Development and application of two independent real-time PCR assays to detect clinically relevant Mucorales species

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PCR-based detection of Mucorales species could improve diagnosis of suspected invasive fungal infection, leading to a better patient outcome. This study describes two independent probe-based real-time PCR tests for detection of clinically relevant Mucorales, targeting specific fragments of the 18S and the 28S rRNA genes. Both assays have a short turnaround time, allow fast, specific and very sensitive detection of clinically relevant Mucorales and have the potential to be used as quantitative tests. They were validated on various clinical samples (fresh and formalin-fixed paraffin-embedded specimens, mainly biopsies, n=17). The assays should be used as add-on tools to complement standard techniques; a combined approach of both real-time PCR assays has 100% sensitivity. Genus identification by subsequent sequencing is possible for amplicons of the 18S PCR assay. In conclusion, combination of the two independent Mucorales assays described in this study, 18S and 28S, detected all clinical samples associated with proven Mucorales infection (n=10). Reliable and specific identification of Mucorales is a prerequisite for successful antifungal therapy as these fungi show intrinsic resistance to voriconazole and caspofungin.

INTRODUCTION

Invasive fungal diseases (IFDs) are life-threatening complications that most commonly occur in immunocompromised patients (Meletiadis & Rolides, 2013). Improved diagnostics and treatment have led to a decrease in the number of infections caused by Candida and Aspergillus, and to a rise of so-called rare fungal pathogens, including members of the order Mucorales (Bitar et al., 2009; Kontoyiannis et al., 2005; Kwon-Chung, 2012; Auberger et al., 2012). Mucorales is a large order of genetically very heterogeneous fungi, and the most common human pathogens are associated with the genera Rhizopus and Lichtheimia (formerly known as Absidia or Mycocladus). Species of other genera, such as Mucor, Rhizomucor and Cunninghamhamella, are less common (Lanternier et al., 2012). Fungi of the families Saksenaceae and Syncephalastraceae are uncommon pathogens and are typically restricted to tropical areas, affecting immunocompetent hosts (Petrikkos et al., 2012). Whereas the exact incidence of mucormycosis is unclear and probably underestimated, an Austrian observational study recently reported 28% of their collected invasive mould infections were caused by Mucorales, equal to 1.2% of at-risk patients (Perkhofer et al., 2010). Other studies reported up to 13% incidence in high-risk patients, and risk of IFD was mainly associated with haematological malignancies and diabetes mellitus (Petrikkos et al., 2012).

Invasive mucormycosis is a fast-progressing and often fatal fungal infection and is difficult to diagnose. Standard culture is insensitive and microscopy often unspecific. There are no serological markers as Mucorales do not possess the cell wall components galactomannan or β-glucan used to detect other IFDs. Clinical symptoms and radiographical imaging cannot differentiate between different causes of IFD, especially between invasive aspergillosis and mucormycosis.

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Abbreviations: FF-PET, formalin-fixed paraffin-embedded tissue; IFD, invasive fungal disease; LoD, limit of detection; qPCR, quantitative PCR.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
As Mucorales are not susceptible to voriconazole, the classical treatment drug in invasive aspergillosis, fast identification of the causative agent is mandatory for effective treatment and good patient outcome. Rapid and reliable identification of Mucorales is key to guide patient treatment. Additional genus or species identification is of interest, e.g. for epidemiological surveys.

The drug recommended for first-line treatment is liposomal amphotericin B (AmB), and for second-line treatment posaconazole or liposomal AmB in combination with caspofungin (Skiada et al., 2013). Breakthrough invasive mucormycosis has been observed in patients receiving voriconazole or echinocandins (Kontoyiannis et al., 2005; Trifilio et al., 2007), and also for posaconazole (Kang et al., 2015; Mousset et al., 2010), showing reduced sensitivity of some Mucorales species (Almyroudis et al., 2007).

DNA detection via PCR is a rapid and sensitive tool to achieve the aim of Mucorales identification. There are reports using different sources for detecting Mucorales DNA from fresh or formalin-fixed paraffin-embedded tissue (FF-PET), bronchoalveolar lavages or serum samples (Bialek et al., 2005; Rickerts et al., 2006; Kasai et al., 2008; Millon et al., 2013). Also, different methods to detect Mucorales by PCR have been described, including conventional, semi-nested and real-time (DNA intercalating dyes, molecular-beacon or hydrolysis probe-based) PCR (Millon et al., 2013; Bialek et al., 2005; Voigt et al., 1999; Hata et al., 2008; Bernal-Martínez et al., 2013; Hrncirova et al., 2010; Lengerova et al., 2014); each suffers from different disadvantages such as high turnaround time, vulnerability to contamination or limited detection of selected species or genera.

Here we describe, for the first time to our knowledge, two rapid, probe-based real-time assays to specifically detect DNA from a broad range of clinically relevant Mucorales species, and their application to clinical specimens.

**METHODS**

**Fungal cultures.** Clinically relevant Mucorales cultures (Table 1) were identified by standard phenotypic procedures. Confirmatory genotypic identification was performed using the Mi croSeq D2 rDNA Fungal Identification System (Applied Biosystems).

**DNA isolation from fungal cultures.** Approximately 0.5 cm² samples were taken from fungal cultures on agar for DNA extraction and suspended in 1 ml deionized water. Samples were centrifuged at 5000 g for 5 min, and the supernatant was removed. Ceramic beads (MagNA Lyser Green beads; Roche Diagnostics) were added to the pellet and vigorously vortexed for 4 min to lyse the fungal cells. DNA was purified by using the High Pure PCR Template Preparation kit (Roche Diagnostics) according to the manufacturer’s instructions. Briefly, 200 µl binding buffer and 50 µl reconstituted proteinase K were added to the vortexed beads, and incubated for 15 min at 70 °C. 2-Propanol was added and the total volume was transferred to the spin column. After several washes the elution volume was adjusted to 100 µl. Extracted DNA was measured using a NanoDrop (ND-1000; Peqlab) and stored at −20 °C. To determine the limit of detection (LoD) of quantitative PCR (qPCR) assays, DNA was serially diluted (10-fold).

**Deparaffinization and DNA extraction of FF-PET samples.** For a detailed description see Babouee Flury et al. (2014).

**DNA extraction from clinical tissue specimens and purification.** DNA was extracted and purified using the QiAmp Mini kit (Qiagen) according to the manufacturer’s instructions, with the exception of an additional heating step of 95 °C for 12 min after pipetting 200 µl AL buffer. Fresh tissue and deparaffinized tissue samples were incubated

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**Table 1.** Mucorales isolates, genome sizes and LoDs for the two Mucorales-specific PCR assays (fg per 5 µl PCR input, and mean Cq values, respectively)

<table>
<thead>
<tr>
<th>Species</th>
<th>Genome size (Mb)*</th>
<th>1 gE (fg)</th>
<th>18S</th>
<th>28S</th>
<th><em>Cq</em>18</th>
<th><em>Cq</em>28</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apophysomyces elegans</strong></td>
<td>38.5</td>
<td>42</td>
<td>4</td>
<td>4</td>
<td>38.2</td>
<td>36.5</td>
</tr>
<tr>
<td><strong>Apophysomyces variabilis</strong></td>
<td>ND</td>
<td></td>
<td>5</td>
<td>5</td>
<td>39.2</td>
<td>36.8</td>
</tr>
<tr>
<td><strong>Cokeromyces recurvatus</strong></td>
<td>29.3</td>
<td>32</td>
<td>4</td>
<td>4</td>
<td>39.1</td>
<td>37.6</td>
</tr>
<tr>
<td><strong>Cunninghamella bertholletiae</strong></td>
<td>31.1</td>
<td>34</td>
<td>52</td>
<td>52</td>
<td>40.9</td>
<td>39.9</td>
</tr>
<tr>
<td><strong>Lichtheimia corymbifera</strong></td>
<td>36.6</td>
<td>40</td>
<td>19</td>
<td>19</td>
<td>39.2</td>
<td>35.7</td>
</tr>
<tr>
<td><strong>Mucor fragilis</strong></td>
<td>ND</td>
<td></td>
<td>29</td>
<td>29</td>
<td>39.7</td>
<td>35.7</td>
</tr>
<tr>
<td><strong>Mucor racemosus</strong></td>
<td>65.5</td>
<td>72</td>
<td>11</td>
<td>11</td>
<td>39.2</td>
<td>36.3</td>
</tr>
<tr>
<td><strong>Rhizomucor pusillus</strong></td>
<td>27.4†</td>
<td>30</td>
<td>32</td>
<td>32</td>
<td>37.1</td>
<td>38.1</td>
</tr>
<tr>
<td><strong>Rhizopus arrhizus</strong></td>
<td>42.4</td>
<td>47</td>
<td>64</td>
<td>64</td>
<td>38.2</td>
<td>35.2</td>
</tr>
<tr>
<td><strong>Rhizopus microsporus</strong></td>
<td>49</td>
<td>54</td>
<td>23</td>
<td>23</td>
<td>38.5</td>
<td>37.0</td>
</tr>
<tr>
<td><strong>Saksenaea oblongispora</strong></td>
<td>42.5†</td>
<td>47</td>
<td>3</td>
<td>3</td>
<td>38.6</td>
<td>37.1</td>
</tr>
<tr>
<td><strong>Syncphalastrum racemosum</strong></td>
<td>29.6</td>
<td>33</td>
<td>NA</td>
<td>20</td>
<td>NA</td>
<td>37.2</td>
</tr>
</tbody>
</table>

ND, Not determined; NA, not applicable.

*Median genome sizes according to NCBI (http://www.ncbi.nlm.nih.gov/pubmed/).
†Genome size of Rhizomucor mihei.
‡Genome size of Saksenaea vasiformis.
in proteinase K and ATL buffer at 56 °C for up to 12 h. The elution step was performed with 100 µl AE buffer and extracted DNA was stored at −20 °C.

Clinical samples. Three FF-PET samples and 14 fresh samples, mainly biopsies, from 14 patients (age 21 to 74) were collected in University Hospital Basel from 2009 to 2014 (for details see Table 2). According to EORTC/MSG criteria (De Pauw, 2005), all were classified as having IFD proven either by positive culture or by histology. Owing to molecular identification using DNA eluates of tissue samples, six patients (eight samples) were affected by Mucorales infections (Babouee et al., 2013).

Development of two real-time PCR assays to detect clinically relevant Mucorales DNA. For optimal design of primers and probes, multiple alignments of Mucorales reference sequences (Table S1, available in the online Supplementary Material) using ClustalW2 software (European Molecular Biology Laboratory, EMBL-EBI) (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were performed (Vitale et al., 2012; Voigt et al., 1999). In addition, reference sequences of non-Mucorales (Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Alternaria alternata, Malassezia globosa, Trichosporon mycotoxinivorans, Fusarium oxysporum and Penicillium marneffei) were included in the alignment.

18S-based Mucorales-specific real-time PCR. For the 18S-based assay, the inner fragment of the semi-nested conventional PCR of approximately 175 bp length described by Bialek et al. (2005) was adapted for real-time PCR by designing a specific probe. Based on multiple sequence alignment, the original primers were modified and a novel locked nucleic acid probe (Sigma Life Science) was designed for sensitive and specific detection of all clinically relevant Mucorales organisms (Tables 3 and S1). According to Bialek et al. (2005), sequencing of the amplicon allowed identification to the genus level.

Table 2. Characteristics of clinical specimens (proven IFD)

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Specimen no.</th>
<th>Sex</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Site</th>
<th>Histology</th>
<th>Culture</th>
<th>Molecular identification*</th>
<th>28S PCR</th>
<th>18S PCR</th>
<th>18S sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>F</td>
<td>57</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Rhizomucor pusillus†</td>
<td>33.7</td>
<td>35.6</td>
<td>Rhizomucor sp.†</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>F</td>
<td>57</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Rhizomucor pusillus†</td>
<td>37.6</td>
<td>39.3</td>
<td>Rhizomucor sp.†</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>M</td>
<td>69</td>
<td>AA</td>
<td>Liver</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Lichtheimia corymbifer†</td>
<td>35.3</td>
<td>32.4</td>
<td>Lichtheimia sp.†</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>M</td>
<td>69</td>
<td>AA</td>
<td>Liver</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Lichtheimia corymbifer†</td>
<td>33.9</td>
<td>Neg.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>F</td>
<td>21</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Lichtheimia corymbifer†</td>
<td>25.0</td>
<td>22.9</td>
<td>Lichtheimia sp.†</td>
</tr>
<tr>
<td>6</td>
<td>F6‡</td>
<td>F</td>
<td>44</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Rhizopus microsporus/aizygospor†</td>
<td>27.2</td>
<td>45.0</td>
<td>Rhizopus sp.†</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>F</td>
<td>51</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Rhizopus oryza†</td>
<td>37.5</td>
<td>38.9</td>
<td>Rhizopus sp.†</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>M</td>
<td>55</td>
<td>AML</td>
<td>Lung</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Rhizomucor pusillus†</td>
<td>39.5</td>
<td>Neg.</td>
<td>Rhizomucor sp.†</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>M</td>
<td>65</td>
<td>AML</td>
<td>Brain</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>30.5</td>
<td>35.5</td>
<td>Rhizomucor sp.</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>M</td>
<td>65</td>
<td>AML</td>
<td>Brain</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>36.0</td>
<td>37.0</td>
<td>Rhizomucor sp.</td>
</tr>
</tbody>
</table>

AA, Aplastic anaemia; ALL, acute lymphatic leukaemia; AML, acute myeloid leukaemia; COPD, chronic obstructive pulmonary disease; NA, not applicable (positive cryptococcal antigen test from cerebrospinal fluid); Neg., negative; Pos., positive.

*Molecular identification by ITS-based panfungal PCR as described by Babouee et al. (2013).

‡Species were identified by the panfungal and the 18S Mucorales assay (18S PCR); sequencing revealed the same genus level.

‡‡F, Formalin-fixed sample.

Table 3. Oligonucleotides used for Mucorales-specific 18S- and 28S-based real-time PCR assays

<table>
<thead>
<tr>
<th>Name</th>
<th>28S Sequence (5’–3’)*</th>
<th>18S Name</th>
<th>Sequence (5’–3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>WB28-1m</td>
<td>tttgggatgcagacct</td>
<td>ZM1mo</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>WB28-2</td>
<td>tcaRagtctctattaWctttcct</td>
<td>ZM3mod</td>
</tr>
<tr>
<td>Probe†</td>
<td>p-WB28</td>
<td>cgaRaRaccagatgcRacaaatgcccgt</td>
<td>p-ZM/LNA</td>
</tr>
</tbody>
</table>

*Oligonucleotides in upper case are wobble nucleotides: R stands for a or g; W for a or t; Y for c or t; B for g, c or t.

†Both probes are FAM-labelled at the 5’ end and BHQ1 at the 3’ end.

‡Parentheses indicate nucleotide with locked nucleic acid modification.
An Amplicons were purified using the MinElute PCR purification kit (Qiagen) according to manufacturer’s instructions. The elution volume was 15 μl. Sequencing was done by a commercial company (LCG, Berlin, Germany). Sequences were identified through alignment with reference sequences using BLAST analysis (National Center of Biotechnology Information, Washington DC; www.ncbi.nlm.nih.gov/BLAST).

**28S-based Mucorales-specific real-time PCR.** Multiple sequence alignment of the 28S rRNA gene (Table S1) allowed the design of primers and probe targeting a 107 bp fragment of the 28S (described in Table 3). The probe (Sigma Life Science) was designed for detection of all clinically relevant Mucorales organisms (Tables 3 and S1). The fragment is located at positions 230 to 336 of the reference sequence of Rhizopus oryzae (accession no. HM849659). With this assay Mucorales DNA can be detected semiquantitatively or quantitatively. Further characterization of the fragment by sequencing may not be successful owing to the shortness of the PCR fragment and the relatively conserved region of the 28S rRNA gene within this group of organisms.

For both assays, qPCR amplifications were performed in a 25 μl mixture using a StepOnePlus thermocycler (Applied Biosystems). Each reaction mixture contained 12.5 μl 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM forward and reverse primer, 200 nM probe (for nucleotide sequence details see Table 3) and 5 μl extracted DNA. Amplification was carried out for both tests, applying a standard thermocycling protocol: uracil-DNA glycosylase activation was at 50 °C for 2 min, and initial Taq polymerase activation was at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 60 s. In each run, negative and positive controls were included. Using 10-fold dilutions of genomic DNA, standard curves were generated. Screening of recent publications on Mucorales infections (Alvarez et al., 2009; Rüping et al., 2010; Skiaeda et al., 2011; Lanternier et al., 2012; Roden et al., 2005) led to the identification of the three most common Mucorales species in each manuscript. In total, we found five different Mucorales species, which were further investigated by serial dilution. For these, five different dilutions (10-fold) close to the LoD were tested. Real-time PCR efficiency rates (% and linear regression analysis ($R^2$)) using DNA of Cunninghamhamella bertholletiae, Lichtheimia corymbifera, Mucor fragilis, Rhizomucor pusillus and Rhizopus arrhizus were calculated by using StepOne software (version 2.2).

**ITS-targeting panfungal PCR.** Panfungal PCR was routinely performed directly from extracted tissue samples as described before (Babouee et al., 2013; Babouee Flury et al., 2014). Primers ITS5 and ITS4, targeting ITS regions 1 and 2, were used for fresh tissue samples, and primers ITS3 and ITS4, amplifying ITS region 2, were applied to FF-PET extracts. Five and 0.5 μl of extracted DNA were used in a total volume of 30 μl in two separate PCRs. The PCR fragments were run and visualized on precast 3% ReadyAgarose gels (Bio-Rad).

The PCR products were purified and sequenced. After purification the sequencing products were detected in an ABI PRISM 3100 genetic analyser (Applied Biosystems). The sequences were edited using SeqMan sequence analysis software (DNASTAR) and compared with reference sequences using BLAST analysis (National Center of Biotechnology Information, www.ncbi.nlm.nih.gov/BLAST) as well as the search tool of the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl/collections/BioloMICSSequences.aspx).

**Histology.** Specimens were obtained during procedures performed in a sterile operating theatre. The samples for histology were fixed in 4% buffered formalin in sterile containers. After paraffin-embedding, biopsies were cut into 4 μm sections and stained (haematoxylin and eosin, elastic Van Gieson and alcian blue periodic acid–Schiff, and, if fungal infection was suspected, Grocott’s methamine silver).

**In vitro-simulating experiment (mixed infection).** To mimic a mixed infection, DNA of two different fungi was mixed in vitro, using a fixed amount of Alternaria DNA (50 fg) with different amounts of Rhizopus DNA that were close to the LoD at the lowest concentration used (10-fold dilutions ranging from 32 fg to 32 pg), respectively. All combinations of these DNA amounts were tested in a panfungal PCR assay (Einsele et al., 1997) and in the Mucorales-specific 18S assay. Amplicons were purified, sequenced and aligned as described above for the 18S-based Mucorales-specific PCR assay.

**RESULTS**

**Analytical sensitivity, PCR efficiency, and linear regression**

DNA from 12 Mucorales strains (Apophysomyces variabilis, Apophysomyces elegans, Cokeromyces recurvatus, Cunninghamhamella bertholletiae, L. corymbifera, Mucor fragilis, Mucor racemosus, Rhizomucor pusillus, Rhizopus arrhizus, Rhizopus microsporus, Saksenaea oblongispora and Syncephalastrum racemosum) was serially diluted and tested by each qPCR assay. Both assays performed similarly for most species tested (for details see Table 1 and Fig. S1). *Syncephalastrum racemosum* was detected only by the 28S PCR.

The LoD ranged between 3 and 64 fg per PCR (5 μl input; Table 1); $C_b$ values ranged between 35.2 and 40.9. Approximately 30 to 70 fg corresponds to one genome equivalent of Mucorales organisms.

PCR efficiency ranged between 80 and 90% and between 88 and 92% for the 18S and 28S assays, respectively (Table S2). Linear regression ($R^2$) ranged between 0.994 and 1 (Table S2).

**Assay specificity**

The analytical specificity of both PCR assays was tested by adding 2.5 ng genomic DNA from the following clinically relevant fungi to the PCR assays: Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Aspergillus niger, Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei, Candida parapsilosis, Fusarium solani, F. oxysporum, Cryptococcus albidus, Cryptococcus humicola, Alternaria alternata, Penicillium chrysogenum and Scedosporium sp. This amount of genomic DNA represents several thousand copies of prospective target molecules and should be detectable by all assays, if one of these species is cross-reactive with an assay. Fifty nanograms of human DNA (four different donors) was also tested.

No cross-reactivity with non-Mucorales species and human DNA was observed by either the 28S or 18S assay.

**Analysis of clinical specimens**

Seventeen different samples from 14 patients ($n=14$ fresh specimens and $n=3$ FF-PET samples) with proven IFD were analysed by the two different real-time PCR assays to detect Mucorales DNA. Ten samples originated from...
patients with proven Mucorales IFD. Seven proven non-Mucorales IFD were used to test the specificity of the new assays in a clinical context. A panfungal PCR assay was initially used to detect fungal DNA as part of the standard routine in University Hospital Basel, enabling an identification of fungal species in 88% (15/17) of specimens (Babouee et al., 2013; Babouee Flury et al., 2014). In only two samples could no pathogen be detected despite positive histology showing fungal hyphae (patient 7; Table 2). Overall, histology showed evidence for the proven IFD in almost all samples (15/16, 93.8%; as there were no cultural or histology data available for the patient affected by Cryptococcus neoformans, this case was excluded from this calculation), whereas culture had diminished sensitivity but detected one additional case of proven invasive aspergillosis (6/16, 37.5%). One proven control case was identified by the cryptococcal antigen test; no further cultural or histology data were available.

All samples (frozen DNA eluates) were tested using the new Mucorales-specific assays. The 28S assay was positive in nine of ten samples of Mucorales IFD (90%), and signals were detected for six of seven affected patients (86% sensitivity). No signals were detected in the non-Mucorales control samples (8/8, 100% specificity). The 18S assay behaved similarly, detecting nine out of ten samples of Mucorales IFD (90%) and seven out of seven affected patients (100% sensitivity). Each assay failed for a single sample (patients 2 and 6; Table 2). Sequencing of the 18S PCR amplicons confirmed seven out of eight genera identified previously by panfungal PCR (Table 2) and identified two additional ones, which were negative by the panfungal approach but showed fungal hyphae in histology. The eighth case was only positive with the 28S assay. No sequence could be obtained here for comparison. The specificity of the 18S assay was reduced to 87.5%, showing contradictory results identifying DNA of Rhizopus and Alternaria by sequencing. The panfungal assay revealed Alternaria DNA in this sample. Cq values of the 18S assay ranged between 22.9 and 45.0, and those of the 28S assay between 25.0 and 37.6.

**DISCUSSION**

PCR is an established, very sensitive molecular technique, which has shown its potential to detect pathogens in many fields, leading to earlier diagnosis and improved patient outcome. Here we describe the modification (in the case of the 18S assay), development and evaluation of two independent Mucorales-specific real-time PCR assays based on different regions of the multicopy ribosomal operon. The design of the tests was based on multiple sequence alignments including all clinically relevant Mucorales species. As Mucorales consists of very heterogeneous taxonomic families, such as Mucoraceae, Lichtheimia and Cunninghamellaceae, the development of such a broad-range assay is complex. Both assays have a short turnaround time (approximately 3 h for DNA extraction and detection), allow fast, specific and very sensitive detection, and were validated on clinical samples (Table 2). A single assay detected only nine out of ten samples, while the combination of both assays detected all, by the second assay either confirming the results of the first or complementing it. Furthermore, sequencing of amplicons from the 18S assay allowed characterization of Mucorales strains to genus level, as shown by Bialek et al. (2005), which provided further confirmation of the specificity of the assay. No cross-reactivity to non-Mucorales strains or human DNA was observed in either assay. In contrast to the original 18S assay, the modified real-time assay did not detect Syncephalastrum racemosum, but this species was detected by the 28S assay complementing the 18S qPCR assay. Syncephalastrum sp. was isolated only once from 465 patients affected by zygomycosis, corresponding to 0.2% of proven cases, and is therefore considered a very rare fungus (Roden et al., 2005). All other tested Mucorales species showed very high sensitivity. Bialek et al. (2005) detected a minimum amount of 0.1 fg L. corymbifera plasmid DNA, which corresponds to five genome equivalents (gE). As one genome weighs approximately 40 fg, 5 gE correspond to 200 fg genomic DNA. Both assays described here detected in general less than 64 fg, and in the best case only 3 fg; for L. corymbifera the LoD was 20 fg, reflecting a 10-fold improvement of sensitivity.

As real-time PCR format is used here, both assays are faster, less prone to contamination and easier to handle than the previously published 18S assay, as there is no need to perform gel electrophoresis and pipette samples between different PCR runs (no nested PCR).

There were three patients for which the two Mucorales assays showed contradictory results (one positive and one negative). Patient 6 had a negative result with the 28S assay but the sequencing result of the 18S assay confirmed the panfungal result; both identified Rhizomucor DNA. Patient 8 revealed DNA of Alternaria and Rhizopus by the panfungal and 18S Mucorales assays, respectively. The 28S assay gave a negative result. The reason might be a double infection with both fungi in one sample from patient 8. This possibility was mimicked in vitro by mixing a fixed amount of Alternaria DNA with different amounts of Rhizopus DNA, the lowest concentration used being close to the LoD. All combinations of DNA amounts mentioned in Methods (In vitro-simulating experiment) were tested in a panfungal PCR assay, enabling detection of fungal DNA, and in the Mucorales-specific 18S assay. Interestingly, despite the presence of two different DNAs in one tube it was only possible to detect (and determine by sequencing) the DNA of Alternaria by broad-range PCR if a higher amount of Alternaria DNA was present. Rhizopus DNA was not detected. Nevertheless, Rhizopus DNA in the presence of an excess of Alternaria DNA was detected by the Mucorales-specific assay. Therefore, having available only the result of the broad-range assay, one would miss the presence of the second, under-
represented DNA, which can be detected if a specific assay is used. This confirms the possibility of a double infection in patient 8.

The third case of contradictory results is presented by patient 2, with positive panfungal and 28S assays. A confirmation of both assay amplicons by sequencing was not possible.

Low fungal load could be an explanation for ambivalent PCR results, since Cq values of above 37 were detected in some samples. Detection at late Cq values is not reproducible and sample replicates can be ambivalent, showing positive and negative results. Owing to a low volume of the DNA extracts (which were previously used for prospective routine screening tests), only a single PCR per assay could be performed.

Previously described assays to detect Mucorales DNA have clear disadvantages. First, (semi-)nested and/or conventional PCR assays are highly prone to contamination and cannot quantify fungal load (Voigt et al., 1999; Bialek et al., 2005; Hrncirova et al., 2010), whereas real-time assays can be run in a closed system, minimizing contamination risk. As quantification is possible, monitoring of Mucorales DNA, e.g. in serum samples, can guide treatment effectively and improve patients’ outcomes. Furthermore, other real-time assays detect either a single Mucorales genus (Bernal-Martinez et al., 2013; Lengerova et al., 2014) or only groups of Mucorales pathogens, missing other clinically relevant species (Millon et al., 2013; Hata et al., 2008). In detail, Voigt et al. (1999) used taxon-specific primer pairs in an end point PCR which would require performing 13 different assays. The assay published by Bialek et al. (2005) detects a broad range of Mucorales in a single assay but uses a semi-nested end point format, which is prone to contamination (Bialek et al., 2005). Hata et al. (2008) described a real-time PCR assay with high resolution melt (HRM) analysis differentiating some Mucorales but missing others (Rhizomucor, Conidiobolus and Syncephalastrum). The assay described by Hrncirova et al. (2010) based on the 18S assay published by Bialek et al. (2005) still has a semi-nested format but has the advantages of real-time PCR, enabling strain differentiation by HRM analysis. Bernal-Martinez et al. (2013) used a multiplex qPCR assay designed to detect only the genera Rhizopus and Mucor. Also very specific assays were used by Millon et al. (2013). Three real-time assays are necessary to detect the four different genera (Lichtheimia, Rhizopus, Rhizomucor and Mucor).

To our knowledge, a method for broad-range detection of all clinically relevant Mucorales genera in a format with reduced risk of contamination has not been published previously. Both assays described here are suitable for specific detection and confirmation of Mucorales when used in combination, reaching 100% sensitivity in clinical specimens. They are faster and more straightforward than end point PCR assays, with a lower risk of contamination. In the future, these tests might also be used as a screening tool, including the analysis of blood specimens (Millon et al., 2013). However, currently, inter-laboratory standardization seems to be difficult since (i) Mucorales contains a large variety of different fungal species, (ii) Mucorales infections occur with a relatively low incidence (but are probably underestimated) and therefore few specimens are collected, (iii) different clinical material is used for the detection of Mucorales, and (iv) different poorly standardized in-house protocols are used for DNA extraction and PCR amplification. Thus, inclusion of Mucorales detection in the revised EORTC/MSG criteria for IFD will be a difficult and lengthy process. In consequence, numerous multi-centre studies with international patient recruitment and standardized protocols for sampling and processing are highly warranted. Recently, standardization of Aspergillus PCR has been extensively achieved by the European Aspergillus PCR Initiative (EAPCRI), leading to the conclusion that the use of standardized DNA extraction and PCR protocols improves assay performance, including assay specificity (White et al., 2015). Like Aspergillus PCR, the Mucorales PCR assay described in our study provides high analytical specificity and in consequence a high degree of aetiological certainty at the genus level.

In summary, we present two real-time PCR assays, which in combination allow substantially improved and fast detection of Mucorales in clinical specimens. Reliable and specific identification of Mucorales is a prerequisite for successful antifungal therapy, as these fungi show intrinsic resistance to voriconazole and caspofungin, representing first-line and salvage drugs for invasive aspergillosis (Walsh et al., 2008; Vitale et al., 2012). Early detection of Mucorales is highly desirable, as mucormycosis is rapidly progressive (Petrikkos et al., 2012), and, thus, adequate and punctual antifungal therapy will substantially improve patient management (Chamilos et al., 2008).

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