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BRIEF REPORT

Is It Time to Include CT "Reverse Halo Sign" and qPCR Targeting Mucorales in Serum to EORTC-MSG Criteria for the Diagnosis of Pulmonary Mucormycosis in Leukemia Patients?

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In 23 leukemia patients with proven (n = 17) or possible (n = 6) pulmonary mucormycosis (PM), the presence of reversed halo sign on computed tomography was strongly associated with the positivity of quantitative polymerase chain reaction assays targeting Mucorales in the serum, confirming the value of these two tools for the diagnosis of PM in this setting.

Keywords. early diagnosis; leukemia; pulmonary mucormycosis; quantitative polymerase chain reaction; reversed halo sign.

Recent studies have reported an increase in the incidence of mucormycosis [1, 2], especially in patients with hematological malignancies in whom mucormycosis ranks the third most common invasive fungal infection (IFI) after candidiasis and aspergillosis [3]. In addition, pulmonary mucormycosis (PM) is the most common clinical form, accounting for 22%–44% of all cases of mucormycosis in patients with hematological malignancies [4]. Considering (i) the incidence and the high rate of mortality in patients with PM [5], (ii) the difficulties in clinically and radiologically distinguishing PM from invasive pulmonary aspergillosis (IPA), (iii) the reduced susceptibility of zygomycetes to voriconazole that is frequently used as a first-line treatment for invasive molds infections, and (iv) the lack of sensibility of noninvasive techniques for conventional diagnosis (ie, culture, direct examination), new tools were needed for the early and specific diagnosis of PM aimed at distinguishing PM from IPA to initiate appropriate therapy and improve the outcome of patients suffering from PM.

Recently, two publications provided attractive tools that could be useful in the specific diagnosis of PM in at-risk patients. First, a study by our team concluded that the reversed halo sign (RHS) on computed tomography (CT) scan was strongly associated with PM in the particular setting of neutropenic leukemia patients with pulmonary infections [6]. Second, Millon et al reported the usefulness of the screening of at-risk patients using specific quantitative polymerase chain reaction (qPCR) targeting circulating DNA of Mucorales in the serum for the early diagnosis of invasive mucormycosis [7]. The aim of the present study was to investigate the usefulness of combining CT scan and qPCR screening for the early diagnosis of PM in a retrospective series of neutropenic leukemia patients with proven or possible PM.

All of the patients included in this study were selected as previously described [6]. Briefly, leukemia patients with prolonged neutropenia (>10 days) who were treated for proven or possible PM between 2004 and 2014 at the Clinical Hematology Department, University Hospital of Dijon, France, based on the presence of an RHS on CT were retrospectively enrolled. The occurrence of fever, cough, chest pain, or hemoptysis triggered a CT scan. Day 0 (D0) of proven or possible PM was defined as the day with the first evidence of the RHS on CT performed after the onset of clinical symptoms. The RHS was considered to be present on the CT if a focal ground-glass opacity surrounded by a solid ring of consolidation was observed as previously described [6, 8].

For each of the patients, proven or possible PM was defined according to the revised criteria of the EORTC/MSG [9]. Proven PM was established based on positive mycological data pathological examination or molecular methods using PCR and direct sequencing on tissue biopsy or culture [10].

For each of the patients included, sequential sera prospectively collected between D − 9 and D + 9 of the diagnosis of PM and stored at −20°C were retrospectively tested for the detection of circulating DNA from the most common species of Mucorales using three qPCR assays targeting *Mucor*/*Rhizopus*, *Lichtheimia*, and *Rhizomucor* species as previously described [7]. Briefly, 1 mL of each of the sera tested was used for automatic DNA extraction using protocol specific B on a Nuclisens EasyMAG device (BioMérieux, Marcy l'Etoile, France). Elution was performed in a 50-µL volume. Detection of fungal DNA was performed on a Roche LC480 II (Roche Diagnostics, Meylan,

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France) with 9 µL of extract, using each of the 3 qPCR assays as described elsewhere [7]. 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. According to Millon et al [7], qPCR results were considered positive or negative when Cq ≤ 44 cycles or Cq ≤ >46 cycles/no signal was detected, respectively. When 44 < Cq < 46 cycles, results were considered equivocal.

Twenty-three patients with acute leukemia were enrolled in this study, among which 13 of them were previously reported [6]. All of them were first diagnosed as putative PM since presenting an RHS on CT (Table 1) that subsequently triggered a specific antifungal therapy to zygomycosis. Sixteen patients (group 1) had proven PM, and the 7 additional patients (group 2), although considered as putative PM patients based on the presence of an RHS on CT, were classified as "possible PM with CT RHS" (Table 1). All of the qPCR data obtained from sera for each of the patients are described in Supplementary Table 1.

Between D − 9 and D + 9, a median number of 8 [3–10] sera per patient were tested for the detection of circulating DNA of Mucorales using the 3 qPCR assays as previously described [7]. Overall, 21 of 23 patients (91%) had at least 1 positive qPCR on serum (15/16 and 6/7 in group 1 and group 2, respectively). Among the 175 performed tests, 88 (50%) were positive without any significant difference between the 2 groups (63/124 in

Table 1. Characteristics of 23 Leukemia Patients With Pulmonary Invasive Fungal Infection

Clinical, Radiological and Mycological Characteristics of the Patients	Proven PM (n = 16)	Possible PM With RHS on CT (n = 7)	All Patients (n = 23)
Clinical features before pulmonary IFI diagnosis			
Median age, y (range)	58 (32–75)	64 (54–75)	61 (32–75)
Sex ratio, M/F	2.2	1.33	1.88
No. of AML and ALL patients	10 and 6	7 and 0	17 and 6
Fever >38.5°C	14 (88%)	5 (71%)	19 (83%)
Cough	6 (37%)	2 (29%)	8 (35%)
Thoracic pain	13 (81%)	5 (71%)	18 (78%)
Hemoptysis, including hemoptoic sputum	5 (31%)	2 (29%)	7 (30%)
Patients with neutropenia at time of IFI diagnosis	16 (100%)	6 (86%)	22 (96%)
Median No. of days of neutropenia before IFI diagnosis, (range)	14 (5–100)	10 (0–70)	13 (0–100)
No. of days between 1st clinical signs and IFI diagnosis (range)	1 (0–4)	1 (0–3)	1 (0–4)
Initial thoracic CT scan findings			
Solitary lesion	15 (94%)	6 (86%)	21 (91%)
Presence of RHS	16 (100%)	7 (100%)	23 (100%)
Mycological and pathological data associated with IFI			
Positive culture of BAL for Mucorales strain	3/15	0/3	3/18
Positive tissue biopsy (pathological exam/culture/PCR)	17/17[a]	0/1	17/18
Positive CT-guided transthoracic needle biopsy	6/6	0/1	6/7
Positive surgical pulmonary resection	8/8	ND	8/8
Positive cutaneous biopsy	2/2	ND	2/2
Positive culture of pleural puncture	1/1	ND	1/1
Detection of circulating Mucorales DNA by qPCR on serum	16	7	23
Median no. of tests per patient (range)	8 (4–10)	8 (3–10)	8 (3–10)
No. of patients with at least 1 positive test	15 (94%)	6 (86%)	21 (91%)
Between D − 9 and D0 since IFI diagnosis	13/13 (100%)	6/6 (100%)	19/19 (100%)
Between D + 1 and D + 9 since IFI diagnosis	11/16 (69%)	3/6 (50%)	14/22 (64%)
Median no. of positive tests per patient (range)	4 (0–8)	5 (0–8)	4 (0–8)
No. of days between 1st positive PCR and IFI diagnosis (range)	−4 (−7, 1)	−4 (−7, 0)	−4 (−7, 1)
Isolated or detected strains of Mucorales (culture or PCR)			
Rhizomucor sp.	11	4	15
Lichtheimia sp.	4	1	5
Rhizopus sp.	1	1	2
Hematological response and outcome			
Complete hematological response after IFI	9 (57%)	4 (57%)	13 (57%)
Median No. of days of neutropenia after IFI, (range)	8.5 (2–38)	4 (0–65)	7.5 (0–65)
Survival at 3 mo after IFI	13 (81%)	3 (43%)	16 (70%)

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CT, computed tomography; IFI, invasive fungal infection; ND, not done; PCR, polymerase chain reaction; PM, pulmonary mucormycosis; RHS, reversed halo sign.

[a] One patient had 2 biopsy procedures.

group 1 vs 25/51 in group 2). For the positive patients, the first positive qPCR was detected at a median time of 4 days (ranging from D − 7 to D + 1) before D0 (Table 1) confirming the sensitivity of the qPCR assays, which were positive earlier than RHS on CT in most of the patients.

Interestingly, before diagnosis of the pulmonary IFI (between D − 9 and D0), 19 of the 23 patients were tested, and all of them had at least 1 positive qPCR on serum. Indeed, 13 of 13 patients with proven PM had positive tests (33/60 positive sera), confirming that the qPCR assays are a valuable tool in the monitoring and the early diagnosis of PM in patients at risk of IFI, as recently reported by Millon et al [11]. Identically, in the same period, 6 of 6 patients with possible PM with CT RHS (group 2) had positive qPCR (19/27 positive sera).

Conversely, after the diagnosis of IFI (D + 1 to D + 9), 22 of the 23 patients were tested, and only 14 of 22 (64%) had positive results (11/16 patients in group 1 and 3/6 patients in group 2), and in these patients 36 of 88 tested sera (41%) were positive (30/64 sera in group 1 and 6/24 sera in group 2). Because all of the patients received active antifungal therapy (Ambisome alone or in combination with Posaconazole in 20 cases; Amphotericin B deoxycholate combined with Posaconazole in 3 cases) as soon as D0, it is possible that the initiation of antifungal treatment could have modified the kinetics of positivity of the qPCR assays.

The molecular identification of the causal agent at the genus level from the serum of patients is of great interest since specifying the epidemiology of mucormycosis, especially in the setting of neutropenic leukemia patients for whom (i) the frequent use of empiric antifungal therapies may suppress the growth of the causal agent from BAL or biopsy samples and (ii) realization of lung biopsies is often limited. Indeed, *Lichteimia* sp. is considered the most common genus responsible for PM in France [12] and Europe [4] in the overall population of patients at risk of mucormycosis. In our series of consecutive leukemia patients, *Rhizomucor* sp. was the most common genus, identified in 68% of the patients (15/22), whereas *Lichteimia* sp. was the causal agent in only 23% of our patients (5/22) (Table 1). These observations point out the usefulness of such molecular tools in highlighting the particular epidemiology of PM in specific populations of patients. Additional prospective studies targeting larger cohorts of specific populations of patients aimed at specifying the epidemiology of PM will be necessary.

In conclusion, in the particular setting of leukemia patients, our retrospective study shows that all of the patients with proven PM (i) displayed the presence of RHS on CT and (ii) experienced positive qPCR targeting Mucorales when qPCR tests were performed before the first CT showing RHS. In addition, in the setting of suspected (possible) PM with presence of RHS on CT, most of the patients also had positive qPCR on serum (reaching 100% of cases when tests were done before the first CT showing RHS). Consequently, in leukemia patients, these findings enhance the value of CT RHS for PM diagnosis.

Finally, prospective studies including larger cohorts of patients will help to (i) confirm the value of RHS on CT combined with qPCR targeting Mucorales in the early diagnosis of PM and (ii) specify the prognosis value of the qPCR assays in monitoring the outcome of patients diagnosed and treated for PM.

Supplementary Data

Supplementary material is available online at *Open Forum Infectious* Diseases online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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