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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Comparison of real-time PCR, nested PCR, and galactomannan antigen detection by enzyme-linked immunosorbent assay in sera for diagnosis of invasive aspergillosis

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Summary Conventional methods for diagnosis of invasive aspergillosis (IA) lack sensitivity and specificity. Serological methods still have many cases of cross-reactivity. However, molecular techniques seem to arise as a rapid approach, specific and direct that could be used in the diagnosis of IA. In this study, we analyzed 88 serum samples from patients of having IA using GM-ELISA test, nested PCR with primers for the rRNA 18S of *Aspergillus* genus and real time PCR specific for *A. fumigatus*. Among the 88 samples, 64 of them had positive GM titers and 23 had positive nested PCRs; 18 of the 23 PCR-positive samples were also GM-positive. On the other hand, 18 samples were detected positive by reel time PCR; 13 positive samples were also detecting positive by nested PCR. QPCR revealed 26 % of the patients with IA, while nested PCR and galactomannan ELISA revealed respectively 34 % and 94 % of the patients with IA. Probable IA was diagnosed in 18 and possible IA was diagnosed in 6 episodes. Forty-four episodes were defined as not having IA. The positive and negative predictive values were respectively 100 %, and 88 % for QPCR, 100 % and 97 % for nested PCR, and 28 % and 60 % for GM test. These results suggesting that combined use of methods might improve the diagnosis of IA.

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Introduction

Invasive aspergillosis (IA) is now a major cause of infectious morbidity and mortality in immunocompromised hosts particularly those treated with chemotherapy for hematologic malignancies or those receiving high-dose corticosteroids [1,2]. Most of these infections are due to *Aspergillus fumigatus*. This fungal species releases a high number of conidia to the atmosphere. Due to its presence in the atmosphere, outbreaks of nosocomial aspergillosis have often been associated in the past with an increase in the concentration of airborne spores [3,4]. The poor prognosis of this infection is usually associated with a complicated and late diagnosis. Traditional diagnostic methods, such as histopathology and culture, which are still considered the gold standards, have low sensitivity, which underscores the need for the development of new means of detecting *Aspergillus* [1,4]. To improve the diagnosis, laboratory tests have been developed to detect circulating galactomannan antigens ''GM'' for rapid diagnosis. GM major constituent of the *Aspergillus* cell may be detectable circulating, in serum, bronchoalveolar lavage (BAL) specimens and urine using a commercial sandwich enzyme linked immunosorbent assay (Platelia Biorad®) [5—7]. However, detection of circulating GM lacks sensitivity and to some extent, specificity, so diagnosis can be delayed [4,5,8,9]. Recently, the development of DNA-based methods for detection of *Aspergillus* nucleic acids in pathologic samples (bronchoalveolar lavage specimens ''BAL'', serum and blood) provides an alternative and potentially more sensitive means for diagnosing IA [2,7,10]. A number of studies have described probes, restriction fragment length polymorphism, nested PCR to detect and identify ribosomal DNA (rDNA) sequences of *Aspergillus* genus [21—23]. PCR methods are particularly promising because of their simplicity, specificity, and sensitivity [5,11,12].

In this study, we describe rapid and sensitive methods for the detection of *Aspergillus* DNA in serum samples:

- nested PCR based on amplification of the 18S rRNA gene using two sets of primers specific of five *Aspergillus* species (*A. fumigatus*, *A. niger*, *A. nidulans*, *A. flavus*, *A. terreus*).
- QPCR for amplification of A. fumigatus DNA using commercializing kit ''*A. fumigatus* real time PCR kit, Genekam biotechnology AG''. Indeed, detection of *A. fumigatus* DNA is down with a pair of fluorescing probes FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-teremethyl-rhodamine) in the Light Cycler 480 instrument. Then, molecular results were compared with those obtained by the sandwich ELISA test.

Specimens

In this study, we tested 88 serum samples from 68 patients (61 % man and 49 % women) hospitalized in reanimation, department of organ transplantation, pulmonary and internal medicine services of Tunisian Military hospital. Samples were collected between January 2013 and mars 2016 at the request of a consulting clinician for routine GM diagnostic.

GM test

ELISA for GM was performed with a commercial kit (Platelia *Aspergillus*; BioRad®) according to the manufacturer's instructions. The ELISA for GM uses a rat monoclonal antibody directed against the GM molecule as both the capture and detection antibodies.

The GM testing was considered positive when the ratio of optical density ''OD'' (OD sample/OD control) was $\geq$ 0.5. An index below 0.5 was considered negative.

Extraction of *Aspergillus* DNA

Extraction of DNA from serum samples was performed using a house protocol using proteinase K and phenol chloroform solutions. In the first step, 200 µL of the serum sample was combined with an equal volume of the lysis buffer containing ''10 mM Tris, 15 mM NaCL, 5 mM EDTA, 1 % SDS, 0.2 mg of gelatin per ml and 0.9 % polysorbate 20''. 10uL of Proteinase K was added to the mixture was incubated for 60 min at 65 °C Proteinase K was then inactivated by heating the mixture to 100 °C for 10 min. In the next step, the supernatant was transferred to a 2 mL microcentrifuge tube, a double volume of phenol-chloroform was added, and the mixture was centrifuged at 16,000 g for 30 min at 4 °C. The supernatant was transferred to a fresh tube, 2 volumes of ethanol stored à −20 °C were added. The mixture was centrifuged at 16,000 g for 30 min at 4 °C, and the pellet was allowed to dry. After rinsing with 70 % ethanol at 4 °C, the extracted DNA was dissolved in 70 µL of TE (10 mM Tris, 1 mM EDTA) were added.

Nested PCR

Nested PCR was performed according to the protocol described by yamakami in 1996 [13], using two sets of primers; the outer set consisted of asp5 (5'GATAACGAACGAGACCTCGG3') and asp8 (5'TGCCAACTCCCCTG AGCCAG3'), which together amplify a 384 bp sequence. The inner primer set consisted of asp1 (5′CGGCCCTTAAATAGCCCGGTC3′) and asp7 (5'CCTGAGCCAGTCCGAAG GC C3'), which together amplify a 357 bp sequence. Each PCR contained a positive control ''*Aspergillus* DNA extracted from culture'' and negative control ''sterile water''.

The first amplification consisted of 5 µL buffer solution (1x), 1 µL dNTPs (10 mM), 1 µL of each external primer (25 mM), 1U of DNA polymerase and 1 µL (50 ng) of DNA. PCR was conducted in an automatic thermal cycler ''Biometra''. PCR was performed under the following conditions: denaturation at 94 °C for 3 min, annealing at 56 °C for 1 min, and extension at 72 °C for 60s for 30 cycles. The last round of PCR was performed at 72 °C for 7 min. The second amplification was accomplished by using 2 µL of the first product as a template. PCR reaction was performed in 50 µL of PCR solution containing 5 µL of buffer solution (1 ×), 2 µL of MgCl2 (25 mM), 1 µL dNTPs (10 mM), 1 µL of each primer and 1U of DNA polymerase. The amplification process was performed under the following conditions: 30 cycles of 94 °C for 30 s for denaturation, 63 °C for 45s for annealing, and extension at 72 °C for 60s for 30 cycles. The nested PCR products were electrophoresed on a 1.5 % agarose gel

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