Open Forum Infectious Diseases

BRIEF REPORT

Exhaled Breath Condensate Surveillance for *Aspergillus* in Acute Leukemia—a Pilot Trial

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Invasive fungal infections in patients with leukemia carry a high mortality rate, but early diagnosis has the potential to modify this natural history. A novel screening method using *Aspergillus* droplet-digital polymerase chain reaction in exhaled breath condensate may have a similar performance to serum galactomannan screening. Larger studies, including other molds, are necessary.

Keywords. acute leukemia; droplet-digital PCR; exhaled breath condensate; invasive pulmonary aspergillosis; invasive mold infections.

Invasive fungal infections, most commonly *Aspergillus*, are associated with a high mortality rate in patients undergoing intensive chemotherapy for acute leukemia [1, 2]. This has prompted many centers to implement routine antimold prophylaxis for patients with acute myeloid leukemia (AML) undergoing intensive chemotherapy [2, 3]. Unfortunately, permissive use of antifungals leads to resistance [4], severe toxicity [5], drug–drug interactions [6], and increased costs.

Current diagnostic methods are suboptimal. Studies assessing blood galactomannan (GM) or polymerase chain reaction (PCR)-based screening in high-risk patients have shown imperfect results [7, 8]. A superior diagnostic method, allowing for early diagnosis of invasive aspergillosis, can potentially change the natural history of this disease.

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Few studies evaluated exhaled breath condensate (EBC) in fungal diagnostics with variable success [9, 10], though these were not based on fungal PCR. We aimed to assess the diagnostic accuracy of twice-weekly screening with *Aspergillus* PCR in EBC for the development of invasive pulmonary aspergillosis (IPA) and compare it to that of serum GM monitoring.

METHODS

Study Design

We conducted a prospective observational pilot study from October 2021 to December 2022. Adult inpatients with AML/lymphoblastic leukemia (ALL) undergoing high-intensity chemotherapy (ie, with expected prolonged [>7 day] and profound [$<0.5 \times 10^9$/L] neutropenia), and who could provide EBC were eligible. We excluded patients with acute promyelocytic leukemia; patients receiving amphotericin B or mold-active azoles as treatment or prophylaxis; and patients in the intensive care unit. The study was approved by our institutional Ethics Board (REB#21-5321), and all participants provided informed consent.

Antifungal Prophylaxis

Routine antifungal prophylaxis for patients with acute leukemia receiving intensive chemotherapy in our center consists of fluconazole. However, patients who receive novel targeted therapies (ie, venetoclax, midostaurin, gilteritinib) or ALL protocols (which include vincristine), are prescribed an echinocandin because of drug–drug interactions with azoles.

Study Procedures

EBC was collected using Rtube (Respiratory Research, Austin, TX), a portable and disposable device with a 1-way valve to collect exhaled air; the condensate was stored at −80 °C. The procedure is risk-free, has no major contraindications, and does not require highly trained personnel. EBC was collected at enrollment and then twice weekly if the patient was neutropenic ($\leq 0.5 \times 10^9$/L), not receiving a mold-active azole or amphotericin B, and hospitalized.

Twice-weekly serum GM testing was done as part of the standard of care in our center but compliance was not enforced. GM was tested for using an immune-enzymatic sandwich microplate assay (Platelia, Bio-Rad, Hercules, CA). Cutoff for positivity was 0.5.

Detecting *Aspergillus* DNA in EBC by Droplet Digital PCR

Fungal DNA was extracted from EBC using the Fungi/Yeast Genomic DNA Isolation Kit© (Norgen Biotek Corp, Thorold,

Ontario, Canada). A set of pan-*Aspergillus* primers and probes capable of detecting the 18 s rDNA of the full spectrum of *Aspergillus* pathogens and a set of *Aspergillus fumigatus*–specific primers and probes targeting only the *A. fumigatus benA* gene were used [11]. The droplet digital PCR (ddPCR) reactions were performed in the Bio-Rad QX200-system (Bio-Rad). After droplet generation, the PCR was performed in the Bio-Rad C1000-thermocycler (Bio-Rad). Fluorescence of droplets was measured with the QX200-Droplet-Reader (Bio-Rad), and results were analyzed using Bio-Rad QuantaSoft software (Bio-Rad). Each sample was run in duplicate with a positive control, DNA from *A. fumigatus* strain NRRL-163 (ATCC, Manassas, VA, USA), and a negative control, nuclease-free water. Wells containing >10 000 droplets were accepted, and results were reported in copies/20 µL. The limit of detection was calculated using serial dilutions of stock *Aspergillus* DNA isolated from human airways and set at 6.5 copies/reaction and 4.95 copies/reaction for *Aspergillus* spp. (pan) and *A. fumigatus* (*benA*), respectively.

Outcomes and Definitions

The primary outcome was the diagnostic characteristics (sensitivity, specificity, positive/negative predictive value and positive/negative likelihood ratio) of surveillance testing using *Aspergillus* ddPCR in EBC compared to serum GM for diagnosing probable/proven IPA during the index hospitalization. IPA was defined using the European Organization for Research and Treatment/Mycoses Study Group criteria [12] and diagnosis was established based on prospective chart review.

Statistical Analysis

The sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio were calculated for EBC *Aspergillus* ddPCR and serum GM per patient and per sample. For the per-patient analysis of EBC *Aspergillus* ddPCR, we conducted 2 separate analyses: 1 where a single positive PCR result (either *benA* or pan-*Aspergillus* genes) was used as a threshold for a positive result, and another where 2 consecutively positive PCR results

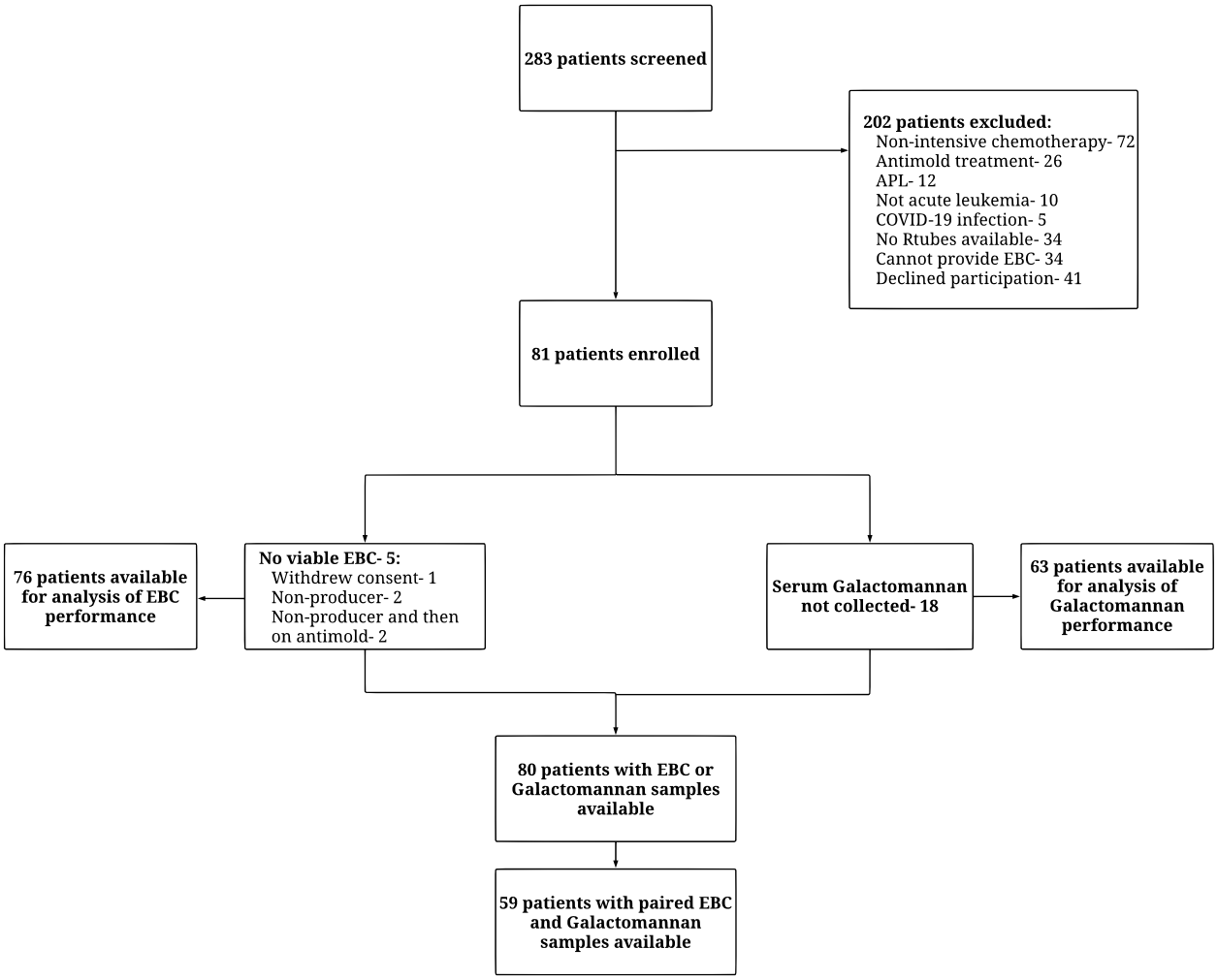

Figure 1. Patient enrollment flow chart.

Table 1. Performance of *Aspergillus* Droplet Digital PCR in EBC and Serum Galactomannan for Diagnosis of Invasive Pulmonary Aspergillosis

A

	Aspergillus ddPCR in EBC Per patient, Threshold 1[a]		Aspergillus ddPCR in EBC Per patient, Threshold 2[b]		Aspergillus ddPCR in EBC Per sample		Serum GM Per patient		Serum GM Per sample	
		95% CI		95% CI		95% CI		95% CI		95% CI
Sensitivity	50%	3.8%–96.2%	50%	3.8%–96.2%	37.5%	11%–71%	50%	3.8%–96.2%	16.7%	1%–55.4%
Specificity	73%	62.2%–82.2%	90.5%	82.5%–95.8%	92.5%	89.5%–94.8%	96.7%	90.2%–99.4%	98.4%	96.8%–99.3%
Positive predictive value	4.8%	.3%–19.3%	12.5%	.8%–44.5%	9.4%	2.4%–22.5%	33.3%	2.3%–83.9%	12.5%	.8%–44.5%
Negative predictive value	98.2%	92.2%–99.9%	98.5%	93.7%–99.9%	98.6%	97%–99.5%	98.3%	92.9%–99.9%	98.8%	97.5%–99.6%
Positive likelihood ratio	1.9	.4–7.8	5.3	1.1–25	5	1.9–13	15.3	2.2–106.5	10.1	1.5–70.2
Negative likelihood ratio	0.7	.2–2.8	0.6	.1–2.2	0.7	.4–1.2	0.5	.1–2.1	0.9	.6–1.2

B

	Aspergillus ddPCR in EBC Per patient, Threshold 1[a]		*Aspergillus* ddPCR in EBC Per patient, Threshold 2[b]		Aspergillus ddPCR in EBC Per sample		Serum GM Per patient		Serum GM Per sample	
		95% CI		95% CI		95% CI		95% CI		95% CI
Sensitivity	50%	3.8%–96.2%	50%	3.8%–96.2%	37.5%	11%–71%	50%	3.8%–96.2%	16.7%	1%–55.4%
Specificity	70.2%	57.6%–81%	89.5%	79.8%–95.7%	91.7%	88.2%–94.4%	96.5%	89.6%–99.4%	98.2%	96.6%–99.2%
Positive predictive value	5.6%	.3%–22.3%	14.3%	.9%–49.4%	10.7%	2.8%–25.5%	33.3%	2.3%–83.9%	12.5%	.8%–44.5%
Negative predictive value	97.6%	89.7%–99.9%	98.1%	91.8%–99.9%	98.2%	96.2%–99.4%	98.2%	92.4%–99.9%	98.7%	97.3%–99.5%
Positive likelihood ratio	1.7	.4–7.1	4.8	1–23	4.5	1.7–11.9	14.3	2–99.4	9.4	1.4–65.1
Negative likelihood ratio	0.7	.2–2.9	0.6	.1–2.2	0.7	.4–1.2	0.5	.1–2.1	0.9	.6–1.2

A. Including all available samples. B. Including only patients with paired EBC-GM samples.

Abbreviations: ddPCR, droplet digital polymerase chain reaction; EBC, exhaled breath condensate; GM, galactomannan.

[a]Using 1 positive sample as the threshold for a positive result.

[b]Using 2 consecutively positive samples as the threshold for a positive result.

(from samples obtained on different days, either *benA* or pan-*Aspergillus* genes) were required to define a positive result. For serum GM, we used a single positive sample as the threshold for a positive result. Performance indices and confidence intervals were calculated using a generalized linear model in SPSS software version 28 (IBM Corp, Armonk, NY).

RESULTS

Eighty-one patients were enrolled and 80 were included in the analyses (Figure 1). Median age was 59 years (interquartile range [IQR] 44–64) and 46.3% (37/80) were female. Most patients had AML (75%, 60/80) and cytarabine and daunorubicin (7 + 3) was the most common chemotherapy regimen (37.5%, 30/80). Median duration of neutropenia was 31.5 days (IQR 21–42.8). Echinocandin prophylaxis was given to 58.8% (47/80) of patients for a median duration of 16 days (IQR 7–22).

Seventy-six patients (95%) had 393 EBC samples available for analysis with a median of 5 samples per patient (IQR 3–7). Sixty-three patients (78.8%) had 444 serum GM tests done with a median of 6 samples (IQR 4–10) per patient. Three patients (3.8%) were diagnosed with probable/proven invasive mold infections: 1 probable fusariosis and 2 probable IPAs. *Aspergillus* ddPCR in EBC correctly identified 1 of 2 patients with probable IPA. The true-positive result was obtained from a patient with febrile neutropenia, pulmonary nodules with surrounding ground-glass opacities on chest computed tomography, and serum GM of 1.25. In fact, ddPCR from EBC was positive 10 days before the diagnosis of IPA. The falsely negative result was seen in a patient with febrile neutropenia, consolidation with adjacent ground-glass opacity, and GM of 1.94 in bronchoalveolar lavage. The diagnostic characteristics of EBC *Aspergillus* ddPCR and serum GM for the diagnosis of IPA are detailed in Table 1. Sensitivity and specificity, analyzed per patient, were 50% (3.8%–96.2%) and 90.5% (82.5%–95.8%) for EBC *Aspergillus* ddPCR (using 2 consecutively positive samples to define a positive result) and 50% (3.8%–96.2%) and 96.7% (90.2%–99.4%) for serum GM, respectively. Comparable results were seen when analyzing per sample. When including only 59 patients who had paired EBC/GM samples, results were similar (Table 1). A detailed description of the clinical characteristics of patients with a falsely positive *Aspergillus* ddPCR in EBC is provided in Supplementary Table 1.

DISCUSSION

We describe a novel method for screening high-risk patients with hematological malignancies for IPA. Our test demonstrated high specificity, similar to serum GM screening, which suggests that a positive test can be used to rule in IPA.

Although the utility of GM is limited to *Aspergillus* infections, EBC ddPCR is noninvasive and has the potential to diagnose any invasive fungal infection with a simple modification of the primers used. This offers a significant advantage because studies have reported delayed diagnosis of non-*Aspergillus* mold infections when implementing serum GM screening [7]. In addition, there may be overestimation of the performance of serum GM because of incorporation bias resulting from serum GM being part of the diagnostic criteria for invasive aspergillosis, which means that EBC *Aspergillus* ddPCR may actually perform better than serum GM.

There are several limitations to our study. The small sample size and low event rate lead to unreliable estimates and very wide CIs. Therefore, further studies in larger cohorts are needed. The inclusion of patients on echinocandin prophylaxis may have decreased the diagnostic performance of our study test and analyzing only patients who did not receive echinocandin prophylaxis was impossible in our small cohort. We also included patients with ALL who are usually considered at lower risk for invasive mold infection compared to AML; this may have contributed to the low event rate. Last, we could not assess in this small cohort the time to test positivity to see whether 1 of the tests offers an advantage. Although in the single case of IPA in which we had both positive EBC and GM, EBC *Aspergillus* ddPCR was positive 10 days before serum GM.

In summary, screening high-risk patients with EBC *Aspergillus* ddPCR had similar diagnostic accuracy to serum GM in our small cohort. Further larger studies are needed to assess the performance of EBC ddPCR for screening for IPA and other mold infections.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. Concept and design: R.B., S.H. Data collection: R.B. Microbiology sample processing and testing: R.B., S.A., C.F.N., L.W., S.Z.A., T.S. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: R.B., S.H. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: R.B., M.L.L.

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Potential conflicts of interest. R.B. and S.H. report receiving the 2021 Princess Margaret Cancer Centre Catalyst Grant in support of this study. V.G. reports receiving grant support from AbbVie and Novartis, consulting fees from Novartis, BMS Celgene, Daichii Sankyo, AbbVie, GSK, and Pfizer, honoraria from Novartis, BMS Celgene, AbbVie, and GSK, and participation in DSMB/advisory board for BMS Celgene, Incyte, and GSK, all not directly related to this study. All other authors report no potential conflicts.

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