INTRODUCTION

The frequency of invasive fungal infections (IFIs) and invasive aspergillosis (IA) is rising due to the increasing number of patients receiving aggressive chemotherapy regimens and immunosuppressive agents (Perlroth et al., 2007). In particular, IA is a major cause of morbidity and mortality in patients with hematologic malignancy or chronic obstructive pulmonary disease, and solid-organ and hematopoietic stem cell transplant recipients. Despite the availability of newer antifungal drugs, the outcome for patients with IA continues to be poor, in large part due to delayed diagnosis and initiation of appropriate antifungal therapy (Nucci et al., 2013). The mortality rate for bone marrow transplant patients with IA is greater than 70% (Nucci et al., 1997; Reichenberger et al., 2002; López-Medrano et al., 2016). Diagnosis of IA is complicated because blood cultures are almost always negative and culture-based techniques are too insensitive or provide results after too long, delaying treatment with an effective antifungal agent. Most patients with suspected disease are treated empirically with voriconazole (Walsh et al., 2008). Moreover, resistance to AmB as well as itraconazole has been reported for several Aspergillus species, although the number of isolates studied in each case was limited (Sampson et al., 1994; Oakley et al., 1997; Chamilos et al., 2005).

This lack of diagnostic tools has led to the development and evaluation of non-cultural diagnostic approaches including imaging, PCR-based detection of Aspergillus DNA, and antigen detection, particularly of galactomannan and β-D glucan in serum and bronchoalveolar lavage (BAL) (Alonso et al., 2012). An example of non-cultural diagnostic tools based on the detection of specific Aspergillus antigens located in the cell wall is the use of galactomannan in the diagnosis of invasive aspergillosis. Another serum marker for the presence of IFIs is (1→3)-β-D-glucan (BDG), which has been included in the relevant European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) diagnostic criteria (Karageorgopoulos et al., 2011). Various molecular approaches have been used for the detection of Aspergillus from clinical samples (Yamakami et al., 1996; White et al., 2011). Targets for the genus level detection of Aspergillus have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions (Henry et al., 2000).

This paper describes the development and evaluation of PCR and Multiplex PCR methods for the detection of the six most common Aspergillus species DNA in BAL specimens from hematology and intensive care unit (ICU) patients at risk IA. Sequences of the SCW4 gene of the six different Aspergillus species (A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus, A. clavatus, A.烦bicans) were amplified using the Multiplex PCR method. The species-specific PCR primers were able to amplify only Aspergillus DNA but not that belonging to other fungal genera tested. The species-specific PCR primers allowed differentiation of each Aspergillus species by the amplicon length produced. The methods described in this study are rapid (less than 4 h), reproducible, simple and specific and demonstrate potential application in the clinical laboratory.

SUMMARY

Aspergillus species are the cause of invasive mold infections in immunocompromised patients: Aspergillus fumigatus, A. flavus and A. terreus account for most cases of invasive aspergillosis (IA). As certain species are associated with higher mortality and vary in their resistance to antifungal therapy, diagnosis requires increasingly rapid molecular methods that enable sensitive detection and species discrimination. We have developed PCR and Multiplex PCR assays for the detection of six medically important Aspergillus species DNA in bronchoalveolar lavage (BAL) specimens from hematology and intensive care unit (ICU) patients at risk of IA, using different species and genus-specific PCR primers, selected within the SCW4 gene, encoding a cell wall glucanase of A. fumigatus, similar to mannoprotein Mp65 of Candida albicans. The genus-specific PCR primers were able to amplify only Aspergillus DNAs but not that belonging to other fungal genera tested. The species-specific PCR primers allowed differentiation of each Aspergillus species by the amplicon length produced. The methods described in this study are rapid (less than 4 h), reproducible, simple and specific and demonstrate potential application in the clinical laboratory.

Key words: Molecular diagnosis, Clinical samples, Aspergillus species, Multiplex PCR, SCW4 gene.

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February 29, 2016
Received
Accepted June 20, 2016
Accepted
terreus and A. versicolor), were determined and six different primer pairs were used in the same PCR reaction to produce different specific amplicons dependent on the target present in the sample. Moreover, we developed three types of Multiplex PCR: in the first, we added a mix of six primer pairs and a single Aspergillus species DNA to each reaction tube; in the second, we added a mix of six different Aspergillus species DNA and a single species-specific primer pair to each reaction tube; in the third, all six primer pairs were added to each tube with all six Aspergillus spp. DNAs. In all three types of Multiplex PCR, the species-specific PCR primers allowed differentiation of each of six Aspergillus species by the amplicon length produced.

MATERIALS AND METHODS

Microbial strains
All microbial strains used in this study are listed in Table 1. All yeast and bacterial isolates were identified by the API system (Biomerieux Italia S.p.A., Firenze, Italy) and by conventional morphological methods (Tsuchiya et al., 1974; Agatensi et al., 1991; Murray et al., 1999).

Processing of BAL fluid specimens and DNA extraction
Patients with a clinical syndrome compatible with pneumonia were enrolled if they had at least two of the following criteria: fever refractory for at least three days to broad-spectrum antibiotics, pleuritic chest pain or physical finding of pleural rub, pulmonary infiltrates, or dyspnea. In addition, at least one host factor (hematological malignancy, COPD, cirrhosis, cancer receiving chemotherapy, solid organ transplant recipient, HIV positive, steroid use, or recipient of T-cell immunosuppressant) was required.

Upon inclusion, all patients underwent a standard bronchoscopy with BAL that was processed and then tested as described below (Torelli et al., 2011). BAL fluid specimens were divided into two aliquots within 1 h of collection after being shaken to ensure their homogeneous mixing.

One of them (5 ml) was centrifuged, and the suspended pellet (500 μl) was directly inoculated on conventional fungal medium (Sabouraud dextrose agar: SDA) supplemented with chloramphenicol (40 μg/ml) and blood agar; and incubated at 37°C (only blood agar plates) for 48 h at 30°C and the other one was frozen at -80°C and then used in PCR. Fungal isolates grown from cultures were identified by morphological and/or molecular methods (Posteraro et al., 2011; Torelli et al., 2011). For direct microscopic examination, a smear from each specimen was made and subjected to a Gomori stain to detect hyphal elements (Torelli et al., 2011).

Genomic DNA preparation
DNAs from Aspergillus were obtained by the needle inoculation of 50 ml of Sabouraud dextrose broth (SAB) (Difco Laboratories, Detroit, MI, USA) with conidia from a seven-day culture in SAB agar and incubation for 72 h at 30°C. The hyphae were recovered on a 0.45 μm-pore size filter and washed with sterile saline. Aliquots of 500 μl of the fungal hyphae were stored frozen at -80°C until use. Prior to lysis, the hyphae were thawed and suspend-

Table 1 - Yeasts, bacteria and other genera tested.

<table>
<thead>
<tr>
<th>Genomic DNA</th>
<th>Number of strains tested</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus</td>
<td>8</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>7</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>6</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>5</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus versicolor</td>
<td>6</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus sydowii</td>
<td>3</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>3</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>9</td>
<td>UCSC clinical isolates**</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>5</td>
<td>SA clinical isolates*</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>4</td>
<td>SA clinical isolates*</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>7</td>
<td>SA clinical isolates*</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>7</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>5</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>6</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>7</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>4</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>4</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Chlamydia spp.</td>
<td>3</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Trichinella spiralis</td>
<td>2</td>
<td>ISS collection°</td>
</tr>
<tr>
<td>Leishmania spp.</td>
<td>6</td>
<td>ISS collection°</td>
</tr>
</tbody>
</table>

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**Università Cattolica del Sacro Cuore, Rome, Italy.

DNA extraction from BAL fluid specimens
Total genomic DNA was obtained from clinical specimens as follows: a minimum of 2 ml of frozen BAL fluid specimen was processed using the MycXtra fungal DNA extraction kit (Myconostica Ltd.), which utilizes a mechanical disruption step, and in the case of viscous specimens, the processing was preceded by an N-acetyl-cysteine-sodium hydroxide treatment. DNAs extracts were frozen at -20°C until PCR was performed.
Sequence analysis


The Mp65p homologous proteins and genes were compared using the BLAST algorithm and the MP65 gene sequence (AJ010064).

Figure 1 - ClustalW multiple sequence alignment of six Aspergillus SCW4 genes. A. flavus (NW_002477248); A. fumigatus (XP_751116); A. nidulans (XP_661331); A. niger (An06g01530); A. terreus (XP_001212458); A. versicolor (Aspve1_0053210). *Denote identity between the corresponding nucleotides. Fla f-Fla r: orange; Fum f-Fum r: pink; Nid f-Nid r: green; Nig f-Nig r: light blue; Ter f-Ter r: lavender; Vers f-Vers r: red; All6 f-All6 r: blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
pared using Mac Vector ClustalW Software with default settings (Figures 1 and 2).

**Primer selection**

All the PCR primers (as shown in Table 2) were designed using the Mac Vector program by analyzing the conserved and variable regions highlighted after alignment of the different homologous MP65 genes for all species.

**Genus-specific PCR**

The PCR reaction was performed in a volume of 25 μl containing 50 mM KCl, 50 mM Tris pH 9.00, 2 mM MgCl2, 200 mM of deoxynucleotide, 50 pmol of primer (All6 f-All6 r) and 1 U Taq polymerase (Gotaq, Promega, Milan, Italy). 100 ng of each DNA tested were used as templates and PCR was performed in an automated thermal cycler (Eppendorf, Milan, Italy) using the following protocol: one cycle with initial denaturation at 94°C for 5 min; 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. Control PCR reactions were performed by omission of the template (negative).

**Species-specific PCR and determination of its sensitivity in six genomic DNAs of different Aspergillus species**

We prepared six sets of PCR primer pairs that differed from each other by target species tested (Table 2). Each PCR re-
RESULTS

Sequence analysis

Initially, by querying Aspergillus fumigatus database with MP65 gene of C. albicans (AJ010064), we found one entry encoding Scw4 cell wall glucanase of A. fumigatus (Scw4p: XP_751116). As shown in Figures 2 and 3, the aminoacidic and nucleotidic alignments had good identity and similarity scores.

Then, by querying different Aspergillus genomes/proteomes databases with SCW4 gene, we found eight entries encoding for protein similar to Scw4p. Aminoacidic or nucleotide sequences of these entries were therefore analyzed by performing ClustalW alignment (Figures 1 and 4).

We designed all the PCR primer pairs that were used for the different PCRs (Table 2 and Figure 1) according to the conserved and variable regions.

PCR detection of the target gene in Aspergillus spp.

Various isolates of Aspergillus species as well as other yeasts and genera (such as bacterial, murine, human and protozoan) were assayed by PCR for the presence of the homologous MP65 gene sequences. All purified genomic DNAs (100 ng) were used as a template in these experiments: an amplification product with the expected length (201 bp) was observed only in all Aspergillus isolates (A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus and A. versicolor) but not in the other ones (Figure 5).

We used the same PCR assays to detect six Aspergillus spp. DNA (A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus and A. versicolor) from BAL specimens (data not shown). By this method it was possible to detect the six species tested even if the bands were more faint than the genomic one.

PCR detection of six different Aspergillus spp.

DNAs from six Aspergillus species (A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus and A. versicolor) were assayed by PCR for the presence of the specific amplicon.

**Table 2** - Sequence and localization of the oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>5’ to 3’sequence</th>
<th>Localization</th>
<th>Score</th>
<th>Locus</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fla f</td>
<td>GTGCTACATGAGAAGAGAG</td>
<td>80-99</td>
<td>20</td>
<td>NW-002477248</td>
<td>237</td>
</tr>
<tr>
<td>Flar</td>
<td>CGCCTCTTATGCGGAGACG</td>
<td>299-282</td>
<td>1811111118</td>
<td>NW-002477248</td>
<td>72°C for 10 min.</td>
</tr>
<tr>
<td>Fum f</td>
<td>ATCGAGACTAAGTGTCACCT</td>
<td>1-20</td>
<td>20</td>
<td>XP-751116</td>
<td>1241</td>
</tr>
<tr>
<td>Fum r</td>
<td>TTACTCCCGATCTGCT</td>
<td>1224-1007</td>
<td>18</td>
<td>XP-751116</td>
<td>72°C for 10 min.</td>
</tr>
<tr>
<td>Nig f</td>
<td>CAAAAGCAGCGCTCAG</td>
<td>280-297</td>
<td>18</td>
<td>An06g01530</td>
<td>808</td>
</tr>
<tr>
<td>Nig r</td>
<td>AAGCTCGGAATCGCCCTT TT</td>
<td>1070-1053</td>
<td>18</td>
<td>An06g01530</td>
<td>808</td>
</tr>
<tr>
<td>Ter f</td>
<td>ATGAAGTGGTTCATCCTCCT</td>
<td>51-74</td>
<td>24</td>
<td>XP-001212458</td>
<td>1012</td>
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<tr>
<td>Ter r</td>
<td>TCAACTCGGTTGATGAAATGC</td>
<td>1043-1024</td>
<td>20</td>
<td>XP-001212458</td>
<td>1012</td>
</tr>
<tr>
<td>Vers f</td>
<td>ATGAAGTGGATTATCCTCCTTGT</td>
<td>1-25</td>
<td>25</td>
<td>Asptu1-0187193</td>
<td>611</td>
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<tr>
<td>Vers r</td>
<td>ATCACTGAGACTGGAAATGGA</td>
<td>590-569</td>
<td>22</td>
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<td>611</td>
</tr>
<tr>
<td>Nid f</td>
<td>ATGAAGTGGATGCGGCTCCTG</td>
<td>1-23</td>
<td>23</td>
<td>XP-661331</td>
<td>400</td>
</tr>
<tr>
<td>Nid r</td>
<td>AGAGATGCGGAAAGATTTGATG</td>
<td>379-360</td>
<td>20</td>
<td>XP-661331</td>
<td>400</td>
</tr>
<tr>
<td>All6 fb</td>
<td>RTSSGMAAYGARCTTSCARC</td>
<td>21</td>
<td></td>
<td></td>
<td>201</td>
</tr>
<tr>
<td>All6 rb</td>
<td>RTCTTAAAGGYYRTGRCAGTT</td>
<td>21</td>
<td></td>
<td></td>
<td>201</td>
</tr>
</tbody>
</table>

Oligonucleotide position is referred to the coding sequences of the different Aspergillus SCW4 homologous genes. All6 f-All6 r localization depending on the alignment, is not shown because it’s different in each specie analyzed.
SCW4 gene primers PCR for aspergillosis diagnosis

Figure 3 - A: ClustalW sequence alignment of AJ010064 (MP65 gene of C. albicans) and XP_751116 (SCW4 gene of A. fumigatus). The symbols* denotes identity between the corresponding nucleotide. B: Table showing of identity and similarity scores percentages created with Mac Vector Software.
product, using six different and species-specific primer pairs (Table 2). Purified fungal genomic DNA (100 ng) was used as a template in these experiments. Under the reaction conditions described in: "Species-specific PCR and determination of its sensitivity in six genomic DNAs of different Aspergillus species" of Materials and Methods, different DNA fragments of variable lengths were observed, in each PCR performed, in the different species without cross amplification (data not shown). We used the same PCR assays to detect the six Aspergillus spp. DNA from BAL (Figure 6). By this method it was possible to detect the six species tested even if the bands were more faint than the genomic one.

**PCR sensitivity in six genomic Aspergillus DNAs**

We assayed by PCR, 100, 10, 1, 0.1 and 0.01 ng DNA from six Aspergillus spp. (A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus and A. versicolor) for the presence of the specific amplicon product; under the reaction conditions described above, different DNA fragments of variable intensity were observed in the different species with a very good sensitivity up to 0.01 ng (Figure 7). We used the same PCR assays to test the sensitivity in six Aspergillus spp. DNA from BAL; by this method it was possible to detect the six species tested (0.01 ng for A. fumigatus and A. nidulans, 0.1 ng for A. flavus, A. terreus and A. versicolor and 1 ng for A. niger) (Figure 8).

**Multiplex PCRs**

In order to identify different Aspergillus species in a single reaction, we developed three simple step-by-step protocols of Multiplex PCR (Henegariu et al., 1997; Arancia et al., 2004). The first Multiplex PCR was tested for its specificity to detect each species of Aspergillus, when all six primer pairs were added to each tube with DNA from a single Aspergillus spp. An amplicon of expected length was obtained for each Aspergillus species tested (data not shown). The second Multiplex PCR was tested for its specificity to

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**Figure 4** - Tables showing the identity and similarity scores percentages created with Mac Vector Software of nine SCW4 genes (A) and nine Scw4p proteins (B) of Aspergillus.

A. flavus (NW-002477248); A. fumigatus (XP_751116); A. nidulans (XP_661331); A. niger (An06g01530); A. oryzae (BAE 54672); A. sydowii (Aspsy1_0044122); A. terreus (XP_001212458); A. tubingenisis (Asptu1_0187193); A. versicolor (Aspve1_0053210).
detect six *Aspergillus* spp. DNA, when a single species-specific primer pair was added in each PCR mix with all six *Aspergillus* spp. DNAs. An amplicon of the expected length was obtained for each *Aspergillus* species tested (data not shown). In the third PCR, all six primer pairs were added to each tube with all six *Aspergillus* spp. DNAs. In this case, six expected length amplicons were obtained for each *Aspergillus* species (data not shown). We used the same PCR assays to detect the six *Aspergillus* spp. DNA from BAL; by this method it was possible to detect the six species tested (Figure 9a, b, c).

**DISCUSSION**

The increasing frequency of invasive fungal infections and the high mortality associated with these diseases have highlighted the need for a rapid identification of the species causing infection. In particular, the number of cases of IA found at autopsy has increased 14-fold since 1978 (Lehrnbecher et al., 2010). Early identification and initiation of antifungal therapy is crucial in reducing the mortality, as the progression of invasive disease from detection to death is typically less than 14 days (Denning et al., 1998; Nucci et al., 2013). Currently, immunosuppressed patients with sign and symptoms consistent with a fungal infection are treated with voriconazole and AmB empirically. However, the resistance of certain *Aspergillus* species to antifungal agents complicates empiric treatment for invasive disease (Denning et al., 1998; Chamilos et al., 2005; Walsh et al., 2008).

Thus, rapid diagnosis and recognition of the species causing infection and treatment with the most active antifungal therapy may be important in the reduction of the mortality of immunosuppressed patients with IA. Microbiological diagnosis of IA remains elusive, and detection of *Aspergillus* in lower respiratory tract samples is one of the main findings that indicate the disease. However, identification of isolates is subject to an unavoidable
delay. Accelerating the detection and identification of Aspergillus would allow clinicians to start appropriate antifungal treatment earlier. As culture of the fluid collected during BAL typically has a low sensitivity (Yeo et al., 2002; Hope et al., 2005), evaluation of the performance of newer molecular techniques requires alternative diagnostic information, which differs according to the type of specimen analyzed (i.e. blood, serum or BAL fluid). Although bronchoscopy is an “invasive” procedure for obtaining specimens (Tuon, 2007), BAL is a direct sampling of the affected organ and is therefore important in the diagnosis of pulmonary fungal infections (Knox et al., 2009).

As emphasized in other published papers (Dupont et al., 1991; Morace et al., 1997), the use of PCR for detection of pathogenic opportunistic yeasts provides a potential-

Figure 6 - PCR amplification products of six Aspergillus species from BALs using six different species-specific primer pairs. A: a) Fum f-Fum r of A. fumigatus; b) Fla f-Fla r of A. flavus; B: c) Nig f-Nig r of A. niger; d) Nid f-Nid r of A. nidulans; C: e) Ter f-Ter r of A. terreus; f) Vers f-Vers r of A. versicolor.

A-C: A. fumigatus (lane 1), A. flavus (lane 2), A. niger (lane 3), A. nidulans (lane 4), A. terreus (lane 5), A. versicolor (lane 6), 1Kb plus DNA ladder (lane 8), A. fumigatus (lane 9), A. flavus (lane 10), A. niger (lane 11), A. nidulans (lane 12), A. terreus (lane 13), A. versicolor (lane 14).
ly more specific, sensitive and rapid test with respect to the current microbiological, biochemical and serological diagnostic methods. Consequently, several methods have been proposed both for genus and species identification. In particular, identification of the infecting *Aspergillus* species may be relevant for the choice of therapy required. In fact, *Aspergillus* species may widely vary in their susceptibility to antimycotics. For this reason, various molecular approaches have been used for the detection of *Aspergillus* from clinical samples (Yamakami et al., 1996; White et al., 2011). Targets for the genus level detection of *Aspergillus* have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions (Henry et al., 2000). A prerequisite for the use of PCR as a routine clinical laboratory application is that it is faster, more sensitive and specific.

**Figure 7** - PCR sensitivity in six genomic *Aspergillus* DNAs. Ten fold serial dilutions (lane 1: 100 ng; lane 2: 10 ng; lane 3: 1 ng; lane 4: 0.1 ng; lane 5: 0.01 ng) of six different *Aspergillus* species DNAs were used as templates in PCR assay with specie-specific primer pairs. A: *A. fumigatus*, B: *A. flavus*, C: *A. niger*, D: *A. nidulans*, E: *A. terreus*, F: *A. versicolor*.
On the basis of these considerations, we have developed PCR and Multiplex PCR assays for the detection of six medically important *Aspergillus* spp. species DNA in BAL specimens from hematology and intensive care unit (ICU) patients at risk IA using different species and genus-specific PCR primers, selected within the SCW4 gene, encoding a cell wall glucanase of *A. fumigatus*, similar to mannoprotein Mp65 of *Candida albicans* and never tested before. The genus-specific primers amplified all *Aspergillus* species DNA tested (100% positivity) giving a band of the expected length (201 bp). DNAs of all other fungal genera tested were not amplified. The species-specific primer pair enabled us to differentiate all the six medically important *Aspergillus* species tested, without cross amplification.

The method described in this study is simple, rapid, specific and reproducible. Even if the bands amplified using...

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**Figure 8** - PCR sensitivity in six genomic *Aspergillus* DNAs from BALs. Ten fold serial dilutions (lane 1: 100 ng; lane 2: 10 ng; lane 3: 1 ng; lane 4: 0.1 ng; lane 5: 0.01 ng) of six different *Aspergillus* species DNAs were used as templates in PCR assay with species-specific primer pairs. A: *A. fumigatus*, B: *A. flavus*, C: *A. niger*, D: *A. nidulans*, E: *A. terreus*, F: *A. versicolor.*
DNA from the BALs were more feeble than the genomic one, obtained from cultures of aliquot of the same clinical samples, it was possible to identify the species. The total time required for the procedure is less than 4 h to obtain the PCR results. The ability to detect and distinguish among the various clinically relevant Aspergillus species is of great diagnostic value, as certain species vary in their resistance to antifungal therapy and are associated with increased virulence and higher mortality. This is mainly the case for A. terreus and A. nidulans, which are frequently resistant to amphotericin B. Further clinical studies are needed to elucidate the true potential of such new techniques for different patient groups. We have drawn primer-pairs specific for six Aspergillus species and we developed different PCR methods. The methods are cost-effective because they only require PCR compo-
nents, DNA isolation reagents, and gel electrophoresis equipment.

In conclusion, since certain *Aspergillus* species vary in their resistance to antifungal therapy and are associated with increased virulence and higher mortality, the ability to detect and distinguish among the various clinically relevant *Aspergillus* species is of great diagnostic value. Further investigations with a higher number of serial samples from patients with invasive aspergillosis and validated by clinical output could supply an important contribution to the IA diagnosis. In particular, the possibility of Aspergillus detection directly from samples (whole blood or urine or serum), taken from patients with IA, is currently under investigation and efforts are being made to validate the applicability of this method in the diagnosis of IA.

Acknowledgments
This research has been partially supported by “Fondazione Roma”, Istituto Superiore di Sanità.

Many thanks are due to Prof. Laura Popolo for help in primer selection, to Dr. Giuseppina Mandarino for help in the preparation of the manuscript and to Mr. Luigi Nicoletti for help in figures preparation.

Authors contributions
Silvia Arancia and Silvia Sandini contributed to the planning of the experimental work and to the interpretation of the data obtained. Moreover, they carried out the PCR assays. Elena De Carolis and Antonietta Vella performed the extraction, purification and characterization of DNA samples used in this study. Sandro Norelli contributed to manuscript drafting and the preparation of the figures. Maurizio Sanguinetti and Flavia De Bernardis, thank to their well known experience in the clinical and experimental mycology, coordinated the different experimental investigations and supported the interpretation and discussion of the obtained results.

Conflict of interest
The authors declare no conflicts of interest.

References


