Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease

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Abstract

The performance of a pan-fungal PCR-based technique was evaluated to assess the aetiology of invasive fungal diseases (IFDs). A total of 89 formalin-fixed paraffin-embedded biopsy samples from 84 patients with proven IFD were studied. Culture of tissue was performed in 68 (81%) patients. The sensitivities of the PCR-based technique and microbiological culture of tissues were 89% and 56%, respectively ($p < 0.01$). According to PCR results, *Aspergillus* species accounted for 67%, *Candida* species for 13%, zygomycetes for 11%, and rare and emerging fungi for 9%. *Aspergillus* species were significantly associated with lung samples (79.6%, $p < 0.01$), Mucorales were associated with skin/subcutaneous samples, and *Candida* species were associated with gastrointestinal samples. Regarding biopsy samples with *Aspergillus* species, *Aspergillus fumigatus* DNA was detected in 43 of 50 (86%), and *Aspergillus flavus* in six of 50 (12%). PCR was positive in 24 of 30 (80%) cases with negative culture. In nine of the 84 patients, the PCR technique failed to amplify the DNA. Six also had negative cultures, and in the remaining three cases culture was positive (*Rhizopus microsporus*, *Rhizopus arrhizus*, and *Sakseneae vasiformis*), suggesting that the PCR technique was not as effective in amplifying the DNA of some species of Zygomycetes. In five cases, there was no correlation between culture results and those obtained with DNA amplification, indicating the possibility of a mixed infection or the presence of colonizer/contaminant microorganisms. In summary, PCR-based techniques for DNA amplification should be implemented in histopathology and microbiology departments, as they appear to be complementary to conventional methods for IFD detection.

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Introduction

The diagnosis of invasive fungal diseases (IFDs) continues to be problematic [1,2]. Classical methods such as culture, where several days are required to observe growth, are not effective for early diagnosis. In addition, the specificity of culture is moderate, as fungi are habitual contaminants in the laboratory, and form part of the saprophytic flora. A retrospective study by Chamilos *et al.* [3] described 60% of IFDs in which hyphae were observed in the tissues and cultures were negative.

Microscopic examination and histopathological studies have significant drawbacks; it is possible to observe fungal structures under the microscope only when the infection is at a very advanced stage, and sensitivity depends on the level of magnification of the microscope, as well as on the number of fields examined. Histopathological studies can distinguish IFDs caused by hyaline moulds from those caused by black fungi, and sometimes zygomycetes can also be identified, although it is not always easy to distinguish these when there is a scarcity of hyphae [4]. The most important limitation of tissue observation is the impossibility of differentiating the species, which is

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essential for determining therapy, given the differences in sensitivity to antifungal agents among emerging fungi [4,5].

PCR-based molecular techniques enable the fast and sensitive identification at a species level by detecting small amounts of DNA in serum and blood [4,6,7]. Although these techniques have yet to be standardized, many recent studies have shown the usefulness of them in the amplification of fungal DNA in tissues, such as the detection of the ITS region of rDNA [8,9], of mitochondrial regions [5], or of the 28S region [10]. Real-time PCR techniques have also been described for the detection of less frequent species, such as *Scedosporium* [11], Zygomycota [12,13] and *Fusarium* species in biopsy samples [14]. The sensitivity and specificity results of these studies are variable, but in all cases there is an improved sensitivity over classical methods, and results are obtained in a quick and specific manner [15,16].

This article reports a retrospective study using an automated real-time PCR-based technique on formalin-fixed paraffin-embedded biopsy samples from two Spanish hospitals. The performance of the real-time PCR-based procedures was analysed, and comparisons with those of culture and histopathological studies were performed. The ability of the molecular techniques to detect and to identify the fungal DNA at the species level was evaluated. The exercise was designed to amplify fungal DNA of common species such as *Candida* and *Aspergillus* species, and also of emerging and rare fungal pathogens.

Materials and Methods

A total of 89 formalin-fixed paraffin-embedded biopsy samples from 84 different patients were analysed, originating from the departments of pathology and microbiology of two hospitals from Madrid, Spain: Hospital Universitario 12 de Octubre (H1) (population: 550 000 inhabitants), a 1300-bed tertiary general hospital that performs 300 solid organ transplants and 50 haematopoietic cell transplants per year; and Hospital Universitario La Paz (H2) (population: 855 000 inhabitants), a 1300-bed tertiary general hospital that performs an average of 55 haematopoietic cell transplants and 150 solid organ transplants per year. Members of the hospital departments reviewed their databases, looking for the results of all samples of tissue biopsies analysed in last 10 years that had been classified as IFD, invasive fungal infection, invasive mycosis, fungal infection or mycosis after histopathological examination by Gomori–Grocott methenamine silver stain and/or haematoxylin and eosin stain.

After database review, a total of 89 tissue biopsy samples were selected and sent to the Spanish Mycology Reference Laboratory of the National Centre for Microbiology for PCR analysis. The H1 centre sent 39 paraffin biopsy samples from 35 different patients. The H2 centre sent 50 paraffin biopsy samples from 49 different patients. Clinical data of patients and the results of microbiological cultures, microscopic examinations and histopathological studies were also reviewed.

For PCR analysis, the paraffin blocks were cut into 10-μm sections. Three to ten cuts were used to extract the DNA, with the number of cuts being varied in order to obtain approximately 25 mg of tissue. Biopsy samples in paraffin were deparaffined by lavage with 1.5 mL of xylene (100%) followed by two lavages with 1.2 mL of ethanol (96–100%). After incubation of the tissue at 37°C to evaporate the remains of the ethanol, DNA was extracted with the QiAmp Tissue DNA Mini Kit (Qiagen, Izasa, Madrid, Spain), according to the manufacturer's instructions. Fifty microlitres of buffer was used for elution. Two microlitres of DNA extracted from each sample was used for each PCR reaction.

PCR reactions performed in an LC480 unit (Roche, Madrid, Spain). The ITS1 region of the rDNA was amplified with the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) [17]. The 2 × Sensimix DNA (Quantace, Ecogen, Madrid, Spain) kit was used, according to the manufacturer's instructions. The PCR reaction (20 μL) contained 0.8 μM each primer, 4.5 mM $MgCl_2$, and SYBR Green I binding dye (Quantace). The conditions for the PCR reaction included a first phase of pre-incubation (enzyme activation) and DNA denaturation at 95°C for 10 min. The next steps included a 45-cycle amplification programme, as follows: denaturation for 10 s at 95°C, 5 s of annealing at 54°C, and 30 s of extension at 72°C. A melting program from 65°C to 97°C was also performed. Melting curves were analysed to discriminate unspecific amplifications from true amplicons, as a PCR amplification control. Four controls were included (DNA of the *Aspergillus fumigatus* CNM-CM-237 strain) at various concentrations (2 pg to 2 fg per 20 μL) with each sample set, as well as negative controls. Agarose gel electrophoresis was performed at 1.5% (Sigma-Aldrich Quimica, Madrid, Spain) with Tris–acetate–EDTA, following the usual protocols [18], in order to verify amplification and the quality of the amplified products. PCR products were sequenced (ABI 3730 XL; Applied Biosystems, Madrid, Spain) with ITS1 and ITS2 primers. The sequences obtained were compared with the GenBank database (http://www.ncbi.nih.gov/Genbank/) and with the database belonging to the Department of Mycology of the Spanish National Centre for Microbiology, which holds 5000 sequences from strains belonging to 270 different fungal species. This database was designed by the Spanish National Centre for Microbiology, and has restricted access. The

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complete procedure was repeated two times on different days.

Descriptive and comparative analyses were performed. Differences in proportions of positive PCR, culture and histopathological examination results were determined with Fisher's exact test or by chi-square analysis. The analysis of concordance between histopathological, microbiological and PCR results was performed with the *gamma* ordinal probabilistic measure. It summarizes, on a −1 to +1 scale, the extent to which higher categories (based on the data codes used) of one variable are associated with higher categories of the second variable. The data codes used for results were as follows: negative = 0, hyaline hyphae with septae/aspergillosis = 1, non-septated hyphae/mucormycosis = 2, yeasts/candidosis = 3, and fungal structures = 4. A p-value of <0.01 was considered to be statistically significant. Statistical analysis was performed with IBM SPSS 19.0 (SPSS Ibérica, Madrid, Spain).

Results

Clinical characteristics

A total of 84 patients diagnosed as having IFD were reviewed. Table 1 shows underlying diseases and sites of localization of IFD. The most common underlying conditions were onco-haematological diseases (29/84, 34.5%), followed by chronic respiratory diseases (21/84, 25%) and solid organ transplantation (12/84, 14.3%). The most frequent localizations of IFD were lung diseases in 54 (64.3%) patients who presented with respiratory symptom infection such as lung infiltrates, nodules, and cavitary lesions. The second most common infection was skin/subcutaneous infection (nine cases, 10.7%). Other patients had sinonasal infections, oropharyngeal symptoms, gastrointestinal diseases, and urinary infections (Table 1).

TABLE 1. Underlying diseases and fungal infections of 84 patients included in the study

Underlying disease	Fungal infection	Number of cases
Onco-haematological	Lung	21
	Sinonasal	2
	Skin/subcutaneous	1
	Oropharyngeal	3
	Vascular	1
	Cerebral	1
	Total	29 (34.5%)
Respiratory	Lung	21
	Total	21 (25%)
Solid organ transplant	Lung	5
	Sinonasal	2
	Skin/subcutaneous	2
	Gastrointestinal	2
	Urinary	1
	Total	12 (14.3%)
Polytrauma	Skin/subcutaneous	4
	Oropharyngeal	1
	Total	5 (5.9%)
Others	Lung	7
	Sinonasal	2
	Skin/subcutaneous	2
	Gastrointestinal	3
	Urinary	1
	Oropharyngeal	1
	Vascular	1
	Total	17 (20.2%)

Histopathological data

A total of 89 tissue biopsy samples from 84 different patients were analysed. Tissue sampling was performed according to the main symptoms of patients: 64.3% of biopsy samples were lung samples, 10.7% were skin/subcutaneous samples, 7.1% were gastrointestinal samples, and 18% were from other deep sites. Hyphae or fungal structures were observed in all of them by analysis of the tissues under the microscope.

Tissue lesions were classified as being caused by hyaline hyphae with septae, by non-septated hyphae, by yeast, and by fungal structures. Black fungi were not found. Anatomical pathologists reported 58.3% (49/84 cases) to be mycoses caused by hyaline species with septae, and suggested *Aspergillus* as the possible agent responsible for infection in 25 cases (25/49, 51%). Filamentous species without septae were reported in 18 of 84 (21.4%) cases, being suggested mycoses caused by Mucorales species in 14 of 18 (77.8%) patients. Yeasts were found in six cases (6/84, 7%), with histopathological reports stating that *Candida*-like forms had been seen, accompanied by evidence of associated tissue damage in 100% (6/6) of cases. Fungal structures were observed in only 11 of 84 patients (13%), and microscopic examinations were unable to provide any further data (Table 2).

Microbiological cultures

Cultures were performed for 68 of 84 (81%) patients. The samples collected were sent for histopathological and cytological evaluation, and not for microbiological study, in the remaining 19% of cases. Cultures were negative in 30 of 68 (44%) cases, and the sensitivity of cultures can therefore be considered to be 56% relative to histopathological results. With regard to fungal species, *Aspergillus* species were the most common among culture-positive cases (21/68, 30.9%), and Mucorales and *Candida* species were the second (9/68, 13.2%) and third (7/68, 10.3%) most common species. Some significant epidemiological differences ($p < 0.01$) were observed between hospitals; for example, Mucorales species were isolated much more frequently among patients of centre H1 (25.8%) than among patients of centre H2 (3%). A bias in this observation should be noted, as almost one-fifth of cases were not referred to microbiological departments for culture.

With regard to type of tissue lesion according to the histopathological report, microbiological cultures were positive in 21 of 38 (55%) of cases caused by hyaline hyphae with septae

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TABLE 2. Histopathological analysis and culture results of samples of patients included in the study

Histopathological report	Number of cases	Culture results	Number of cases	PCR results	Number of cases
Hyaline hyphae with septae	49	*Aspergillus* spp.	18	*Aspergillus* spp.	17
				Phoma spp.	1
		Candida spp.	2	*Aspergillus* spp.	2
		Scedosporium apiospermum	1	*Scedosporium apiospermum*	1
		Negative	17	*Aspergillus* spp.	12
				Zygomycota spp.	2
				Negative	3
		Not done	11	*Aspergillus* spp.	9
				Candida spp.	2
Non-septated hyphae	18	Zygomycota spp.	9	Zygomycota spp.	5
				Candida spp.	1
				Negative	3
		Aspergillus spp.	3	*Aspergillus* spp.	2
				Candida spp.	1
		Candida spp.	1	*Candida* spp.	1
		Negative	5	*Zygomycota* spp.	2
				Aspergillus spp.	1
				Negative	2
Yeasts	6	*Candida* spp.	4	*Candida* spp.	4
		Negative	2	*Candida* spp.	2
Fungal structures	11	Negative	6	*Aspergillus* spp.	4
				Hyaloperonospora parasitica	1
				Negative	1
		Not done	5	*Aspergillus* spp.	3
				Other species[a]	2

[a]*Schizophillum commune* and *Aureobasidium pullulans*.

whose samples were cultured for microbiological evaluation. Sensitivity rates were similar for cases reported as being caused by non-septated hyphae (70%) and by yeasts (66%). Regarding cases reported as fungal structures, microbiological cultures were performed in six cases, and all were negative. No differences were observed by type of tissue biopsy.

PCR results

The PCR-based technique detected fungal DNA in 75 of 84 patients (89.3%) (Table 2). Notably, DNA amplification was positive in 80% (24/30) of patients diagnosed with IFD with negative cultures. In six of nine patients with a negative PCR result, microbiological cultures were also negative. PCR-negative results were not significantly associated with institution, type of patient, tissue biopsy, or fungal species.

The species distribution in positive biopsy samples was as follows: 50 of 75 (66.6%) cases harboured DNA of *Aspergillus* species, 11 of 75 (14.6%) harboured DNA of *Candida* and other yeasts (one *Cryptococcus neoformans* and one *Udeniomyces* species), nine of 75 (12%) harboured DNA of Mucorales species, and five harboured DNA of other mould species. Regarding biopsy samples with *Aspergillus* species, *A. fumigatus* DNA was detected in 43 of 50 (86%), *Aspergillus flavus* in six of 50 (12%), and *Aspergillus niger* in one of 50 (2%). *Aspergillus terreus* DNA was not detected in any case. Regarding Mucorales, five of nine cases harboured DNA of *Rhizopus oryzae* and three harboured DNA of *Lichtheimia* species. Rare fungi were *Scedosporium apiospermum*, *Hyaloperonospora parasitica*, *Schizophillum commune*, *Aureobasidium pullulans*, and *Phoma* species.

With regard to type of tissue biopsy, *Aspergillus* species were significantly associated with lung biopsy, as 43 of 54 (79.6%) lung biopsy samples harboured *Aspergillus* DNA ($p < 0.01$). Mucorales species were associated with skin/subcutaneous samples (5/9, 55.5%), and *Candida* species with gastrointestinal samples (3/6, 50%).

Some significant differences were observed between institutions. The most common species detected with the PCR-based technique were *Aspergillus* species in both centres, but their rate in the H1 centre was 34.5% and their rate in the H2 centre was 86.9% ($p < 0.01$). *Candida* species were the second most common organisms (31%) and Mucorales the third most common (20.7%) in the H1 centre, according to PCR results. Mucorales (6.5%) and *Candida* species (4.3%) were the second and third most common species, respectively, in the H2 centre.

Correlation between PCR results, histopathological reports, and microbiological culture

As stated above, the sensitivity of microbiological culture was 56% and the sensitivity of the PCR-based technique was 89.3% when histopathological reports were used as a reference to compare the performance of culture and DNA amplification in detecting IFDs. Notably, DNA amplification was positive in 80% (24/30) of patients diagnosed with IFD with negative cultures.

The *gamma* probabilistic measure between histopathological microscopic examination, microbiological results and PCR-based identification was statistically significant ($p < 0.01$), taking into account positive cultures and PCR results only. *Gamma*

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index values were 0.65 between histopathological and culture results, 0.68 between microscopic examination and PCR results, and 0.76 between culture and PCR data. These values were significant, but some discrepancies were observed. Hyaline hyphae with septae had been reported in 49 cases, but PCR results were negative in three of 49 (6.1%), and PCR detected Mucorales in another two of 49 (4%) cases. Regarding microscopic examination reporting non-septated hyphae, PCR was not able to detect fungal DNA in five of 18 (27.7%) patients, and PCR identified *Aspergillus* species in three of 18 (16.6%) cases classified as probable mucormycosis by histopathological examination.

Analysis can be performed for the six cases with negative culture and PCR results. They were three cases with hyaline septated hyphae, two cases with non-septated hyphae, and one for which histopathological examination reported fungal structures.

Discussion

The aim of this study was to ascertain the aetiology of IFDs by using a PCR-based molecular technique as a complementary procedure to microscopic examination and microbiological culture. Such techniques have proven to be very useful for the correct identification of fungal species, and have also assisted in the taxonomic reclassification of many groups [1]. Correct identification is essential for the correct choice of antifungal treatment, particularly in cases caused by emerging and rare species, which are often resistant to some antifungal agents. In order to strengthen its representativeness, the study was designed to analyse retrospectively tissue biopsies from two different Spanish tertiary hospitals, including all cases diagnosed with IFD in the last 10 years in both hospitals. Additionally, the study was intended to evaluate a PCR-based method including a commercial extraction method without preliminary fungal cell breakage steps, and an automated quantitative PCR technique that is easy to perform in a clinical laboratory.

One of the first findings was the low sensitivity of cultures, which was estimated to be 56%. No significant differences were observed by institution, tissue biopsy, or fungal species. Several reasons for the poor performance of cultures can be suggested, such as the scarcity of fungal cells in the sample, inhibition by tissue components or antifungal agents, and methodological limitations (lack of inoculation onto a diverse range of culture media, and incubation at both 30°C and 37°C).

The PCR technique evaluated in the exercise showed a sensitivity of 89%, which is significantly higher than that of culture ($p < 0.01$). PCR was positive in 80% of cases with negative culture, and thus showed a remarkably good performance. In only nine cases did the PCR technique fail to amplify the DNA, and, in six of these, microbiological culture was also negative. There were three cases for which cultures were positive and the PCR technique was negative. Those three cases were caused by the Mucorales species *Rhizopus microsporus*, *Rhizopus arrhizus*, and *Sakseneae vasiformis*. This suggests that the ITS1 and ITS2 primers may not be effective in amplifying the species of this group, as has previously been described for certain zygomycetes [8,19].

In five cases, there was no correlation between culture results and those obtained by DNA amplification. Four of those cases were patients suffering from a respiratory infection, and the fifth one was diagnosed with gastric mucormycosis. Cultures from two cases of respiratory infection yielded *Aspergillosis* species, whereas DNA amplification yielded *Candida albicans* and *Phoma* species. Another two cases of respiratory disease had culture yielding *Candida* species, but both PCR results were *Aspergillus* species. *R. microsporus* was cultured from the patient with a gastric infection, whereas DNA of *C. albicans* was amplified by the PCR-based assay performed on the gastric biopsies. Taking into account the clinical entity and the histopathological examination (hyaline septated hyphae were observed in the four cases of respiratory infection and non-septated hyphae in the patient suffering from a gastric infection), the DNA amplification could have been more accurate for two of four cases of respiratory fungal disease. In other cases, the possibility of a mixed infection or the presence of the microorganism detected as the colonizer by PCR cannot be ruled out.

These results show that microbiological culture and molecular techniques can be complementary procedures for IFD detection. It should be stressed that culture was not performed in 19% of cases. Clinical microbiologists should strive to inform the appropriate clinical units to request both histopathology and microbiological/mycological investigations.

Histopathology was considered to be the reference technique in this study, because, in many cases, it is the only method that makes it possible to detect infection [20]. However, microscopy cannot be considered to be an early diagnostic technique or one by means of which it is possible to classify the species causing the fungal infection. Histopathological examinations are able to determine whether the infection is caused by yeasts or by mycelial fungi, and whether one of the latter group is an agent of hyalohyphomycosis, phaeohyphomycosis, or zygomycosis. Expertise is usually needed for interpretation, and the reliability is decreased

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when an inexperienced observer examines the sample. The concordance between the results of microscopy, culture and PCR proves the reliability of histopathological examination. However, some results show its limitations. The DNA amplification technique found seven cases caused by *Aspergillus* species that the histopathologist reported as invasion caused by fungal structures. Six of those cases were negative by culture, which could indicate that it is possible to view fungal structures only when they are very abundant in the sample to be analysed, which is usually the case when the infection is at an advanced stage. Therefore, histopathological diagnosis should be primarily descriptive of the fungus (hyphae and septa), and should include the presence or absence of tissue invasion and the host reaction to the infection.

Regarding epidemiology, the DNA amplification and conventional techniques were complementary to each other, and the PCR-based method helped us with classification at the species level of most IFD cases. The genus *Aspergillus* accounted for 67% of all samples analysed, but it must be pointed out that the percentage of zygomycetes and rare and emerging fungi accounted for 20%.

The PCR-based techniques should be implemented in histopathology and microbiology departments, as they appear to be complementary techniques to conventional methods for IFD detection. The pan-fungal PCR-based procedure evaluated in this exercise showed much higher sensitivity than microbiological culture, and its performance could be regarded as better than that of microscopic examination, as the molecular method was useful for fungal species identification. Limitations should also be considered, such as the facts that pan-fungal PCR could be less sensitive for the detection of some fungal species, and the possibility of a mixed infection or the presence of the microorganism detected as the colonizer or contaminant cannot be ruled out.

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Transparency Declaration

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