiter plates were prepared by the manufacturer (Sanofi Pasteur Diagnostics), and the coating conditions of the plates are proprietary. However, since the coating conditions of the EB-A2 monoclonal antibody on the microtiter plate were different from those reported previously[116], lower optical density values were found.

Results

Aspergillus *ELISA*

Different concentrations of GM (range: 0.25 to 10 ng/ml) were added to 0.9% saline and culture- and PCR-negative BAL fluid samples from low-risk patients and were analyzed by ELISA. The degree of increase in the optical density values of 0.9% saline spiked with increasing concentrations of GM was directly proportional to that found with culture-

and PCR-negative BAL fluid samples spiked with the same concentrations of GM (data not shown). The mean optical density values of the 30 BAL fluid samples from nonneutropenic patients with negative fungal culture and PCR results were used to study the ELISA background. There was a Gausian distribution of the optical density values, allowing a cutoff value of 0.17 to be calculated from the mean optical density value (mean optical density value, 0.09; range, 0.056 to 0.146) plus 4 standard deviations. The optical density of control serum spiked with 1 ng of GM per ml was used as cutoff value for serum (optical density value, 0.17)[116]. The results of *Aspergillus* genus-specific PCR and ELISA for the BAL fluid samples from 19 patients with hematological malignancies and the BAL fluid samples from 35 control patients are given in Table 1.

Clinical data

The characteristics of the 19 patients with hematological malignancies and the results of culture, PCR, and ELISA are given in Table 2. Five of seven BAL fluid samples from patients with probable IPA were positive by PCR and ELISA. GM was detected in serum samples from all patients with an ELISA-positive BAL fluid sample. Moreover, serum samples were available from the period before the bronchoscopy was performed for four patients, and GM could be detected up to 30 days before the BAL fluid sample was obtained (patient A5; Table 2).

Table 1. *Results of* Aspergillus *genus-specific PCR amplification and sandwich ELISA of BAL fluid samples obtained from 19 patients with hematological malignancies and 35 nonneutropenic patients.*

Microbiological analysis result	No. of patients	
	Hematologic patients	Nonneutropenic patients
PCR positive, ELISA positive	5	0
PCR positive, ELISA negative	3	5
PCR negative, ELISA positive	2	2
PCR negative, ELISA negative	9	28
Total	19	35

Shortly after bone marrow transplantation during severe neutropenia, patient A5 developed fever and sinusitis. The administrationof ciprofloxacin and amoxicillin-clavulanic acid did not result in clinical improvement, and amphotericin B (1 mg/kg of body weight per day) was added to the regimen. During recovery of the granulocytes, a chest-roentgenogram showed the development of a nodular infiltrate. Although culture of sinus aspirate and BAL remained negative, PCR and ELISA of BAL fluid samples were positive, and GM was retrospectively detected in the serum at the time that the sinusitis first became apparent.

Both PCR and ELISA were positive for one of two BAL fluid samples from patients with possible IPA, but serum samples were not available for analysis by the ELISA. The PCR was positive for BAL fluid samples from patients C1 and C2, who had no evidence of the presence of IPA, and GM was not detected in the BAL fluid or serum samples from these patients. GM was detected in a PCR-negative BAL fluid sample from patient C3, and two of seven serum samples were also found to be positive. The two ELISA-positive serum samples were obtained on the day that the BAL was performed and 1 day thereafter, respectively. Five consecutive serum samples obtained during 2 weeks following the BAL were all negative, even though severe neutropenia persisted and at the time of the seroconversion only a loading dose of 10 mg of amphotericin B had been administered. Treatment with ciprofloxacin and amphotericin B (1 mg/kg/day) for 2 weeks resulted in resolution of the pulmonary infiltrates.

Nonneutropenic patients

PCR analysis of 35 BAL fluid samples from nonneutropenic patients revealed positive amplification reactions for five samples which were all negative by ELISA[127]. The ELISA was positive for 2 of 30 PCR-negative BAL fluid samples (Table 1).

Table 2. Characteristics of 19 patients with hematological malignancies and results of analysis of BAL fluid samples by culture and Aspergillus genus-specific PCR, and BAL fluid and serum by ELISA.

Category	Patient No	Sex/age (yr)	Underlying disease or condition(s)	BAL fluid analysis			No of samples positive by serum ELISA/ total no. of samples	Time between first positive serum and BAL[a]	Granulocyte count (10^9/liter)	Outcome
				Culture	PCR	ELISA				
A	1	M, 43	NHL	*Aspergillus fumigatus*	+	0.581 (+)	3/3	-4	3.6	Deceased
	2	M, 36	NHL	*Aspergillus fumigatus*	+	0.278 (+)	3/4	-3	5.8	Deceased
	3	M, 48	HL	*Aspergillus fumigatus*	+	0.128 (-)	0/3		2.5	Survived
	4	M, 54	AML	*Stenotrophomonas malthophilia*	+	1.322 (+)	1/1		<0.1	Survived
	5	M, 22	AA, BMT	*Citrobacter freundii, Klebsiella pneumoniae*	+	0.370 (+)	25/28	-30	<0.1	Survived
	6	M, 58	MDS, BMT	Negative	-	0.247 (+)	3/11	-11	2.1	Survived
	7	M, 20	Myelofibrosis	*Pseudomonas aeruginosa, Candida albicans*	-	0.095 (-)	NA		<0.1	Deceased
B	1	M, 59	AML	Negative	+	0.736 (+)	NA		<0.1	Survived
	2	M, 68	NHL	*Candida albicans*	-	0.113 (-)	NA		9.2	Deceased
C	1	M, 48	AML, BMT	*Candida glabrata*	+	0.152 (-)	0/1		<0.1	Survived
	2	F, 36	AML	Negative	+	0.122 (-)	NA		<0.1	Deceased
	3	M, 34	CML, BMT	*Candida albicans*	-	0.220 (+)	2/7		<0.1	Survived
	4	F, 52	MM	Negative	-	0.093 (-)	0/1		<0.1	Deceased
	5	F, 43	ALL, BMT	*Candida albicans*	-	0.075 (-)	0/5		3.8	Deceased
	6	F, 40	AML	Negative	-	0.077 (-)	0/1		2.1	Survived
	7	F, 44	MM	Herpes simplex virus	-	0.087 (-)	0/1		3.5	Survived
	8	F, 62	CLL	*Pneumocystis carinii*	-	0.079 (-)	NA		2.0	Deceased
	9	F, 56	AML	*Candida albicans*	-	0.114 (-)	0/2		<0.1	Deceased
	10	F, 72	AML	Negative	-	0.098 (-)	0/2		<0.1	Deceased

[a]Patients from whom at least one serum sample was taken within six weeks before the BAL was obtained. Values are numbers of days between the first serum sample found positive by ELISA and date of bronchoscopy.

Discussion

The diagnosis of IPA in an early stage of the disease is of great importance since early antifungal treatment is associated with an improved outcome[1]. The early diagnosis of IPA is difficult, and a definitive diagnosis is often made at autopsy. The presence of severe thrombocytopenia in neutropenic patients limits the use of invasive diagnostic procedures to obtain a specimen for culture and histopathological examination. BAL has been advocated as a safe procedure to obtain a specimen for the diagnosis of IPA in immunocompromised patients with pulmonary infiltrates. Although the recovery of *Aspergillus* species from BAL fluid is highly indicative of IPA, the diagnostic yield from culture of BAL fluid samples is only 30%[126]. Therefore, attention has focussed on other techniques for the early diagnosis of IPA, such as *Aspergillus*-specific PCR in BAL fluid[18,78,97,119] and the detection of circulating *Aspergillus* antigen in serum or urine[7,44,47,64,101,102,116,128,137]. Little is known about the utility of *Aspergillus* antigen detection in BAL fluid. One research group has previously described the use of a radioimmunoassay to detect an uncharacterized purified *A. fumigatus* carbohydrate in BAL fluid[4,5,140]. The radioimmunoassay detected *Aspergillus* antigen in 91% of the BAL fluid samples from rabbits with IPA, and in 27% of these animals antigen was detected in BAL fluid only and not in serum[4]. The radioimmunoassay was also used to detect *Aspergillus* antigen in BAL fluid samples from nine immunocompromised patients with pulmonary infiltrates, and two patients were found to be positive[5]. GM was detected by a latex agglutination test (Pastorex *Aspergillus*) in BAL fluid samples from three patients with AIDS and IPA[72]. However, GM could not be detected by the same test under laboratory conditions in BAL fluid samples from 14 untreated rabbits with IPA, while serum samples from 10 (71%) animals were positive[39]. Although the present study was limited by the difficulty in reliably classifying patients with IPA, GM was detected by sandwich ELISA in BAL fluid samples from five of seven patients with radiological evidence of IPA, and therefore, this test may be promising for use in the diagnosis of this infection.

The increase in the level of sensitivity required to diagnose early IPA, however, is hampered by an increase in the number of false-positive results. For this reason, it was

interesting to compare DNA and GM detection, since these two assays have a different target. In the case of diagnosis by PCR, a positive result obtained from the use of a BAL fluid sample can be due to the presence of contaminating conidia without any mycelial development (see, for example, data for patients C1 and C2). Indeed, previous studies found a rate of 15% positive PCR amplification in BAL fluid samples from nonneutropenic patients[119,127]. The occurence of a negative PCR result for patient A6, whose BAL fluid and serum samples were and who had radiological and clinical evidence of IPA, could be due to the presence of inhibitors of the PCR reaction in the BAL fluid[120].

False-positive results were also obtained by the sandwich ELISA. The reasons for the false-positive reactions are unknown, but it has been shown that molecules which are responsible for the false positivity appear to mimic the epitope recognized by anti-GM monoclonal antibodies[116]. In 30 culture- and PCR-negative BAL fluid samples from nonneutropenic patients, a 7% false-positive rate was found, which is similar to the 8% false-positive rate found in serum[116]. A 10-fold concentration of the samples by acetone precipitation resulted in a false-positive rate of 27% (data not shown). Analysis by PCR and ELISA can be done in less than 24 h (PCR, 24 h; ELISA, 4 h), and the results can be forwarded to the clinician on the day following the BAL.

An excellent correlation was found between serum and BAL fluid ELISA results, and GM was detected in the serum even before the BAL was performed. Since a bronchoscopy was performed only after pulmonary infiltrates were present on the chest roentgenogram, GM may be detectable in the serum before infiltrates become visible on the plain chest film. It is has been shown that in neutropenic patients abnormalities are difficult to detect on plain chest film[87] and that other radiologic methods, such as high resolution computed tomography, are more sensitive for the detection of pulmonary infiltrates in these patients[61,62] and may even be used for therapeutic monitoring[136].

This preliminary investigation suggests that both *Aspergillus* genus-specific PCR and sandwich ELISA might be beneficial for use in the early diagnosis of IPA in patients with hematological malignancies. The ELISA is relatively easy to perform, and the results are available within 4 h. Since false-positive results may be found by ELISA of the serum, detection of *Aspergillus* species in BAL fluid by ELISA or PCR may provide additional

evidence for the presence of IPA. The results of the present study also suggest that in some patients GM antigenemia may be detected in serum before pulmonary infiltrates are visible on the chest roentgenogram. However, carefully designed prospective studies need to be performed in order to evaluate the diagnostic value of these assays. We are prospectively monitoring serum GM levels by ELISA in patients during cytotoxic therapy-induced neutropenia. When GM is detected for the first time, confirmation is sought by ELISA with additional serum samples, high-resolution computed tomography scan, and where possible, bronchoscopy and PCR analysis of BAL fluid. Once the presence of GM is confirmed or the signs or symptoms pathognomonic for IPA have developed, treatment with antifungal agents is started preemptively. By following these guidelines, the diagnosis and treatment of IPA may be possible at an early stage of infection.

IMMUNOHISTOCHEMICAL DETECTION OF *ASPERGILLUS*

IMMUNOPEROXIDASE STAINING FOR THE DETECTION OF *ASPERGILLUS* SPECIES IN ROUTINELY PROCESSED TISSUE SECTIONS

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Submitted for publication

Summary

The performance of an immunoperoxidase stain which employs the monoclonal antibody EB-A1 to detect *Aspergillus* species in formalin-fixed, paraffin-embedded tissue was evaluated. The monoclonal antibody EB-A1 directed against galactomannan was used to detect *Aspergillus* species in 23 patients with suspected or proven invasive aspergillosis. Immunostaining was performed on formalin-fixed paraffin-embedded tissue using the streptavidin-biotin method and compared to conventional hematoxylin and eosin, periodic acid-Schiff, and Gomori methenamine silver stain. Results of immunostaining were semi-quantitatively analyzed with regard to both intensity of staining and number of positively staining microorganisms. Tissue sections from 16 patients with proven invasive mycoses due to *Candida* species, *Apophysomyces elegans*, *Rhizopus oryzae*, *Pseudallescheria boydii* and *Histoplasma capsulatum* were used as controls. In 19 out of 23 cases invasive aspergillosis was proven by both histological examination and culture (18x *Aspergillus fumigatus* and 1x *A. flavus*). Immunoperoxidase stains were positive in 17 cases (89%) including one case of disseminated infection due to *A. flavus*. Furthermore, the immunoperoxidase stain was positive in a culture negative tissue section with histological evidence for mycelial development, indicating the presence of *Aspergillus* species. Some cross-reactivity was observed with the highly related fungus *P. boydii*, although the number of mycelial elements staining was low. In general, detection of microorganisms with the conventional methods was more difficult than after immunostaining. Immunoperoxidase staining using the monoclonal antibody EB-A1 performs well on routinely processed tissue sections and allows detection and generic identification of *Aspergillus* species. An additional advantage is that it may help to provide an etiological diagnosis when cultures remain negative.

Introduction

Aspergillus species can cause severe pulmonary infections in immunocompromised patients[13]. The antemortem diagnosis of invasive aspergillosis is difficult and, although

new sensitive diagnostic methods are being evaluated[78,116,130], a definitive etiological diagnosis can be made only by the demonstration of tissue invasion by fungal mycelium and a positive culture with morphological identification of *Aspergillus* species. The diagnosis of invasive aspergillosis by histological examination of tissue sections alone is not reliable, because numerous filamentous fungi cannot be differentiated from *Aspergillus* organisms on morphological criteria. However, a correct etiological diagnosis is important for the correct antimycotic therapy of systemic mycoses and for the study of fungal epidemiology. Furthermore, the necessity for an etiological diagnosis will increase as new fungal pathogens, such as *Alternaria* species[3,142], have been reported to cause invasive infections in immunocompromised patients. To obtain a rapid and accurate generic identification, both poly-[89] and monoclonal[9,58,80,89] antibodies have been employed for direct immunoperoxidase staining of tissue sections. Previous observations suggested that the monoclonal antibody EB-A1 directed to galactomannan was useful in the diagnosis of infections resulting from *Aspergillus* species[9]. In this study we have evaluated the performance of this monoclonal antibody to detect and identify *Aspergillus* species in tissue sections obtained from patients with invasive mycoses.

Materials and Methods

Between 1989 and 1991 pathology and microbiology records from the University Hospital Nijmegen were reviewed for consecutive autopsy cases with invasive aspergillosis. In each case identified, the medical records were reviewed for clinical and radiologic evidence of invasive aspergillosis. In addition, tissue sections were obtained from cases with proven invasive infections due to other filamentous fungi and *Candida* species, in order to evaluate the specificity of the immunoperoxidase stain.

All tissue specimens were fixed in 10% formalin solution, and processed through paraffin. Four μm paraffin sections were cut for conventional stain techniques including hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Gomori methenamine silver (GMS), and immuno-histochemistry. Immunohistochemical staining was performed with the streptavidin-biotin-peroxidase-complex method. In short, sections were cut and

rehydrated, endogenous peroxidase activity was blocked (by incubation in 1% H_2O_2), after which sections were incubated for 20 minutes in normal goat serum (diluted 1:5 in phosphate-buffered saline; PBS). The slides were then incubated with 20 μg/ml of the primary antibody (EB-A1, Eco-Path Aspergillus, Sanofi Pasteur Diagnostics, Genk, Belgium) for 60 minutes at room temperature. As a detection system, we used Multilink I (BioGenex, San Ramon, California, USA) followed by streptavidine-peroxidase conjugate (BioGenex). As a chromogen, we used a diaminobenzadine solution containing 0.65% imidazol. The staining was intensified by incubation in 0.5% $CuSO_4$ and 0.9% NaCl for 5 minutes. Finally, sections were weakly counter-stained with Mayer's hematoxylin, dehydrated and mounted. As a negative control, sections were incubated with PBS instead of the primary antibody.

Tissue sections were examined for the presence of the characteristic brown staining and semiquantitatively evaluated by estimating the number of stained mycelial elements in the following categories: no staining and only a few scattered positive elements (less than 5%) was considered negative (-), staining 5% to 25% of elements (+), staining in 25 to 50% (++), staining in 50% to 75% (+++), and staining in 75% to 100% (++++). The intensity was graded on a scale of negative to +++, (-) indicated absence of staining; mild staining (+); moderate staining (++); and intense staining (+++). Equivocal reactions were considered negative. Furthermore, any background staining including aspecific staining of cells or tissues was noted seperately. All slides were scored independantly by two of the authors (P.V., F.S.)

Results

A total of 40 tissue sections from 39 patients with either suspected or autopsy proven invasive fungal infection were studied. The anatomic sites of fungal infection and the results of histologic examination and culture are listed in Table 1. Twenty-three patients had either a proven or suspected infection with *Aspergillus* species. The clinical characteristics of these patients and the results of culture and immunoperoxidase staining are shown in Table 2.

Table 1. *Histologic findings, microbiological identification, number of cases and sites of involvement of invasive* Aspergillus *infection in 23 autopsy cases and 16 controls.*

Histologic findings	Culture	Number of cases	Sites of infection
mycelial elements	*Aspergillus fumigatus*	18	diss. 6; lung 12
mycelial elements	*A. flavus*	1	diss.
mycelial elements	negative	1	lung
negative	*A. fumigatus*	3	lung
pseudo hyphae	*Candida* spp.[a]	12	diss. 6; lung 5; tongue 1
mycelial elements	*Zygomycetes*[b]	2	lung 1; humerus 1
mycelial elements	*Pseudallescheria boydii*	1	lung
yeast form	*Histoplasma capsulatum*	1	diss.

[a]including *C. albicans* (10 cases), *C. tropicalis* (one case), and *C. parapsilosis* (one case).
[b]including *Apophysomyces elegans* and *Rhizopus oryzae*.

In 19 of 23 cases there was evidence of disease based on histopathology and culture, including seven cases of disseminated infection (cases 1 to 7). All tissue sections obtained from the cases with disseminated infection showed intense hyphal immunostaining (++++/+++, Figure 1A), including one case of *A. flavus* infection (case 7, Figure 1C). One patient (case 8) with acute myeloblastic leukemia died of respiratory insufficiency, and invasive pulmonary aspergillosis was diagnosed at necropsy. The presence of *Aspergillus* species was confirmed by both culture and immunoperoxidase stain. However, in this patient histologic examination of a pulmonary thrombus revealed mycelial elements. Culture yielded *Rhizopus microsporum var rhizopodiformis* and the immunostaining was negative. Another patient (case 11) with chronic myeloid leukemia died of septicemia and respiratory insufficiency. At autopsy a bronchopneumonia was diagnosed in the lower lobe of the left lung with mycelial elements in the GMS stain. Although cultures remained negative, the immunostaining indentified *Aspergillus* species.
A total of 18 out of 20 (90%) tissue sections with mycelial development were positive by the immunoperoxidase method. In three patients (cases 21 to 23) a bronchopneumonia was diagnosed at autopsy but, although culture of the lung tissue yielded *A. fumigatus*,

Table 2. *Clinical characteristics of 23 patients with invasive aspergillosis and results of culture and immunohistochemistry.*

Case	Sex/Age	Underlying disease	*Aspergillus* Infection	Culture	Immuno-peroxidase
1.	M/41	Myelodysplastic syndrome	Diss	*Aspergillus fumigatus*	++++/+++
2.	M/51	ALL	Diss	*A. fumigatus*	++++/+++
3.	F/1.5	Pearsons' Syndrome	Diss	*A. fumigatus, C. albicans*	++++/+++
4.	F/66	Grawitz tumor	Diss	*A. fumigatus*	++++/+++
5.	M/72	AML	Diss	*A. fumigatus*	++++/+++
6.	F/45	AML, BMT	Diss	*A. fumigatus*	++++/+++
7.	F/29	SLE	Diss	*A. flavus*	++++/+++
8.	M/60	AML	lung	*A. fumigatus, Rhizopus microsporon var rhizopodiformis*	++++/+++
9.	F/15	Agammaglobulinemia	lung	*A. fumigatus*	-
10.	M/58	AML	lung	*A. fumigatus*	-
11.	M/51	CML	lung	negative	++++/+++
12.	M/32	AML	lung	*A. fumigatus*	+++/+++
13.	M/59	Adenocarcinoma lung	lung	*A. fumigatus*	+++/+++
14.	F/15	NHL, BMT	lung	*A. fumigatus*	+++/+++
15.	M/11	CGD	lung	*A. fumigatus*	+++/+++
16.	M/57	Hepatocellular carcinoma	lung	*A. fumigatus*	+++/+++
17.	M/60	Myelodysplastic Syndrome	lung	*A. fumigatus*	++++/+++
18.	M/58	AML	lung	*A. fumigatus*	+++/+++
19.	F/44	AML, BMT (2x)	lung	*A. fumigatus*	+++/+++
20.	M/29	AML	lung	*A. fumigatus*	++++/+++
21.	M/61	HL	lung	*A. fumigatus*	-
22.	M/55	Rheumatoid arthritis, Kidney transplant	lung	*A. fumigatus*	-
23.	M/50	AML	lung	*A. fumigatus*	-

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; BMT, bone marrow transplantation; SLE, systemic lupus erythematodus; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; CGD, chronic granulomatous disease; HL, Hodgkin lymphoma.

mycelial elements could not be detected by conventional fungal staining. In all three cases the immunostaining was also negative.

The cross-reactivity of the monoclonal antibody EB-A1 was tested by staining tissue sections obtained from 16 patients with non-*Aspergillus* mycoses (Table 1), including 12 histopathological and culture proven cases with invasive candidiasis (*C. albicans*, 10 cases; *C. tropicalis*, one case; and *C. parapsilosis*, one case), a patient with pulmonary pseudallescheriasis[113], a patient with *Apophysomyces elegans* osteomyelitis of the humeral bone[77], a patient with a *Rhizopus oryzae* bronchopneumonia, and a patient with a disseminated *Histoplasma capsulatum* infection. No cross-reactive immunostaining was observed with *Candida* species, *A. elegans* (Figure 1B, (-/-)), *R. oryzae* (Figure 1D, (-/-)) and *H. capsulatum*. Immunostaining was observed in *P. boydii* mycelial elements, although the majority of elements showed no reactivity. Background staining was observed to be mild in infections in which there was frank necrosis, and did not hamper the detection of the microorganisms. In vital lung tissue cross-reactivity of the antibody was observed in epithlial cells lining the alveoli and in alveolar macrophages. Immunostaining in these specimens was sometimes quite intense, however, mycelial elements were easily identified on the basis of their very different morphology.

Fungal elements were detected in PAS and GMS staining in all but 2 positively immunostaining cases. In general, detection of microorganisms with the conventional methods was more difficult than after immunostaining.

Discussion

An accurate identification of fungal elements in tissue sections utilizing conventional fungal stains requires considerable knowlegde of fungal morphology, and remains difficult when based on morphologic features alone. This is particulary difficult for histopathologists who are usually not well versed in fungus morphology. Especially the histological differentiation of *Aspergillus* species from *Fusarium* species and *P. boydii* is difficult since these fungi may induce similar clinical features and exhibit filamentous development in host tissue.

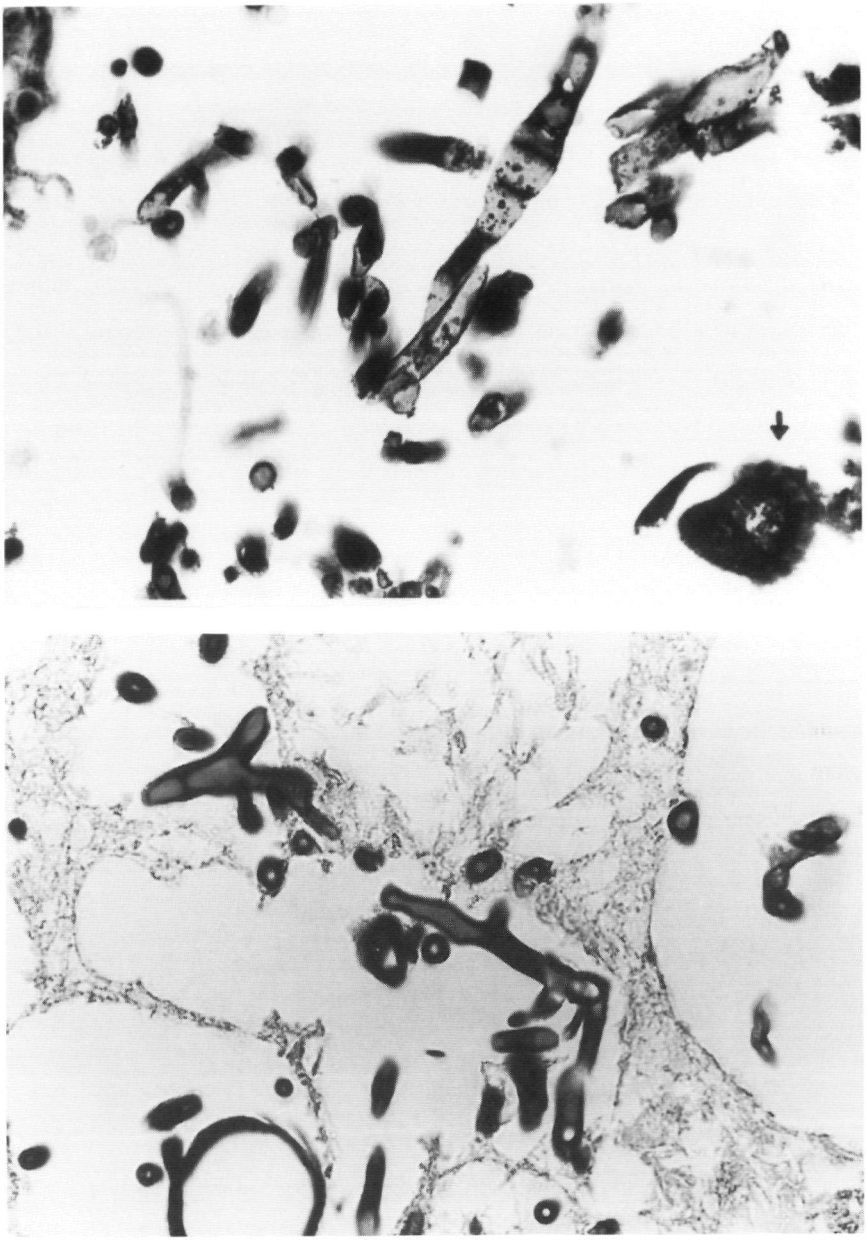
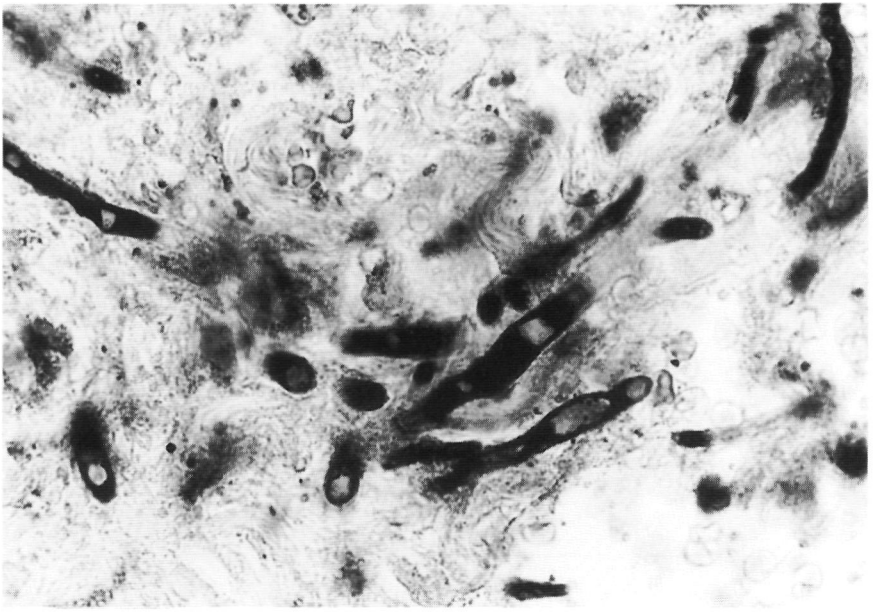


Figure 1. *Immunostaining of formalin-fixed, paraffin-embedded tissue sections with the monoclonal antibody EB-A1. A,* Aspergillus fumigatus *in lung tissue (++++/+++), note intense immunostaining of conidiophore (arrow); B,* Apophysomyces elegans *in necrotic bone*

C



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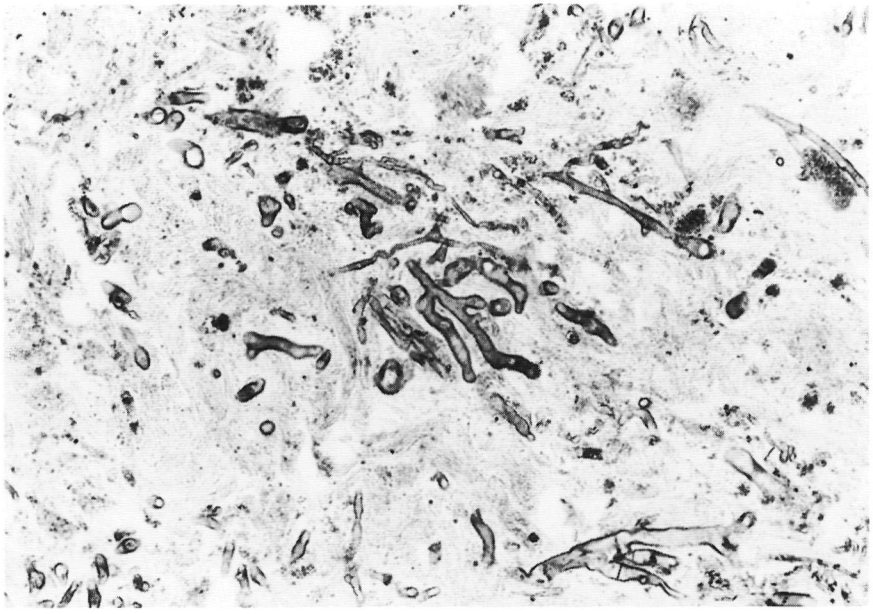

tissue (-/-), microorganism stains due to counterstain with H&E; C, Aspergillus flavus *in lung tissue (++++/+++); D,* Rhizopus oryzae *in lung tissue. No immunostaining (-/-), counterstain with H&E highlights fungal elements.*

Although subtle morphologic features, such as dichotomous branching at 45° of *Aspergillus* mycelium, may help to differentiate this fungus from other filamentous fungi, only a presumptive diagnosis can be made when cultures remain negative. Immunohistochemistry using mono- or polyclonal antibodies capable of detecting specific fungi in tissue sections has been shown to be helpful in this respect[9,10,58,80,89,91]. A drawback however is that only genera of fungi can be differentiated. An advantage of immunostaining is that formalin-fixed, paraffin-embedded sections are used and that the staining method is relatively easy to perform. In the present study, immunoperoxidase staining with EB-A1 proved useful for the generic identification of *Aspergillus* species in tissue sections. The presence of *Aspergillus* organisms was confirmed in 17 of 19 culture positive tissue sections and in one case fungal mycelium was identified as *Aspergillus* species in a culture negative tissue section. Cross-reactivity with three *Candida* species, two *Zygomycete* species, and *H. capsulatum* was not observed.

An important factor in the evaluation of any immunohistochemical method is the estimation of background staining in relation to positively staining microorganisms. We did note low levels of background staining in proteinecious edema fluid in the lungs and also sometimes in exsudates as well as alveolar macrophages. The levels of background staining were low and usually did not deter identification of the fungi. In some cases, however, macrophages did demonstrate higher levels of immunostaining, reflecting the high concentrations of endogenous peroxidases within these cells, again this staining did not make fungal identification more difficult.

Immunofluorescence studies have shown that *Aspergillus*, *Fusarium*, and *P. boydii* are antigenically closely related[54]. Previous observations indicated that the monoclonal antibody EB-A1 does not cross-react with *Fusarium* species[9], but the reactivity with *P. boydii* was not ascertained. Our results suggest that EB-A1 can also differentiate *Aspergillus* organisms from *P. boydii*. Although some cross-reactive staining occured with the *P. boydii* hyphae, the intensity of immunostaining was low and the majority of elements were not immunodecorated.

The monoclonal antibody EB-A1 has been shown to cross-react with *Penicillium marneffei*[9,10] which has some immunodominant epitopes in the cell wall which are

identical to those of *Aspergillus* species[85]. Invasive infections with both *Aspergillus* species[30] and *P. marneffei*[90] have been reported in AIDS patients and therefore the monclonal antibody EB-A1 cannot be used to differentiate these fungi. Since, the tissue form of *P. marneffei* develops as a yeast-like structure[28], differentiation from *Aspergillus* species can be made by histologic examination with conventional fungal stains. However, histologic examination should be interpreted with caution because elongated forms or filamentous development of *P. marneffei* have been reported to occur in the stratum corneum of the skin[10], in liver and lung tissue specimens of an AIDS patient[48], and in pulmonary cavities[28].

In conclusion, immunoperoxidase staining with EB-A1 in formalin-fixed and paraffin-embedded tissue allows a generic diagnosis of invasive infections due to *Aspergillus* species in the case of negative cultures. In routine laboratory practice this method is superior to conventional PAS and GMS staining. However, the results should be interpreted in the whole clinical context of the patient, because false negative results and cross-reactivity with *P. marneffei* may occur.

Acknowlegdement

We thank M. Jeunink for excellent technical assistance.

Chapter 4

CLINICAL IMPLICATIONS

PROSPECTS FOR THE EARLY DIAGNOSIS OF INVASIVE ASPERGILLOSIS IN THE IMMUNOCOMPROMISED PATIENT

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Reviews in Medical Microbiology (in press).

Summary

In the last several years there has been a measure of progress in diagnosing invasive aspergillosis at an early stage of disease. Polymerase chain reaction (PCR) assay's have been found to be more sensitive than culture in detecting *Aspergillus* species in bronchoalveolar lavage (BAL) fluid specimens but the diagnostic reliability may be diminished by false-positive reactions obtained from patients colonized with *Aspergillus* conidia or by laboratory contamination. *Aspergillus* antigens can be detected by latex agglutination (LA) test but the commercial Pastorex *Aspergillus* (Sanofi Diagnostics Pasteur, France) was found to be too insensitive to diagnose invasive aspergillosis early. This same company therefore developed a sandwich ELISA using the same monoclonal antibody to galactomannan which was almost 10 times more sensitive than the LA test, and detected *Aspergillus* antigen in the serum at an earlier stage of infection. The development of sensitive diagnostic methods such as these offers an opportunity for optimising diagnosis and improving the management of invasive aspergillosis. Patients at high risk can be identified by regular, serial monitoring of serum samples during the period of greatest risk, and, if positive, the diagnosis can be substantiated by pulmonary CT-scan and PCR analysis of BAL fluid and treatment with antifungal agents can be initiated pre-emptively. By allowing selective targeting of patients for antifungal therapy to pre-empte invasive aspergillosis, the outlook for patients at risk should be improved in terms of both morbidity and mortality.

Introduction

The fungus *Aspergillus* is an unbiquitous saprophyte which can cause opportunistic infections in immunocompromised patients. *Aspergillus fumigatus* is the most common species encountered in human disease. Before infections can occur, *Aspergillus* conidia must be inhaled and lodge in the airways. Germination can occur in the paranasal sinuses but most often takes place in the alveoli of the lung when the number of alveolar macrophages is decreased or their ability to kill is severely impaired. Once mycelia develop and invade the adjacent tissue, pulmonary infection results which may dissemina-

te to other organs. Those at high risk of invasive aspergillosis include patients who are neutropenic as a result of cytotoxic therapy, those receiving prolonged courses of high-dose corticosteroids, recipients of organ transplants, and patients with AIDS. The incidence of infection and the period of greatest risk are listed in Table 1 although the overall incidence of invasive aspergillosis, even in those most predisposed to infection is variable and often unpredictable. Nonetheless, the disease is potentially fatal and is therefore a serious complication especially when it threatens the survival of patients with an otherwise good life expectancy.

Several strategies can be adopted to reduce the morbidity and mortality associated with invasive aspergillosis most important of which, is to reduce exposure of immunocompromised patients to conidia by air filtration[110]. The administration of antifungal agents for prophylaxis in order to prevent germinated *Aspergillus* conidia from invading tissue may also be effective although convincing data are lacking[143]. Attempts should be made to reduce other risk factors, such as restricting the use of broad-spectrum antibiotics[2], or minimising the duration of granulocytopenia by administering hematopoietic growth factors[15].

Table 1. *Incidence of invasive aspergillosis in susceptible patient groups and time period of greatest risk of infection.*

Host group	Incidence of invasive aspergillosis	Time period of highest risk (days)	Reference
Allogeneic bone marrow transplantation	3.8 - 8.7%	0 - 90 P[a]	49,76
Autologous bone marrow transplantation	0 - 4.5%	0 - 90 P	49,76
Peripheral stem-cell bone marrow transplantation	5.6%	0 - 90 P	49
Kidney transplantation	0 - 2.8%	30 - 90 P	121,138
Liver transplantation	1.5 - 14.7%	14 - 100 P	25,65,66
Heart-lung transplantation	3 - 19%	9 - 90 P	60
Heart transplantation	0 - 24%	12 - 45 P	46
HIV/AIDS	0.3%	-[b]	30,56,72,93
Cytotoxic-therapy induced granulocytopenia	up to 70%	> 22	41,109

[a]P = posttransplant.
[b]Advanced stages of AIDS in addition to specific risk factors.

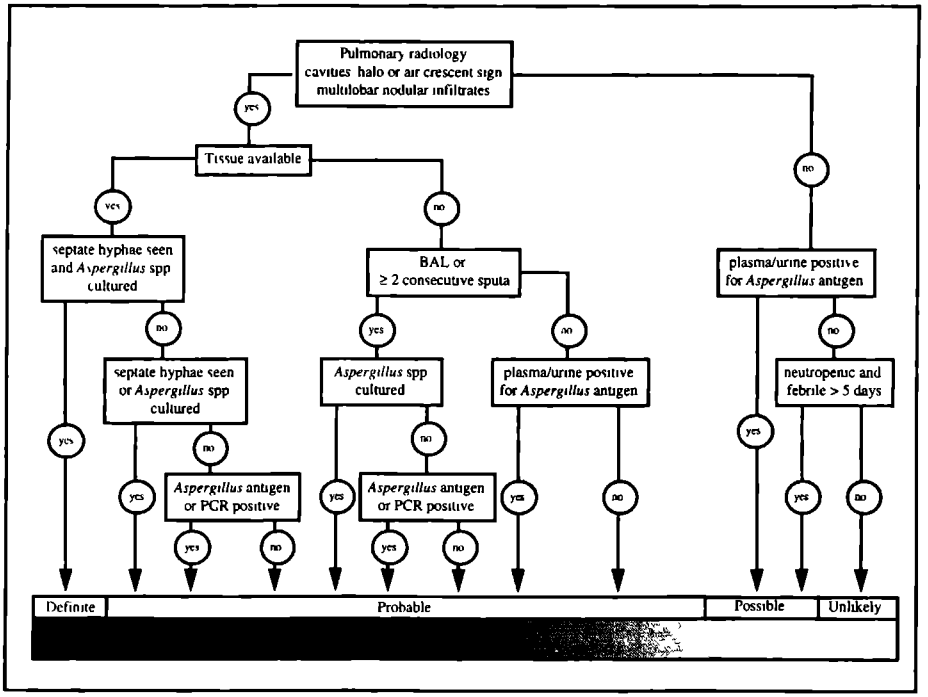
More potent, and less toxic antifungal agents are also being developed and investigated[70].Timely diagnosis presents the main obstacle to managing invasive aspergillosis espacially as treating infection as early as possible is essential for treatment to prove effective. However, clinical signs and symptoms, and plain chest X-rays are neither specific nor sensitive and a definitive diagnosis can only be made by demonstrating histologically invasion of tissues by the fungus and recovering *Aspergillus* species in culture. Unfortunately, neutropenia induced by cytotoxic therapy is usually accompanied by severe thrombocytopenia which often precludes the use of invasive methods for diagnosis. Culture of sputum or expectorated bronchial secretions is regarded as being useful in diagnosing invasive aspergillosis in neutropenic patients[146], even though the sensitivity is only 25%[81]. Specimens for microbiological analysis may be obtained by fiber-optic bronchoscopy in some cases and, although the recovery of *Aspergillus* species from bronchoalveolar lavage (BAL) fluid is highly indicative of invasive aspergillosis, the sensitivity of the technique (ranges from 40-58%)[71,104,112,146] and the diagnostic yield is low, especially in patients with focal pulmonary lesions[76]. The difficulty in establishing the diagnosis of invasive aspergillosis has led to the definition of categories which reflect the reliability of diagnosis (Figure 1).

The diagnosis of invasive aspergillosis should ideally be made at an early stage of infection because successful treatment depends upon intervening before fungal proliferation becomes overwhelming[1]. Within the past three years several methods have been developed to assist the early detection of *Aspergillus* species. Among these methods, those that detect the antigen galactomannan and DNA in clinical specimens from immunocompromised patients appear most promising.

Detection of Aspergillus *DNA by polymerase chain reaction*

A large burden of fungal mycelium is required to successfully diagnose invasive aspergillosis which renders culture of BAL fluid samples relatively insensitive and the fungus is often only detected at an advanced stage of the disease. The use of oligonucleotide primers to amplify specific fragments of *Aspergillus* DNA in BAL fluid by polymerase chain reaction (PCR) may allow small amounts of *Aspergillus* DNA to be detected so

Figure 1. *Key diagnostic features for the diagnosis of invasive aspergillosis in relation to the spectrum of probability.*



permitting invasive aspergillosis to be diagnosed at an earlier stage of infection. Several PCR assay's have been developed including those which amplify DNA fragments from *Aspergillus fumigatus* alone[97,111] and those which react with the DNA of a wider range of the *Aspergillus* species involved in human disease[18,78,119]. PCR assay of BAL fluid from patients suspected of having invasive aspergillosis was found to be more sensitive than culture of the same material[18,78,97,111,119]. PCR can detect *Aspergillus* specifically but needs to be combined with Southern-blot hybridization[18,78,97,111,119] and restriction enzyme analysis[78], to differentiate the fungus from other genetically closely related filamentous fungi such as *Penicillium marneffei* and *Paecilomyces variotii*[78,111,131]. Such PCR assays might prove valuable in diagnosing invasive aspergillosis early.

However, the reliability of the PCR may be reduced by the occurrence of false-positive or false-negative results or by positive results due to colonization of the respiratory tract without infection. For instance, positive amplification reactions were observed in 6 - 23% of BAL fluid samples from immunocompromised patients who had no evidence of invasive aspergillosis[18,78,119] and in up to 15% of similar specimens from non-neutropenic patients[119,127]. False positive reactions can result from contamination by *Aspergillus* conidia of biological samples or reaction buffers and can be overcome, partly, by ensuring that each step in the process is strictly segregated[67] and that reaction buffers are stored in small volumes and used only once. *Aspergillus* species may colonize the respiratory tract of some patients, such as smokers, those with cystic fibrosis[83], those receiving corticosteroids, and patients with AIDS[93]. We found a significant higher number of positive PCR results in BAL fluid samples from smokers and non-neutropenic patients receiving corticosteroids, when compared to those from patients without these risk factors[127]. Therefore, the PCR result should be interpreted cautiously and always in the clinical context of the patient. False-negative results also occur due to the presence of residual inhibitors of the amplification reaction[18,127] and can be minimised by using competitive PCR[18] or several dilution steps.

If the PCR assay is to prove helpful in diagnosing invasive aspergillosis early, the optimum method and technique and schedule for obtaining samples need to be defined and the diagnostic value has to be established in prospective studies.

Detection of Aspergillus *antigens in body fluids*

Since the detection of specific antibodies to *Aspergillus* is very insensitive[145], attention has been increasingly focussed upon the development of tests for detecting antigen. Several in-house serological methods have been developed which employ uncharacterized polysaccharides and (glyco)protein antigens of *Aspergillus* species[4,45,101,102,117] which can be detected in patient serum, urine, and in BAL fluid samples[4]. However, most research has focussed on galactomannan (GM), a major cell wall component in *Aspergillus* species that is released during growth in vivo and may also be secreted into the culture medium under certain circumstances[45]. Rat monoclonal antibodies raised against *Aspergillus* GM have

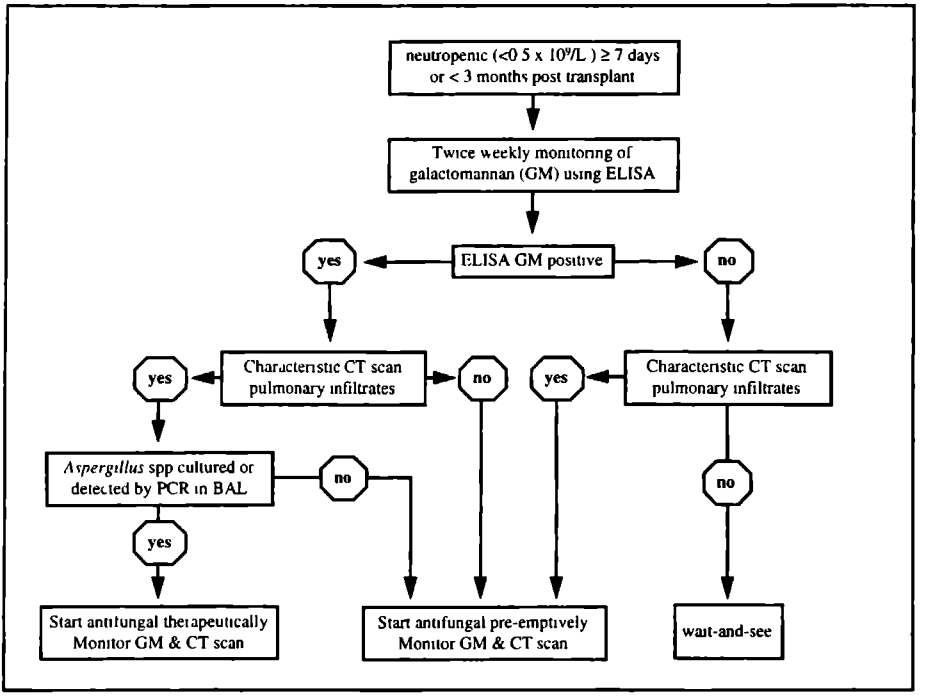
been applied to diagnose invasive aspergillosis serologically[32,98] and immunohistochemically[91]. The rat monoclonal antibody EB-A2 is the basis of the commercial Pastorex *Aspergillus* latex agglutination (LA) test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France)[114] which has been evaluated extensively using serum and urine[7,31,44,47,57,75,123,128,137] as well as BAL fluid[39,72]. Several reports suggested that the sensitivty of the LA test in serum samples from patients with a high index of suspicion for invasive aspergillosis approached 95%[31,44] and that antigen could be detected earlier than was possible using conventional tests in 68% of patients with proven invasive aspergillosis[44]. These observations were not confirmed by others[7,47,57,75,128,137]. Moreover, the latex test yielded a sensitivity of only 38% in one series[75] and was found to yield positive results only during advanced stages of infection in other studies[47,128]. The LA test was found positive in BAL fluid samples from three patients with AIDS and invasive aspergillosis[72]. However, under experimental conditions, no GM was detected by the LA test in BAL fluid from 14 untreated rabbits with invasive pulmonary aspergillosis, while serum samples were found positive in 10 (71%) animals[39]. Nevertheless, the LA test appears very specific although significant cross-reactivity with the antigens of certain other fungi including airborne contaminants may occur[52]. Thus, the LA test may contribute to the diagnosis of invasive aspergillosis when cultures remain negative provided that serial serum samples are obtained, but the test does not allow early diagnosis. Recently, a sandwich ELISA was developed which employs the same monoclonal antibody as the LA test[116] but was more sensitive because the antibody functioned both as a captor and detector thereby allowing the detection limit to be lowered 10-fold to 0.5 - 1.0 ng/ml. The ELISA test made it possible to detect GM in serum samples from more patients than was achieved with the LA test yielding a sensitivity of 90% when a series of serum samples was employed[129]. Furthermore, GM was detected by ELISA at an earlier stage of infection, even before clinical signs and symptoms had become apparent[116]. The increase in sensitivity of the ELISA also allowed detection of GM in BAL fluid samples from patients suspected of invasive aspergillosis, and an excellent correlation was found between these results and those found in corresponding serum samples[130]. GM was detected in serum of four patients from whom samples had been obtained within six weeks before bronchoscopy

was performed, which suggests that GM may be detected by ELISA in the serum before pulmonary infiltrates are visible on plain chest X-ray[130]. Although the increase in sensitivity of the ELISA test was accompanied by a false-positives rate of 8%, a specificity of 84% was obtained when a series of serum samples were employed[129].

Prospects for early diagnosis of invasive aspergillosis

The diagnosis of invasive aspergillosis requires a multi-disciplinary approach. The development of new sensitive microbiological and imaging techniques, including ultrafast computerized tomography[62,136], has implications for the optimal diagnostic work-up and improved management of invasive aspergillosis. Since the prognosis of invasive aspergillosis is uniformly poor and early diagnostic procedures for establishing a reliable diagnosis are generally lacking, the practice of initiating early empiric antifungal therapy to high-risk patients has become widespread in order to treat occult fungal infections promptly[29]. This approach appears benefitial to febrile granulocytopenic patients who remain febrile despite broad-spectrum antibacterial therapy[1,20,21,33,92,135,136]. However, fever is a non-specific sign of infection and a considerable group of granulocytopenic patients who do not have fungal infection will receive antifungal treatment, usually with amphotericin B desoxycholate which is relatively toxic. Less toxic lipid formulations of amphotericin B are available but are so costly that treatment has to be limited to those patients with proven disease or to those showing signs of toxic reactions to amphotericin B desoxycholate. On the other hand, persistant fever may be due to concurrent infections caused by other microorganisms and may obscure the presence of invasive aspergillosis, thus delaying diagnosis further[82]. Patients at high risk of deep fungal infections can be identified by the presence of specific risk factors predisposing to fungal infection and guidelines for recognizing high-risk patients most likely to benefit from the empiric use of amphotericin B during neutropenia or after bone marrow transplantation have been proposed by Fraser and Denning[40]. However, risk factors for fungal infections, including invasive aspergillosis, have yet to be established for other patient groups such as solid-organ recipients who are also at high-risk[88].

Figure 2. *Suggested guidelines for identification of patients at high risk for invasive aspergillosis and use of pre-emptive antifungal therapy during neutropenia induced by cytotoxic therapy or following organ transplantation with a high risk for invasive aspergillosis (e.g. bone marrow, heart-lung and liver transplantation).*



The development of the sandwich ELISA technique to detect GM allows an alternative approach to identifying patients at high risk for invasive aspergillosis even whether or not specific risk factors are identified. A single test is not sufficiently sensitive to identify all patients at risk so regular, serial monitoring of plasma or serum during the entire period of greatest risk is required. This is likely to prove the most productive approach to diagnosing invasive aspergillosis early thereby allowing more selective targeting of empiric antifungal treatment. The most efficient approach for each patient group will depend upon several factors including the underlying disease, the type and degree of immunosuppressive therapy, the incidence of invasive aspergillosis in that particular patient population, the environment, the availability and sophistication of radiological

facilities and, the possibility of using invasive diagnostic procedures. Guidelines which may be applied to patients with hematological malignancies during the period of neutropenia induced by cytotoxic-therapy or to transplant recipients with a high risk for invasive aspergillosis (e.g. bone marrow, heart-lung and liver transplantation) are shown in Figure 2. Plasma or serum from each patient is monitored regularly for the presence of GM using the ELISA technique and key clinical signs and symptoms, other laboratory results, and radiological features of the lung are registered systematically. When GM is detected for the first time, further confirmation should be sought by obtaining additional plasma or serum samples for ELISA. In addition, a CT-scan should be performed and, where possible, BAL fluid should be obtained for PCR analysis. Once the presence of GM is confirmed or, there are signs of infection almost pathognomonic for invasive aspergillosis such as the 'crescent sign' on a pulmonary CT-scan, treatment with antifungal agents should be started pre-emptively. In order to identify when treatment is failing to control the infection[67], the response should be monitored by following the course of GM levels using the ELISA technique[116] and by regular CT scans[136].

A more individual approach needs to be adopted for patients at a lower risk of invasive aspergillosis and for those for whom the risk is not predictable such as recipients of kidney transplants, patients receiving a prolonged course of high-dose corticosteroids, and those with AIDS. For instance, plasma or serum might be monitored for GM levels only when fever persists for more than 4 days despite an adequate trial of broad-spectrum antibiotics or when the risk of *Aspergillus* infection is increased by anti-rejection therapy.

By combining novel approaches to the early diagnosis of invasive aspergillosis with pre-emptive therapy, the outlook for patients at risk of invasive aspergillosis should be improved in terms of both morbidity and mortality. Moreover, the cost/benefit ratio may prove more favourable when only those at greatest risk of invasive aspergillosis are treated as the others for whom the risk is negligible would no longer receive antifungal treatment empirically. The results of prospective studies now underway are therefore eagerly awaited since, if this approach proves successful, it may be possible to implement similar guidelines for the early diagnosis of other deep fungal infections in immunocompromised patients.

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The microbiological diagnosis of invasive aspergillosis by conventional methods lacks sensitivity and positive results are often obtained only during advanced infection. In this thesis two methods are descibed and evaluated which may allow the diagnosis at an early stage of disease. Chapter 1 describes the development and evaluation of a PCR assay which detects *Aspergillus* DNA fragments. For this assay the nucleotide sequences of the genes encoding for the 18S rRNA of four *Aspergillus* species were elucidated. Since cross-reactivity with other fungi might impair the specificity of the assay, the nucleotide sequences were also elucidated for three related filamentous fungi. Phylogenetic analysis and comparison was performed with the sequenced fungi and the 18S rRNA sequences of 23 other eukaryotes. By this analysis, the genera *Aspergillus*, *Paecilomyces* and *Penicillium* were divided into two coherent clusters and showed a close intergeneric relationship which is in keeping with the existing morphological and taxonomic classification (chapter 1.1). For *Aspergillus* specific detection genus-specific sequences were selected in the V7 to V9 region of the 18S rRNA. By using hot-start PCR, Southern blot hybridization, and restriction enzyme analysis, *Aspergillus*-specific and -sensitive determination was achieved (chapter 1.3). A positive amplification reaction was found by *Aspergillus* genus-specific PCR with 11 of 72 (15%) BAL fluid samples from non-neutropenic patients at low risk for invasive aspergillosis. A positive PCR result was associated with corticosteroid therapy and cigarette smoking ($p < 0.05$)(chapter 1.2). In immunocompromised patients positive PCR results were obtained from culture-negative BAL fluid samples from two patients suspected of invasive aspergillosis and three neutropenic patients with fever and pulmonary infiltrates (chapter 1.3). These results indicate that PCR analysis of BAL fluid samples may allow an early diagnosis of invasive aspergillosis, but prospective evaluation is needed to determine the diagnostic value.

Aspergillus antigen detection may be used to diagnose invasive aspergillosis (chapters 2 and 3). The monoclonal antibody EB-A2 raised against *Aspergillus* galactomannan is the basis of a commercially available latex agglutination (LA) test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, France). Galactomannan was detected by the LA test in serum samples from eight patients which clinical, radiological or histopathological evidence for invasive aspergillosis (chapter 2.1). However, in most of these patients a positive LA test

was obtained only during advanced disease. Furthermore, the LA test failed to detect galactomannan in serum samples from two patients with a proven *Aspergillus* infection. A good reproducibility of the LA test was maintained when serum samples were stored at -70°C. A sandwich ELISA which employs the same monoclonal antibody as the LA test was evaluated in serum samples obtained from 61 immunocompromised patients (chapter 2.2). The ELISA gave positive results earlier in the course of infection than did the LA test. A sensitivity of 90% and a specificity of 84% were obtained for the ELISA when a series of serum samples was employed. Galactomannan was also detected by ELISA in BAL fluid samples from patients receiving treatment for hematological malignancies with fever and pulmonary infiltrates (chapter 2.3). An excellent correlation was found between ELISA results in serum and BAL fluid. Moreover, in four patients galactomannan was detected in serum samples which were collected within six weeks before the BAL was obtained. These results indicate that *Aspergillus* antigen detection in serum may allow the diagnosis of invasive aspergillosis at an early stage of infection.

The demonstration of tissue invasion in biopsy specimens or at autopsy and a positive culture for *Aspergillus* species allows a definitive etiological diagnosis of invasive aspergillosis. A diagnosis by histological examination of tissue sections alone may not be reliable because several filamentous fungi cannot be differentiated from *Aspergillus*. An immunoperoxidase stain which employs the monoclonal antibody EB-A1 allowed detection and generic identification of *Aspergillus* species in tissue sections from patients with a proven invasive aspergillosis and therefore may help to establish an etiological diagnosis (chapter 3). Since cross-reactivity with *Penicillium marneffei* has been reported, immunoperoxidase staining should be related to fungal morphology and interpreted in the clinical context of the patient.

The diagnosis of invasive aspergillosis requires a multi-disciplinary approach. The development of these sensitive microbiological diagnostic methods has implications for the optimal diagnostic work-up and management of invasive aspergillosis (chapter 4). Regular monitoring of the galactomannan level in serum samples from patients during the period of highest risk may allow early identification and selection of patients for antifungal treatment. When a positve ELISA result is confirmed by ELISA with additional serum

samples, pulmonary CT scan and PCR analysis of BAL fluid, pre-emptive antifungal therapy can be initiated. By using this approach the outlook for patients at risk of invasive aspergillosis should be improved in terms of both morbidity and mortality, and the results of prospective studies now underway are therefore eagerly awaited.

De microbiologische diagnose van invasieve aspergillose met behulp van conventionele methoden is weinig sensitief en positieve resultaten worden vaak pas verkregen tijdens een vergevorderd stadium van infectie. In dit proefschrift worden twee methoden beschreven en geëvalueerd die mogelijk kunnen bijdragen aan het vroegtijdig stellen van de diagnose. In hoofdstuk 1 wordt de ontwikkeling en de resultaten van de evaluatie van een PCR test beschreven, waarmee *Aspergillus* DNA aangetoond kan worden. Hiertoe werd de nucleotide volgorde bepaald van de genen die coderen voor het 18S rRNA van vier *Aspergillus* species. Omdat kruisreactiviteit met andere filamenteuze schimmels een lage specificiteit tot gevolg kan hebben, werd tevens de nucleotide volgorde bepaald van drie nauw verwante schimmels. Phylogenetisch onderzoek werd verricht met de onderzochte schimmels en de 18S rRNA sequenties van 23 andere eukaryoten. Hierbij werden de genera *Aspergillus*, *Penicillium* en *Paecilomyces* verdeeld in twee clusters die een nauwe intergenerische verwantschap vertoonden, wat in overeenstemming is met de huidige morfologische en taxonomische classificatie (hoofdstuk 1.1). *Aspergillus* specifieke detectie werd bereikt door genus specifieke nucleotide sequenties in het V7 - V9 regio te selecteren. Met behulp van hot-start PCR, Southern blot hybridizatie, en digestie met restrictie enzymen was het mogelijk met een hoge specificiteit en sensitiviteit *Aspergillus* aan te tonen (hoofdstuk 1.3). In bronchoalveolair lavage (BAL) vloeistof monsters van 70 niet-neutropene patiënten, die een laag risico hadden om een invasieve *Aspergillus* infectie door te maken, werd in monsters van 11 (15%) patiënten een positieve amplificatie gevonden. Een positieve PCR uitslag was geassocieerd met corticosteroid therapie en het roken van sigaretten ($p<0.05$)(hoofdstuk 1.2). Bij immuungecompromitteerde patiënten werd *Aspergillus* DNA aangetoond in BAL vloeistof van twee patiënten die verdacht werden voor invasieve aspergillose en van drie patiënten met koorts en longinfiltraten (hoofdstuk 1.3). Deze resultaten wijzen erop dat de PCR bruikbaar is voor de vroege diagnose van invasieve aspergillose, maar de diagnostische waarde dient geëvalueerd te worden in prospectief onderzoek.

Het aantonen van *Aspergillus* antigenen kan gebruikt worden voor de diagnostiek van invasieve aspergillose (hoofdstuk 2 en 3). Het monoclonale antilichaam EB-A2, gericht tegen het galactomannan, vormt de basis van een commercieel verkrijgbare latexagglutina-

tie test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, Frankrijk). Met behulp van deze test kon galactomannan aangetoond worden in serum monsters van acht patiënten met klinische, radiologische of microbiologische aanwijzingen voor invasieve aspergillose (hoofdstuk 2.1). Echter, in de meeste patiënten werd de test voor het eerst positief in een vergevorderd stadium van de infectie. Bij twee patiënten met een bewezen *Aspergillus* infectie werd geen galactomannan aangetoond in het serum. Een goede reproduceerbaarheid van de test werd bereikt door de serum monsters te bewaren bij -70°C. Een sandwich ELISA die gebruik maakt van hetzelfde monoclonale antilichaam als de latex test werd geëvalueerd in serum monsters van 61 immuungecompromitteerde patiënten (hoofdstuk 2.2). In vergelijking met de latex test kon met behulp van de ELISA galactomannan aangetoond worden in serum monsters die in een vroegtijdig stadium van infectie waren afgenomen. Een sensitiviteit en specificiteit van respectievelijk 90% en 84% werd bereikt wanneer meerdere achtereenvolgende serum monsters werden getest. Galactomannan kon met de ELISA ook aangetoond worden in BAL vloeistof van patiënten, die behandeld werden voor hematologische maligniteiten, met koorts en longinfiltraten (hoofdstuk 2.3). Een zeer goede correlatie werd gevonden tussen de ELISA resultaten in BAL vloeistof en de bepalingen in het serum. Bij vier patiënten werd galactomannan aangetoond in serum monsters die afgenomen waren binnen zes weken voorafgaande aan de BAL. Deze resultaten wijzen erop dat deze ELISA gebruikt kan worden om vroegtijdig invasieve aspergillose te diagnostiseren.

Een definitieve etiologische diagnose van invasieve aspergillose kan alleen gesteld worden door het aantonen van schimmeldraden in weefselcoupes én een positieve *Aspergillus* kweek. Het stellen van de diagnose op basis van histologie alleen kan onbetrouwbaar zijn omdat met routine kleuringen het microscopisch beeld van *Aspergillus* niet onderscheiden kan worden met dat van andere filamenteuze schimmels. Met behulp van een immunoperoxidase kleuring, die gebruik maakt van het anti-galactomannan antilichaam EB-A1, was het mogelijk *Aspergillus* schimmeldraden aan te tonen in weefselcoupes van patiënten met een bewezen invasieve aspergillose en daarmee een generische identificatie te verkrijgen (hoofdstuk 3). Omdat het monoclonale antilichaam kruisreacties vertoonde met *Penicillium marneffei* dient de immunoperoxidase kleuring gerelateerd te worden aan de

morfologie en de klinische gegevens.

Het stellen van de diagnose invasieve aspergillose vereist een multi-disciplinaire benadering. Het beschikbaar komen van deze gevoelige microbiologische diagnostische methoden heeft implicaties voor de diagnostische benadering en behandeling van patiënten met invasieve aspergillose (hoofdstuk 4). Het regelmatig monitoren van galactomannan spiegels in serum monsters van patiënten tijdens de periode waarin het risico het hoogst is zou een vroegtijdige diagnose en selectie van patiënten voor behandeling tot gevolg kunnen hebben. Wanneer een positieve ELISA titer gevonden wordt dient deze bevestigd te worden met ELISA bepalingen met nieuwe serum monsters, CT scan van de thorax, en PCR onderzoek van de BAL vloeistof. Na confirmatie kan pre-emptieve therapie met antifungale middelen gestart worden. Met deze benadering zou mogelijk de morbiditeit en letaliteit van invasieve aspergillose verbeterd kunnen worden, maar dienen eerst de resultaten van prospectief verricht onderzoek afgewacht te worden.

DANKWOORD

Het feit dat slechts één auteursnaam op de voorzijde van dit proefschrift vermeld staat, doet geen recht aan de bijdrage die velen hebben geleverd aan de totstandkoming ervan. Omdat ik niet iedereen persoonlijk kan noemen, wil ik een ieder die een bijdrage heeft geleverd aan dit proefschrift daarvoor hartelijk bedanken. Enkele personen wil ik met name noemen:

Mijn promotor en opleider, Prof. Dr. Mieke Hoogkamp-Korstanje, wil ik bedanken voor de gelegenheid en het vertrouwen dat ze mij gegeven heeft om dit onderzoek uit te voeren. Ik ervaar het als een groot voorrecht om haar "eerstgeborene" te zijn.

Veel dank ben ik verschuldigd aan mijn beide co-promotores. Dr. Jacques Meis heeft mij enthousiast gemaakt voor de klinische mycologie en heeft een zeer grote bijdrage geleverd aan dit onderzoek. Doordat hij bij elke werkbespreking niet alleen veel ideeën voor onderzoek inbracht, maar ook de daarbij behorende deadline voor de uitvoering en de publicatie ervan, was het mogelijk deze onderneming voorspoedig te voltooien. Dr. Ben de Pauw ben ik zeer dankbaar voor zijn begeleiding en voor de gelegenheid die ik kreeg om gedurende enkele maanden kennis te maken met de dilemma's en met de overwegingen, die gemaakt worden aan het ziekbed van neutropene patiënten met koorts. Ik beschouw deze ervaring als zeer waardevol en wil ook beide chefs de clinique, Dr. Ton Schattenberg en Dr. John Raemaekers, alsmede alle verpleegkundigen van de afdeling A51 bedanken voor hun begeleiding. Ik hoop dat de vruchtbare samenwerking tussen de afdelingen medische microbiologie en hematologie ook in de toekomst voortgezet zal worden.

Vanuit het laboratorium heb ik veel steun gehad van Dr. Willem Melchers die mij in de gelegenheid heeft gesteld kennis te maken met de geheimen van de moleculaire biologie, dat wil zeggen, de PCR techniek. De praktische vaardigheden van deze techniek heeft Patrick van den Hurk mij met veel geduld bijgebracht, zodat het uiteindelijk ook mij lukte de negatieve controle daadwerkelijk negatief te krijgen. Bij veel van de serologische bepalingen heeft Ton Rijs mij welwillend bijgestaan. Dank geldt ook Jan Zoll voor zijn prachtige "bomen", Dr. J. Peter Donnelly, "the Scot who contributed much even though I failed to catch him early", én Dr. Jean-Paul Latgé en Mark Tabouret voor de plezierige samenwerking.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 31 augustus 1962 te 's Gravenhage. In 1981 behaalde hij het diploma Atheneum ß aan het Rijnlands Lyceum te Wassenaar. Hierna studeerde hij gedurende één jaar Biologie aan de Rijksuniversiteit te Leiden. In 1982 begon hij aan dezelfde universiteit met de studie Geneeskunde. Het doctoraal- en artsexamen werden behaald in respectievelijk 1988 en 1990. In 1990 en 1991 was hij werkzaam als reserve eerste-luitenant arts bij de geneeskundige instructie groep te Hilversum. Van januari 1992 tot januari 1996 was hij in opleiding tot medisch microbioloog op de afdeling Medische Microbiologie van het Sint Radboud Ziekenhuis te Nijmegen (hoofd: Prof. Dr. J.A.A. Hoogkamp-Korstanje).

Stellingen
bij het proefschrift

MICROBIOLOGICAL DIAGNOSIS OF INVASIVE ASPERGILLOSIS

I

De voorwaarde voor vroegtijdige diagnose van invasieve aspergillose, met behulp van de sandwich ELISA, is regelmatige bemonstering.

II

De op handen zijnde kostenexplosie na de registratie en ten gevolge van onterecht gebruik van liposomale antifungale middelen, vereist een protocollaire multidisciplinaire diagnostiek van invasieve aspergillose.

III

Preventie van invasieve aspergillose met behulp van HEPA-filters op patiëntenkamers is zinloos, indien deze filters niet ook geplaatst worden in onderzoekkamers, gangen en toiletten.

IV

De relatie tussen bouwactiviteiten in ziekenhuizen en het optreden van invasieve infecties met *Aspergillus* species veronderstelt dat het opschrift "Wij bouwen aan de toekomst" niet van toepassing is op patiënten, die behandeld worden voor een hematologische maligniteit.

V

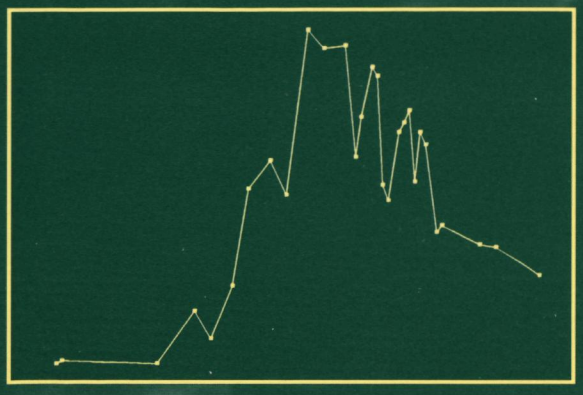
"Beter één *Aspergillus* spore in de hand dan tien in de lucht" is wel van toepassing op de neutropene patiënt, maar niet op de moleculaire diagnostiek van *Aspergillus* infecties.

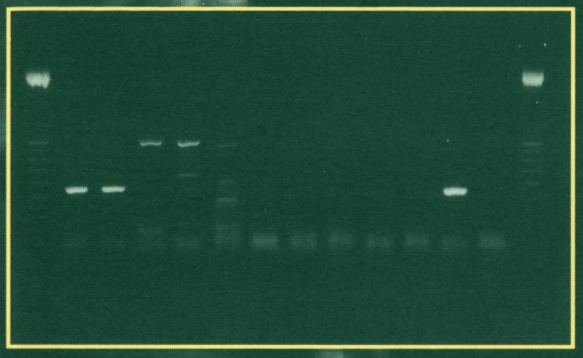
VI

The essence of wisdom is to make empiric decisions on inadequate evidence.

VII

Gezien de inhoud van de biobak tijdens warme zomermaanden, kan de betekenis van de afkorting GFT (Groente- Fruit- en Tuinafval) beter vervangen worden door Gevaarlijke Fungi en Toxinen.



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