Discrimination of Aspergillosis, Mucormycosis, Fusariosis, and Scedosporiosis in Formalin-Fixed Paraffin-Embedded Tissue Specimens by Use of Multiple Real-Time Quantitative PCR Assays

Elham Salehi,[a,b] Mohammad T. Hedayati,[b,c] Jan Zoll,[d] Haleh Rafati,[e] Maryam Ghasemi,[f] Atosa Doroudinia,[g] Mahdi Abastabar,[b,c] Ali Tolooe,[h] Eveline Snelders,[d] Henrich A. van der Lee,[d] Antonius J. M. M. Rijs,[d] Paul E. Verweij,[d] Seyedmojtaba Seyedmousavi,[c,d*] Willem J. G. Melchers[d]

Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran[a]; Department of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran[b]; Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran[c]; Department of Medical Microbiology, Radboud University Medical Centre, and Centre of Expertise in Mycology Radboudumc/CWZ, Nijmegen, The Netherlands[d]; Department of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands[e]; Department of Pathology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran[f]; Transplantation Research Center, National Research Institute for Tuberculosis and Lung Disease, Department of Pathology Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran[g]; Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran[h]

In a retrospective multicenter study, 102 formalin-fixed paraffin-embedded (FFPE) tissue specimens with histopathology results were tested. Two 4- to 5-μm FFPE tissue sections from each specimen were digested with proteinase K, followed by automated nucleic acid extraction. Multiple real-time quantitative PCR (qPCR) assays targeting the internal transcribed spacer 2 (ITS2) region of ribosomal DNA, using fluorescently labeled primers, was performed to identify clinically important genera and species of *Aspergillus*, *Fusarium*, *Scedosporium*, and the *Mucormycetes*. The molecular identification was correlated with results from histological examination. One of the main findings of our study was the high sensitivity of the automated DNA extraction method, which was estimated to be 94%. The qPCR procedure that was evaluated identified a range of fungal genera/species, including *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium solani*, *Scedosporium apiospermum*, *Rhizopus oryzae*, *Rhizopus microsporus*, *Mucor* spp., and *Syncephalastrum*. *Fusarium oxysporum* and *F. solani* DNA was amplified from five specimens from patients initially diagnosed by histopathology as having aspergillosis. *Aspergillus flavus*, *S. apiospermum*, and *Syncephalastrum* were detected from histopathological mucormycosis samples. In addition, examination of four samples from patients suspected of having concomitant aspergillosis and mucormycosis infections resulted in the identification of two *A. flavus* isolates, one *Mucor* isolate, and only one sample having both *R. oryzae* and *A. flavus*. Our results indicate that histopathological features of molds may be easily confused in tissue sections. The qPCR assay used in this study is a reliable tool for the rapid and accurate identification of fungal pathogens to the genus and species levels directly from FFPE tissues.

The frequency of invasive fungal diseases (IFDs) has increased significantly over the past 3 decades, and they are associated with excessive morbidity and mortality in immunocompromised hosts, patients hospitalized with severe underlying diseases (e.g., acute myelogenous leukemia), those requiring complex surgical procedures (e.g., trauma patients), and individuals who require support in intensive care units (1–5).

The most well-known cause of opportunistic filamentous mycoses is *Aspergillus fumigatus* (6). However, the epidemiology of IFDs due to filamentous fungi has been expanded well beyond *A. fumigatus*, including non-*fumigatus* species of *Aspergillus*, the mucormycetes, *Fusarium* and *Scedosporium* species, and a wide variety of melanized fungi (4, 7).

Given the complexity of the population of patients at risk and the diverse and increasing arrays of fungal pathogens, which show significantly different antifungal susceptibilities, (3) early and reliable detection of a causative fungal pathogen is crucial to guide the appropriate and successful treatment of IFDs (8, 9).

Formalin-fixed paraffin-embedded (FFPE) tissues obtained from patients with proven IFDs are frequently used to detect the etiology of invasive mycoses (10–12). While histopathology can prove invasive fungal infections by the demonstration of fungal elements in tissue specimens, genus- or species-level identification due to morphological characteristics is limited. Apart from this, despite detection of fungal elements in the specific stained histological samples, fungal cultures from tissue biopsy specimens often remain negative in a substantial number of cases (13, 14).

Despite a lack of standardization with respect to optimal sam-

Received 1 June 2016 **Returned for modification** 6 July 2016
Accepted 1 September 2016

Accepted manuscript posted online 7 September 2016

Citation Salehi E, Hedayati MT, Zoll J, Rafati H, Ghasemi M, Doroudinia A, Abastabar M, Tolooe A, Snelders E, van der Lee HA, Rijs AJMM, Verweij PE, Seyedmousavi S, Melchers WJG. 2016. Discrimination of aspergillosis, mucormycosis, fusariosis, and scedosporiosis in formalin-fixed paraffin-embedded tissue specimens by use of multiple real-time quantitative PCR assays. J Clin Microbiol 54:2798–2803. doi:10.1128/JCM.01185-16.

Editor: D. W. Warnock, University of Manchester

Address correspondence to Seyedmojtaba Seyedmousavi, Seyedmousavi@nih.gov, or Willem J. G. Melchers, Willem.Melchers@radboudumc.nl.

* Present address: Seyedmojtaba Seyedmousavi, Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

ple type, primer selection (panfungal, genus specific, or species specific), and PCR formats (qualitative, quantitative, real-time) (2, 15–17), PCR-based techniques provide a promising alternative for the species-level identification of fungal agents in FFPE tissues (1, 5, 18–22).

In the present study, we developed and evaluated a real-time quantitative PCR (qPCR) assay targeting the multicopy internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) (23), to detect and identify genus and species of *Aspergillus*, the *Mucormycetes*, *Fusarium*, and *Scedosporium* directly from FFPE tissue specimens obtained from patients with histologically proven IFDs.

(Part of this research was presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases, 9–12 April 2016, Amsterdam, The Netherlands, [24].)

MATERIALS AND METHODS

Samples. One hundred two tissue specimens (from 102 patients) collected from 2008 to 2014 with positive histopathology results were used for this study. In addition, six FFPE tissue specimens from patients without IFDs were used as controls. The tissue blocks were archived in the tissue registry at multiple university hospitals in Iran and had already been processed per routine care. The Review Board of Mazandaran University Medical Center in Iran approved the use of these blocks for this study.

Review of histopathology. The original histopathology results were confirmed by two independent reviewers for a total of three interpretations. Both secondary reviewers were blind to the initial diagnoses and had access to various histochemical stains, including hematoxylin-eosin (H&E) and Gomori-methenamine-silver (GMS), to aid in the diagnostic process. If all histopathology results from all three independent reviewers were the same, they were considered in agreement. If a consensus was not reached among all three histopathology reviewers, the result was considered discordant.

Tissue processing for DNA extraction. Prior to obtaining tissue sections for qPCR testing, we produced a single slide (5 μm) stained with H&E from the surface of the block to ascertain that adequate tissue and fungal elements were present. The microtome blade was switched between blocks to prevent carryover of nucleic acid from one block to the next. Two 4- to 5-μm FFPE tissue sections from each specimen were used for DNA extraction.

Nucleic acid extraction. Nucleic acid extraction was performed on samples by use of an automated EZ1 extraction instrument (Qiagen, Venlo, The Netherlands) and a DNA tissue kit, according to the manufacturer's recommendations. Tissue sections of paraffin-embedded tissues were digested with proteinase K, followed by automated nucleic acid extraction. Briefly, each tissue specimen was transferred into a 1.5-ml sample tube. A 190-μl volume of buffer G2 was added and incubated for 5 min at 75°C, with vigorous mixing (in a shaking thermomixer). The temperature was then reduced, and samples were cooled to 56°C. Ten microliters of proteinase K solution was added, and the tubes were mixed gently. This mixture was incubated overnight at 56°C, with continuous vigorous mixing.

To validate the presence of amplifiable DNA and the absence of inhibitory substances, a PCR targeting the endogenous human beta-globin gene fragment (nucleotides 70400 to 70667; accession number NG_000007.3) was performed using the primer set G1 (5′-GAA GAG CCA AGG ACA GGT AC-3′) and G2 (5′-CAA CTT CAT CCA CGT TCA CC-3′). Samples lacking amplifiable beta-globin DNA were considered to contain inadequate cellular material or inhibitors of the PCR assay.

Primer and probe design for qPCR. Different real-time qPCR assays were designed for the detection of *Aspergillus*, *Fusarium*, and *Scedosporium* species and *Mucorales* (sub)genera, as described previously (23). Briefly, species-specific forward primer-probe combinations were selected within the ITS2 region of the rDNA gene. Primers and probes were selected based on the alignment of at least five ITS2 sequences per species of *Aspergillus*, *Scedosporium*, or *Fusarium*. For the detection of *Mucorales* (sub)genera, at least five ITS sequences per (sub)genus was used for the selection of primers and probes. The input DNA sequences were obtained from NCBI's reference sequence (RefSeq) database and were aligned by using the ClustalW program. In all assays, a single reverse primer corresponding to the 3′ end of 28S rDNA was used. The primer and probe sequences are shown in Table 1.

***Aspergillus* PCR.** Primer and probe combinations were chosen to detect the species *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus nidulans/A. versicolor*. Additionally, a pan-*Aspergillus*-detecting probe was constructed. Cross-reactivity was determined for all primer and probe combinations. The pan-*Aspergillus* assay detected all known *Aspergillus* species, including *Aspergillus tubingensis*, *Aspergillus clavatus*, *Aspergillus tatrazonus*, *Aspergillus candidus*, *Aspergillus orchraceus*, *Aspergillus ustus*, and *Aspergillus glaucus*.

***Fusarium* PCR.** Primer and probe combinations were chosen to detect the species *Fusarium oxysporum*, *Fusarium subglutinans*, *Fusarium solani*, and *Fusarium dimerum*. Cross-reactivity was determined for all primer and probe combinations.

***Scedosporium* PCR.** Primer and probe combinations were chosen to detect the species *Scedosporium apiospermum*, *Scedosporium aurantiacum*, and *Scedosporium prolificans*. Additionally, a pan-*Scedosporium*-detecting probe was constructed. Cross-reactivity was determined for all primer and probe combinations.

Mucorales PCR. Primer and probe combinations were chosen to detect the (sub)genera *Rhizopus microsporus*, *Rhizopus oryzae*, *Mucor*, *Cunninghamella bertholletiae*, *Lichtheimia*, *Syncephalastrum*, and *Rhizomucor*. Cross-reactivity was determined for all primer and probe combinations.

Real-time PCR. The real-time PCR was performed using a Roche LightCycler 480 Instrument II system. The PCR mixture formulation of a 350 nM concentration of either forward or reverse primer, a 250 nM concentration of the TaqMan probe, and 5 μl of sample DNA in the Roche LightCycler 480 probe master mix was used according to the manufacturer's protocol. The *Aspergillus* PCR was performed with two triplex assays. Triplex 1 contained the probes for *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus nidulans/A. versicolor*. Triplex 2 contained the probes for *Aspergillus niger*, *Aspergillus flavus*, and the pan-*Aspergillus* probe. The *Mucorales* PCR was performed with one quadriplex assay and one triplex assay. The quadriplex assay contained probes for *Rhizopus microsporus*, *Rhizopus oryzae*, *Mucor*, and *Cunninghamella bertholletiae*. The *Mucorales* triplex assay contained probes for the detection of *Lichtheimia*, *Syncephalastrum*, and *Rhizomucor*. *Fusarium* and *Scedosporium* PCR assays were both performed in one quadriplex PCR.

Thermal cycling was performed with an initial decontamination program for 10 min at 40°C, followed by hot-start activation and initial DNA denaturation for 10 min at 94°C. Template DNA was amplified in a two-step cycling program of 50 cycles consisting of denaturation for 10 s at 94°C and annealing and extension for 1 min at 60°C. The positive cutoff value was determined at C_T values of <40.

Analytical specificity. For validation of the multiple qPCR assays, the cross-reactivity and interference were investigated.

Statistical analysis. All data analyses were performed using GraphPad Prism, version 5.3, for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a P value of <0.05 (two-tailed).

RESULTS

Overall, mold hyphae were detected histopathologically in 102 of the tissue specimens from patients identified as having aspergillosis (59 specimens), mucormycosis (29 specimens), concomitant aspergillosis and mucormycosis (4 specimens), or infections that could not be further specified by histological analysis (10 specimens).

One of the main findings of our study was the high sensitivity of the DNA extraction method, which was estimated to be 94%. No fungal DNA was amplified from 6 control tissue specimens obtained from patients without IFDs.

TABLE 1 Primers and probes sequences used for qPCR assays

Assay target	Primer or probe	Sequence (5′–3′)[a]
Panfungal	Reverse primer[b]	ATATGCTTAAGTTCAGCGGGT
Pan-*Aspergillus*	Forward primer[c]	GCGTCATTGCTGCCCTCAAGC
Aspergillus fumigatus	Afum probe	6FAM-CAGCCGACACCCAACTTTATTTTT-BBQ
Aspergillus terreus	Ater probe	LC610-GCTTCGTCTTCCGCTCCGTAG-BBQ
Aspergillus nidulans	Anid/Aver probe	Cy5-AGCCG+GCGT+C+TCCAAC+C+TTATT-BBQ[d]
Aspergillus niger	Anig probe	6FAM-ATGCTCTGTAGGATTGGCCGG-BBQ
Aspergillus flavus	Afla probe	LC610-TGATTTGCGTTCGGCAAGCGC-BBQ
Pan-*Aspergillus*	pAsp probe	Cy5-TCCTCGAGCGTATGGGGCTT-BBQ
Fusarium	Forward primer	TGAATCATCGAATCTTTGAACGC
F. oxysporum	Foxy probe	6FAM-ACTCGCGTTAATTCGCGTTCCYC-BBQ
F. solani	Fsol probe	LC610-CATTACAACCCTCAGGCCCCCG-BBQ
F. oxysporum/*F. subglutinans*	Foxy/subg probe	6HEX-TGATTGGCGGTCACGTCGAG-BBQ
F. solani/*F. dimerum*	Fsol/dim probe	Cy5-TACGCTCCGTCCCAGTGCG-BBQ
Scedosporium	Forward primer	GAGCGTCATTTCAACCCTCG
S. apiospermum	Sapi probe	6FAM-TAAGTCTCTTTTGCAAGCTCGCATTGG-BBQ
S. aurantiacum	Saur probe	6HEX-AAAAGTCTTCTTTTGCAAGCTTCGCATTGG-BBQ
S. prolificans	Spro probe	LC610-TTACAAGCCCAAGGATCGGTGTTGG-BBQ
Pan-*Scedosporium*	panSce probe	Cy5-TCGCATTGGGTCCCGGCGGA-BBQ
Mucorales quadriplex	Forward primer 1	TGAATCATCRARTCTTTGAACGCA[e]
Mucorales triplex	Forward primer 2	GAATCATCGARTTCTYGAACGCA[e]
Rhizopus microsporus	Rmic probe	6FAM-ATTGYCTAAAATACAGCYTC+T+T+T+GT-BBQ[e]
Rhizopus oryzae	Rory probe	Cy5-GGCTTGCTAGGCAGGAATATTACGCT-BBQ
Mucor	Mucor probe	6HEX-TGASYACGCCTGTTTCAGTATCARAA-BBQ[e]
Cunninghamella bertholletiae	Cber probe	LC610-ATTCCA+TAAGGTACG+TCTGTTTCAGTACC-BBQ
Lichtheimia	Licht probe	6FAM-TTGATGGCATTYAGTTGCTGTCATG-BBQ[e]
Syncephalastrum	Sync probe	LC610-CTTGTCCTTKGGGTATGCTTGTTTCAG-BBQ[e]
Rhizomucor	Rhizom probe	Cy5-CTTTGGATTTGCGGTGCTGATGG-BBQ

[a] Dyes used as 5′ fluorophores were as follows: 6FAM, 6-carboxyfluorescein; LC610, LightCycler Red 610 dye; Cy5, cyanine 5; 6HEX. The 3′ quencher used was BlackBerry quencher (BBQ).
[b] The panfungal reverse primer was used in all assays.
[c] The pan-*Aspergillus forward primer was used in all Aspergillus* assays. Triplex 1 contains the Afum, Ater, and Anid/Aver probes. Triplex 2 contains the Anig, Afla, and pAsp probes.
[d] +N indicates an LNA residue.
[e] Degenerated bases are indicated as follows: R (purine), G or A; Y (pyrimidine), C or T; S (strong), G or C; or K (keto), G or T.

The qPCR procedure evaluated in this study showed an overall sensitivity of 64% for the identification of fungi from FFPE. Among 59 qPCR-positive specimens, the identification was in agreement between PCR and histopathology in 47 specimens (80%). As shown in Table 2, a range of fungal genera/species were identified, including *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, *F. oxysporum*, *F. solani*, *Scedosporium apiospermum*, *Rhizopus oryzae*, *R. microsporus*, *Mucor* spp., and *Syncephalastrum* spp. (Table 2). In total, 62% of the fungal species detected belonged to the *Aspergillus* genus, with *A. flavus* being the most frequently observed species (24 of 35 specimens; 68%). The most frequently detected fungi causing mucormycosis belonged to the species *Rhizopus oryzae*, which represented 62% of the proven samples, followed by isolates identified as the genus *Mucor* (31%).

Fusarium oxysporum and *F. solani* DNA were amplified from three specimens from patients initially diagnosed as having aspergillosis by histopathology.

Aspergillus flavus, *S. apiospermum*, and *Syncephalastrum* were

TABLE 2 Results of histopathological analysis and real-time qPCR assay targeting the ITS region of rDNA in 102 biopsy specimens obtained from patients with invasive fungal diseases

Diagnosis from histopathology (no. of specimens)	Species or genus detected by real-time qPCR assay (no. of specimens)	Sensitivity of qPCR assay [no. of specimens with identified species or genus/total no. (%)]
Aspergillosis (59)	*A. fumigatus* (9), *A. flavus* (20), *A. terreus* (1), *A. niger* (1), *F. oxysporum* (4), *F. solani* (1), *R. oryzae* (1)	37/59 (63)
Mucormycosis (29)	*R. oryzae* (7), *Mucor* (3), *R. microsporus* (1), *A. flavus* + *Mucor* (2), *A. flavus* + *R. oryzae* (1), *A. flavus* (2), *Syncephalastrum* (1), *Scedosporium* (1)	18/29 (62)
Concomitant aspergillosis and mucormycosis (4)	*A. flavus* (2), *Mucor* (1), *R. oryzae* + *A. flavus* (1)	4/4 (100)
Unknown (10)		
Total	59	59/92 (64)

detected from mucormycosis samples. In addition, examination of four samples from patients suspected as having concomitant aspergillosis and mucormycosis infections resulted in detection of one *R. oryzae* and three *A. flavus* isolates.

The qPCR assay used, however, failed to detect fungal DNA in 9 samples, possibly as a result of the destruction of DNA before paraffin wax embedding. For 5 of these specimens, DNA was extracted from tissue sections in which only scant fungal hyphae were seen upon histopathological examination. All of these specimens had histopathological characteristics of fungal elements indicative of either *Aspergillus* spp. or mucormycetes. All 5 samples were also PCR negative for human beta-globulin.

Moreover, all 10 samples that could not be specified by histopathology were also negative by the qPCR assay, which suggests that application of a panfungal assay might be beneficial.

No cross-reactivity was observed for non-*Aspergillus* fungal species with the pan-*Aspergillus* probe except for *Paecilomyces variotii*. The species-specific probes, however, showed cross-reactivity with closely related (sub)species. For non-*Fusarium* fungal species with the species-specific probes, no cross-reactivity was observed. The pan-*Scedosporium* assay detected all known *Scedosporium* species. No cross-reactivity was observed for non-*Scedosporium* fungal species with the pan-*Scedosporium* probe. No cross-reactivity was observed for non-*Mucorales* fungal genera. The species-specific probes, however, showed cross-reactivity with closely related (sub)species.

DISCUSSION

In the current study, a specific qPCR assay targeting the ITS region of rDNA genes of *Aspergillus*, *Fusarium*, *Scedosporium*, and mucormycetes proved to be a reliable tool for the rapid and accurate identification of fungal species directly from FFPE tissues. Notably, among the regions of the ribosomal cistron, the ITS region has been shown to have the highest probability of successful identification for the broadest range of fungi (25, 26) in the presence of human DNA (27).

Rapid and accurate identification of fungal pathogens to the species level is critical to improve the management of IFDs (9, 28, 29). Several studies targeting various regions of rDNA genes reported the validity and clinical applicability of molecular techniques for identification of the fungal agents in FFPE tissues (1, 8, 13–14, 18–22, 30–32).

Similar to our finding, in a retrospective study by Buitrago et al., the ability of the real-time PCR procedures targeting ITS1-ITS2 in the rDNA gene was evaluated to detect and identify the fungal DNA at the species level (32). A total of 89 FFPE biopsy samples from 84 patients with proven IFDs were tested. In 9 of the 84 patients, the PCR technique failed to amplify the DNA. *Aspergillus fumigatus* DNA was detected successfully in 43 of 50 patients (86%), and *Aspergillus flavus* was detected successfully in 6 of 50 patients (12%). PCR was positive in 24 of 30 (80%) cases with a negative culture. In another study, Lau et al. used a panfungal PCR assay targeting the ITS1 region of the rDNA gene cluster to detect fungal DNA in fresh and FFPE tissue specimens from patients with culture-proven and/or histologically proven IFDs. The assay used successfully detected and identified the fungal pathogen in 93.6% and 64.3% of culture-proven and solely histologically proven cases of IFDs, respectively (18). Using an ITS panfungal PCR assay, Babouee Flury et al. also found a total sensitivity of 53.8% in culture-positive specimens obtained from patients with proven IFDs, classified according to the EORTC criteria (21). The ITS2 PCR targeting the shorter fragment, however, showed a higher detection rate with a sensitivity of 53.8%, in comparison to the PCR targeting the ITS1-ITS2 fragment (sensitivity, 38%).

Furthermore, it is worth pointing out that in other studies by Bialek et al. (20) and Rickerts et al. (13, 14), PCR assays targeting the mitochondrial aspergillosis DNA and the 18S rDNA of zygomycetes were found to support the histopathological diagnosis and to identify the infecting species. Landlinger et al. also evaluated the clinical potential of separate real-time qPCRs targeted a highly conserved region of the 28S rRNA multicopy gene, covering a wide spectrum of molds, yeasts, and the *Mucormycetes* (33). The sensitivity of the assay was revealed to be 96% (95% confidence interval, 82% to 99%).

Our results also showed that the Qiagen EZ1 extraction instrument in combination with the DNA tissue kit is a reliable method for the detection of intact double-stranded DNA, with significantly higher sensitivity (94%) than that reported previously. Importantly, DNA extraction from FFPE tissues is difficult and requires special protocols in order to extract small amounts of DNA suitable for amplification. Most described methods report an amplification success rate between 60 and 80% (11, 34). However, one should consider that in addition to utilizing commercial kits for DNA extraction, employing a bead beating step and/or automatic homogenizer may lead to better results.

As a limitation, detection of fungal DNA from fFFPE tissue might be challenging due to the degradation of DNA and the presence of PCR inhibitors in these samples (35). In addition, environmental contamination of specimens and/or the PCR master mixture by ubiquitous fungal spores is a possible cause of "false positives" (36).

In conclusion, the qPCR assays targeting the ITS2 region proved to be useful tools for the molecular identification of fungal species and have been shown to amplify DNA from a broad range of fungi in the presence of human DNA (12). By using this method, the time to diagnosis can be greatly reduced. Our results also indicate that histopathological features of molds may easily be confused in tissue sections. The qPCR assay used in this study therefore represents a highly sensitive and promising alternative/adjunctive tool that can be easily incorporated into clinical mycology laboratories.

ACKNOWLEDGMENTS

This publication was prepared as a collaborative study between the Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran (research project fund no. 859) and the Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands.

S.S. has received research and travel grants from Astellas Pharma B.V. P.E.V. has served as a consultant and has received research grants from Astellas, Basilea, Gilead Sciences, Merck, and Pfizer. The other authors have no conflict of interests.

FUNDING INFORMATION

This work, including the efforts of Elham Salehi and Mohammad T. Hedayati, was funded by Mazandaran University of Medical Sciences (MazUMS) (859).

This publication was prepared as a collaborative study between the Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran (research project fund no. 859), and the Department of Medical

Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands.

REFERENCES

1. **Procop GW.** 2010. Molecular diagnostics for invasive fungal infections: a call for refinement and implementation. J Mol Diagn **12:**17–19. http://dx.doi.org/10.2353/jmoldx.2010.090173.
2. **Oren I, Paul M.** 2014. Up to date epidemiology, diagnosis and management of invasive fungal infections. Clin Microbiol Infect **20**(Suppl 6)**:**1–4. http://dx.doi.org/10.1111/1469-0691.12642.
3. **Pfaller MA, Pappas PG, Wingard JR.** 2006. Invasive fungal pathogens: current epidemiological trends. Clin Infect Dis **43:**S3–S14.
4. **Slavin M, van Hal S, Sorrell TC, Lee A, Marriott DJ, Daveson K, Kennedy K, Hajkowicz K, Halliday C, Athan E, Bak N, Cheong E, Heath CH, Orla Morrissey C, Kidd S, Beresford R, Blyth C, Korman TM, Owen Robinson J, Meyer W, Chen SC, Australia New Zealand Mycoses Interest Group.** 2015. Invasive infections due to filamentous fungi other than Aspergillus: epidemiology and determinants of mortality. Clin Microbiol Infect **21:**490.e1-10. http://dx.doi.org/10.1016/j.cmi.2014.12.021.
5. **Auberger J, Lass-Florl C, Ulmer H, Nogler-Semenitz E, Clausen J, Gunsilius E, Einsele H, Gastl G, Nachbaur D.** 2008. Significant alterations in the epidemiology and treatment outcome of invasive fungal infections in patients with hematological malignancies. Int J Hematol **88:** 508–515. http://dx.doi.org/10.1007/s12185-008-0184-2.
6. **Kwon-Chung KJ, Sugui JA.** 2013. Aspergillus fumigatus—what makes the species a ubiquitous human fungal pathogen? PLoS Pathog **9:**e1003743. http://dx.doi.org/10.1371/journal.ppat.1003743.
7. **Parize P, Rammaert B, Lortholary O.** 2012. Emerging invasive fungal diseases in transplantation. Curr Infect Dis Rep **14:**668–675. http://dx.doi.org/10.1007/s11908-012-0296-y.
8. **Cordonnier C.** 2004. Fungal infections: current diagnosis and treatment. Hematol J **5**(Suppl 3)**:**S59–62. http://dx.doi.org/10.1038/sj.thj.6200424.
9. **Kontoyiannis DP.** 2012. Invasive mycoses: strategies for effective management. Am J Med **125:**S25–38. http://dx.doi.org/10.1016/j.amjmed.2011.10.009.
10. **Tarrand JJ, Lichterfeld M, Warraich I, Luna M, Han XY, May GS, Kontoyiannis DP.** 2003. Diagnosis of invasive septate mold infections. A correlation of microbiological culture and histologic or cytologic examination. Am J Clin Pathol **119:**854–858.
11. **Munoz-Cadavid C, Rudd S, Zaki SR, Patel M, Moser SA, Brandt ME, Gomez BL.** 2010. Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: comparison of five tissue DNA extraction methods using panfungal PCR. J Clin Microbiol **48:**2147–2153. http://dx.doi.org/10.1128/JCM.00459-10.
12. **Kasai M, Harrington SM, Francesconi A, Petraitis V, Petraitiene R, Beveridge MG, Knudsen T, Milanovich J, Cotton MP, Hughes J, Schaufele RL, Sein T, Bacher J, Murray PR, Kontoyiannis DP, Walsh TJ.** 2008. Detection of a molecular biomarker for zygomycetes by quantitative PCR assays of plasma, bronchoalveolar lavage, and lung tissue in a rabbit model of experimental pulmonary zygomycosis. J Clin Microbiol **46:**3690–3702. http://dx.doi.org/10.1128/JCM.00917-08.
13. **Rickerts V, Khot PD, Myerson D, Ko DL, Lambrecht E, Fredricks DN.** 2011. Comparison of quantitative real time PCR with sequencing and ribosomal RNA-FISH for the identification of fungi in formalin fixed, paraffin-embedded tissue specimens. BMC Infect Dis **11:**202. http://dx.doi.org/10.1186/1471-2334-11-202.
14. **Rickerts V, Mousset S, Lambrecht E, Tintelnot K, Schwerdtfeger R, Presterl E, Jacobi V, Just-Nubling G, Bialek R.** 2007. Comparison of histopathological analysis, culture, and polymerase chain reaction assays to detect invasive mold infections from biopsy specimens. Clin Infect Dis **44:**1078–1083. http://dx.doi.org/10.1086/512812.
15. **Alanio A, Bretagne S.** 2014. Difficulties with molecular diagnostic tests for mould and yeast infections: where do we stand? Clin Microbiol Infect **20**(Suppl 6)**:**36–41. http://dx.doi.org/10.1111/1469-0691.12617.
16. **Herbrecht R, Patterson TF, Slavin MA, Marchetti O, Maertens J, Johnson EM, Schlamm HT, Donnelly JP, Pappas PG.** 2015. Application of the 2008 definitions for invasive fungal diseases to the trial comparing voriconazole versus amphotericin B for therapy of invasive aspergillosis: a collaborative study of the Mycoses Study Group (MSG 05) and the European Organization for Research and Treatment of Cancer Infectious Diseases Group. Clin Infect Dis **60:**713–720. http://dx.doi.org/10.1093/cid/ciu911.
17. **Mengoli C, Cruciani M, Barnes RA, Loeffler J, Donnelly JP.** 2009. Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. Lancet Infect Dis **9:**89–96. http://dx.doi.org/10.1016/S1473-3099(09)70019-2.
18. **Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P, Halliday C.** 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol **45:**380–385. http://dx.doi.org/10.1128/JCM.01862-06.
19. **van Diepeningen AD, de Hoog GS.** 2016. Challenges in Fusarium, a trans-kingdom pathogen. Mycopathologia **181:**161–163. http://dx.doi.org/10.1007/s11046-016-9993-7.
20. **Bialek R, Konrad F, Kern J, Aepinus C, Cecenas L, Gonzalez GM, Just-Nubling G, Willinger B, Presterl E, Lass-Florl C, Rickerts V.** 2005. PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue. J Clin Pathol **58:**1180–1184. http://dx.doi.org/10.1136/jcp.2004.024703.
21. **Babouee Flury B, Weisser M, Prince SS, Bubendorf L, Battegay M, Frei R, Goldenberger D.** 2014. Performances of two different panfungal PCRs to detect mould DNA in formalin-fixed paraffin-embedded tissue: what are the limiting factors? BMC Infect Dis **14:**692. http://dx.doi.org/10.1186/s12879-014-0692-z.
22. **White PL, Wingard JR, Bretagne S, Loffler J, Patterson TF, Slavin MA, Barnes RA, Pappas PG, Donnelly JP.** 2015. Aspergillus polymerase chain reaction: systematic review of evidence for clinical use in comparison with antigen testing. Clin Infect Dis **61:**1293–1303. http://dx.doi.org/10.1093/cid/civ507.
23. **Mohammadi F, Hashemi SJ, Zoll J, Melchers WJ, Rafati H, Dehghan P, Rezaie S, Tolooe A, Tamadon Y, van der Lee HA, Verweij PE, Seyedmousavi S.** 2016. Quantitative analysis of single-nucleotide polymorphism for rapid detection of TR34/L98H- and TR46/Y121F/T289A-positive Aspergillus fumigatus isolates obtained from patients in Iran from 2010 to 2014. Antimicrob Agents Chemother **60:**387–392. http://dx.doi.org/10.1128/AAC.02326-15.
24. **Salehi E, Melchers W, Zoll J, Hedayati M, Rafati A, van der Lee H, Verweij P, Seyedmousavi S.** 2016. 26th Eur Congr Clin Microbiol Infect Dis, 9 to 12 April 2016, Amsterdam, The Netherlands, poster 1630.
25. **Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List.** 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A **109:**6241–6246. http://dx.doi.org/10.1073/pnas.1117018109.
26. **Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC.** 2014. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud Mycol **78:**141–173. http://dx.doi.org/10.1016/j.simyco.2014.07.004.
27. **Khot PD, Ko DL, Fredricks DN.** 2009. Sequencing and analysis of fungal rRNA operons for development of broad-range fungal PCR assays. Appl Environ Microbiol **75:**1559–1565. http://dx.doi.org/10.1128/AEM.02383-08.
28. **Al-Hatmi AM, Meis JF, de Hoog GS.** 2016. Fusarium: molecular diversity and intrinsic drug resistance. PLoS Pathog **12:**e1005464. http://dx.doi.org/10.1371/journal.ppat.1005464.
29. **Seyedmousavi S, Verweij P.** 2015. Azole resistance in Aspergillus fumigatus: mechanisms, route of resistance selection, and clinical implications, p 1–17. *In* Gotte M, Berghuis A, Matlashewski G, Wainberg M, Sheppard D (ed), Handbook of antimicrobial resistance. Springer, New York, NY.
30. **Buitrago MJ, Bernal-Martinez L, Castelli MV, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2014. Performance of panfungal- and specific-PCR-based procedures for etiological diagnosis of invasive fungal diseases on tissue biopsy specimens with proven infection: a 7-year retrospective analysis from a reference laboratory. J Clin Microbiol **52:**1737–1740. http://dx.doi.org/10.1128/JCM.00328-14.
31. **Hofman V, Dhouibi A, Butori C, Padovani B, Gari-Toussaint M, Garcia-Hermoso D, Baumann M, Venissac N, Cathomas G, Hofman P.** 2010. Usefulness of molecular biology performed with formaldehyde-fixed paraffin embedded tissue for the diagnosis of combined pulmonary invasive mucormycosis and aspergillosis in an immunocompromised patient. Diagn Pathol **5:**1. http://dx.doi.org/10.1186/1746-1596-5-1.
32. **Buitrago MJ, Aguado JM, Ballen A, Bernal-Martinez L, Prieto M, Garcia-Reyne A, Garcia-Rodriguez J, Rodriguez-Tudela JL, Cuenca-**

Estrella M. 2013. Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease. Clin Microbiol Infect **19:**E271–277. http://dx.doi.org/10.1111/1469-0691.12110.
33. **Landlinger C, Preuner S, Baskova L, van Grotel M, Hartwig NG, Dworzak M, Mann G, Attarbaschi A, Kager L, Peters C, Matthes-Martin S, Lawitschka A, van den Heuvel-Eibrink MM, Lion T.** 2010. Diagnosis of invasive fungal infections by a real-time panfungal PCR assay in immunocompromised pediatric patients. Leukemia **24:**2032–2038. http://dx.doi.org/10.1038/leu.2010.209.
34. **Heydt C, Fassunke J, Kunstlinger H, Ihle MA, Konig K, Heukamp LC, Schildhaus HU, Odenthal M, Buttner R, Merkelbach-Bruse S.** 2014. Comparison of pre-analytical FFPE sample preparation methods and their impact on massively parallel sequencing in routine diagnostics. PLoS One **9:**e104566. http://dx.doi.org/10.1371/journal.pone.0104566.
35. **Cabaret O, Toussain G, Abermil N, Alsamad IA, Botterel F, Costa JM, Papon JF, Bretagne S.** 2011. Degradation of fungal DNA in formalin-fixed paraffin-embedded sinus fungal balls hampers reliable sequence-based identification of fungi. Med Mycol **49:**329–332. http://dx.doi.org/10.3109/13693786.2010.525537.
36. **Greer CE, Wheeler CM, Manos MM.** 1994. Sample preparation and PCR amplification from paraffin-embedded tissues. PCR Methods Appl **3:**S113–122. http://dx.doi.org/10.1101/gr.3.6.S113.