JOURNAL OF CLINICAL MICROBIOLOGY, June 2010, p. 2043–2046 Vol. 48, No. 6
0095-1137/10/$12.00 doi:10.1128/JCM.02319-09

Molecular Detection and Identification of *Zygomycetes* Species from Paraffin-Embedded Tissues in a Murine Model of Disseminated Zygomycosis: a Collaborative European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Fungal Infection Study Group (EFISG) Evaluation▽

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Received 25 November 2009/Returned for modification 8 January 2010/Accepted 30 March 2010

The present study was performed to assess the interlaboratory reproducibility of the molecular detection and identification of species of *Zygomycetes* from formalin-fixed paraffin-embedded kidney and brain tissues obtained from experimentally infected mice. Animals were infected with one of five species (*Rhizopus oryzae*, *Rhizopus microsporus*, *Lichtheimia corymbifera*, *Rhizomucor pusillus*, and *Mucor circinelloides*). Samples with 1, 10, or 30 slide cuts of the tissues were prepared from each paraffin block, the sample identities were blinded for analysis, and the samples were mailed to each of seven laboratories for the assessment of sensitivity. A protocol describing the extraction method and the PCR amplification procedure was provided. The internal transcribed spacer 1 (ITS1) region was amplified by PCR with the fungal universal primers ITS1 and ITS2 and sequenced. As negative results were obtained for 93% of the tissue specimens infected by *M. circinelloides*, the data for this species were excluded from the analysis. Positive PCR results were obtained for 93% (52/56), 89% (50/56), and 27% (15/56) of the samples with 30, 10, and 1 slide cuts, respectively. There were minor differences, depending on the organ tissue, fungal species, and laboratory. Correct species identification was possible for 100% (30 cuts), 98% (10 cuts), and 93% (1 cut) of the cases. With the protocol used in the present study, the interlaboratory reproducibility of ITS sequencing for the identification of major *Zygomycetes* species from formalin-fixed paraffin-embedded tissues can reach 100%, when enough material is available.

The diagnosis and management of zygomycosis remain difficult tasks. Indeed, there are no clinical or radiological signs specific for zygomycosis, and standardized serological or antigen detection tests are not currently available (10, 27). Classically, the diagnosis of invasive zygomycosis relies on direct examination or the histopathology of a clinical sample demonstrating typical broad nonseptate hyphae and, less commonly, on culture (23). The presence of hyphae associated with tissue damage in samples from a normally sterile site is diagnostic of proven invasive filamentous fungal infection even if the culture result is negative (9). Nevertheless, differentiation between zygomycosis and infection due to hyalohyphomycetes (such as *Aspergillus* spp.) may be difficult when hyphal elements are scant or not typical. A precise diagnosis is of paramount importance because the medical treatments for zygomycosis and aspergillosis are radically different. Moreover, the hyphal morphology is not specific to a species or even a genus. Identification of the causative species of *Zygomycetes* may be important to uncover epidemics or to trace the source of nosocomial infection (22). Cultures are negative in a significant number of cases of zygomycosis (24), and frequently, a formalin-fixed paraffin-embedded (PE) tissue sample is the only material available for use for diagnosis in the clinical setting. In these cases, molecular techniques may be the tools of choice for the identification of the causative organisms. Nevertheless, there are currently no standardized techniques, and few studies have been performed to test molecular tools for the identification of the species from these samples.

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▽ Published ahead of print on 7 April 2010.

TABLE 1. *Zygomycetes* isolates used in this study

Species	Isolate[a]	GenBank accession no. of ITS sequence
Rhizopus oryzae	CNRMA 03.918	DQ119025
Rhizopus microsporus	IP 1123.75	DQ119013
Lichtheimia corymbifera	CNRMA 03.697	DQ118984
Rhizomucor pusillus	CNRMA 04.210	DQ118999
Mucor circinelloides	CNRMA 03.154	DQ118987

[a] Culture collection abbreviations: CNRMA, Centre National de Référence Mycologie et Antifongiques, Institut Pasteur, Paris, France; IP, Collection des Champignons, Institut Pasteur, Paris, France.

To date, several molecular approaches, mainly based on PCR assays, have been used for the diagnosis of zygomycosis by the use of such material. Since DNA damage associated with formalin fixation can compromise the performance of this approach, the aim of the study described here was to assess the interlaboratory reproducibility and sensitivity of the molecular identification of *Zygomycetes* species from PE tissues from experimentally infected mice. This study was performed by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Fungal Infection Study Group (EFISG).

(This work was presented in part at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2007.)

MATERIALS AND METHODS

Isolates. Five isolates of *Zygomycetes* (one isolate each of *Rhizopus oryzae*, *Rhizopus microsporus*, *Lichtheimia corymbifera* [formerly *Absidia corymbifera*] [14]), *Mucor circinelloides*, and *Rhizomucor pusillus* were used in the study. These isolates were previously identified by both phenotypic and molecular methods (26). The isolate numbers and the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 sequence accession numbers in GenBank are shown in Table 1. The isolates were stored as spore suspensions in 20% glycerol at −80°C until they were used.

Animal model of zygomycosis. Tissue sample preparation was centralized at the National Reference Center for Mycology and Antifungals (NRCMA), Institut Pasteur, Paris, France. To obtain infected tissue samples, an animal model of disseminated zygomycosis in nonimmunosuppressed mice was used. In this model, the two main target organs are the brain and kidneys (8). Female specific-pathogen-free outbred OF1 mice (age, 7 weeks; weight, 22 to 24 g; Charles River Laboratories, L'Arbresle, France) were used for the experiments. The mice were given food and water *ad libitum*. The animal studies were performed according to the recommendations of the European Community (Directive 86/609/EEC, 24 November 1986) and were approved by the Ethics Committee of the Pasteur Institute.

Subculture of isolates and inoculum preparation were performed as described previously (8, 26). Briefly, stock spore suspensions were prepared on the day of infection in 2 ml of sterile 0.9% NaCl with 0.05% Tween 80 by swabbing the surface of the cultures with a sterile cotton swab. The suspensions were filtered with a syringe through a nylon filter (pore size, 11 μm) to get rid of hyphal fragments. After appropriate dilution, the spore suspensions were counted in a hemacytometer and the concentrations were adjusted in sterile saline to those needed for infection. Ten mice in total (two mice for each fungal species) were infected by injection of 0.1 ml of the spore suspension into a lateral tail vein (10^5 or 10^6 spores per mouse).

Processing of samples. The animals were killed 3 to 4 days after infection to obtain an invasive infection characterized by hyphae in tissues. For each of the five fungal species, two brains and two kidneys were collected, leading to a total of 20 organs. The organs were aseptically removed, fixed in 10% formalin for at least 48 h, and then included in paraffin. Sections of 10 μm were cut from the blocks; and 2-ml polypropylene tubes (Eppendorf, Le Peck, France) containing either 1 cut (i.e., corresponding to standard histopathology), 10 cuts, or 30 cuts were prepared. The section starting each series of slide cuts prepared for each laboratory was microscopically examined after it was stained with Gomori-methenamine silver (GMS) to confirm the presence of hyphae. Quantification of the fungal burden in the organs before fixation was not attempted, as it has been demonstrated that determination of the numbers of CFU for *Zygomycetes* is not a reliable marker of infection in this model (8). The organs from uninfected mice were processed similarly and were used as negative controls. Therefore, 32 tubes (3 tubes for each organ infected by one of the five species, plus 2 tubes containing uninfected tissue samples), blinded as to the species, were provided to each of the seven participating laboratories.

TABLE 2. PCR results obtained from the seven laboratories for the different species and target organs

Species	Organ	No. of samples positive/total no. tested
R. oryzae	Brain	7/7
	Kidney	7/7
L. corymbifera	Brain	6/7
	Kidney	6/7
R. pusillus	Brain	7/7
	Kidney	5/7
R. microsporus	Brain	7/7
	Kidney	7/7
Total	Brain	27/28
	Kidney	25/28

Molecular study. A common protocol (DNA extraction and PCR) was supplied to each participant laboratory.

DNA extraction. DNA extraction was performed as previously described by Bialek et al., with modifications (3). A first step of deparaffinization was performed by adding 1,500 μl of xylene to the 2-ml tubes containing the tissue section(s). After vortexing and incubation of the tubes for 5 min at room temperature, the tubes were centrifuged at 20,000 × *g* for 5 min. After removal of the supernatant, 1,500 μl of 100% ethanol was added, the tubes were incubated for 5 min at room temperature and centrifuged at 20,000 × g for 5 min, and the supernatant was removed. Both steps were repeated once, and the tubes were incubated at 37°C on a heating block until the total evaporation of the ethanol. DNA was then extracted by using a QIAamp DNA minikit (Qiagen), as follows: 140 μl of ATL buffer and 20 μl of proteinase K were added to the tubes. After complete lysis at 56°C, the tubes were boiled in water for 5 min. An additional step of incubation in liquid nitrogen for 1 min, followed by incubation in boiling water for 2 min, was repeated twice. All of the following steps except the last step were performed according to the manufacturer's instructions (the protocol for DNA purification from tissues); in the last step, elution was done twice with 30 μl of buffer AE (Qiagen).

PCR amplification and sequencing. The rDNA ITS1 region was amplified with the fungal universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). The amplification mixtures (100 μl) contained 5 μl of the extracted genomic DNA, 2.5 μl of each primer at 20 μM, 10 μl of 2.5 mM deoxynucleoside triphosphate (Roche Diagnostics GmbH, Mannheim, Germany), 10 μl of 25 mM $MgCl_2$ (Applied Biosystems), 1.25 μl of AmpliTaq Gold polymerase at 5 U/μl (Applied Biosystems), 10 μl of 10× PCR Gold buffer (Applied Biosystems), and 58.75 μl of water. Amplification of the PCR products was done with the following cycling parameters: an initial step of denaturation of 10 min at 94°C; 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and elongation for 30 s at 72°C; and a final extension for 10 min at 72°C. Both strands of the PCR products were sequenced with primers ITS1 and ITS2 on automated capillary DNA sequencers. Identification was achieved in each laboratory by comparing the sequences obtained with those in the GenBank database by use of the BLAST algorithm.

RESULTS

PCR results. The PCR results obtained for 30 cuts of tissues, for four species, and for the two tested organs (a total of 56 samples) are indicated in Table 2. Overall, a positive PCR result was obtained for 52 of the 56 samples (93%). There were no major differences depending on the organ (89% and 96% positive PCR results for kidney and brain tissues, respectively)

TABLE 3. PCR positivity and identification results from different quantities of tissues used for DNA extraction

Tissue quantity (no. of cuts)	No. of tissue samples/total no. tested (%)	
	Positive by PCR	With correct identification
30	52/56 (93)	52/52 (100)
10	50/56 (89)	49/50 (98)
1	15/56 (27)	14/15 (93)

or depending on the species (100% positive PCR results for *R. oryzae* and *R. microsporus* and 86% positive PCR results for *L. corymbifera* and *R. pusillus*). Similarly, the proportions of positive PCR results ranged from 75% to 100%, depending on the laboratory.

Of note, even though the organs were histologically found to be infected, the tissues infected with *M. circinelloides* were PCR negative in 93% of the cases for the samples with 30 cuts and were always negative for the samples with 10 cuts and 1 cut. Therefore, the data for *M. circinelloides* were excluded from the analysis of the results.

Identification results. Identification to the species level was correct for all the 52 PCR-positive samples corresponding to 30 cuts (Table 3). Of note, several possible identifications (up to four different species) were reported by two laboratories. In one case, corresponding to one *R. oryzae*-infected tissue sample, the reported identification based on sequence homology analysis with the GenBank entries was *Rhizopus oryzae*/*Rhizopus japonicus*/*Amylomyces rouxii* (the last two names could be considered synonyms of *R. oryzae* [16, 25]). In two other cases, both of which corresponded to *R. microsporus*-infected tissues, the identifications reported were *Rhizopus microsporus*/*Rhizopus azygosporus*/*Rhizopus sexualis*/*Rhizopus oryzae* and *Rhizopus microsporus* var. *rhizopodiformis*/*Rhizopus oryzae*, respectively.

Sensitivity. To broadly assess the sensitivity of the technique, different quantities (1, 10, and 30 cuts) of tissues were provided for each sample. The PCR results and sequence-based identification to the species level are shown in Table 3. There was no major difference in the proportions of positive PCR results when the quantity of tissue decreased from 30 cuts (93% of the samples of which had positive PCR results) to 10 cuts (89% of the samples of which had positive PCR results). In contrast, when the quantity decreased to 1 cut, positive PCR results were obtained in less than 30% of the cases. The proportion of correct identifications also decreased with a decreasing amount of tissue but remained >90% even when 1 cut was used.

DISCUSSION

The aim of the study described here was to test the reproducibility of a molecular method for the detection and identification of the causative species in histopathologically proven zygomycosis by the use of PE tissue samples. Identification of the species may be important to gain better knowledge of the epidemiology of zygomycosis and because the different species of *Zygomycetes* exhibited various antifungal susceptibilities (1, 6, 7). Species identification may thus be important to guide the choice of antifungal therapy. The method was assessed by testing the same samples in seven different laboratories known for their involvement in the diagnosis of invasive fungal infections. Overall, amplification of fungal DNA was obtained in 75 to 100% of the cases, but we had to remove the results for one of the five species tested (*M. circinelloides*).

The ITS region was chosen as the target sequence since sequence-based identification of this region by PCR amplification with panfungal primers has been recommended as the method of first choice for the identification of *Zygomycetes* to the species level from pure culture (2). A similar approach targeting rDNA has been shown to be reliable for the identification of different species of *Zygomycetes* from fresh specimens in experimental models of zygomycosis (15, 26) or in patients (17–19). However, for *M. circinelloides*, almost all PCR results were negative, even though the tissue samples were infected, as demonstrated by the presence of hyphae on the GMS-stained slides. The failure of amplification of *M. circinelloides* has several possible explanations. The first one is the presence of PCR inhibitors in the extracted DNA. This seems unlikely, as the other paraffin cuts with the other species were processed similarly and led to the correct amplification in the seven laboratories. However, this possibility should be excluded in further quality control by using an internal control of the amplification (5). A second explanation is the unequal efficiency of the primer set used to amplify the five species evaluated. Indeed, the different species of *Zygomycetes* exhibit very divergent sequences in the ITS region (26). When DNA extracted from pure culture or from frozen sections is used (26), this lower yield is not evidenced and does not hamper sequencing. However, when *M. circinelloides* DNA is mixed with huge quantities of eukaryotic DNA, the yield of the amplification could be insufficient to obtain amplified DNA suitable for sequencing. This underlines the need for complementary studies to evaluate other primer sets and the need for quantification of the results by real-time quantitative PCR (5). Several primer sets could be designed and used in separate assays or combined in a multiplex assay. As DNA from PE tissues is fragmented during the fixation process, PCR amplification of the shortest sequence possible may improve the yield of the test. An optimum should then be found between a sufficiently short target for good amplification and a sufficiently long target for precise identification.

Difficulties with the identification of *Zygomycetes* in PE tissues have been highlighted in previous studies. Roughly half of the sequence-based identifications from PE tissues used failed whatever the PCR method or the DNA target for amplification was (4, 12, 17).

DNA amplification from formalin-fixed PE tissues is difficult due to cross-linking and the subsequent fragmentation of DNA during the fixation process. Therefore, there is a need for a more standardized procedure for the fixation of PE tissue specimens and more standardized storage conditions for PE tissue specimens (13). The common protocol for DNA extraction used in the present study may not be optimal. The importance of obtaining good-quality DNA for amplification and sequencing in large quantities is also evidenced by our finding that with 1 cut, amplification was obtained in less than 30% of the cases. Increasing the amount of tissue tested dramatically improved the yield, which suggests that at least 10 cuts of a regular-size biopsy specimen should be used. The use of laser microdissection to procure precisely the fungal cells in a tissue

specimen together with real-time PCR could represent the next step in how we can improve the material recovered for analysis (21).

Additionally, the interpretation of sequences may be difficult not only because of the poor quality of the chromatograms obtained but also because of the pollution of public databases with unidentified or badly identified microorganisms. Indeed, it has been shown that up to 20% of the entries in public international databases such as GenBank may be erroneously identified to the species level, especially for uncommon species (20). For the *Zygomycetes*, errors may be related to the presence of sequences incorrectly assigned to a given species (e.g., the ITS sequences of *R. microsporus* annotated as *R. oryzae*). Moreover, the distinction between some closely related species (e.g., *R. microsporus* and *R. azygosporus*) is not resolved by sequencing of the ITS region. Finally, the currently unstable taxonomy of this group of fungi also complicates identification, as some species may appear with different names within a few months due to synonymy (11, 14). All these issues highlight the importance of comparing the sequences obtained in a study to those of reference strains, ideally type strains of the suspected species.

In summary, the present study demonstrates that a consensual sequence-based identification approach for the mycological diagnosis of zygomycosis is possible, although several points warrant improvement. First, the DNA extraction yield and better standardization could be achieved through the use of commercial devices and kits. However, formalin will always damage DNA, highlighting the need for means of systematic culture and storage of frozen unfixed tissues when invasive fungal infections are suspected. Second, different primer designs are needed to identify the widest spectrum of *Zygomycetes* species as possible. Nevertheless, despite several limitations, the technique described here can be used to initiate further studies with samples from patients with proven or probable zygomycosis.

ACKNOWLEDGMENTS

Patrick Schwarz was supported by an educational grant from Gilead Sciences, Paris, France.

We thank Françoise Dromer for careful review of the manuscript.

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