Clinical evaluation of a Mucorales-specific real-time PCR assay in tissue and serum samples

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Molecular diagnostic assays can accelerate the diagnosis of fungal infections and subsequently improve patient outcomes. In particular, the detection of infections due to Mucorales is still challenging for laboratories and physicians. The aim of this study was to evaluate a probe-based Mucorales-specific real-time PCR assay (Muc18S) using tissue and serum samples from patients suffering from invasive mucormycosis (IMM). This assay can detect a broad range of clinically relevant Mucorales species and can be used to complement existing diagnostic tests or to screen high-risk patients. An advantage of the Muc18S assay is that it exclusively detects Mucorales species allowing the diagnosis of Mucorales DNA without sequencing within a few hours. In paraffin-embedded tissue samples this PCR-based method allowed rapid identification of Mucorales in comparison with standard methods and showed 91% sensitivity in the IMM tissue samples. We also evaluated serum samples, an easily accessible material, from patients at risk from IMM. Mucorales DNA was detected in all patients with probable/proven IMM (100%) and in 29% of the possible cases. Detection of IMM in serum could enable an earlier diagnosis (up to 21 days) than current methods including tissue samples, which were gained mainly post-mortem. A screening strategy for high-risk patients, which would enable targeted treatment to improve patient outcomes, is therefore possible.

INTRODUCTION

In Europe and the USA, invasive fungal diseases (IFD) are still a life-threatening complication for immunocompromised patients (Meletiadis & Rolides, 2013). In particular, infections by members of the order Mucorales are difficult to detect and to manage. Improved diagnosis and the application of yeast- and mould-active prophylaxis in many centres has led to a reduced incidence of disease due to Candida and Aspergillus. However, previously rare infectious fungal agents such as Fusarium and Mucorales are on the rise (Kontoyiannis et al., 2005; Bitar et al., 2009; Auburger et al., 2012; Kwon-Chung, 2012). The order Mucorales represents a large and genetically very heterogeneous taxon. In Europe, the most commonly identified human pathogenic genera are Rhizopus and Lichtheimia (synonyms: Absidia or Mycocladus) followed by Mucor, Rhizomucor and Cunninghamella (Lanternier et al., 2012).

However, epidemiological data on mucormycosis are limited, and incidences might be underestimated. Some studies have indicated an incidence of invasive mucormycosis (IMM) of up to 13% in high-risk patient populations; major risk factors for developing IMM are haematological malignancies and uncontrolled diabetes mellitus with accompanied ketoacidosis (Petrikkos et al., 2012).

The diagnosis of IMM is challenging because of non-specific symptoms, clinical signs and radiographic manifestations,
which can be easily confused with other invasive fungal infections such as aspergillosis or fusariosis. Standard diagnostic testing such as culture and microscopy are insensitive or non-specific (Lackner et al., 2014). There are no commercially available serological antigen screening tests for Mucorales as these fungi do not possess sufficient quantities of cell-wall antigens (galactomannan or beta-glucan) that are detected by existing serological tests for IFD. Also, no standardized and reliable PCR-based tests for IMM are currently available.

Reliable, fast and early identification of the causative agent is necessary for effective treatment of IMM and for optimal patient outcome (Chamilos et al., 2008). PCR-based diagnostic tests can be a rapid, sensitive and specific solution for the detection and identification of infectious fungi from clinical specimens, capable of detecting minute amounts of Mucorales DNA (a minimum of 3 fg) (Springer et al., 2016a). The key to effective molecular diagnosis of IFD is efficient DNA extraction from the patient sample especially if the load of pathogen is low (White et al., 2010). Detection of Mucorales DNA from fresh or formalin-fixed, paraffin-embedded tissue (FFPET), bronchoalveolar lavages (BAL), and serum samples has been achieved using several different protocols (Bialek et al., 2005; Rickerts et al., 2006; Kasai et al., 2008; Millon et al., 2013, 2015; Alario et al., 2015).

Unlike pathogens such as bacteria or viruses that can be easily detected in serum samples, the load of fungi in blood is very low (Springer et al., 2012). Therefore, detection of fungal DNA by PCR is often close to the limit of detection, which requires significant assay optimization. DNA detection from fresh or FFPET samples has been the most direct way to reliably identify the causative agents of IMM, but biopsying severely ill patients is connected with significant comorbidities. At present, most cases of mucormycosis are diagnosed post-mortem by autopsies with the probability of pre-mortem diagnosis estimated to be as low as approximately 50% (Antinori et al., 2009).

Mucorales are not susceptible to voriconazole, the first-line antifungal drug for invasive aspergillosis (IA). Therefore, a fast identification of the causative agent is mandatory for effective treatment and improved patient outcome. Rapid and reliable identification of Mucorales is key to guide patient treatment.

The aim of the current study was to assess the clinical value of a recently described Mucorales-specific real-time PCR assay (Springer et al., 2016a) in tissue and serum samples from patients at risk from invasive mucormycosis. This assay (Muc18S) could provide an add-on tool to diagnose mucormycosis in tissue and in serum samples enabling a less invasive and an earlier diagnosis of IMM than post-mortem tissue samples.

**METHODS**

**Study design.** (1) Archived FFPET blocks were Grocott stained to select block regions containing fungal elements. DNA was extracted from slices adjacent to positively stained regions; these extracts were analysed by a Mucorales-specific PCR assay (Muc18S) and an internal control assay detecting Bacillus DNA (Springer et al., 2016b). (2) The in-house Muc18S assay was also used to retrospectively analyse sera from patients suffering from IMM.

**Clinical tissue samples.** Sixteen tissue specimens, obtained from biopsies or during autopsies, from 15 patients (age 19 to 71) were collected at University Hospital Innsbruck from 2010 to 2014 (Table 1) and stored as paraffin-embedded tissue blocks. Patients were classified according to the EORTC/MSG criteria (De Pauw et al., 2008); 11 of the patients selected were classified with proven IMM by positive histology and tissue availability. Histological suspicion of IMM was indicated when non-septate or sparsely septate hyphae of variable width (6–25 µm) were found. Moreover, the hyphae of Mucorales in tissue have branching angles ≥90° and show an irregular, ribbon-like appearance. Four patients showed no signs of infection (C12–C15, Table 1). The underlying conditions of patients included in this study were haematological disorders, trauma or having undergone solid organ transplantations. Due to reasons of patient privacy regulations, the precise underlying disease is not available.

**Histology.** Specimens were obtained during procedures performed in a sterile operating theatre. The samples for histology were fresh (calcofluor staining) or fixed in 4% buffered formalin (haematoxylin and eosin, elastic Van Gieson and alcian blue periodic acid–Schiff and if suspicious for fungal infection, Grocott’s methenamine silver staining) in sterile containers. After paraffin-embedding, biopsies were cut into 10 µm sections and stained. For DNA extraction, adjacent slices (n=3) to Grocott-positive sections showing fungal hyphae were used.

**DNA extraction from FFPET.** DNA from three 10 µm tissue sections per sample was extracted using the BioRobot EZ1 (Qiagen) according to manufacturer’s instructions (protocol: Purification of DNA from Paraffin-Embedded Tissue). Elution volume was adjusted to 50 µl. PCR-positive samples were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. The elution volume was adjusted to 30 µl using ultrapure water.

**Mucorales-specific real-time PCR assay (Muc18S).** For the Mucorales-specific assay (Muc18S) quantitative real-time PCR (qPCR) amplifications were performed as described previously with the following modification, the reaction volume was reduced to 20 µl using 10 µl Taqman GenEx master mix (Applied Biosystems) (Springer et al., 2016a). 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 (Springer et al., 2016a). According to Bialek et al. (2005), sequencing of the amplicons allowed identification at least to the genus level. We evaluated the new assay by testing 12 different Mucorales strains (Springer et al., 2016a). Amplicons were purified and sequenced as described previously (Springer et al., 2016a). Sequences were identified through alignment with reference sequences using BLASTN analysis (National Center of Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST) andalthrough the search tool of the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl/collections/BioloMICSSequences.aspx).

**Clinical samples – serum.** Serum specimens (n=268) from allogeneic haematopoietic stem cell transplant recipients and patients receiving myeloablative chemotherapy were collected during hospitalization in the period 2008 to 2012 at the University Hospitals of Innsbruck (MUI) and Wuerzburg (UKW). Sera were collected prospectively once a week at MUI and twice weekly at UKW. Patients suffered from the following underlying diseases: acute myelogenous leukaemia (n=17), multiple myeloma (n=10), acute lymphoblastic leukaemia (n=5),
osteomyelofibrosis (n=2) and non-Hodgkin’s lymphoma (n=1). This cohort comprised nine women (mean age 47.8, range 34 to 72) and 26 men (mean age 53.6, range 27 to 71). Patients were classified according to the EORTC/MSG criteria (De Pauw et al., 2008); proven/probable IFD was determined by either histology or positive culture from BAL. The pure cultures were identified by microscopy and PCR/sequencing. Selected serum samples were analysed with the Mucorales-specific qPCR assay (Muc18S). These sera were selected retrospectively according to the patients’ EORTC/MSG classification and availability. Serum samples from cases of possible IFD were selected prior to the date of the patients’ first positive computerized tomography (CT) scan. The study was approved by the local ethics committees of the MUI (UN4529) and UKW (41/06).

DNA extraction from serum samples. DNA was extracted from 1 ml of serum using the QIAamp UltraSens virus kit (Qiagen) with the following modifications: (a) no carrier RNA was used (addition of cRNA caused unspecific amplification), (b) lysate centrifugation was adjusted to 3000 g, and (c) the elution volume was adjusted to 35 µl. The elution buffer was incubated on the column for 1 min prior to centrifugation. In each DNA extraction run, one negative control (human serum) and one Bacillus-positive serum (spiked with 10 000 plasmid copies Bacillus DNA) were included as quality controls (Springer et al., 2016b). PCR reagents and consumables were quality controlled regularly to exclude contamination from the reagents and materials. In addition, sera from healthy persons were regularly tested as DNA extraction controls. Serum was extracted in more than 30 DNA extraction procedures simultaneously to the patient samples. All control sera remained PCR-negative.

Data analysis. Patients with proven or probable IFD according to the EORTC/MSG criteria (De Pauw et al., 2008) were classified as true positives, unclassified controls as true negatives. Diagnostic performance parameters were calculated as described previously (Armitage et al., 2002).

RESULTS

Analyzing tissue samples

Tissue samples showing histology typical for Mucorales (n=12) and four control samples (without signs or symptoms typical for IFD) (Table 1) were used to test the Mucorales-specific qPCR assay. This assay targeting the 18S ribosomal DNA region (Muc18S) detected 10 of 11 IMM infections. Mucorales DNA was confirmed by sequencing of the PCR products (Table 1).

The qPCR assay Muc18S was not able to detect one infection, which was found to be positive by histology and culture (no. 8). No PCR inhibition was detected within this DNA eluate. The control samples were negative. The Mucorales-specific assay showed a good diagnostic performance; sensitivity was 91 % (Table 2).

Analyzing serum samples

Two hundred and sixty-eight serum samples from 35 patients were collected by MUI and UKW. Using the EORTC/MSG criteria (De Pauw et al., 2008), five patients were classified as probable/proven for IFD by culture and 17 patients were classified with possible IFD. Thirteen patients showed no signs of infection (Table 3). Serum samples were analysed for the presence of Mucorales DNA by

<table>
<thead>
<tr>
<th>Sample ID*</th>
<th>Sex</th>
<th>Age</th>
<th>Site</th>
<th>Histology†</th>
<th>Culture</th>
<th>Muc18S‡</th>
<th>Muc18S sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>m</td>
<td>58</td>
<td>Groin</td>
<td>Pos</td>
<td>Pos</td>
<td>33.2; 33.6</td>
<td>Rhizopus sp.</td>
</tr>
<tr>
<td>2a</td>
<td>f</td>
<td>57</td>
<td>Stomach</td>
<td>Pos</td>
<td>Neg</td>
<td>25.3; 25.6</td>
<td>Rhizomucor sp.</td>
</tr>
<tr>
<td>2b</td>
<td>f</td>
<td>57</td>
<td>Gut</td>
<td>Pos</td>
<td>ND</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>f</td>
<td>71</td>
<td>Lung§</td>
<td>Pos</td>
<td>Pos</td>
<td>39.1; 39.7</td>
<td>Lichtheimia sp.</td>
</tr>
<tr>
<td>4</td>
<td>f</td>
<td>58</td>
<td>Cheek§</td>
<td>Pos</td>
<td>Pos</td>
<td>26.9; 27.5</td>
<td>Lichtheimia sp.</td>
</tr>
<tr>
<td>5</td>
<td>m</td>
<td>57</td>
<td>Groin</td>
<td>Pos</td>
<td>Pos</td>
<td>30.4; 30.7</td>
<td>Rhizopus sp.</td>
</tr>
<tr>
<td>6</td>
<td>m</td>
<td>48</td>
<td>Lung§</td>
<td>Pos</td>
<td>ND</td>
<td>30.9; 31</td>
<td>Rhizomucor sp.</td>
</tr>
<tr>
<td>7</td>
<td>m</td>
<td>32</td>
<td>Lung§</td>
<td>Pos</td>
<td>Pos</td>
<td>31.6; 31.8</td>
<td>Lichtheimia sp.</td>
</tr>
<tr>
<td>8</td>
<td>f</td>
<td>43</td>
<td>Heart§</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>f</td>
<td>19</td>
<td>Pleura§</td>
<td>Pos</td>
<td>Pos</td>
<td>30.4; 30.8</td>
<td>Rhizopus sp.</td>
</tr>
<tr>
<td>10</td>
<td>f</td>
<td>73</td>
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<td>Pos</td>
<td>Neg</td>
<td>32.2; 32.5</td>
<td>Lichtheimia sp.</td>
</tr>
<tr>
<td>11</td>
<td>m</td>
<td>58</td>
<td>Lung§</td>
<td>Pos</td>
<td>Neg</td>
<td>36.9; 37.9</td>
<td>Rhizomucor sp.</td>
</tr>
<tr>
<td>C12</td>
<td>m</td>
<td>29</td>
<td>Kidney</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>C13</td>
<td>m</td>
<td>49</td>
<td>Liver</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>C14</td>
<td>m</td>
<td>69</td>
<td>Liver</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>C15</td>
<td>m</td>
<td>68</td>
<td>Gut</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td></td>
</tr>
</tbody>
</table>

Pos, positive; Neg, negative; m, male, f, female.
*C, non-Mucorales IFD; samples 2a and 2b are samples from the same patient.
†Pos, positive for Mucorales; for details see Methods.
‡Quantification cycle (Cq) values are given if positive.
§Sample gained by autopsy.
the Muc18S qPCR assay. The highest percentages of positive results for the Muc18S assay, 25% of sera and 100% of patients, were detected in patients with probable/proven IFD (Table 3). Sequencing revealed DNA from two different genera of Mucorales (Lichtheimia, Rhizopus) and confirmed the pathogen identified by conventional methods in four of five cases (in one case species was not identified by conventional methods; Table 4). All positive sera from an individual patient contained the same fungal species.

qPCR detected DNA of the causative pathogen up to 21 days (mean —8.25 days; Table 4) before the first positive signs by culture or microscopy. Day zero was defined as the time point of the EORTC/MSG classification (positive BAL, positive culture or positive tissue).

Possible IFD was defined by host factor and radiological signs being non-specific for the causative fungal pathogen (De Pauw et al., 2008). Sera from patients affected by possible IFD were selected by the presence of a positive CT scan and analysed by the Muc18S assay. Five of 17 possible IFD cases (29%) had Mucorales-positive sera (Table 4). Sequencing revealed DNA from the genera Mucor and Rhizomucor. Two patients had more than one positive sample. Whereas serum from patient ‘po6’ was only positive for Rhizomucor DNA, serum of patient ‘po21’ contained Mucor and Rhizomucor DNA in different serum samples (Table 4).

Only two sera out of 98 sera obtained from 13 high-risk haematological control patients without an EORTC/MSG classification (unclassified control samples) were positive (2%). DNA from Mucor and Actinomucor was detected (Table 4). Mucorales DNA might be present in clinical specimens even though no infection is ongoing as the causative agent might be treated with appropriate antifungal prophylaxis/therapy or the infection is controlled by the patient’s immune system. The contamination of PCR reagents and consumables can be widely excluded.

Survival was analysed by using the day of diagnosis of fungal infection established by EORTC/MSG classification (positive culture, histology or CT scan) as a reference point. All patients with proven/probable IFD died within 36 days from diagnosis of IFD, whereas all patients with possible IFD and a positive Mucorales PCR survived at least 100 days after diagnosis with IFD (data not shown). Patients with possible IFD showed a maximum of three positive results for the Muc18S assay, 25% of sera and 100% of patients, were detected in patients with probable/proven IFD (Table 3). Sequencing revealed DNA from two different genera of Mucorales (Lichtheimia, Rhizopus) and confirmed the pathogen identified by conventional methods in four of five cases (in one case species was not identified by conventional methods; Table 4). All positive sera from an individual patient contained the same fungal species.

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<table>
<thead>
<tr>
<th>EORTC/MSG</th>
<th>Pat. (no.)</th>
<th>Sera (no.)</th>
<th>Range samples per pat.</th>
<th>Pos. sera</th>
<th>Pos. sera (%)</th>
<th>Mean Cq (range)</th>
<th>Pos. pat. (n)</th>
<th>Pos. pat. (%)</th>
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</thead>
<tbody>
<tr>
<td>Probable/</td>
<td>5</td>
<td>52</td>
<td>4–21</td>
<td>13</td>
<td>25</td>
<td>34.8 (24.7–45.6)</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>proven</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Possible</td>
<td>17</td>
<td>118</td>
<td>3–16</td>
<td>8</td>
<td>7</td>
<td>39.4 (37.6–41)</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>Unclassified</td>
<td>13</td>
<td>98</td>
<td>4–13</td>
<td>2</td>
<td>2</td>
<td>40.7 (39.1–41.5)</td>
<td>2</td>
<td>15</td>
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</table>

pat., Patient; Pos., PCR positive in Muc18S assay; Cq, quantification cycle.

Table 2. Diagnostic performance of molecular assay Muc18S

<table>
<thead>
<tr>
<th>Muc18S</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>LR+</th>
<th>LR−</th>
<th>DOR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.91</td>
<td>1.00</td>
<td>0.09</td>
<td>81.0</td>
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</table>

Mucorales-PCR assay results and became negative in all cases. This is in contrast to cases with proven IMM where only two of five patients survived 100 days. Here, the Muc18S PCR results stayed positive until the time of diagnosis. No follow-up samples were available, mainly due to the death of the patient.

DISCUSSION

The aim of this study was to assess a probe-based Mucorales-specific real-time PCR assay (Muc18S) (Springer et al., 2016a) in clinical specimens (tissue and serum). The Muc18S assay showed 91% sensitivity in histologically proven tissue samples and was able to identify the fungal pathogen via sequencing of the PCR product. The specificity of the Muc18S assay for Mucorales species allows rapid diagnosis of IMM DNA without sequencing.

In comparison with standard methods, the Muc18S assay failed to detect one of 11 patients. The tissue samples for DNA extraction were carefully controlled to ensure that they contained microscopically visible fungal elements. Therefore, DNA extracts from areas without fungal elements can be excluded as reasons for negative molecular results. However, due to the methodology, the presence of fungal nuclei in the slices extracted cannot be proven, which would implicate fungal elements without extractable DNA. Due to appropriate controls and an internal control, PCR inhibition can be excluded as a reason for false-negative molecular results. Additional PCR replicates might have
Table 4. Characteristics of patients with positive serum samples using Mucorales 18S assay (Muc18S)

<table>
<thead>
<tr>
<th>Pat. code</th>
<th>Age</th>
<th>Sex</th>
<th>Disease</th>
<th>Site</th>
<th>Culture (no.)</th>
<th>Sera (no.)</th>
<th>Available sera*</th>
<th>Pos. Muc18S sera (no.; mean Cq)</th>
<th>Sequencing Muc18S</th>
<th>Pos. sera (days)</th>
<th>AM (D-30)†</th>
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<tbody>
<tr>
<td>PR1</td>
<td>64</td>
<td>m</td>
<td>MM</td>
<td>Sinus</td>
<td>(Mucorales)</td>
<td>7</td>
<td>3; 40, 42.8, 39.4</td>
<td>Rhizopus sp.</td>
<td>−8§</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>PR2</td>
<td>67</td>
<td>m</td>
<td>MM</td>
<td>BAL</td>
<td>Lichtheimia sp.</td>
<td>15</td>
<td>2; 39.3, 42.6</td>
<td>Lichtheimia sp.</td>
<td>−1§</td>
<td>VRC</td>
<td></td>
</tr>
<tr>
<td>PR3</td>
<td>42</td>
<td>f</td>
<td>MM</td>
<td>Liver, lung, kidney</td>
<td>Rhizopus sp.</td>
<td>21</td>
<td>6; 36.4, 36.2, 32, 31.9, 27.2, 24.7</td>
<td>Rhizopus sp.</td>
<td>−21§</td>
<td>CAS; VRC</td>
<td></td>
</tr>
<tr>
<td>PR4</td>
<td>54</td>
<td>m</td>
<td>MM</td>
<td>Cheek</td>
<td>Rhizopus sp.</td>
<td>5</td>
<td>1; 38.8</td>
<td>Rhizopus sp.</td>
<td>0</td>
<td>VRC/ANID; VRC</td>
<td></td>
</tr>
<tr>
<td>PR5</td>
<td>58</td>
<td>f</td>
<td>ALL</td>
<td>Cheek</td>
<td>Lichtheimia sp.</td>
<td>4</td>
<td>1; 30.5</td>
<td>Lichtheimia sp.</td>
<td>−3</td>
<td>PSC</td>
<td></td>
</tr>
<tr>
<td>po6</td>
<td>65</td>
<td>m</td>
<td>AML</td>
<td>NA</td>
<td>NA</td>
<td>15</td>
<td>2; 37.6, 39.1</td>
<td>Rhizomucor sp.</td>
<td>−6§</td>
<td>VRC</td>
<td></td>
</tr>
<tr>
<td>po9</td>
<td>34</td>
<td>f</td>
<td>ALL</td>
<td>NA</td>
<td>NA</td>
<td>5</td>
<td>1; 38.9</td>
<td>Rhizomucor sp.</td>
<td>106</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>po14</td>
<td>50</td>
<td>m</td>
<td>OMF</td>
<td>NA</td>
<td>NA</td>
<td>16</td>
<td>1; 39.7</td>
<td>Mucor sp.</td>
<td>−23</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>po18</td>
<td>63</td>
<td>m</td>
<td>AML</td>
<td>NA</td>
<td>NA</td>
<td>10</td>
<td>1; 39.7</td>
<td>Mucor sp.</td>
<td>−250</td>
<td>VRC</td>
<td></td>
</tr>
<tr>
<td>po21</td>
<td>64</td>
<td>m</td>
<td>AML</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
<td>3; 40.8, 39.7, 38.8</td>
<td>Mucor sp.</td>
<td>−13§</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>uncl24</td>
<td>37</td>
<td>f</td>
<td>NHL</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
<td>1; 41.5</td>
<td>Mucor sp.</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>uncl33</td>
<td>62</td>
<td>m</td>
<td>AML</td>
<td>NA</td>
<td>NA</td>
<td>8</td>
<td>1; 39.1</td>
<td>Actinomucor sp.</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Pat., patient; PR, proven/probable IMM; po, possible IFD; uncl, unclassified; m, male; f, female; MM, multiple myeloma; ALL, acute lymphoblastic leukaemia; AML, acute myelogenous leukaemia; OMF, osteomyelofibrosis; NHL, non-Hodgkin’s lymphoma; Cq, quantification cycle; ANID, anidulafungin; CAS, caspofungin; PSC, posaconazole; VRC, voriconazole; ‘†’ indicates usage of a different antifungal drug at the same time.

*Days in relation to EORTC/MSG classification; ‘−’ indicates days before.
†Antifungal treatment (AM) 30 days prior to EORTC/MSG classification.
‡Positive sera are adjacent to each other.
§Earliest positive serum samples.
||Samples gained by autopsy.
¶Positive sera within 11 days.
increased the sensitivity. Also, fixation of tissue for a longer period than 1 week can hamper amplification (Rogers et al., 1990). As the same species (Rhizopus arrhizus) was cultured from samples 8 and 9 (Table 1), we conclude that R. arrhizus is detectable by our PCR system.

Analysing serum samples for the presence of cell-free circulating DNA was successfully trialled in several studies to detect Aspergillus and also Mucorales (Millon et al., 2013; Springer et al., 2013; Legrand et al., 2016). Millon et al. (2013) published data for serum analyses of two different French patient cohorts affected by IMM, whereas Legrand et al. (2016) published data for plasma analyses of a French patient cohort affected by invasive wound mucormycosis. Using different extraction and detection methods, we detected Mucorales DNA in sera from an Austrian/German cohort of patients at risk for IMM. A single assay is able to detect specifically Mucorales DNA. Our data indicate that a screening approach would have enabled earlier diagnosis (up to 21 days) in 80% of probable/proven cases, which would have facilitated targeted treatment (Table 4). Ideally, sera should be collected continuously, but due to the patient’s availability there were temporal gaps between some of our patient samples. Serum samples of particular interest were those collected adjacent to ones that tested positive. Assuming collection twice a week, sera should be available every 3 to 4 days. This was not the case for, e.g. patient PR2 (Table 4), where Mucorales DNA was detected at day –1. No additional serum samples were available until day –26. As we could not analyse these missing samples, PCR positivity could have been detected at an earlier time point and would improve early diagnosis even more. A similar situation is given for the only patient where serum analysis showed no temporal advantage (PR4, Table 4). In this patient, sera between days 0 and –10 were not available for testing. Nevertheless, serum tests enable longitudinal and prospective testing of high-risk patients and are less invasive than taking biopsies, easier to perform and to repeat, and mostly provide earlier positive results than conventional methods. Therefore, it can lead to better treatment and patients’ outcome.

We found the highest percentage of PCR-positive sera and the highest DNA content in sera from patients with probable/proven invasive mucormycosis in comparison with sera from unclassified patients or those with possible IFD (Table 3). Mucorales DNA was detected in all patients with probable/proven IMM. In three of five IMM patients, multiple consecutively positive sera were detected identifying the same causative agent within a patient (Table 4). Sera from patients with possible IFD also contained Mucorales DNA, with 29% of these patients being positive. Therefore, Mucorales spp. are a possible causative agent in possible IFD. However, in contrast to patients with probable/proven IMM, the mean quantification cycle (Cq) value was higher in patients with possible IFD (34.8 vs 39.4, respectively; Table 3) indicating a lower DNA load in possible IFD patients. Similar Cq values for probable/proven IMM were recently reported (Millon et al., 2015). This reported Cq value (Cq 34) reflects a higher DNA load in patients with IMM than in patients affected by IA and may be caused by extensive angioinvasion by Mucorales (Ibrahim et al., 2012; Millon et al., 2015) reflecting blood as a suitable diagnostic material.

In one patient (po21, Table 4), DNA of two different Mucorales species was detected, suggesting a co-infection in this patient. The number of co-infections may be underestimated in general and in our study as another studies reported up to 33% of fungal co-infections (Kontoyiannis et al., 2000).

In contrast to probable/proven IMM cases, only two of the five PCR-positive possible IFD cases showed more than one consecutive positive PCR result. Subclinical infections may be a reason for this reduced PCR positivity as antifungal treatment or leucocyte recovery might prevent a clinically

**Fig. 1.** Proposed diagnostic workflow using the Muc18S assay. DNA from clinical specimens such as tissue or serum samples would be isolated and analysed. A positive PCR test would trigger a patient’s antifungal treatment as Mucorales species show intrinsic resistance to some antifungals, e.g. voriconazole. Optional sequencing (dashed lines) can identify the genus of the causative pathogen.
detectable infection (de Pauw & Viscoli, 2011; Hammond et al., 2011).

Survival for patients with possible IFD was superior in comparison with that for patients with probable/proven IFD. This confirms the data by Millon et al. (2015) showing a longer survival when PCR became negative in comparison with permanently positive PCR results (Millon et al., 2015). Interestingly, in probable/proven IFD, only Lichtheimia sp. or Rhizopus sp. was identified, whereas in patients with possible IFD Mucor sp. or Rhizomucor sp. was identified. As our number of cases is limited, the distribution in this data set could be by chance. More cases are needed to evaluate this in greater detail.

As we performed a specific PCR, this enabled a fast and specific detection of Mucorales species only. A positive test result could be used to trigger or re-evaluate an antifungal regimen because all members of the order Mucorales show intrinsic resistance to, e.g. voriconazole, the first-line treatment for IA. Remaining treatment options are limited and should be applied as early as possible. A proposed workflow is shown in Fig. 1 for a diagnostics and treatment protocol for IMM based on use of the Muc18S assay. An optional sequencing step of Mucorales-positive specimens can reveal the genus of the causative pathogen to study epidemiology and potentially help to select the best antifungal treatment option. In the case of a negative Muc18S assay, other molecular assays can be performed, e.g. to detect other moulds like Aspergillus.

In summary, we evaluated the clinical value of our recently described Mucorales-specific qPCR assay in tissue and serum samples. This probe-based assay is able to detect a broad spectrum of clinically relevant Mucorales species in a single PCR reaction and can be used as an add-on tool to confirm or enable quickly and specifically the diagnosis of mucormycosis and to screen high-risk patients. Depending on the clinical specimen and the protocol used, diagnosis can be performed within 4 h after patient material reaches the laboratory. This method is faster and more specific than standard methods and can help to distinguish between infections caused by Aspergillus and Mucorales, which is relevant for antifungal regimens and patient outcome.

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REFERENCES


