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Incidence of Pulmonary Aspergillosis and Correlation of Conventional Diagnostic Methods with Nested PCR and Real-Time PCR Assay Using BAL Fluid in Intensive Care Unit Patients

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Background: Although the incidence of invasive aspergillosis in the intensive care unit (ICU) is scarce, it has emerged as major problems in critically ill patients. In this study, the incidence of pulmonary aspergillosis (PA) in ICU patients has evaluated and direct microscopy and culture has compared with nested polymerase chain reaction (PCR) and real-time PCR for detection of *Aspergillus fumigatus* and *A. flavus* in bronchoalveolar lavage (BAL) samples of the patients. *Methods:* Thirty BAL samples obtained from ICU patients during a 16-month period were subjected to direct examinations on 20% potassium hydroxide (KOH) and culture on two culture media. Nested PCR targeting internal transcribed spacer ribosomal DNA and TaqMan real-time PCR assay targeting β-tubulin gene were used for the detection of *A. fumigatus* and *A. flavus*. *Results:* Of 30 patients, 60% were men and 40% were women. The diagnosis of invasive PA was probable in 1 (3%), possible in 11 (37%), and not IPA in 18 (60%). Nine samples were positive in nested PCR including seven samples by *A. flavus* and two by *A. fumigatus* specific primers. The lowest amount of DNA that TaqMan real-time PCR could detect was ≥40 copy numbers. Only one of the samples had a positive result of *A. flavus* real-time PCR with C_t value of 37.5. *Conclusions:* Although a significant number of specimens were positive in nested PCR, results of this study showed that establishment of a correlation between the conventional methods with nested PCR and real-time PCR needs more data confirmed by a prospective study with a larger sample group. J. Clin. Lab. Anal. 27:181–185, 2013.

Key words: Pulmonary aspergillosis; ICU; *Aspergillus flavus*; *Aspergillus fumigatus*

INTRODUCTION

The major risk factors for pulmonary aspergillosis (PA) are congenital or acquired immunodeficiencies (1, 2). Nevertheless, a broad group of patients who are admitted to intensive care units (ICUs) may also be susceptible to infections. This heterogeneous patient population is highly susceptible to healthcare-associated fungal infections (3, 4). In addition, during recent years, a rising incidence of PA in non-neutropenic critically ill patients has been reported (5, 6). However, estimates about the incidence of invasive aspergillosis (IA) among critically ill patients are sparse and variable. Definitive

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diagnosis requires histopathological evidences of deep-tissue invasion or a positive culture from normally sterile sites (7). Although the diagnostic value of bronchoalveolar lavage (BAL) is controversial, it is used in many cases as an accessible specimen in clinical practice. The evaluation of multiple findings from microscopy and culture of BAL samples is helpful in the effort to improve the diagnosis of fungal disease (8).

Molecular diagnostic techniques such as nucleic acid detection by PCR are emerging as potentially more sensitive and rapid alternatives to conventional techniques for the diagnosis of PA (9).

We performed this study to estimate the incidence of PA in ICU patients and also to evaluate the correlation between conventional methods with nested PCR and real-time PCR to detection of *Aspergillus fumigatus* and *A. flavus* in BAL samples of ICU patients in Tehran, Iran.

MATERIALS AND METHODS

Between June 2009 and October 2010 a total of 30 BAL fluid specimens were obtained from ICU patients at the Shariati Hospital and the Medical Mycology laboratory in Tehran University of Medical Sciences, Tehran, Iran. BAL fluid (4–7 ml) were obtained by experienced physicians according to standardized techniques (10) and collected in sterile vessels without conservation media and transferred to the laboratory. Samples were centrifuged at 3,000 rpm for 20 min and the pellet was vortexed and resuspended in 400–600 μl of supernatant. An aliquot of supernatant was kept at −20°C until DNA extraction.

Direct Examination and Culture

A 150 μl aliquot of the centrifuged sediment was treated with a drop of 15% potassium hydroxide (KOH) preparation, covered with 24 × 50 mm coverslip and examined with 400× magnification of microscope. A 75 μl aliquot of samples were plated on Sabouraud glucose (4%) agar and brain heart infusion agar (Difco, USA) and were incubated at 30°C for 3–7 days. The results were subsequently compared with clinical data.

DNA Extraction

For cell lysis of BAL fluids, each sample was subjected to freezing in liquid nitrogen and thawing in boiling water for four times. For sticky and viscous samples Sputasol (dithiothreitol 2%) (Wako, Japan) was added and sonicated for 2–5 min (11). DNA was extracted from each BAL sample by conventional phenol–chloroform method (12).

Nested PCR

Specific primer pairs for nested PCR (Table 1) were used for amplification of partial internal transcribed spacer (ITS) region as described by Sugita et al. (13). The primer pairs amplify a 305 bp and 308 bp fragments in *A. flavus* and *A. fumigatus*, respectively. The PCR mixture contained 12.5 μl of 2× premix (Amplicon, Denmark), 15 pmol of each primer, 2 μl of DNA template solution, and enough water up to the total reaction volume of 25 μl. The mixture was heated at 95°C for 5 min and PCR was performed with the following program by a thermal cycler (Takara PCR Thermal Cycler Dice™ mini, TP100, Tokyo, Japan): 94°C for 45 s; 60°C for 1 min; and 72°C for 1 min; all repeated for 30 cycles. Thermal cycling was terminated by polymerization at 72°C for 7 min. One microliter of 1/100 diluted of the first PCR product was used as a template for the second (nested) PCR. Each mixture was heated at 95°C for 5 min and PCR was performed in 94°C for 50 s; 58°C for 40 s, and 72°C for 45 s for 25 cycles followed by polymerization at 72°C for 8 min. The PCR products were electrophoresed onto 2% agarose gel in TBE (Tris 0.09 M, boric acid 0.09 M, EDTA 0.002 M) and visualized using UV transilluminator after staining with ethidium bromide (0.5 μg/ml).

Real-time PCR

The real-time PCR primers and probes were designed based on multiple alignments of various sequences of partial β-tubulin gene by Geneious and Genscript softwares as described previously (14). Real-time PCR assay was performed with a 7500 Real-Time PCR System (Applied Biosystems, CA, USA) based on TaqMan chemistry. The specific primers and probes were used in a specific multiplex PCR reaction for *A. fumigatus* and *A. flavus* (Table 1).

Each run was performed using EagleTaq Master Mix (Roche, Basel, Switzerland) according to the manufacturer recommended protocol. The reaction mixture contained 2 μl of template DNA solution, 0.2 μM of each probe, 0.4 μM of each primer, and 10 μl of master mix and enough water up to the final volume of 20 μl. Following an initial denaturation step at 95°C for 10 min, PCR amplification was performed for 45 cycles consisting of 95°C for 3 s and 60°C for 30 s. DNA isolated from *A. fumigatus* (TIMM 3968) and *A. flavus* (TIMM 2912) as the positive controls and water as the negative control were used in each run. For quantitation, *A. fumigatus* and *A. flavus* specific DNA was cloned using a TA cloning kit and pCR 2.1 vector (Invitrogen, CA, USA) and the plasmid with cloned insert was sequenced (ABI PRISM™

TABLE 1. The Sequence of Primers and Probes Used for Specific Real-Time PCR and Nested PCR Detection of *A. fumigatus* and *A. flavus* in This Study

Species	*Aspergillus fumigatus*	*Aspergillus flavus*
FP for RT-PCR	5′-ATCCTCCCAATTGAGAAAG	5′-ACGACGACCATATGGCATTA
RP for RT-PCR	5′-AGCGAGTCAGATCGTGAGATG	5′-GGAGAAGCGGTCAGGAGTT
SP for RT-PCR	FAM-CCATCAGACACGCGTCCGCTT	VIC-CCTTGCCGTCAGATCCATTCCA
FP for 1st n-PCR	5′-CAGCGAGTACATCACCTTGG	5′-CAGCGAGTACATCACCTTGG
RP for 1st n-PCR	5′-CCATTGTTGAAAGTTTTAACTGATT	5′-CCATTGTTGAAAGTTTTAACTGATT
FP for 2nd n-PCR	5′-ACTACCGATTGAATGGCTCG	5′-ACTACCGATTGAATGGCTCG
RP for 2nd n-PCR	5′-CATACTTTCAGAACAGCGTTCA	5′-TTCACTAGATCAGACAGAGT

FP, forward primer; RP, reverse primer; SP, specific probe; RT-PCR, real-time PCR; n-PCR, nested PCR.
FAM and VIC, commercial reporter dyes.

ABI-3730 Genetic Analyzer, Applied Biosystem) to confirm the insertion of a single copy of amplicon.

The specificity of the *Aspergillus* PCR assays was assessed by testing genomic DNA extracted from several reference strains including *Aspergillus*, *Penicillium*, *Fusarium*, *Candida*, and *Trichosporon* species.

RESULTS

We examined 30 BAL specimens obtained from ICU patients from which 60% were men ($n = 18$) with average age of 52, and 40% were women ($n = 12$) with average age of 40.8. According to the EORTC/MSG 2008 criteria (15), 2 (7%) of the patients were designed as probable PA, 10 (33%) as possible PA, and 18 (60%) as without PA.

Progressive acute angle dichotomous branching hyphae were not seen in any BAL samples in direct microscopy examination but one BAL sample had a positive culture by *A. flavus*.

From the 30 samples, nine samples were positive in nested PCR (Fig. 1) including seven samples by *A. flavus* specific primers and two by *A. fumigatus* specific primers. Of two samples with probable PA, only one of them had positive results with *A. fumigatus* nested PCR. Of the ten samples with possible PA, two (20%) of them had positive results of *A. flavus* nested PCR but eight (80%) of them had negative nested PCR.

We considered samples with threshold cycle (C_t) of <38 as positive real-time PCR. Only one of samples had a positive result of *A. flavus* real-time PCR with C_t value of 37.5 (Fig. 2).

In this study, the lowest amount of DNA that TaqMan real-time PCR could detect was ≥35 copy numbers for *A. fumigatus* and ≥40 copy numbers for *A. flavus*.

DISCUSSION

As a cross-sectional study, during a screening program, direct examination, culture, nested PCR, and real-time PCR were evaluated for diagnosis of PA in ICU patients by using BAL samples. The assay was applied to analysis of 30 BAL fluid specimens from patients at risk of PA.

In the ICU patients, molds infections are much less common than the infections caused by *Candida* species. In a nonselected patient population at an academic hospital, the prevalence of invasive fungal infections increased from 2.2% to 5.1% over a 12-year period that is partly in association with an increase in the rate of *Aspergillus* infection (16). However, estimates about the incidence of PA among critically ill patients are sparse and variable. In a large study, 127 (6.9%) of 1,850 hospitalized patients had microbiologic or histopathologic evidences of aspergillosis during their ICU stay, including 89 cases (70%) in which there was not an underlying hematological malignancy (17). Valles et al. (18) reported 13 (19%) of 67 episodes of PA with pathologic and microbiologic evidence of aspergillosis in a cohort of patients with severe

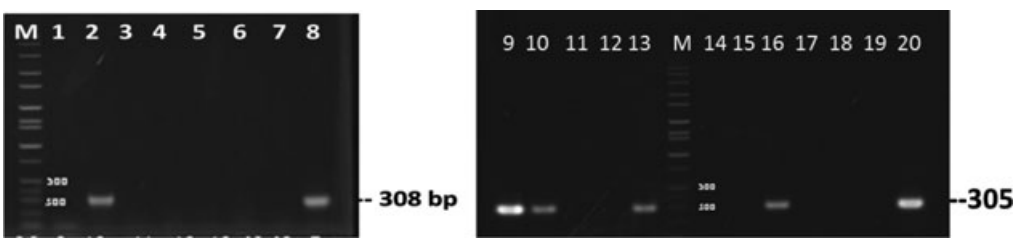

Fig. 1. Agarose gel electrophoresis of nested PCR amplification of DNA extracted from BAL samples by specific primers for *Aspergillus flavus* (right) and *A. fumigatus* (left): Lanes 2, 9, 10, 13, and 16 are positive sample; lanes 8 and 20 are positive controls; lanes 6, 7, 18, and 19 are negative controls; lanes M are DNA size marker.

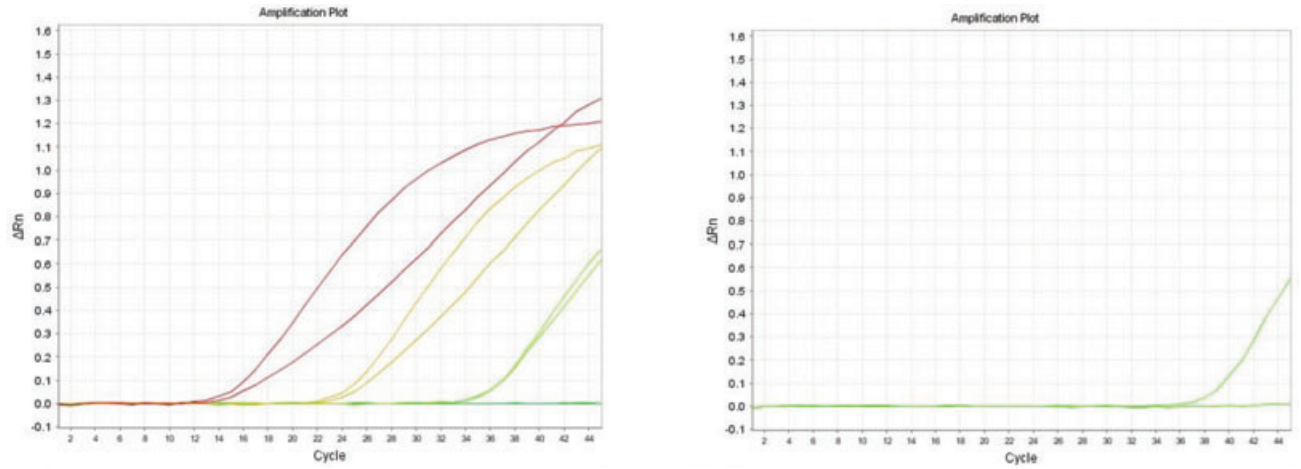

Fig. 2. Examples of amplification plots of real-time PCR. Left: standards (duplicate) of *A. flavus* with 40×10^6 copy numbers, 40×10^3 copy numbers, and 40 copy numbers. Right: the amplification plot of *A. flavus* and *A. fumigatus* in a BAL sample.

hospital-acquired pneumonia who had been admitted to the ICU.

Cultivation of *Aspergillus* has poor sensitivity and deep tissue biopsy specimens are difficult to obtain. Furthermore, fungal culture is often confounded by antifungal treatment, as empirical treatment is a common practice in patients with hematological malignancy and fever unresponsive to antibacterial agents. Another problem is that, isolation of fungus from respiratory specimens may also reflect colonization of the airway instead of invasive infection, or even environmental contamination of the culture (19). Although in this study, patients without PA had five (27.8%) positive nested PCR for *A. flavus*, one (5.5%) for *A. fumigatus*, and one (5.5%) positive real-time PCR for *A. flavus*, however it may not be able to differentiate between invasive infection and colonization of the respiratory tract and/or environmental contamination.

In our study, *A. flavus* was the most common cause among *Aspergillus* species in culture and PCR, unlike other parts of the world that *A. fumigatus* is dominant agent specially in immunocompromised hosts (20, 21). It may be due to higher occurrence of the fungus in the environment samples like air, water in the geographic locations. Similarly, there is an agreement on higher prevalence of *A. flavus* isolation from patients (22) and air (23) in Iran. In this study, clear correlation between results of conventional methods, nested PCR and/or real-time PCR tests, and the final classification of invasive aspergillosis using the consensual definitions (24) was not found. Exact measurement of fungal DNA in BAL fluids would be difficult (25). Although in this study nested PCR had higher sensitivity (nine positive cases), but it seems this method has false-positive results more than the other methods. Some of discrepancies in results obtained from DNA-based method may be because of rigidity of spores and degenerated hyphae that may influence the DNA purification step.

The diagnosis of PA infection in ICU patients still remains challenging. As definitive diagnosis of pulmonary fungal infections in severely immunocompromised patients is difficult, and a high index of suspicion is necessary in patients with risk factors for invasive disease (26). Histopathological diagnosis, by examining lung tissue obtained by thoracoscopy or open-lung biopsy, remains the "gold standard" in the diagnosis of IPA (15, 27). However, lung biopsies are not performed very often because of concern about complications in patients who are often thrombocytopenic and in respiratory distress. Unfortunately, in this study we did not have any biopsy tissue for examination and hence lacking golden standard was the drawback of our project. Nevertheless, in immunocompromised patients with the characteristic clinical presentation, demonstration of *Aspergillus* in culture or stain, even if obtained from sputum or BAL fluid (a nonsterile site), has a high predictive value for IPA and for most of practical purposes it is informative for diagnosis and treatment (4).

Our study suggests that molecular diagnostic methods, conventional methods, and even sampling conditions need more definite standards to improve the diagnosis of PA and the data need to be confirmed by a prospective study with a larger sample group.

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