

## ORIGINAL ARTICLE

# PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue

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*J Clin Pathol* 2005;58:1180–1184. doi: 10.1136/jcp.2004.024703

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Accepted for publication  
24 March 2005

**Background:** Invasive fungal infections are often diagnosed by histopathology without identification of the causative fungi, which show significantly different antifungal susceptibilities.

**Aims:** To establish and evaluate a system of two seminested polymerase chain reaction (PCR) assays to identify and discriminate between agents of aspergillosis and mucormycosis in paraffin wax embedded tissue samples.

**Methods:** DNA of 52 blinded samples from five different centres was extracted and used as a template in two PCR assays targeting the mitochondrial aspergillosis DNA and the 18S ribosomal DNA of zygomycetes.

**Results:** Specific fungal DNA was identified in 27 of 44 samples in accordance with a histopathological diagnosis of zygomycosis or aspergillosis, respectively. *Aspergillus fumigatus* DNA was amplified from one specimen of zygomycosis (diagnosed by histopathology). In four of 16 PCR negative samples no human DNA was amplified, possibly as a result of the destruction of DNA before paraffin wax embedding. In addition, eight samples from clinically suspected fungal infections (without histopathological proof) were examined. The two PCR assays detected a concomitant infection with *Absidia corymbifera* and *A fumigatus* in one, and infections with *Rhizopus arrhizus* and *A fumigatus* in another two cases.

**Conclusions:** The two seminested PCR assays described here can support a histopathological diagnosis of mucormycosis or aspergillosis, and can identify the infective agent, thereby optimising antifungal treatment.

Filamentous fungi of the genus *aspergillus* and the order Mucorales cause life threatening diseases in immunocompromised patients and require early and effective antifungal treatment.<sup>1–2</sup> Different mycotic infections cannot be distinguished by clinical and radiological signs, but the causative fungi show significantly different antifungal susceptibilities in vitro and in vivo.<sup>1–6</sup> Identification of the infecting fungus can be achieved by culture, but yield is low.<sup>7–10</sup> Invasive fungal infections are diagnosed most often by histopathology without genus or species identification.

“Different mycotic infections cannot be distinguished by clinical and radiological signs, but the causative fungi show significantly different antifungal susceptibilities in vitro and in vivo”

Polymerase chain reaction (PCR) assays have been introduced successfully for the identification of fungal species.<sup>11–12</sup> Fungal cultures are identified by targeting multi-copy genes such as ribosomal DNA (rDNA). However, amplification and identification of fungal rDNA directly from clinical specimens is impaired by similarities between RNA sequences from pathogenic and contaminating fungi, animals, and humans.<sup>13</sup> The increasing size of databases containing fungal rDNA sequences reveals that some formerly published primers and hybridisation probes can no longer be regarded as species or genus specific.<sup>14–15</sup> To circumvent these problems, genes encoding specific proteins can be targeted, but specific proteins are unknown for most of the numerous species causing mucormycosis.<sup>16–19</sup>

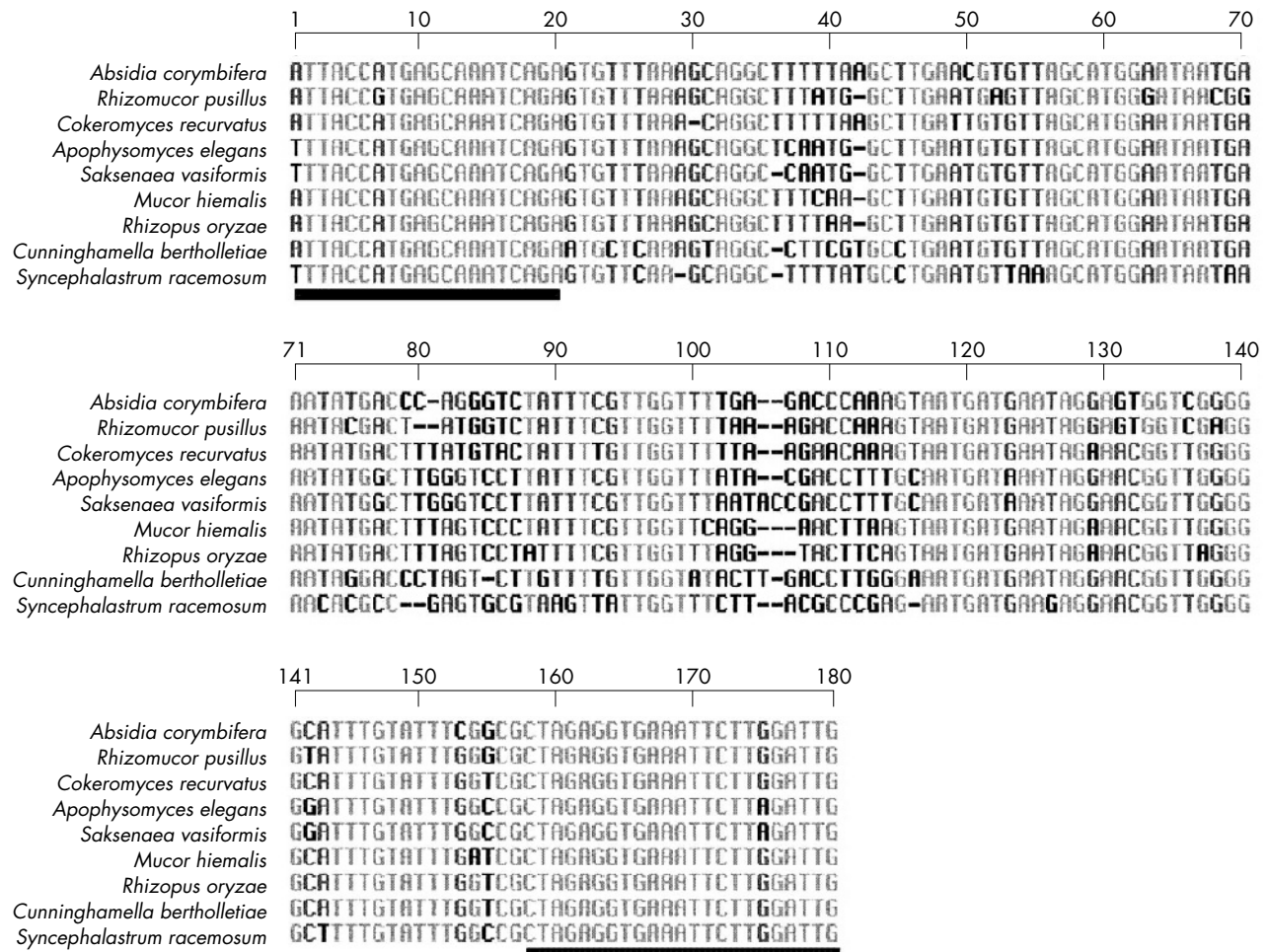
We developed a seminested PCR assay targeting the 18S rDNA of zygomycetes, which does not amplify genes of *aspergillus* species, and we modified a PCR assay targeting the mitochondrial DNA of *Aspergillus* spp, which does not amplify DNA from zygomycetes.<sup>20–22</sup> The aim of our study was to evaluate the identification and discriminatory efficacy of the two PCR assays in paraffin wax embedded tissue sections originating from different laboratories.

## MATERIALS AND METHODS

Forty two paraffin wax blocks and 10 paraffin wax embedded tissue sections in sterile tubes from 52 patients at risk for invasive fungal infections were sent blinded to our laboratory in Tübingen, Germany. In total, 28 biopsies of paranasal sinuses, orbita, nasal mucosa, palate, or facial bones (summarised as sinus biopsies), 15 lung biopsies, five biopsies of the central nervous system, three liver biopsies, and one skin biopsy were screened for fungal DNA.

Xylene (1 ml) was added to an Eppendorf tube containing two 5 µm sections of paraffin wax embedded tissue. The paraffin wax was removed by incubation and washing with xylene and ethanol.<sup>18</sup> DNA was extracted using the QIAamp tissue kit as recommended by the manufacturer (Qiagen, Hilden, Germany), modified by adding three cycles of freezing in liquid nitrogen for one minute and boiling for five minutes to disrupt the fungal cells.<sup>16</sup>

**Abbreviations:** PCR, polymerase chain reaction; rDNA, ribosomal DNA



**Figure 1** Alignment of 18S ribosomal RNA sequences of zygomycetes amplified by inner primer set ZM1 and ZM3 based on a program according to Corpet (<http://prodes.toulouse.inra.fr/multalin/multalin.html>).<sup>24</sup> The complimentary primer regions are indicated by black bars. Black letters indicate low or missing consensus in contrast to grey letters, which indicate high consensus at a given nucleotide position. Species and GenBank accession numbers used from top to bottom: *Absidia corymbifera* (AF113407), *Rhizomucor pusillus* (AF113434), *Cokeromyces recurvatus* (AF113416), *Apophysomyces elegans* (AF113412), *Saksenaeva vasiformis* (AF113442), *Mucor hiemalis* (AF113428), *Rhizopus oryzae*, now named *R. arrhizus* (AF113440), *Cunninghamella bertholletiae* (AF113421), and *Syncephalastrum racemosum* (X89437).

Alignment of 18S rDNA sequences from *Rhizopus arrhizus* (GenBank accession number AF113440) and *Aspergillus fumigatus* (GenBank AB008401) by special BLAST program (bl2seq) at the National Center for Biotechnology Information, Washington, DC, USA, revealed significant differences within the V4 and V5 variable regions.<sup>11</sup> Primers within this region show homologies to 18S rDNA of agents of zygomycosis deposited in GenBank.<sup>23</sup> The outer primers ZM1 (5'-ATT ACC ATG AGC AAA TCA GA-3') and ZM2 (5'-TCC GTC AAT TCC TTT AAG TTT C-3') are complementary to nucleotide positions 711 to 730 and 1117 to 1096 (*R. arrhizus*, AF113440), respectively. Products of the seminested reaction using primers ZM1 and ZM3 (5'-CAA TCC AAG AAT TTC ACC TCT AG-3') are 175 to 177 bp long, depending on the species (fig 1).<sup>24</sup>

To enhance the sensitivity of a published *A. fumigatus* PCR, a third primer was added to run a seminested PCR assay.<sup>20-22</sup> P2 (5'-CTT TGG TTG CGG GTT TAG GGA TT-3') and Asp2 (5'-GGG AGT TCA AAT CTC CCT TGG G-3') are complementary to nucleotide positions 938-916 and 738-759, respectively, of *A. fumigatus* mitochondrial transfer RNA (GenBank accession number L37095). The product of the seminested reaction using P1 (5'-GAA AGG TCA GGT GTT CGA GTC AC-3') and P2 is 135 bp long.<sup>20</sup>

The reaction mixtures of the primary PCRs consisted of 10 µl previously extracted DNA in a total volume of 50 µl, with final concentrations of 10mM Tris/HCl (pH 8.3), 50mM KCl, and 2.5mM MgCl<sub>2</sub> (10× Perkin-Elmer buffer II plus MgCl<sub>2</sub> solution; Roche Molecular Systems, Branchburg, New Jersey, USA); 1µM of each primer of the outer primer sets (ZM1/ZM2 and P2/Asp2, respectively; Roth, Karlsruhe, Germany); 1.5 U of AmpliTaq DNA polymerase (Roche); and 100µM of each dNTP (Promega, Madison, Wisconsin, USA). Identical reaction mixtures were used for the nested PCRs except that 1 µl of the first reaction, 50µM of each dNTP, and 1µM of each inner primer (ZM1/ZM3 and P1/P2) were used. All reaction mixtures were thermally cycled once at 94°C for five minutes, 35 times at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for one minute, and then once at 72°C for five minutes.

To validate the presence of amplifiable DNA and absence of inhibitory substances a PCR was performed using the primer set G1 (5'-GAA GAG CCA AGG ACA GGT AC-3') and G2 (5'-CAA CTT CAT CCA CGT TCA CC-3') targeting the human β globin gene (nucleotides 70400-70667; accession number, NG\_000007.3). The conditions were as described above except that 5 µl DNA extract and 3mM MgCl<sub>2</sub> were used, and the extension time was reduced from one minute to 45

seconds. When the result was negative, DNA extraction was repeated if enough material was available.

All PCRs were run in a Primus PCR thermocycler Tc 9600 (MWG Biotech, Ebersberg, Germany). PCR products were analysed by electrophoresis in ethidium bromide stained 1.8% agarose gels.

Amplicons of the primary PCRs using DNA extracted from laboratory strains of *A fumigatus* and *Absidia corymbifera* were cloned as described in detail previously.<sup>18</sup> Serial dilutions of cloned DNA were used to determine the lower detection limit of the nested PCR assays.

Cloned plasmid DNA (10 µl containing 100 fg (~ 5000 genome equivalents)) was used as a positive control in every PCR assay. Sterile water was included in the DNA extraction after every fifth sample and used in the PCR assays, and reaction mixtures without DNA were run in the first and nested PCRs to monitor contamination.

The nested PCR products were sequenced by a semiautomated system (Applied Biosystems Division, Perkin-Elmer Biosystems, Foster City, California, USA) and sequences were used for a BLAST search of the GenBank database.<sup>18</sup> A PCR assay was considered positive if the product obtained from the aspergillus PCR was identical to the sequence in GenBank, and if the product amplified by zygomycetes PCR showed 100% homology to an 18S rDNA sequence of a zygomycete.

Results of PCR assays not meeting these criteria were recorded as negative.

The diagnoses and results of microscopy were unblinded for further analysis.

## RESULTS

The aetiological agent was identified by PCR in 18 of the 24 biopsies with amplifiable human DNA from the sinus, orbita, palate, or other site related to the nasal cavities, in six of the 15 lung biopsies, in all five brain biopsies, in one of the three liver biopsies, and in the one skin biopsy. Seventeen of 48 biopsies with amplifiable human DNA remained negative by both fungal PCR assays.

As shown in table 1, a genus or species identification was achieved in 14 of 23 samples diagnosed as zygomycosis by histopathology. The corresponding aspergillus PCR assays were negative. PCR products of *Rhizomucor pusillus* and *R miehei* are identical using the primers described in this study, and recorded as *Rhizomucor* spp. In one sample zygomycetes PCR was negative, but *A fumigatus* specific

DNA was amplified. Nine samples were negative in both PCR assays. In four of them no human β globin DNA could be detected by the control PCR.

*Aspergillus fumigatus* specific DNA was amplified from 11 of 17 samples with histologically diagnosed aspergillosis. The corresponding zygomycetes PCR assays were negative. No DNA was amplified from the remaining six samples. A concomitant infection was diagnosed in four samples by histopathology but verified by PCR in only one.

Another eight samples were obtained from suspected invasive fungal infections—that is, clinical data were ambiguous, fungi were grown from other sites, or the histopathological findings were consistent with a fungal infection but not further specified. Identification was accomplished in three samples, and five samples gave negative fungal PCR results.

No cross contamination was seen because all controls remained negative. A nested PCR product was obtained from all positive controls indicating optimal reaction conditions.

A PCR product was detected using a minimum amount of 0.1 fg plasmid DNA. Assuming up to 40 copies/genome, a detection limit of five genome equivalents was calculated for both assays.

## DISCUSSION

In 25 of 40 samples with amplifiable human DNA, results obtained by the two PCR assays were in accordance with the histopathological findings, and the aetiological agent was identified to the genus or species level. In three additional samples, specific DNA was identified. Hayden *et al* examined biopsies from tissues with culture confirmed zygomycosis (13 samples) and aspergillosis (37 samples), by in situ hybridisation using five nucleotide probes.<sup>25</sup> According to these authors, several cases of zygomycosis were not interpretable, and species identification was not possible. Because of similarities between fungal ribosomal genes, species identification can only be accomplished by sequencing the PCR product. However, even targets of 280 bp within the 18S rDNA might not be sufficient to discriminate between genera.<sup>26</sup> Immunohistochemistry has been used to identify and discriminate aspergillosis and mucormycosis, in addition to candidosis, scedosporiosis, and fusariosis, but species identification of zygomycetes was not achieved.<sup>27–29</sup>

Agents of mucormycosis and aspergillosis are considered ubiquitous fungi that might be found in nasal mucous.<sup>30</sup> Although we mainly examined biopsies from airways, only a

**Table 1** Results of PCR assays correlated to the type of biopsy tissue and histopathological diagnosis

Diagnosis by histopathology	PCR results and identified species	Tissue and number of PCR results					Total
		Sinus*	Lung	Brain	Liver	Skin	
Zygomycosis (n = 23)	<i>Rhizomucor</i> spp†	5	1	1	–	1	8
	<i>Mucor hiemalis</i>	2	–	–	–	–	2
	<i>Absidia corymbifera</i>	1	1	–	–	–	2
	<i>Rhizopus arrhizus</i>	–	–	1	–	–	1
	<i>Aspergillus fumigatus</i>	1	–	–	–	–	1
	Negative (including 4 β globin PCR negative DNA extracts)	9	–	–	–	–	9
Aspergillosis (n = 17)	<i>Aspergillus fumigatus</i>	6	3	2	–	–	11
	Negative	–	6	–	–	–	6
Mixed infection (n = 4)	<i>Rhizomucor</i> spp and <i>A fumigatus</i>	1	–	–	–	–	1
	<i>Rhizomucor</i> spp	2	–	–	–	–	2
	Negative	1	–	–	–	–	1
Suspected invasive fungal infection (n = 8)	<i>Rhizopus arrhizus</i> and <i>A fumigatus</i>	–	–	1	–	–	1
	<i>Absidia corymbifera</i>	–	–	–	1	–	1
	<i>A fumigatus</i>	–	1	–	–	–	1
	Negative	–	3	–	2	–	5
Total		28	15	5	3	1	52

\*Biopsies of paranasal sinuses, nasal mucosa, orbita, palate, or facial bones; †*Rhizomucor pusillus* and *R miehei* were not distinguishable by sequencing, and results were reported as *Rhizomucor* spp.

limited number of samples were identified with DNA from fungi not detected by histopathology. *Aspergillus fumigatus* specific DNA was identified from only one case of histopathological diagnosis of zygomycosis; this might have been the result of contamination of the sinuses. However, a misdiagnosis by histopathology cannot be excluded; this has been described even in culture confirmed cases.<sup>25</sup> The numerous biopsies in our study with only one type of fungal DNA identified by PCR or with negative results by both PCR assays argue against a high rate of contamination of the biopsies or during paraffin wax embedding. Specimens from culture confirmed cases are necessary to determine the specificity and sensitivity of the PCR assays.

“Because of similarities between fungal ribosomal genes, species identification can only be accomplished by sequencing the polymerase chain reaction product”

No specific fungal DNA was amplified from 16 of 44 biopsies with fungal infection identified by histopathology. The failure to amplify specific DNA might result from fungal DNA concentrations below detection limits, a focal infection with varying amounts of fungal elements within the tissue, or hyalohyphomycosis caused by *Pseudallescheria boydii* or fusarium species. According to Bretagne *et al*, DNA of several aspergillus species will be amplified by the primers, but sequences for comparison are not deposited in GenBank.<sup>20</sup> In four cases, no amplifiable DNA was detected, probably because of the destruction of DNA during formalin fixation.<sup>31</sup>

A dual infection diagnosed by histopathology was detected by PCR in only one of four cases, but also in a biopsy of suspected fungal infection. There are few reports on concomitant fungal infections.<sup>32</sup> Because of the differences in antifungal susceptibilities, the identification of both agents is mandatory to institute appropriate antifungal treatment. Histopathology appears to be the most sensitive method for the detection of invasive fungal infection.<sup>7</sup> The two PCR assays described here can be used for individual cases, for prospective therapeutic studies, and also for retrospective analysis to determine species specific levels of in vivo susceptibility and case fatality rates of infection with mucorales species.<sup>9–33</sup> Species identification might be important to identify local differences of infecting mucorales.<sup>34</sup> *Cunninghamella* specific DNA was not amplified from the samples in our study and *Mucor hiemalis* specific DNA was detected only in sinus biopsies from one centre in Monterrey, Mexico.

In conclusion, a two PCR assay system for the discrimination and identification of agents of mucormycosis and *A. fumigatus* was evaluated in paraffin wax embedded tissue samples, and found to support the histopathological diagnosis and identify the infecting species. It is intended to be

used for epidemiological and diagnostic purposes, and to guide antifungal treatment.

## ACKNOWLEDGEMENTS

We thank Dr H Mair, Department of Pathology, University Innsbruck, Dr R Sedivy, Department of Clinical Pathology, University Vienna, Dr G Tucek, Department of Pathology, Kaiser Franz Josef Krankenhaus, Vienna, and J Beck-Managetta, Department of Oral and Maxillo-Facial Surgery, Landeskrankenhaus Salzburg, Austria for providing paraffin wax embedded tissue samples. The authors wish to thank Dr JM Scotter for her thoughtful review of the manuscript.

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The data presented here are part of the doctoral thesis of F Konrad.

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## Take home messages

- We describe two seminested polymerase chain reaction assays for use on paraffin wax embedded samples that can be used to support a histopathological diagnosis of mucormycosis or aspergillosis
- These assays were also useful in identifying the infective agent in a high proportion of cases, and would be useful for optimising antifungal treatment
- These assays could be used for both epidemiological and diagnostic purposes

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*J Clin Pathol* 2005 58: 1180-1184  
doi: 10.1136/jcp.2004.024703

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