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High negative predictive value diagnostic strategies for the reevaluation of early antifungal treatment: A multicenter prospective trial in patients at risk for invasive fungal infections

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Summary Early antifungal therapeutic strategies are proposed during invasive fungal infection (IFI), but antifungal stewardship programs should institute a systematic reevaluation of prescriptions, particularly in the context of empirical treatment. Here, we aimed to evaluate the performances and particularly the negative predictive value (NPV) of diagnostic strategies, including a whole blood panfungal quantitative PCR assay (PF-qPCR) in a high risk population for IFI. The first step was to standardize and optimize a new PF- rtPCR targeting ITS2 region. Then, this method was evaluated in a multicenter prospective study including 313 patients with suspected IFI for whom an early antifungal treatment was prescribed. All patients enrolled at day 0 of their treatment benefited from serum *Aspergillus* galactomannan (GM) antigen detection twice a week, weekly PF-qPCR assay, and when indicated and feasible, CT-scan and mycological sampling. In total, 125 of 313 patients were diagnosed with IFI:

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Antifungal stewardship program

68 invasive aspergillosis (eight proven, 48 probable and 12 possible), one fusariosis, 47 candidemia, three disseminated candidiasis and six cryptococcosis. Globally, the sensitivity of the PF-qPCR assay was only 40%, but the specificity, PPV and NPV were 96%, 88% and 69%, respectively. In the population of patients at high risk for invasive aspergillosis who also benefited from *Aspergillus* GM detection, the sensitivity and the NPV of the combined detection reached to 78% and 84%, respectively. Even higher NPV were obtained when combining negative PF-qPCR and CT scan (95%) as well as negative GM and CT scan (93%), thus allowing to rationalize and re-evaluate the prescription of empirical treatment in such highly selected population.

Introduction

Invasive fungal infections (IFI) cases has considerably increased over the last two decades, with an enlarged population of patients at risk.[1] In particular, IFI are an important cause of morbidity and mortality among patients with haematological malignancies, in transplanted patients and in patients hospitalized in intensive care unit. Mortality due to fungemia is estimated to be 30–40%[2–5] and more than 50% for invasive aspergillosis.[6–8] The survival rate depends on early diagnosis[9,10] and rapid initiation of appropriate antifungal treatment.

The emergence of new antifungal drugs and strategies have improved the prognosis of IFI.[11–13] The use of empirical treatment (ET) for refractory fever in high-risk patients is one of these strategies.[14] Because antifungals are costly and occasionally responsible for side effects and selection of resistant isolates, reliable diagnostic strategies would be welcome to re-evaluate unnecessary antifungal treatment as part of a policy of an antifungal stewardship program.[15] Therefore, consensual definitions for proven, probable and possible IFI, based on host factors, clinical and mycological criteria have been proposed.[16,17] PCR is not still included in these criteria while considered as a practical tool for the onset of antifungal treatment in routine,[18] because some pitfalls need to be ruled out.

In recent years, many PCR-based detection assays for fungal DNA have been debated. Some authors reported that PCR is a sensitive tool for the early diagnosis of IFI using total blood whereas others prefer to use serum.[19–21] PCR techniques have shown high sensitivity in respiratory samples and tissue biopsies.[22,23] In blood, even if the fungal load is low, PCR appears more sensitive than serum as shown for the detection of *Aspergillus* DNA.[21,24] However, the diversity of fungi responsible for IFI and the wide range of methods in used led to controversial results using molecular tools in early screening of infected patients.[25]

In this context, the first objective of our study was to design and evaluate a new panfungal quantitative PCR (PF-qPCR) assay in three reference laboratories, and to define its analytical sensitivity and specificity. Our second goal was to evaluate the performances of this assay as long as other diagnostic tools in the framework of a multicenter clinical trial enrolling patients at high risk for IFI who received early antifungal treatment. The final objective was to identify the best strategy that would allow to reevaluate antifungal prescriptions and to stop potentially unnecessary treatment.

Methods and patients

Panfungal quantitative PCR assay (PF-qPCR)

Evaluation of a DNA extraction method

Various DNA extraction protocols were tested in the 3 laboratories involved, using serial dilutions of artificially infected blood samples. Aliquots of 1 ml of whole blood from healthy individuals, collected on sample devices using potassium EDTA as anticoagulant, were spiked under sterile conditions with various inoculums of *Aspergillus fumigatus* (Longbottom strain), *Candida albicans* (IP4872 = ATCC10231) or *Cryptococcus neoformans* (#CNR87213) achieving $0/1/10^1/10^2/10^3/10^4/10^5$ conidia/ml. At first step, DNA from 1 ml of each sample using MagNa Pure Compact device (Roche Diagnostics) was extracted without previous mechanical lysis. The poor sensitivity obtained conducted us to further insert a lysis of conidia.

Thus, a volume of 500 μl of each blood sample was incubated with 500 μl of lysis buffer (MagNA Pure Bacteria Lysis Buffer, Roche Diagnostics) in tubes containing ceramic microbeads (MagNA Lyser Green Beads, Roche Diagnostics), then crushed in a Magna Lyser apparatus (Roche Diagnostics). The fungal DNA extraction efficiency was compared among 3 different volumes of lysate supernatant i.e. 400 μl, 700 μl and 1 ml. They were recovered and extracted using MagNa Pure Compact device and Magna Pure Compact Nucleic Acid Isolation Kit I-Small volume (400 μl and 700 μl) or Magna Pure Compact Nucleic Acid Isolation Kit I-Large Volume (1 ml) (Roche Diagnostics). The samples used by both kits were extracted by external lysis protocol and an elution volume of 100 μl, according to the manufacturer's instructions.

During each series of extraction, a negative control (whole blood without fungal DNA) was tested.

Real-time amplification method

Serial dilutions of the artificially infected blood samples were extracted and DNA were amplified using homemade PF-qPCR; Primers were designed after alignment of the nucleotide sequences of ITS2 region of multiple yeasts and filamentous fungal rDNA as published previously: MITS-2A