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Original article

Early diagnosis and monitoring of mucormycosis by detection of circulating DNA in serum: retrospective analysis of 44 cases collected through the French Surveillance Network of Invasive Fungal Infections (RESSIF)

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ABSTRACT

The main objective of this study was to assess the diagnostic performance of a set of three *Mucorales* quantitative PCR assays in a retrospective multicentre study. Mucormycosis cases were recorded thanks to the French prospective surveillance programme (RESSIF network). The day of sampling of the first histological or mycological positive specimen was defined as day 0 (D0). Detection of circulating DNA was performed on frozen serum samples collected from D−30 to D30, using quantitative PCR assays targeting *Rhizomucor*, *Lichtheimia*, *Mucor/Rhizopus*. Forty-four patients diagnosed with probable ($n = 19$) or proven ($n = 25$) mucormycosis were included. Thirty-six of the 44 patients (81%) had at least one PCR-positive serum. The first PCR-positive sample was observed 9 days (range 0–28 days) before diagnosis was made using mycological criteria and at least 2 days (range 0–24 days) before imaging. The identifications provided with the quantitative PCR assays were all concordant with culture and/or PCR-based identification of the causal species. Survival rate at D84 was significantly higher for patients with an initially positive PCR that became negative after treatment initiation than for patients whose PCR remained positive (48% and 4%, respectively; $p < 10^{-6}$). The median time for

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complete negativity of PCR was 7 days (range 3–19 days) after initiation of L-AmB treatment. Despite some limitations due to the retrospective design of the study, we showed that *Mucorales* quantitative PCR could not only confirm the mucormycosis diagnosis when other mycological arguments were present but could also anticipate this diagnosis. Quantification of DNA loads may also be a useful adjunct to treatment monitoring. **L. Millon, CMI 2016;22:810.e1–810.e8**

Introduction

Mucormycosis is associated with high mortality rates, especially in haematological patients (>50%) and remains difficult to diagnose [1–3]. The early distinction with invasive aspergillosis is of utmost importance because the antifungal treatment for each is different. Unfortunately, the underlying conditions of both infections are similar and the imaging findings are not specific enough to ascertain the diagnosis [4]. Moreover, detection of circulating antigens such as galactomannan and β-D-glucan provides no help for diagnosing mucormycosis, and cultures are often delayed or negative, preventing early management. Delayed directed antifungal treatment impacts the outcome of mucormycosis (i.e. initiating an amphotericin B-based therapy ≥6 days after diagnosis resulted in a twofold increase in mortality rate at 12 weeks after diagnosis) [5]. Other important benefits of early diagnosis are less extensive or disfiguring surgery and reduced suffering for rhinocerebral localizations [6].

Species identification is also of interest because *Mucorales* may have different susceptibility to azoles. Newer azoles (posaconazole, isavuconazole but not voriconazole) may be active on *Mucorales* and can be given for long-term oral maintenance therapy or in the case of intolerance to liposomal amphotericin B therapy [7,8]. The most common genera in invasive mucormycosis are *Rhizopus*, *Rhizomucor*, *Lichtheimia* and *Mucor*, accounting for 90% of all cases [1,2,9]. Other genera (*Cunninghamella*, *Apophysomyces*, *Saksenaea*, *Cokeromyces*, *Actinomucor* and *Syncephalastrum*) species are individually responsible for <1% to 5% of reported cases of mucormycosis [10].

We have already reported a quantitative PCR (qPCR) test targeting the most clinically relevant species of *Mucorales* for early diagnosis of mucormycosis in serum [11]. The test is based on a combination of three qPCR assays targeting 18S rDNA from *Mucor/Rhizopus*, *Lichtheimia* and *Rhizomucor*. We detected DNA in serum samples from nine of ten patients before (median 10 days; range 3–68 days) histopathology and/or culture. Recent case reports using the same qPCR assays confirmed the early detection, and suggested that fluctuation of DNA load can be used to predict disease progression [12,13].

The goal of the present study was to extend our qPCR results to include ten other centres of the French network for surveillance of invasive fungal infection (RESSIF, Institut Pasteur, Paris). We also aimed to assess the correlation between DNA load and treatment efficacy and outcome.

Patients and Methods

Clinical and biological data

A prospective surveillance programme (RESSIF for 'Réseau de Surveillance des Infections Fongiques Invasives') was implemented in January 2012 by the National Reference Centre for Invasive Mycoses and Antifungals (NRCMA, Institut Pasteur, Paris, France) with the participation of 25 tertiary-care teaching hospitals. All new mucormycosis cases were recorded by each local microbiologist. Each case was notified through a secured website using a standardized questionnaire and analysed by a local committee. Demographics, underlying conditions, diagnostic tools, date of hospitalization, first-line antifungal therapy and outcome at day 90 were recorded. Episodes were classified as probable or proven mucormycosis according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) definitions for invasive fungal diseases [18].

For each proven or probable case with positive culture, strains were sent to the NRCMA where they were identified according to a polyphasic approach combining morphological and molecular features [14]. The sequences for the whole ITS1-5.8S-ITS2 region were determined and similarity searching was achieved using both the online database MYCOBANK (www.mycobank.org) and the NRCMA database.

If the culture was negative while hyphae suggestive of *Mucorales* were seen in tissues, molecular identification on tissues was performed at the University Hospital of Besançon by PCR sequencing targeting 18S ribosomal DNA (18S rDNA), as described by Bialek et al. [15], and/or internal transcribed spacer [16]; for two patients from another centre (Saint Louis University Hospital—APHP, Paris), identification was performed on tissues using PCR electrospray-ionization mass spectrometry [17].

Microbiologists who had notified a case to the NRCMA were asked to send 1 mL of all available frozen serum samples from D−30 to D30 of the mycological diagnosis for each patient. They were also asked for the date of the first pulmonary CT scan showing lesions suggestive of invasive mould infection (condensation and/or nodules), or of the first sinus scan showing fungal infection.

Quantitative PCR analysis

The serum samples were sent frozen in dry ice to the University Hospital of Besançon where all the qPCR assays were performed as previously described [11], with a modification in the reverse primer of Muc assay (MucR1-A: 5′-CCT AGT TTG CCA TAG TTC TCT GCA G-3′). In short, DNA was extracted from 1 mL of serum, eluted in 50 μL, and 9 μL was used for each of the qPCR assays targeting *Rhizomucor* (assay name: Rmuc), *Lichtheimia* (assay name: Acory), *Mucor/Rhizopus* (assay name: Muc), respectively.

Quantitative results were expressed by determining the detection threshold, or quantification cycle (Cq) that marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Hence, the higher the Cq, the smaller the amount of DNA in the sample.

Statistical analysis

Survival was analysed using the Kaplan–Meier method, and the log-rank test was used to test for differences. Survival was