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PCR-Based Approach Targeting Mucorales-Specific Gene Family for Diagnosis of Mucormycosis

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ABSTRACT Mucormycosis is an aggressive, life-threatening infection caused by fungi in the order Mucorales. The current diagnosis of mucormycosis relies on mycological cultures, radiology and histopathology. These methods lack sensitivity and are most definitive later in the course of infection, resulting in the prevention of timely intervention. PCR-based approaches have shown promising potential in rapidly diagnosing mucormycosis. The spore coating protein homolog encoding *CotH* genes are uniquely and universally present among Mucorales. Thus, *CotH* genes are potential targets for the rapid diagnosis of mucormycosis. We infected mice with different Mucorales known to cause human mucormycosis and investigated whether *CotH* could be PCR amplified from biological fluids. Uninfected mice and those with aspergillosis were used to determine the specificity of the assay. *CotH* was detected as early as 24 h postinfection in plasma, urine, and bronchoalveolar lavage (BAL) samples from mice infected intratracheally with *Rhizopus delemar*, *Rhizopus oryzae*, *Mucor circinelloides*, *Lichtheimia corymbifera*, or *Cunninghamella bertholletiae* but not from samples taken from uninfected mice or mice infected with *Aspergillus fumigatus*. Detection of *CotH* from urine samples was more reliable than from plasma or BAL fluid. Using the receiver operating characteristic method, the sensitivity and the specificity of the assay were found to be 90 and 100%, respectively. Finally, *CotH* was PCR amplified from urine samples of patients with proven mucormycosis. Thus, PCR amplification of *CotH* is a promising target for the development of a reliable, sensitive, and simple method of early diagnosis of mucormycosis.

KEYWORDS Mucorales, diagnosis, *CotH*, *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*

Mucormycosis is an aggressive life-threatening infection caused by fungi in the order Mucorales (1). These fungi are ubiquitous in the environment, proliferating in soil and on decaying materials, generally propagating with the production of a large number of air dispersed spores (2, 3). Although considered an uncommon disease,

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mucormycosis is classified as an emerging infection by the National Institutes of Allergy and Infectious Diseases (NIAID) that is often misdiagnosed due to the lack of a reliable diagnostic assay. Mucormycosis is the third most common fungal infection in patients with hematologic malignancies (4). Among all Mucorales, *Rhizopus* spp. are responsible for almost 70% of all cases of mucormycosis, with the *Mucor* and *Lichtheimia* species being the next most common (1).

Major predisposing risk factors for mucormycosis consist of an impaired immune system, generally caused by neutropenia, corticosteroid treatment, and uncontrolled diabetes with or without ketoacidosis (1, 3, 5). However, patients with severe trauma and burns, without prior immune deficiency, are also at increased risk of developing mucormycosis, mostly as cutaneous disease (1, 3, 5). For example, outbreaks of mucormycosis have been associated with natural disasters such as the Indian Ocean tsunami of 2004 (6) and the Joplin tornado (7–9) and with soldiers subjected to blast injuries during combat operations (10, 11). Despite the drastic surgical removal of infected foci combined with antifungal therapy, the mortality rates associated with all forms of mucormycosis are approximately 50% (12). In certain situations, including patients with prolonged neutropenia, disseminated disease, or cerebral involvement, mortality can be unavoidable (13–16). The rapid progression of the disease and the current lack of early and effective diagnostic methods contribute to the high mortality rates.

Current diagnosis of mucormycosis relies heavily on mycological culture and radiological and histopathological examination. Blood cultures or those from biopsy specimens are mostly negative, and positive cultures can occur sometimes due to contamination rather than true infection. Radiological methods also lack sensitivity, because it is difficult to differentiate imaging findings from aspergillosis, a disease that is treated differently from mucormycosis (17, 18). In contrast, histology can be definitive and sensitive but requires experienced personnel to differentiate between mucormycosis and aspergillosis. Another major drawback of the current mucormycosis diagnostic methods is that they can only be definitive at a later stage of the disease, which prevents timely intervention. Delaying amphotericin B treatment was shown to be associated with a 2-fold increase in mortality due to mucormycosis (19). Thus, a reliable and rapid diagnostic assay would likely improve outcomes due to the earlier introduction of proper treatment.

In general, PCR-based molecular techniques have high potential for accurately diagnosing infection in early stages of the disease (20, 21). The amplified target usually is species specific and allows the diagnosis with high fidelity. We have discovered the gene family of spore coating encoding proteins (*CotH*) as the first identified mold invasins that allow Mucorales to penetrate host cells (22). *CotH* genes are universally and uniquely present in mucoralean fungi (22, 23). Thus, we investigated the feasibility of using *CotH* as targets for early diagnosis of mucormycosis using two clinically relevant mouse models of mucormycosis and by testing a limited number of human samples.

MATERIALS AND METHODS

Fungal strains. Five Mucorales fungi were used to identify consensus sequence in *CotH* genes. These are the most commonly clinically isolated organisms, including *Rhizopus delemar* 99-880, *R. oryzae* 99-892, and *M. circinelloides* f. *jenssenii* DI15-131 (all from the fungal testing laboratory of the University of Texas Health Science Center at San Antonio); *L. corymbifera* 008-0490 (an isolate obtained from a patient that was enrolled in the DEFEAT Mucor clinical trial [24]); and *Cunninghamella bertholletiae* 182 (kindly provided by Thomas Walsh, Weill Cornell Medical College). For validation of the specificity of the assay, *Rhizomucor* (DEFEAT Mucor clinical trial [24]) and *Apophysomyces* elegans ATCC 90757 were subsequently included. *A. fumigatus* AF293 was used as a negative control. All fungi were grown on potato dextrose agar at 37°C until confluent sporulation was observed.

Primer design. Because each Mucorales harbors several copies of *CotH* (23) (Fig. 1A), a preliminary step involved the categorization of the various gene sequences within one organism into groups that were then used to create a phylogenetic tree (Fig. 1B) to identify groups with the highest sequence similarity between species. The selected sequences (Fig. 1C) were then aligned using available on-line tools (https://www.ebi.ac.uk/Tools/msa/muscle/). Several candidate primers were designed in various highly conserved regions. A BLAST search was performed with the whole *CotH* sequences, as well as the

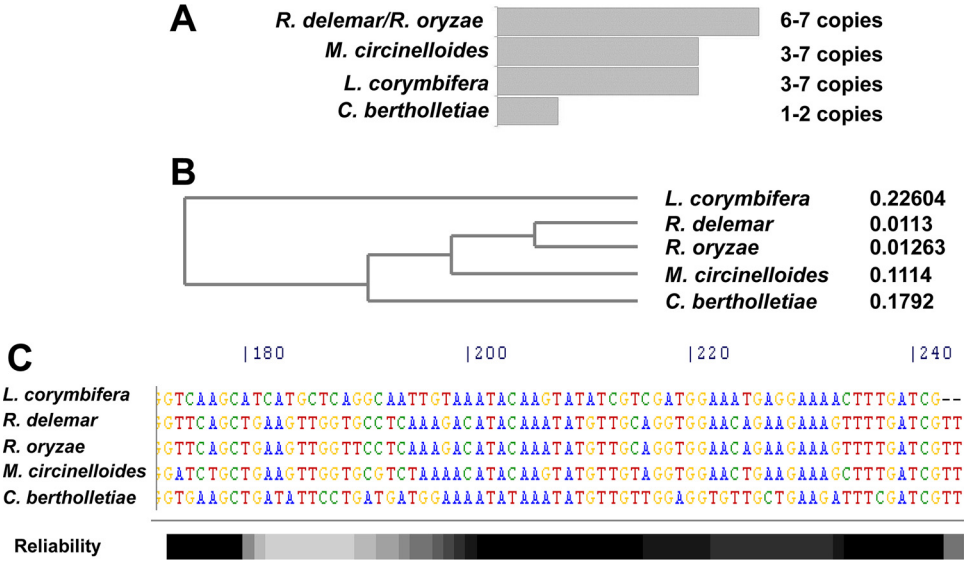

FIG 1 Design of *CotH*-specific primers. (A) Different Mucorales harbor one to seven copies of *CotH* genes, which would allow amplification of a PCR signal. (B) A phylogenetic tree was constructed using each species' *CotH* sequence that presented the highest similarity among all others Mucorales. (C) Multialignment analyses were performed with available online tools. The result showed many regions of high similarity or identity, especially in the 5′ region, that were used to design candidate primers.

designed primers, to ensure analytical specificity and to exclude cross-reactivity with other genes. Next, the primers were tested to establish their specificity using gDNA extracted from cultures of the different Mucorales under investigation.

To evaluate the sensitivity of each designed primer, sterile phosphate-buffered saline (PBS), mouse urine samples, and mouse serum samples (from uninfected mice) were spiked with different spore concentrations of *R. delemar*, *M. circinelloides*, *L. corymbifera*, or *C. bertholletiae*. These primers are proprietary to Vitalex Biosciences, a company developing diagnostic methods and immunotherapy against mucormycosis.

DNA extraction and PCR. gDNA was extracted using three different methods. Two methods involved the use of the commercial kits of QIAamp DNA minikit (Qiagen) and the Quik-DNA fungal/bacterial kit (Zymo Research) in which genomic DNA was extracted according to the manufacturer's recommendations. For the third extraction method, we developed a new strategy optimizing the protocol from the Master Pure Yeast DNA purification kit (Epicentre Biotechnologies). Briefly, fungal spores (5×10^6/ml) were grown overnight in potato dextrose broth at 37°C with shaking at 200 rpm. The visible mycelia were collected by filtration using Whatman paper (6-μm pore size) or pelleted by centrifugation at 10,000 $\times$ *g*. The collected mycelia were resuspended in lysis buffer and vortexed for 5 min in the presence of glass beads (425 to 600 μm, acid washed; Sigma-Aldrich). After incubation at 65°C for 30 min, followed by placement of the samples for 5 min on ice, protein precipitation buffer was added, and the samples were centrifuged for 10 min. The DNA in the supernatant was then precipitated with isopropanol, washed with 70% ethanol, resuspended in distilled water, and diluted to a final concentration of 50 to 100 ng/μl. In all of the methods tested, an exogenous DNA of a Mucorales ricin-like gene was used as an internal control to check for the validity of the extraction method and for inhibition of the PCR assay.

PCR was performed in a reaction mixture of 25 μl using 1 μl of the gDNA extracted from culture or 2 μl of gDNA extracted from biological samples as the template, 20 pmol of each primer, and 1$\times$ *Taq* PCR Premix (TP01; Bioland). The PCR conditions consisted of denaturation for 3 min at 95°C, followed by 40 amplification cycles, including denaturation at 95°C for 30 s, annealing at various temperatures according to the selected primers (generally 56 to 64°C) for 30 s, and elongation at 72°C for 30 s. A final extension step of 10 min at 72°C was also included. All 25 μl of the reaction mixture was analyzed by electrophoresis in 1.5% agarose gel in the presence of ethidium bromide and visualized under UV light.

To verify the sequence of the amplified band, the PCR was performed as described above using a proofreading polymerase (i.e., the Phusion High-Fidelity PCR master mix; Thermo Fisher), except for a denaturation step performed at 98°C instead of at 95°C. The amplicon was purified by either elution from agarose gel (QIAquick gel extraction kit; Qiagen) or directly after PCR with a QIAquick PCR purification kit (Qiagen). Purified DNA fragments were ligated into the pGEM-T Easy vector (Promega) and transformed to XL-10 Gold Ultracompetent cells (Agilent). Plasmids were extracted from positive clones and sequenced with M13F universal primer.

Mouse models. CD-1 male mice were infected with invasive pulmonary mucormycosis (IPM) or invasive pulmonary aspergillosis (IPA). For IPM, we used either neutropenic or diabetic ketoacidotic (DKA) mice infected intratracheally (25), while IPA was induced in neutropenic mice via inhalation (26). For

TABLE 1 Human plasma and urine samples collected from patients with proven mucormycosis[a]

Sample	Site(s) of infection	Sample collection period (days)[b]	Origin of sample	Method(s) of diagnosis	Causative Mucorales
6414225	Pansinusitis, skin	3–7	Cologne, Germany	Culture	*Lichtheimia corymbifera*
6470827	Lungs, liver	3–7	Cologne, Germany	Histopathology/PCR[c]	*Rhizomucor pusillus*
700210809	Sinus, lungs	28	AsTeC	Histopathology/culture	*Rhizopus*
700077090	Disseminated[d]	14	AsTeC	Histopathology/culture	*Rhizomucor*

[a]Biological samples were obtained from four patients with proven mucormycosis from a Medical Center in Germany or from the AsTeC sample collection (29).
[b]That is, the sample collection in days from the onset of symptoms.
[c]Identified by amplifying 28S rDNA, followed by large-subunit rDNA D/D2 domain sequencing.
[d]That is, brain, spleen, kidneys, renal pelvis, bladder, ureters.

neutropenia, mice were injected intraperitoneally (i.p.) with cyclophosphamide (200 mg/kg) and subcutaneously with cortisone acetate (500 mg/kg for IPM and 250 mg/kg for IPA) on days −2 and +3 (relative to infection) (25, 26). To render mice diabetic, a single dose of streptozotocin (210 mg/kg) was introduced i.p. 10 days prior to infection (27). Intratracheal instillation of Mucorales spores or inhalation of *A. fumigatus* conidia was conducted as previously described (25, 26). For hematogenously disseminated candidiasis, immunocompetent mice were infected via the tail vein with 2×10^5 blastopores of *C. albicans* SC5314 (28). Some of the Mucorales-infected mice were treated with either liposomal amphotericin B (LAmB; 10 mg/kg/day, given intravenously for 4 days), posaconazole given at 30 mg/kg/day for 4 days, or a single dose of 30 μg of monoclonal antibody targeting the protein CotH3. All treatment started 24 h postinfection. At selected time points postinfection, urine samples were collected by the use of metabolic cages, followed by euthanasia and serum and BAL sample collection. All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Los Angeles Biomedical Research Institute under protocol 30264.

Human samples. To further validate our assay, a number of human biological samples were included in the analysis and obtained from the Clinical Study Center of Infectious Diseases, Department I for Internal Medicine, University Hospital of Cologne, Cologne, Germany (urine and plasma samples obtained from patients with histopathologically proven mucormycosis), and from the Aspergillosis Technology Consortium (AsTeC), NIH-NIAID Clinical Laboratory Diagnostics for Invasive Aspergillosis samples at the University of Florida, Gainesville (29) (Table 1). All samples were collected after obtaining a signed consent form approved by the institution's respective institutional review board and were tested for infection by PCR at LA Biomed under IRB protocol 11671. Samples (500 μl) were used for DNA extraction using the protocol described above optimized for mouse urine samples.

Statistical analysis. Sensitivity and specificity of the PCR assay were evaluated using the receiver operating characteristic (ROC) curve analysis (30, 31). An area under the ROC curve (AUC) of 1.0 represents a perfect curve fit, while an AUC of 0.5 represents random classification (32). Calculations of the ROC curve and the AUC were performed with MedCalc. Using ROC analysis, we determined the sensitivity and specificity of the PCR-based assay. A P value of <0.05 in each of the ROC curves demonstrates that the results are significant and robust.

RESULTS

Testing the specificity and sensitivity of universal primers. To design universal primers that can amplify *CotH* genes from different Mucorales fungi, we constructed a phylogenetic tree of *CotH* sequences of *R. delemar*, *R. oryzae*, *M. circinelloides*, *L. corymbifera*, and *C. bertholletiae* (Fig. 1B). Initially, eight primer sets were designed from short consensus sequences present in most *CotH* genes. These primer sets were tested in preliminary studies using gDNA extracted from *R. delemar*. Three primer sets, amplifying fragments in the 5′ region (Fig. 1C), primer sets #2, #4, and #6, demonstrated the best ability to amplify *CotH* fragments using gDNA extracted from different Mucorales. Specifically, primer sets #2 and #6, but not primer set #4, amplified the desired band (confirmed by size and sequence of the band) from all Mucorales gDNA with different intensities (Fig. 2A). However, primer set #2 was not specific to Mucorales since it also amplified nonrelated bands from *A. fumigatus* gDNA. Primer set #6 was specific in amplifying *CotH* bands from Mucorales, with the highest sensitivity shown toward *R. delemar* and *R. oryzae*. Less sensitivity was demonstrated with samples spiked with *L. corymbifera* and *M. circinelloides*, while the weakest sensitivity was obtained when gDNA from *C. bertholletiae* was used as a template (Fig. 2A).

To have a robust PCR diagnostic assay, an extraction method must ensure the maximum yield of fungal DNA from biological samples. Therefore, we compared three methods of DNA extraction from PBS spiked with 10-fold dilutions of *R. delemar* spores. Using primer set #6, we were only able to detect *CotH* fragment from samples spiked

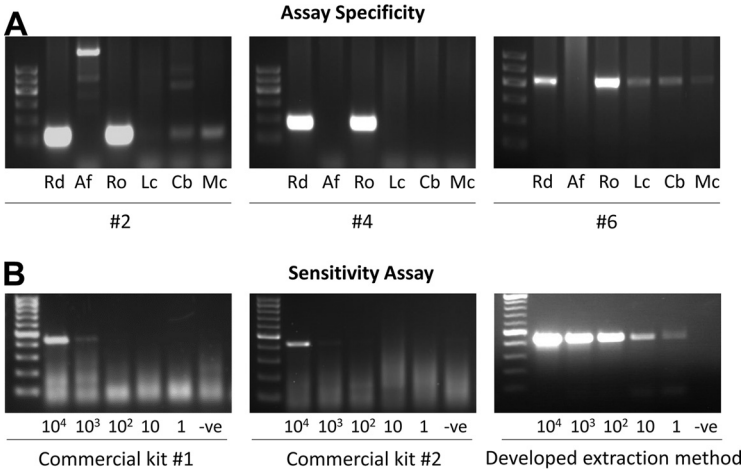

FIG 2 Determination of the specificity and sensitivity of the designed primers. PCR amplification of genomic DNA (gDNA) of *R. delemar* (Rd), *R. oryzae* (Ro), *L. corymbifera* (Lc), *C. bertholletiae* (Cb), and *M. circinelloides* (Mc) was performed using *CotH*-specific primer sets #2, #4, and #6. gDNA from *A. fumigatus* (Af) was included as a negative control. (A) Best candidate primers specifically amplified the desired band from all tested Mucorales but not from *A. fumigatus*. A fast, inexpensive, and reliable method for the extraction of DNA from low-volume/low concentration samples was optimized in our laboratory. (B) The method used for panel A was compared to commercially available kits in extracting gDNA from *R. delemar* prior to conducting PCR using primer set #6. Note the amplification of the *CotH* fragment from cultures spiked with even 1 spore/ml, showing a much higher sensitivity with the optimized DNA extraction method compared to the two commercially available kits.

with no less than 10^4 or 10^3 spores/ml when the two commercial kits were used to extract DNA. In contrast, using our optimized method of DNA extraction, we were able to amplify *CotH* fragment from samples spiked with as low as 1 spore/ml (Fig. 2B). Thus, we subsequently used this optimized DNA extraction method for all of our DNA extractions from mouse or human samples.

Detection of Mucorales infection from mouse biological samples. In a pilot experiment, neutropenic mice were infected intratracheally with 2.5×10^5 spores of *R. delemar*, *L. corymbifera*, *C. bertholletiae*, and *M. circinelloides*. Bronchoalveolar lavage (BAL), plasma, and urine samples were collected at sacrificing time on days +1, +3, and +4 relative to infection for *R. delemar* and *L. corymbifera*; for *C. bertholletiae*, samples were collected only at days +1 and +3 due to mouse mortality. Because *M. circinelloides* in previous experiments showed a late development of the infection (25), samples were collected at days +3, +4, and +7. Qualitative culture analyses were performed with both BAL and plasma samples, but not with urine due to the low volume collected per each mouse. Cultures from plasma resulted in more reliable detection of infection compared to cultures from BAL fluid (Table 2). PCR using primer set #6 was performed with all collected samples after gDNA extraction using our optimized method that contained a bead beating step and lysis buffer (earlier studies without the use of bead beating and/or lysis buffer did not give us consistent results

TABLE 2 Detection of infection in neutropenic mice infected with different Mucorales[a]

	Culture		PCR		
Mucorales	**BAL**	**Plasma**	**BAL**	**Plasma**	**Urine**
R. delemar	+/+/+	+/+/+	+/+/+	–/–/–	+/+/+
L. corymbifera	–/+/+	+/+/+	–/–/–	–/–/–	–/+/–
C. bertholletiae	+/+	+/+	–/+	–/–/–	+/+
M. circinelloides	–/–/–	+/+/–	–/–/–	–/–/–	+/+/+

[a]"+" and "−" represent results for the designated sample collected from a single mouse on days +1, +3, and +4 for *R. delemar*, *L. corymbifera*, or *C. bertholletiae* or on days +3, +4, and +7 for *M. circinelloides*. For example, "+/+/+" means culture or PCR positive on days +1, +3, and +4 for *R. delemar*, *L. corymbifera*, and *C. bertholletiae* or positive on days +3, +4, and +7 for *M. circinelloides*.

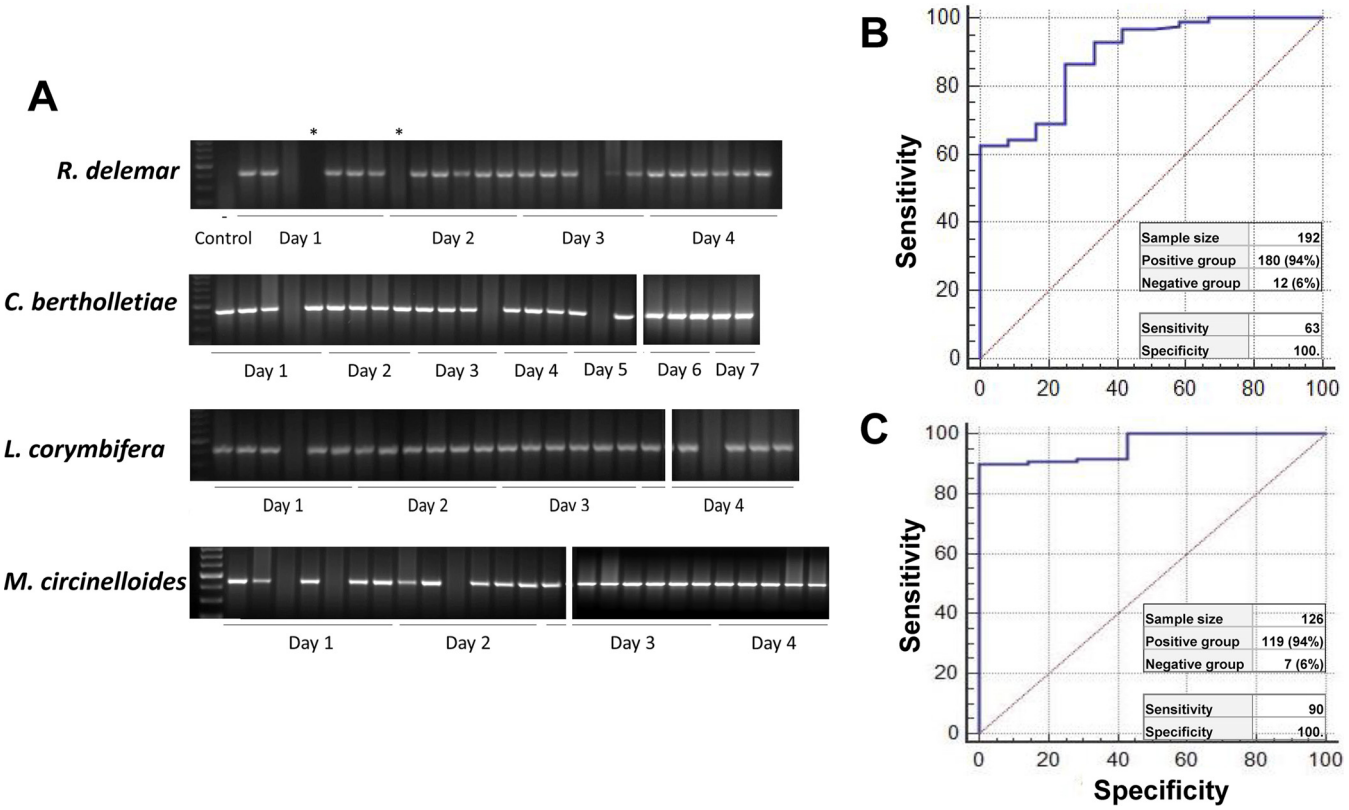

FIG 3 Detection and sensitivity and specificity analysis of *CotH* in urine samples of DKA mice infected with different Mucorales. Urine samples were collected from mice infected with different Mucorales species on different days postinfection. The samples were extracted for DNA and PCR conducted with primer set #6. A larger volume of starting samples (>100 μl) increased the probability of *CotH* detection with an efficiency between 88 and 92% (A). *, DNA extracted from urine samples of <100 μl. Spaces between gels indicate the assembled pictures form more than one gel. The ROC was utilized to determine the sensitivity and specificity of the assay by including the results for urine samples collected from mice infected with Mucorales species or those infected with *A. fumigatus* or with (B) or without (C) including urine samples collected from Mucorales-infected mice that had been treated with antifungal therapy.

in all three biological samples tested [data not shown]). Amplification of *CotH* fragment from urine samples was more sensitive and reliable than amplification from BAL fluid. In contrast, none of the plasma samples showed amplification of the desired band.

It has been reported that heparinized blood could inhibit PCR amplification (33, 34). Thus, we hypothesized that the lack of *CotH* amplification in plasma samples was due to the presence of heparin. Indeed, the introduction of heparin in the PCR of DNA samples extracted from Mucorales spiked specimens, rendered them negative for *CotH* amplification, as well as for the Mucorales ricin-like gene that was used as an internal control (data not shown).

Because the PCR results were more consistent with the urine samples, we focused our subsequent studies on detecting *CotH* genes in mouse urine. Since the collection of urine from neutropenic mice was rather challenging, sample collection significantly improved when we switched to DKA mice coupled with the use of metabolic cages. More than 200 samples from different infection models (infected with Mucorales, *A. fumigatus*, or *C. albicans*) have been collected, including uninfected controls. We noticed that a starting volume of >100 μl of urine increased the probability of *CotH* detection. Figure 3A shows the results from a representative experiment in which we infected DKA mice with different Mucorales and collected urine samples on different days postinfection using metabolic cages. Excluding urine samples that had <100 μl, the assay reached sensitivities of 91% for *R. delemar*, 88% for *C. bertholletiae*, 92% for *L. corymbifera*, and 92% for *M. circinelloides* (Fig. 3A). Some of the amplified bands from different experiments were sent for Sanger sequencing. The presence of single-nucleotide polymorphisms in the *CotH* amplicon allowed the identification of the infection down to the species for all sequenced bands.

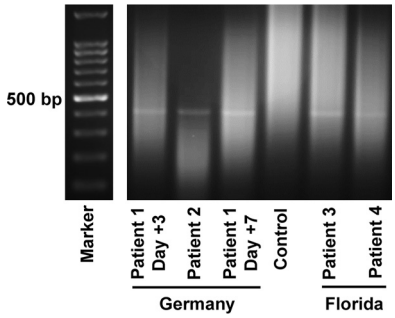

FIG 4 Detection of *CotH* in urine samples from patients with proven mucormycosis. PCR amplification of a 433-bp *CotH* fragment using gDNA extracted from urine samples of four different patients with proven mucormycosis. The space in the gel indicates assembled picture from more than one gel.

ROC analysis. To statistically further validate the sensitivity and specificity of the assay, we performed an area under the ROC curve analysis under two different scenarios. First, we included urine samples collected from uninfected mice, mice infected with Mucorales species, or mice infected with *A. fumigatus*. We also included urine samples from mice infected with Mucorales and treated with antifungal drugs (LAmB or posaconazole) or anti-CotH protein antibodies. For 192 samples analyzed, the AUC value was equal to 0.89, with a sensitivity of 63%, which was driven by some negative samples taken from treated mice indicating successful treatment. The specificity of the assay was at 100% ($P < 0.0001$) (Fig. 3B). In the second scenario, the same samples were analyzed, except this time samples collected from mice treated with antifungal were excluded from the analysis. For the 126 samples, the AUC value was equal to 0.96, with an increased sensitivity to 90%, and the specificity remained at 100% ($P < 0.0001$) (Fig. 3C).

Validation of the assay in human samples with proven mucormycosis. Biological samples (plasma and urine) were obtained from four different patients with proven mucormycosis. The samples came from two patients seen at the University of Cologne, Cologne, Germany, and two patients as part of the AsTeC biological sample collection housed at the University of Florida in Gainesville (Table 1). Plasma and urine samples were collected at several days from the onset of symptoms. None of the plasma samples resulted in positive amplification of *CotH* (data not shown), possibly due to the use of heparin in obtaining plasma. In contrast, at least one urine sample from each patient resulted in amplification of the targeted *CotH* fragment using primer set #6 (Fig. 4). We were able to sequence the PCR bands amplified from the two patients obtained from Germany and the first sample obtained from University of Florida (Table 1), while the fourth sample was not adequate for reliable sequencing. The sequence identities of the bands confirmed infection with *Lichtheimia*, *Rhizomucor*, and *Rhizopus*, respectively.

DISCUSSION

Definitive diagnosis of mucormycosis is frequently delayed because of its current reliance on clinical examination and radiographic features (e.g., reverse halo sign or multiple nodular lesions on CT scans of lungs) (35, 36). These features are only possible to detect at an advanced stage of the disease and in many cases are nonspecific. Alternatively, a recent article described the use of a serum disaccharide by mass spectroscopy showing a high degree of detection of this biomarker in nine of ten mucormycosis serum samples. However, this assay does not discriminate between invasive mucormycosis, aspergillosis, or candidemia because of its panfungal nature (37). It also requires the use of matrix-assisted laser desorption ionization–time of flight mass spectroscopy. Consequently, further diagnostic work is required which often includes invasive procedures such as obtaining BAL fluid and biopsy specimens. Performing these invasive procedures can be challenging in certain clinical scenarios (e.g., severe pancytopenia in hematologic malignancy patients). Even when samples are

obtained from patients, the definitive diagnosis is reliant on culturing methods or histopathological examination. It is known that the culture of biological samples from patients with mucormycosis often yields negative results because of hypha fragmentation during sample processing (38). Even when culturing the organism from a biological sample, the information is rarely sufficient to establish mucormycosis diagnosis because Mucorales are ubiquitous, may colonize healthy people, and are frequent laboratory contaminants (1). Moreover, microscopic/histopathological examination requires some level of expertise to identify Mucorales as ribbon-like aseptate hyphae branching at 90° angles (3). Finally, unlike the galactomannan and β-1,3-D-glucan tests that are commonly used to diagnose aspergillosis (39, 40), there is no approved serological test for mucormycosis. For these collective reasons, definitive mucormycosis diagnosis cannot be established in many cases and a simple, reliable, and rapid assay of mucormycosis diagnosis is required. Such an assay can be used to rapidly diagnose the infection among hospitalized patients who are at high risk for infection (e.g., hematopoietic cell transplant patients, immunosuppressed patients with iron overload, or patients with graft-versus-host disease). Preferably, a quantitative assay (e.g., qPCR) that demonstrates response to treatment can also be routinely used to monitor the progression of the disease burden, inform the use of successful antifungal therapy, and predict relapse. Such an assay has to have high sensitivity/specificity, be simple to perform, preferably be noninvasive, and be affordable.

We exploited the unique and universal presence of *CotH* gene family in Mucorales to develop a rapid and reliable PCR-based diagnosis of mucormycosis. *CotH* genes are also present in multiple copies in most Mucorales, and these fungi are known to be multinucleated (41), a feature that is responsible for augmenting the sensitivity of any PCR-based diagnostic assay. By using proprietary primers to *CotH* genes and optimized DNA extraction method, we are able to show that *CotH* gene fragments can be amplified with a 100% specificity from urine, serum, and BAL fluid samples obtained from mice with mucormycosis. *CotH* DNA was more consistently detected in urine samples than in BAL fluid or serum samples and was totally absent from plasma samples obtained from heparinized blood. The detection of *CotH* DNA in urine samples with higher sensitivity compared to serum or BAL fluid could be attributed to the fact that urine is a less complex biological sample and hence has the smallest amount of interfering substances with the PCR. In this respect, several successful PCR-mediated detection assays have been reported to many pathogens, including *Leishmania*, *Mycobacterium tuberculosis*, and human papillomavirus (42–44).

Detection of infection in urine samples obtained from humans with definitive mucormycosis (albeit the amplified bands were accompanied by an intense smear) appeared to corroborate the high potential of the assay for human use. The presence of smear in the human samples is probably due to the use of a larger volume of urine (500 μl versus $\sim$200 μl for mouse samples) and dictates that further optimization and cleaning of the extraction assay for human samples are needed; this is currently the subject of active investigation with the use of more quantitative assay.

Of particular importance was the high sensitivity ($\sim$90%) of detecting murine mucormycosis in the urine of mice infected with *Rhizopus*, *Lichtheimia*, *Mucor*, or *Cunninghamella* as early as 24 h postinfection, which is likely attributed to the presence of *CotH* as multicopy genes due to the genome duplication and the multinucleated nature of Mucorales (Fig. 1) (41). This finding emphasized the potential use of this PCR-based method for rapid detection of $>$90% of all causes of mucormycosis (45). This rapid detection of *CotH* in the urine of mice infected intratracheally 24 h prior to detection points to the rapid hematogenous dissemination of Mucorales in this model, as verified by culturing the mold from the plasma (Table 2). Although the sensitivity of detecting *CotH* genes in urine was dependent on obtaining sufficient sample volume of at least 100 μl, this limitation should not pose a problem when the assay is further validated in humans. It is equally important to emphasize that the use of urine as the biological sample of choice for diagnosing the infection has the potential to avoid the need for invasive methods. However, a potential drawback for developing a biomarker

assay which is reliant on urine or serum samples is the potential for false-negative diagnosis due to lack of hematogenous dissemination of the organism from the original site of infection (e.g., cutaneous, sinus infection). Another limitation of the developed assay is the lack of quantitation of the amplified DNA and the need to sequence the amplified band to determine the species causing infection. The latter is important due to differences in susceptibility of Mucorales to antifungal therapy (46). Such limitations can be overcome in future developments of qPCR methods, molecular beacons, specific primers to differentiate *CotH* sequence among different Mucorales, and the inclusion of high-resolution melting (HRM) assay. Furthermore, the design of primers for active infection detection using NASBA technology is a possibility given that *CotH* genes are among the most highly expressed genes in *Rhizopus* spores and germlings (23). These future developments are expected to increase the sensitivity of the assay without compromising the current specificity of 100%.

A plethora of PCR-based approaches have shown promise in diagnosing mucormycosis with high sensitivity and specificity. These include PCR assays, PCR/HRM analysis, and PCR/electrospray ionization mass spectroscopy (PCR/ESI-MS). Almost all of these assays targeted the amplification of either internal transcribed spacer 1 (ITS1) or ITS2 (47–49) or 18S-28S ribosomal rRNA genes (50–52). The sensitivity/specific of the primers were determined either from gDNA extracted from Mucorales grown in cultures by using the rolling cycle amplification technique, without confirming the findings in biological samples (47), or with formalin-fixed, paraffin-embedded biopsy specimens (48), which usually reduces the sensitivity of the assay (~56%) (53). The use of unfixed tissue to diagnose mucormycosis using PCR/ESI-MS was recently shown to be more effective in identifying Mucorales to the species level than qPCR, ITS PCR, and 18S PCR (51).

Few studies used fresh biological samples either from animal models or from mucormycosis patients. For example, using PCR to amplify the ITS2 region, followed by an HRM assay, Lengerova et al. screened BAL samples from immunosuppressed patients at risk for developing mucormycosis and reported a sensitivity and a specificity of 100 and 93%, respectively, for detecting infection (54). These researchers concluded that the PCR/HRM assay is a useful rapid screening method for BAL samples due to its high negative predictive value of 99%. It is prudent to mention that a potential problem of targeting the ITS sequence for molecular diagnosis of mucormycosis stems from the reported heterogeneity of the ITS sequence among different rDNA operons of the same strain (55).

Similarly, using a rabbit model of mucormycosis and a qPCR targeting the amplification of 28S rRNA followed by melting-curve analysis to distinguish between different Mucorales, a variable sensitivity was shown for detecting infection in BAL fluid with higher sensitivity compared to plasma samples (50). The use of certain primers to identify the presence of 18S rDNA using real-time PCR showed 100% detection in serum samples from patients with proven or probable mucormycosis and 29% in patients with possible cases (52). They concluded that by using this assay for screening patients, it is possible to diagnose mucormycosis by up to 21 days. Two more studies described the use of real-time PCR assay based on a combination of primers targeting 18S rDNA from *Mucor/Rhizopus*, *Lichtheimia*, and *Rhizomucor* (MucR1) to detect Mucorales circulating DNA in serum samples. In the first study, 36 of 44 patients (81%) with probable or proven mucormycosis had at least one positive PCR test with the first PCR-positive sample observed 9 days median time before diagnosis made with mycological criteria (56). This study also suggested the usefulness of the assay in monitoring the efficacy of treatment. The second study used a modification of the MucR1 primers to detect circulating DNA in the serum of burn patients. At least one positive qPCR test was obtained for all 11 cases that developed invasive wound infection (57). Furthermore, circulating DNA was detected 11 days (range, 4.5 to 15 days) before standard diagnosis, and the assay appears to predict the outcome of antifungal therapy since a persistent positive qPCR after the initiation of treatment was associated with death (57).

In conclusion, *CotH* genes were suitable biomarkers for the development of a rapid,

simple, and reliable diagnostic PCR-based assay for mucormycosis. The amplification of *CotH* DNA from urine 1 day postinfection is also a desirable feature of the assay. Future studies will focus on developing a quantification method that can differentiate between the causes of infection and validate the assay using human samples with proven mucormycosis cases.

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