Diagnostic Microbiology and Infectious Disease 80 (2014) 111–118

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycology

Rapid and specific detection of section *Fumigati* and *Aspergillus fumigatus* in human samples using a new multiplex real-time PCR

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ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 11 June 2014
Accepted 11 June 2014
Available online 19 June 2014

Keywords:
Aspergillus fumigatus
Section *Fumigati*
Multiplex real-time PCR
ITS region
benA gene
BAL samples

ABSTRACT

Invasive aspergillosis is an opportunistic infection caused primarily by *Aspergillus fumigatus*. However, other common fungal pathogens belonging to section *Fumigati* are often misidentified as *A. fumigatus*. Thus, we have developed a multiplex real-time PCR (qPCR) assay with primers and specific TaqMan probes based on internal transcribed spacer regions or *benA* gene to discriminate, in less than 3 h, species of section *Fumigati* and, specifically, *A. fumigatus*. The multiplex qPCR showed a limit of detection of 20 and 50 fg of DNA for section *Fumigati* and *A. fumigatus*, respectively. Moreover, it enabled detection of a single germinated conidia. The inclusion of some PCR facilitators together with the dilution of samples makes it possible to completely avoid PCR inhibitions in all bronchoalveolar lavage (BAL) samples assayed. This technique may be a useful complementary tool in the diagnosis of invasive pulmonary aspergillosis caused by *A. fumigatus* using BAL fluid.

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1. Introduction

Aspergillosis is a group of diseases caused by opportunistic species of the *Aspergillus* genus. These diseases range from allergies to invasive aspergillosis (IA), the most serious being bronchopulmonary allergic aspergillosis, aspergilloma, and IA, which appear in immunocompromised patients, especially those with prolonged neutropenia (Walsh et al., 2008). Among these, IA stands out, with mortality rates of greater than 50%, reaching 95% in certain clinical scenarios (Balloy and Chignard, 2009; Maschmeyer et al., 2007), and *Aspergillus fumigatus* is the major etiological agent, followed by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and other species with a lower incidence. Although the spores of *A. fumigatus* are a small proportion of the airborne spores in hospitals (0.3%), this fungus causes approximately 90% of the systemic infections due to *Aspergillus* (Brakhage and Langfelder, 2002).

Nowadays, it remains relatively difficult to identify the species causing aspergillosis. Classical diagnostic methods are very unreliable, as they are based on morphological features, which depend mainly on growth conditions, and tend to require a high level of experience and/or a long period of incubation. Moreover, there is a risk of misidentifying the etiological agent. The section *Fumigati* that includes *A. fumigatus* provides a prime example of this problem: several species, such as *Aspergillus lentulus*, *Aspergillus viridinutans*, *Aspergillus fumigatiaffinis*, *Neosartorya pseudofischeri*, *Neosartorya hiratsukae*, and *Neosartorya udagawae* having been wrongly identified and reported as *A. fumigatus* (Balajee et al., 2005a, 2005b, 2006; Hong et al., 2008), illustrating how difficult it can be to identify species when morphological examination alone is used. In addition, some of these species (*A. lentulus*, *A. viridinutans*, *N. pseudofischeri*, and *N. udagawae*) have been reported to be resistant *in vitro* to azole antifungals itraconazole, miconazole, posaconazole, ravuconazole, and/or voriconazole (Alcazar-Fuoli et al., 2008), and although their incidence is not high, it would be useful to distinguish *A. fumigatus* from them.

From a clinical point of view, it is essential to detect IA and identify the species responsible as early as possible, to define specific therapeutic strategies (Rüping et al., 2008) and, consequently, improve patient outcome; diagnostic assays targeting fungal biomarkers have been developed (Cuenca-Estrella et al., 2011), and these offer the potential for new paradigms in prevention and early treatment (Almyroudis and Segal, 2009). Among them, detection of circulating galactomannan in serum or bronchoalveolar lavage (BAL) fluid (Hage et al., 2011; Pfeiffer et al., 2006; Rex, 2006) may be a useful complementary diagnostic procedure for IA. Nevertheless, in such tests, false-positive and false-negative results are problematic as well as it being impossible to identify individual species of the *Aspergillus* genus.

For the correct identification of *Aspergillus* clinical isolates, molecular identification is usually recommended. Comparative

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http://dx.doi.org/10.1016/j.diagmicrobio.2014.06.003
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sequence analysis of the internal transcribed spacer (ITS) regions, specifically the ITS1 and ITS2 non-coding regions flanking the 5.8S rDNA, was suggested as appropriate for identifying *Aspergillus* to the level of subgenus/section (Balajee et al., 2007). Meanwhile, amplification followed by the sequencing of some codifying genes, such as actin, calmodulin, rodlet A, and/or β-tubulin, has been used to distinguish *A. fumigatus* from related species (Balajee et al., 2007; Samson et al., 2007). Other techniques, such as analysis based on random amplified polymorphic DNA (Brandt et al., 1998) or restriction fragment length polymorphism (Staab et al., 2009) and a new method based on microsphere-based Luminex assays (Landlinger et al., 2009), may allow molecular identification without sequencing. However, these methodologies are time consuming and laborious, and they require technology that is not available to all clinical mycology laboratories. On the other hand, there are currently a variety of commercial assays based on real-time PCR (qPCR) that can assist in this diagnosis. Among them, MycAssay *Aspergillus* (Myconostica, Cambridge, UK), *Aspergillus* tracer (Affigene, Bromma, Sweden), Septifast (Roche, Madrid, Spain), MycoReal *Aspergillus* (Ingenetix Gmb H, Vienna, Austria), and *Aspergillus* spp. q-PCR Alert (Nanogen, Turin, Italy) should be mentioned. None of these, however, achieve identification at the species level.

The use of multicopy sequences, such as the ITS region or *benA* gene (that codes the β-tubulin protein), together with different strategies to avoid PCR inhibitions, might improve the detection level of *A. fumigatus* DNA in human samples and at the same time distinguish it from other related species, even those in section *Fumigati*. Therefore, the aim of this work was to develop a multiplex qPCR that enables species of section *Fumigati* to be quickly and easily distinguished from other fungi and specifically identifies *A. fumigatus* in a single reaction, avoiding PCR inhibitions.

2. Materials and methods

2.1. Samples to analyze

2.1.1. Collection of microorganisms

The following microorganisms were used: 70 strains of 40 species of the *Aspergillus* genus, 10 species of Mucorales, 3 species of other filamentous fungi (genera *Fusarium*, *Penicillium*, and *Scedosporium*), 5 species of yeast, and 4 species of bacteria (Table 1). The procedures for maintaining and harvesting the microorganisms to obtain DNA were carried out following methods described previously (Abad-Diaz-De-Cerio et al., 2013).

2.1.2. Human samples

Six samples of human DNA were extracted from cells obtained from the oral cavity of healthy volunteers by rinsing with saline (NaCl 0.9%). In addition, 18 BAL samples were obtained from patients of Hospital Universitario y Politécnico La Fe (Valencia, Spain). Four out of these 18 BAL samples were considered as probable IA according to 2008 definitions of the European Organization for Research and Treatment of Cancer and Mycoses Study Group of the U.S. National Institute of Allergy and Infectious Diseases (De Pauw et al., 2008). The BALs were performed with 150 mL of saline solution, and the samples were delivered to the microbiology laboratory within 1 h. There, 10 mL of BAL fluid from each sample was centrifuged at 3000 g for 20 min, and 2 mL of sediment was immediately packaged and frozen at −80 °C for molecular analysis. In addition, another 10 mL of BAL fluid was centrifuged, and the suspended pellet (1–3 mL depending on availability) was directly inoculated on conventional fungal medium (Sabouraud dextrose agar supplemented with 50 μg/mL chloramphenicol) and incubated at 30 °C for 4 weeks. Filamentous fungal isolates were identified by macroscopic and microscopic examination, according to the standard morphological procedures used in this hospital.

DNA extracted from all human samples was analyzed by the multiplex qPCR developed in this study. Furthermore, 10 out of the 13 negative BAL samples, those in which no microorganism was detected by culture, were pooled for studies to address the problem of PCR inhibition.

Table 1
Collection of microorganism tested by multiplex qPCR.

Aspergillus spp.	Mucorales
Eurotium chavelieri (FMR 171)	*Absidia coerulea* (URV 12)
Aspergillus clavatus (CECT 2674)	*Mucor circinelloides* (CBS 195.68)
Aspergillus caelatus (FMR 05-5864)	*Mucor indicus* (CBS 226.29)
Aspergillus bombycis (CBS 117187)	*Mucor hiemalis* (CBS 201.65)
Aspergillus flavus (ATCC 204304, CBS 282.95, CBS 542.69, FMR 97, FMR 10-516)	*Mucor racemosissimus* (CBS 135.65)
Aspergillus nomius (FMR 11907)	*Mucor racemosus* (CBS 260.68)
Aspergillus oryzae (CECT 2094)	*Rhizomucor irregularis* (CBS 103.93)
Aspergillus parasiticus (CECT 2680)	*Rhizomucor pusillus* (FMR 10383)
Aspergillus tamarii (CBS 631.67)	*Rhizopus oryzae* (CBS 112.07)
Petromyces alliaceus (CBS 110.26)	*Rhizopus rhizopodiformis* (FMR 9899)
Aspergillus fumigatus (Af-293, ATCC 204305, CBS 133436, CBS 109032, CBS 120.53, CBS 123.59, CECT 2071, FMR 2, UPV 10-610)	
Aspergillus fumigatiaffinis (CBS 117186)	**Other filamentous fungi**
Aspergillus lentulus (CBS 117885, CBS116886)	*Fusarium incarnatum* (CECT 2224)
Aspergillus viridinutans (CBS 127.56)	*Penicillium chrysogenum* (CECT 20272)
Neosartorya aureola (CBS 106.55)	*Scedosporium prolificans* (UPV 99-059)
Neosartorya coreana (CBS 117059)	
Neosartorya glabra (CBS 118457)	**Yeasts**
Neosartorya fisheri (CBS 118456, FMR 11804)	*Candida albicans* (NCPF 3153)
Neosartorya hiratsukae (CBS 304.93, CBS 109356)	*Candida krusei* (ATCC 6258)
Neosartorya laciniosa (CBS 315.89, FMR 11807)	*Candida parapsilosis* (ATCC 22019)
Neosartorya pseudofischeri (CBS 208.92, CBS 117074)	*Cryptococcus neoformans* (ATCC 90113)
Neosartorya spinosa (CBS 117717, FMR 237)	*Saccharomyces cerevisiae* (CECT 1171)
Neosartorya udagawae (UPV 06-091, UPV 06-092)	
Aspergillus sydowii (CBS 170.63)	**Bacteria species**
Aspergillus versicolor (UPV 99-071)	*Escherichia coli* (CECT 4201)
Emericella nidulans (CECT 2544, FMR 189)	*Pseudomonas aeruginosa* (ATCC 27853)
Aspergillus acidus (CBS 564.65)	*Pseudomonas fluorescens* (CECT 378)
Aspergillus awamori (CBS 557.65)	*Vibrio harveyi* (CECT 525)
Aspergillus brasiliensis (CBS 101740)	
Aspergillus carbonarius (CECT 2086)	
Aspergillus ellipticus (CBS 707.79)	
Aspergillus japonicus (CECT 20386)	
Aspergillus niger (CECT 2091, CBS 513.88, CBS 554.65, FMR 4, FMR 04-1740)	
Aspergillus tubingensis (CECT 2089, FMR 11906, FMR 04-3468)	
Aspergillus ambiguus (CBS 117.58)	
Aspergillus carneus (CBS 111.49)	
Aspergillus neoniveus (CBS 471.91)	
Aspergillus niveus (CBS 115.27)	
Aspergillus terreus (CBS 469.81, CBS 811.96, CECT 2663, CECT 2808, FMR 5)	
Aspergillus calidoustus (FMR 09-479)	

Note: The strain reference numbers for different collections appear in brackets: FMR and URV = Reus Faculty of Medicine, Universitá Rovira I Virgili; CECT = Colección Española de Cultivos Tipo; ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures; UPV = University of the Basque Country (UPV/EHU); NCPF = National Collection of Pathogenic Fungi.
Species with positive results by multiplex qPCR are indicated in bold (complete results are given in Table 3).

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