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Original article

Clinical use of fungal PCR from deep tissue samples in the diagnosis of invasive fungal diseases: a retrospective observational study

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ABSTRACT

Objectives: To assess the clinical use of panfungal PCR for diagnosis of invasive fungal diseases (IFDs). We focused on the deep tissue samples.
Methods: We first described the design of panfungal PCR, which is in clinical use at Helsinki University Hospital. Next we retrospectively evaluated the results of 307 fungal PCR tests performed from 2013 to 2015. Samples were taken from normally sterile tissues and fluids. The patient population was nonselected. We classified the likelihood of IFD according to the criteria of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG), comparing the fungal PCR results to the likelihood of IFD along with culture and microscopy results.
Results: There were 48 positive (16%) and 259 negative (84%) PCR results. The sensitivity and specificity of PCR for diagnosing IFDs were 60.5% and 91.7%, respectively, while the negative predictive value and positive predictive value were 93.4% and 54.2%, respectively. The concordance between the PCR and the culture results was 86% and 87% between PCR and microscopy, respectively. Of the 48 patients with positive PCR results, 23 had a proven or probable IFD.
Conclusions: Fungal PCR can be useful for diagnosing IFDs in deep tissue samples. It is beneficial to combine fungal PCR with culture and microscopy. **M. Ala-Houhala, Clin Microbiol Infect 2018;24:301**

Introduction

Invasive fungal diseases (IFDs) are an emerging clinical dilemma in critically ill patients. The number of patients in an immunosuppressive state (e.g. due to cancer, transplantation, HIV or the use of immunosuppressive agents) has risen substantially in the last few years. Invasive candidiasis and invasive aspergillosis are the most common IFDs diagnosed in immunocompromised patients [1–4]. However, the epidemiology of IFDs has changed in recent years; *Candida* species are still one of the most frequent causes of IFDs, but the incidence of *Aspergillus* species and other filamentous fungi, such as *Zygomycetes, Fusarium* and *Scedosporium* spp., has increased [2,5–9]. IFDs are difficult to treat, and they place a remarkable financial burden on the healthcare system [10–12].

The diagnosis of IFD is challenging. Clinical manifestations are often nonspecific and mild in the early phase of infection. Standard methods of diagnosis, microscopy and culture lack efficiency and have a low sensitivity [13,14]. Microscopic identification requires skilled personnel, and it is demanding to differentiate, for example, *Aspergillus* hyphae from those of *Fusarium* and *Scedosporium* spp. However, a specific identification of the infecting species would be important for implementation of an appropriate antifungal therapy. Molecular-based methods like PCR-based techniques can potentially offer a more rapid diagnosis [14–17]. PCR assays usually have a higher sensitivity than conventional methods, and they allow detection of a small amount of DNA [15,18]. Species-specific designed PCR can be fast and sensitive for diagnosing fungal disease [19], but panfungal techniques cover potentially the range of fungi causing IFDs. Only a few commercial methods are available;

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these have been reviewed by Lackner et al. [20]. In addition, the rapid development of sequencing technologies might change fungal diagnostics in the future [21].

Here we describe the design of a panfungal PCR. We compare the PCR results to the culture and microscopy results as well as evaluate the clinical use of panfungal PCR for diagnosing IFDs.

Materials and Methods

The panfungal PCR described here is in clinical use at Helsinki University Hospital Laboratory (HUSLAB). We investigated the clinical use of this fungal PCR for diagnosing IFDs in southern Finland from 2013 to 2015. The analysis is retrospective and observational. We focused on PCR samples taken from deep tissues and fluids. Blood and bronchoalveolar lavage fluid samples were excluded from this study.

Design of fungal PCR

The PCR was designed to identify ribosomal DNA sequences of fungal chromosomes. The primers were designed with Allele ID and Beacon Designer software (Palo Alto, CA, US) to recognize two target gene regions, using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA; https://blast.ncbi.nlm.nih.gov/Blast.cgi) for searching its database to predict secondary structures. The forward primer oligonucleotide sequence for *its_03* was GCATATCAATAAGCGGAGGA, and the reverse primer was CAGGCATAGTTCACCATCT, both of which amplify fungal 28S rRNA sequence (788 bp). For *its_05,* the forward primer was GATTGAATGGCTTAGTGAGG, and the reverse primer was TTGTTCGCTATCGGTCTC, and they amplify approximately 1000 bp sequences starting from the end of 18S sequence.

PCR was performed using the DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA), and thermocycling was carried out under the following conditions: initial denaturation and activation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 70 seconds and extension at 72°C for 1 minute. The 20 μL reaction contained 0.1 μL Qiagen HotstarTaq (Qiagen, Venlo, the Netherlands), 0.2 mM dNTP, 0.5 μM primers and 5 μL template DNA. PCR-amplified fragments were separated using agarose gel electrophoresis and visualized under ultraviolet light. Amplified fragments were purified with ExoSAP Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) using both the forward and reverse primers and an ABI Prism 3100 genetic analyzer (Thermo Fisher Scientific). Then analysis was performed by BLAST. An inhibition control (amplification of lambda DNA or *Oryza sativa* gene) was tested with each sample, a nontemplate control was tested with each run and the extraction control was tested with each extraction lot. In addition, with each sample, an empty reference container, opened in the sampling site, was used as a negative control.

Fungal strains and clinical samples

The clinical samples were cultured by routine diagnostics at HUSLAB using standard methods [22,23].

For the PCR analyses, fungus cells were collected in 650 μL of water and homogenized in Precellys (Bertin Instruments, Montigny-le-Bretonneux, France), and 100 μL of the homogenized mixture was purified with the NucliSENS kit (bioMérieux, Marcy l'Etoile, France) using the easyMAG automatic nucleic acid purification platform (bioMérieux), as described by the manufacturer. DNA was eluted to 100 μL, of which 1 μL was directly used for PCR amplification. The clinical samples (tissue samples were approximately 3 × 3 mm and fluid samples were >0.2 mL) were homogenized in Precellys solution and purified using the Nordiag Arrow instrument (Isogen Life Science, Utrecht, the Netherlands) with a Viral NA Kit (DiaSorin, Saluggia, Italy) before PCR analysis. All clinical samples were fresh and were stored, if necessary, for a maximum of 4 days at +4°C before analysis.

For medical record review, patients were retrospectively identified from the microbiologic laboratory database at Helsinki University Hospital. The patient population was nonselected. We reviewed the medical records of all patients whose samples underwent a fungal PCR as part of their clinical care from 1 January 2013 to 31 December 2015. Culture and microscopy were performed on the same sample as fungal PCR. We included tissue and fluid specimens taken from normally sterile body sites (e.g. cerebrospinal fluid, pleural effusion, ascites, vitreous body, liver, lung, lymph node, bone). We excluded bronchoalveolar lavage and blood samples from the analysis.

Patient charts were reviewed, and we collected data related to a possible IFD (e.g. age, gender, immunosuppressive state, comorbidities, primary site of the sample, culture and microscopy results, primary antifungal therapy). Patients were considered to be in an immunosuppressive state when they had haematologic malignancy, HIV, genetic immunodeficiency and active cancer for which they had received chemotherapy during the past 30 days; and a history of solid organ transplantation, hematopoietic stem cell transplantation or immunosuppressive medication. Immunosuppressive medications included prednisolone (15 mg per day taken for more than 3 weeks), biological drugs, cyclosporine, tacrolimus mycophenolate, azathioprine and methotrexate. We classified the likelihood of a fungal infection according to the criteria of European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) [24]. We compared the standard diagnostic methods of culture and microscopy to the PCR results.

The study protocol was approved by the research board of the Inflammation Center at the Helsinki University Hospital.

Statistical analysis

Statistical analyses were carried out by SPSS Statistics 22 (IBM SPSS, Chicago, IL, USA). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were identified.

Results

Design and validation of fungal PCR

The PCR method was designed to identify (*its*) regions of the fungal ribosomal gene. The primers were designed to identify all fungus species but not to bind human or bacterial DNA. The PCR assay was optimized and validated using reference strains, including 30 fungal pathogens and two bacteria strains (Supplementary Table 1). All positive control strains were correctly identified, with no false-positive amplifications. All strains were amplified with both primer pairs (*its_03* and *its_05*), except *Rhizopus oryzae* and *Scopulariopsis brevicaulis,* which were only positive with the *its_05* primers (Supplementary Table 1). Upon sequence analysis, all strains had more than 99% homology with the correct target. Thus, the assay attained 100% analytic specificity. The analytic sensitivity of the PCR was defined by tenfold dilutions of the template DNA of *Candida albicans.* The sensitivity with 40 amplification cycles was 100 fg/μL, which corresponds to

approximately 25 genome copies. No nonspecific human DNA was detected in the clinical samples.

Clinical analysis

In total, 632 PCR samples from a nonselected population were obtained during the study period. We excluded 325 of these 632 samples when patient documentation was unavailable or when the material was not sampled from a sterile body site. The remaining 307 samples ($n = 296$ patients) were enrolled onto the study. Culture results were missing for four specimens and microscopy results for five. The patients' median age was 54 years (range, 1–87 years). Twenty-five patients (8.1%) were younger than 18 years, and 182 of the patients were male (59.3%). In total, 127 patients (41%) were immunocompromised, 28 (9.1%) had received a transplant and 57 (18.5%) were diagnosed with a haematologic malignancy (Table 1).

Clinical sensitivity and specificity

Overall, there were 48 positive (16%) PCR results and 259 negative (84%) ones (Table 2). The culture was positive for 21 patients (7%) and negative for 282 (93%); microscopy was positive for 24 patients (8%) and negative for 278 (92%). The concordance rate for the PCR and culture results was 86%, while it was 87% between PCR and microscopy. The sensitivity of PCR compared to culture was 57% and specificity was 88%. In contrast with microscopy, PCR had a sensitivity of 65% and a specificity of 89%. There were nine samples with a positive culture result but a negative PCR outcome. Of these nine samples, culture identified *Aspergillus fumigatus* ($n = 3$), *Candida albicans* ($n = 5$) and *Candida glabrata* ($n = 1$). For five of these samples, there was very little growth in the culture; three of these patients did not have IFDs. For two of the nine samples, the culture and PCR were probably performed using two different tissue samples, which may explain the discordant results.

We calculated the sensitivity, specificity, NPV and PPV of fungal PCR in relation to the likelihood of IFD using the EORTC criteria. The sensitivity of PCR for diagnosing IFD was 60.5%; the specificity was 91.7%, and the NPV and PPV were 93.4% and 54.2%, respectively.

Twenty-two samples had positive PCR results even though culture and microscopy results were negative and the patients did not have an IFD. Most of these PCR results were due to contaminants from skin, environment or laboratory procedure (e.g. 11 *Malassezia* spp.) (Table 3). In 16 of the 22 samples, the PCR results were positive, but the laboratory reported that these results were very likely due to contamination from the skin or environment, a hypothesis with which the clinician agreed. When these 16 samples were excluded from the results, the specificity of fungal PCR for diagnosing IFDs increased to 97.6%.

The sensitivity of culture for diagnosing IFD was 43.9%, while the specificity was 98.8%. Cultures had an NPV of 91.8% and a PPV of 85.7%. IFD could be diagnosed using microscopy with a sensitivity of 50%, a specificity of 98.8% and NPV and PPV of 92.4% and 87.5%, respectively.

Specimens

The quality of the specimens is listed in Table 4. The most frequent specimen sites were cerebrospinal fluid (16%), soft tissue abscess (13%) and lung tissue (12%). Lung samples were most frequently positive on PCR. There were 12 positive (38%) PCR results from lung samples and 15 proven or probable fungal infections. Overall, 43 patients (14%) had IFDs, which were proven ($n = 29$), probable ($n = 10$) and possible ($n = 4$). Of these, 18 patients had a haematologic malignancy, and seven had received a solid or stem-cell transplant. PCR was positive in 23 (59%) of 39 cases, and these patients were diagnosed with a proven or probable IFD.

There were 48 positive (16%) PCR results, and 23 of these had proven or probable IFD. There was a positive correlation between the PCR results and IFD diagnosis (proven/probable) ($p < 0.001$). The species identified by PCR and diagnosed as proven or probable

Table 1
Demographic and clinical characteristics of 307 patients

Characteristic	Variable	Value
Gender	Female	125 (40.7%)
	Male	182 (59.3%)
Age	Median years	54
	0–17 years	25 (8.1%)
	18–65 years	199 (64.8%)
	>65 years	83 (27.0)
Three-month mortality	Alive	284 (92.5%)
	Dead	23 (7.5%)
Immunosuppression	None	180 (58.6%)
	Haematologic malignancy	57 (18.5%)
	HIV	12 (3.9%)
	Genetic immunodeficiency	4 (1.3%)
	Active cancer[a] treated with chemotherapy	12 (3.9%)
	Immunosuppressive medication	14 (4.6%)
Type of transplant	Solid organ	14 (4.6%)
	Stem cell	14 (4.6%)
Transplantation	Yes	28 (9.1%)
	No	279 (90.9%)

[a] Other than haematologic malignancy.

Table 2
Comparison of PCR results with culture and microscopy findings

Mode	Finding	PCR result, *n* (%)	
		Positive	Negative
Culture[a]	Positive	12 (4)	9 (3)
	Negative	34 (11)	248 (82)
Microscopy[b]	Positive	16 (5)	8 (3)
	Negative	31 (10)	247 (82)
Culture + microscopy	Both positive	10 (3)	3 (1)
	One positive	9 (3)	11 (4)
	Both negative	29 (9)	245 (80)

[a] Culture results were missing for four patients (two PCR positive, two PCR negative).
[b] Microscopy results were missing for five patients (one PCR positive, four PCR negative).

Table 3
Summary of specimens without clinical invasive fungal disease identified by PCR ($n = 22$)[a]

Species	No.
Aspergillus fumigatus	1
Aspergillus versicolor	1
Aspergillus conigus	1
Candida albicans	2
Candida parapsilosis	1
Candida sake	1
Cryptococcus albidus	1
Cladosporium spp.	2
Malassezia spp.	11
Rhodotorula spp.	1
Total	22

[a] Fungus samples were tested according to European Organization for Research and Treatment of Cancer/National Institute of Allergy and Infectious Diseases Mycoses Study Group criteria. Findings were negative in these specimens by both microscopy and culture.

Table 4
Findings of different clinical specimens

Site of specimen	All, *n* (%)	Positive PCR specimen, *n* (%)	Proven or probable invasive fungal disease, *n* (%)[a]
Cerebrospinal fluid	49 (16.0)	3 (6.1)	1 (2.0)
Soft tissue abscess	40 (13.0)	6 (15.0)	5 (12.5)
Lung	38 (12.4)	12 (31.6)	15 (39.5)
Pleural effusion	31 (10.1)	6 (19.4)	2 (6.5)
Liver	27 (8.8)	5 (18.5)	7 (25.9)
Bone	23 (7.5)	1 (4.3)	0 (0.0)
Vitreous body	16 (5.2)	2 (12.5)	3 (18.8)
Lymph node	15 (4.9)	2 (13.3)	0 (0.0)
Cerebral tissue	14 (4.6)	2 (14.3)	0 (0.0)
Other tissues	54 (17.6)	9 (16.7)	6 (11.1)
Total	307 (100.0)	48 (15.6)	39 (12.7)

[a] European Organization for Research and Treatment of Cancer/National Institute of Allergy and Infectious Diseases Mycoses Study Group criteria.

IFDs are summarized in Table 5. PCR identified *Aspergillus fumigatus* ($n = 7$), *Candida* spp. ($n = 9$), *Rhizopus* spp. ($n = 2$), *Hormographiella aspergillata* ($n = 1$), *Histoplasma capsulatum* ($n = 1$), *Scedosporium apiospermum* ($n = 1$), *Phoma opuntiae* ($n = 1$) and *Cryptococcus albidus* ($n = 1$). Sixteen patients had a proven or probable IFD but a negative fungal PCR result. These cases are summarized in Supplementary Table 2.

Discussion

In the present study, we describe design of a fungal PCR and evaluate the clinical performance of the PCR for diagnosing IFDs using deep tissue and fluid samples. Our results revealed the concordance between PCR and the conventional methods of culture and microscopy was >85%. In our study, fungal PCR identified fungal pathogens in 23 (59%) of 39 cases with a proven or probable IFD. The specificity of the fungal PCR compared to the likelihood of a fungal infection was 91.7%.

Several studies have examined fungal PCR, mostly from blood samples [25–28]. Our goal was to evaluate panfungal PCR from deep tissue samples. The analytic specificity of the fungal PCR described in this study was excellent, reaching 100%. Our study was performed in a nonselected population. Lass-Flörl et al. [16] evaluated the use of broad-range PCR to diagnose fungal infections in microscopy-negative samples. Their results showed higher figures than our study; the sensitivity of the broad-range PCR for diagnosis of IFDs was 95.6% and the specificity was 96.4%. They analysed their patient population further than our study did and excluded any patients unlikely to have a fungal infection. Trubiano et al. [29] studied the use of panfungal PCR for sterile site specimens and bronchoalveolar lavage specimens, demonstrating that panfungal PCR outperformed culture and histopathology from tissue specimens. Hammond et al. [30] studied the use of PCR in patients with haematologic malignancy and also in hematopoietic stem-cell transplant recipients with proven mucormycosis. They showed that PCR is useful for confirming the diagnosis of mucormycosis.

There were some limitations in our study. This was a single-centre retrospective study. PCR samples were obtained during routine clinical care when there was suspicion of a fungal infection. However, in the end, the likelihood of fungal infection was very low in many patients. Our study population included patients with malignancy, vasculitis, bacterial infections and so on.

In our study, the sensitivity of PCR for diagnosing IFDs remained fairly low (60.5%), even though it was higher than that of culture or microscopy. The sensitivity might have been limited by the DNA extraction method used in the study, which was chosen on the basis of the very low background DNA in reagents and consumables to increase specificity. Sensitivity might be theoretically better with other methods. Developing better extraction chemistry should improve clinical sensitivity in future.

Contaminants can affect the utility of fungal PCR. Twenty-two samples (Table 3) had a positive PCR result in our study, but the corresponding culture and microscopy were negative, and the patients did not have a clinical IFD. In these 22 samples, PCR identified

Table 5
Summary of PCR-positive samples with proven or probable invasive fungal diseases

Site of specimen	Fungus species identified by PCR	Fungus culture result	Fungus microscopy result	Primary antifungal treatment	EORTC/MSG criteria
Lung	*Rhizopus microphorus*	*Rhizopus microphorus*	Negative	Amphotericin B	Proven
Lung	*Hormographiella aspergillata*	Negative	Hypha	Amphotericin B	Proven
Lung	*Aspergillus fumigatus*	*A. fumigatus*	Hypha	Voriconazole	Proven
Lung	*A. fumigatus*	*A. fumigatus*	Hypha	Voriconazole	Proven
Lung	*A. fumigatus*	Negative	Hypha	Voriconazole	Proven
Lung	*Histoplasma capsulatum*	Negative	Negative	No[a]	Proven
Lung	*Rhizomucor* spp.	Negative	Hypha	Amphotericin B	Proven
Lung	*A. fumigatus*	Missing	Hypha	Voriconazole	Proven
Pleural effusion	*Candida albicans*[b]	Negative	Negative	Caspofungin	Proven
Liver	*C. albicans*	Negative	Yeast	Missing	Proven
Liver	*Candida krusei*	*C. krusei*	Yeast	Anidulafungin	Proven
Liver	*A. fumigatus*	*A. fumigatus*	Hypha	Voriconazole	Proven
Liver	*C. albicans/Candida glabrata*	*C. albicans/C. glabrata*	Yeast	Missing	Proven
Liver	*C. albicans*	Negative	Yeast	Caspofungin	Proven
Subcutis	*C. albicans*	*C. albicans*	Yeast	Caspofungin	Proven
Subcutis	*Phoma opuntiae*	*P. opuntiae*	Missing	No	Proven
Subcutis	*Scedosporium apiospermum*	*S. apiospermum*	Hypha	Voriconazole	Proven
Vitreous body	*Candida dubliniensis*	*C. dubliniensis*	Negative	Fluconazole	Proven
Abscess	*Candida parapsilosis*	*Pyrenochaeta romeroi*	Negative	Voriconazole	Proven
Maxillary cavity	*A. fumigatus*	*A. fumigatus*	Hypha	Voriconazole	Proven
Lung	*A. fumigatus*	Negative	Negative	Caspofungin	Probable
Vitreous body	*C. albicans*	Negative	Negative	Fluconazole	Probable
Bone marrow	*Cryptococcus albidus*	Negative	Negative	Fluconazole	Probable

EORTC/MSG, European Organization for Research and Treatment of Cancer/National Institute of Allergy and Infectious Diseases Mycoses Study Group.
[a] PCR, culture and microscopy were performed on lung biopsy samples. Pathology was treated by surgical removal. Diagnosis was confirmed by histopathologic examination of removed tissue.
[b] Lung transplantation. Patient had systemic candidiasis. Culture was positive, with *C. albicans* identified in samples taken from blood, pleural effusion and subcutaneous abscess. Sample for PCR assay was taken during later phase of infection. Culture and microscopy were negative when performed on same sample as PCR.

11 *Malassezia* spp., which were likely contaminations from the patient's skin or the laboratory procedure. PCR also identified *Cladosporium* spp. ($n = 2$), *Cryptosporidium albidus* ($n = 1$), *Aspergillus versicolor* ($n = 1$) and *Aspergillus conigus* ($n = 1$), which were most likely contaminations from the environment. There were two cases in which PCR assay had a very weak, uncertain positive result, identifying *Aspergillus fumigatus* and *Candida albicans.* Culture and microscopy were negative in both cases. The first patient had a mycobacterial infection in his lungs and the other had an oesophageal carcinoma. One patient had an abscess in the submandibular area caused by *Staphylococcus aureus,* but the fungal PCR identified *Candida albicans.* The PCR result might have been a contamination from oral mucosa. In one case, PCR assay identified *Rhodotorula* spp. in a soft tissue abscess with sepsis caused by *Fusobacterium nucleatum.* However, the PCR sample was taken inadequately, which may have influenced the result. In two cases PCR identified *Candida* spp., but neither patient had a clinical fungal infection. A cerebrospinal fluid sample from the first patient revealed *Candida sake,* and a brain biopsy sample of the other revealed *Candida parapsilosis.*

In conclusion, the analytic specificity of the fungal PCR described in our study was excellent (100%). According to the EORTC criteria, the specificity of fungal PCR compared to the likelihood of IFD was 91.7%. Fungal PCR is a useful tool for the diagnosis of IFDs in conjunction with culture and microscopy from deep tissue samples.

Transparency Declaration

Financial support was received from Helsinki University Hospital (EVO grant TYH2016105). MA has received a grant from the Infectious Disease Society of Finland and has been invited to a conference by MSD and Pfizer. VJA has received speaker's honoraria from Astellas, MSD, Pfizer and Roche; has had recent consultancies with MSD and Pfizer; and has participated in studies with Astellas and MSD. JV and JK are employees of Mobidiag Ltd., which is not linked to this study. This study is based on their previous academic work at HUSLAB. PKK and JA report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2017.08.017.

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