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Bronchoalveolar Lavage Fluid Cytology in Culture-Documented Invasive Pulmonary Aspergillosis in Patients with Hematologic Diseases: Analysis of 67 Episodes

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ABSTRACT There is a paucity of studies on the yield of Gomori-methenamine-silver (GMS) staining in bronchoalveolar lavage (BAL) fluid cytology and its comparison with fluorescent dye staining for the diagnosis of invasive pulmonary aspergillosis (IPA) in patients with hematologic malignancies. To that end, we analyzed the yield of direct fungal visualization in BAL fluid cytology with GMS staining, in a series of culture-positive IPA cases in 67 patients with hematologic malignancies, and we compared the results with those of direct examination with calcofluor white staining and BAL fluid galactomannan assays, when available. GMS staining in BAL fluid cytology was positive in 42% of the 67 cases and revealed coinfections in 7 cases. In contrast, only 2/67 (3.6%) BAL fluid samples were positive in direct smears stained with the fluorescent dye calcofluor white. Positive GMS staining results were significantly more frequent in IPA cases with cavitary lesions and IPA cases caused by >1 *Aspergillus* species, but the proportions of positive cytology results among *Aspergillus* species were not different.

KEYWORDS GMS, invasive aspergillosis, hematologic malignancy

The diagnosis of invasive pulmonary aspergillosis (IPA) remains suboptimal, despite improvements in radiology and the development of methods to identify biomarkers, such as *Aspergillus* galactomannan, in serum and bronchoalveolar lavage (BAL) fluid. Bronchoscopy with BAL remains a key procedure for the diagnosis of IPA (1, 2). Specifically, guidelines recommend microscopic examination, including the identification of fungal elements in fresh unconcentrated specimens, such as BAL fluid, by the application of fluorescent dyes (e.g., calcofluor white) (1). BAL fluid cytology with Gomori-methenamine-silver (GMS) staining, performed with highly concentrated specimens, allows direct visualization of fungal elements within hours (3). There is a paucity of studies on the yield of GMS staining in BAL fluid cytology and comparison with fluorescent dyes for the diagnosis of IPA in patients with hematologic malignancies. To that end, we analyzed the yield of direct fungal visualization in BAL fluid cytology with GMS staining in a series of culture-positive IPA cases involving patients with hematologic malignancies, and we compared the results with those of direct examination with calcofluor white and BAL fluid galactomannan assays, when available.

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MATERIALS AND METHODS

Cases studied. We retrospectively analyzed all consecutive cases (between September 1999 and March 2015) of culture-documented IPA (proven or probable) in adult patients with hematologic malignancies at M. D. Anderson Cancer Center, a tertiary-care cancer center in Houston, Texas. All cases had concurrently available results for BAL fluid cytology with GMS staining. Using a standardized case report form, we collected information regarding clinical, histopathologic, and radiologic findings, microbiologic results, treatments, and outcomes (crude mortality rates 42 days following the IPA diagnosis). The study was approved by the M. D. Anderson Cancer Center institutional review board.

Definitions. We followed the European Organization for Research and Treatment of Cancer (EORTC)-Mycoses Study Group (MSG) criteria for the classification of invasive aspergillosis (proven or probable) (4). Cytology results were considered positive when fungal elements consistent with *Aspergillus* (5) were observed. A history of recent neutropenia (absolute neutrophil counts of $<$500 neutrophils/ml) for $>$10 days was defined if it occurred within the 30 days prior to the IPA diagnosis. The use of corticosteroids ($\geq$0.3 mg/kg/day of prednisone equivalent) for $>$3 weeks prior to the IPA diagnosis was considered prolonged use of corticosteroids. Prior immunosuppressant use was defined as the receipt of calcineurin inhibitors, tumor necrosis factor alpha (TNF-α) blockers, alemtuzumab or other cytotoxic monoclonal antibodies, cytarabine, or fludarabine within 90 days prior to the IPA diagnosis. Prior azole exposure was defined as the use of systemically administered azoles during the 12 weeks preceding the BAL fluid culture. *Aspergillus* galactomannan was measured by using a Platelia *Aspergillus* enzyme immunoassay test kit (Bio-Rad, Hercules, CA), in accordance with the manufacturer's instructions. Serum samples that had an optical density index of $\geq$0.5 were considered positive and underwent repeat testing to ensure positive results. BAL fluid samples that had an optical density index of $\geq$1 were considered positive.

Bronchoscopy was performed using a diagnostic video bronchoscope (Olympus, Tokyo, Japan). After intravenous administration of sedation agents and routine inspection of the tracheobronchial tree, the bronchoscope was wedged into a segmental or subsegmental bronchus leading to an area of radiographic concern regarding infection. Aliquots of 20 ml of saline were serially instilled and retrieved until sufficient BAL fluid was recovered for clinically indicated testing (6). Typically, 80 to 140 ml was instilled and 35 to 50 ml was recovered. The BAL fluid was divided, and 15-ml specimens were sent to the microbiology, chemistry/hematology, and cytopathology laboratories. In contrast to BAL, which is performed by wedging the bronchoscope into a single affected segment or subsegment of the lung (typically determined prior to the procedure, on the basis of radiographic appearance) to allow targeted sampling of the distal lung parenchyma in a lung region of interest, bronchial washing is performed by collectively suctioning existing secretions and retrieving instilled saline from the major airways without occlusive wedging into a single bronchus, thereby rendering bronchial washing samples less specific for an individual targeted lung region than BAL fluid samples. Bilateral bronchial washing samples were sent for microbiology at the bronchoscopist's discretion.

GMS staining was performed in the cytopathology laboratory, by centrifuging BAL fluid samples using a high-volume cytocentrifuge. GMS stains were prepared using 5 to 6 ml of BAL fluid per slide (7). Bronchial washing samples were infrequently used for GMS staining. When necessary, bronchial washing samples were first centrifuged in a 50-ml tube to create a cell block pellet. The cytopathology laboratory also routinely performed two modified Giemsa stains (Papanicolaou stain) and the Diff-Quik stain (Romanowsky stain).

Calcofluor white staining was performed in the microbiology laboratory. The 15-ml uncentrifuged BAL fluid specimen was first divided according to sample volume requirements for all ordered tests. Standard order sets included bacteriologic culture and Gram staining (1 ml), *Legionella* culture (1 ml), calcofluor white staining (150 μl), fungal culture (0.5 ml), *Aspergillus* galactomannan assay (1 ml), molecular respiratory panel (1 ml), *Pneumocystis jirovecii* molecular testing (1 ml), virologic culture (3 ml), and acid-fast bacillus (AFB) decontamination assay with culture and staining for AFB (6 ml). Calcofluor white staining samples were centrifuged, heat fixed, and directly stained (Alpha Tec Systems, Vancouver, WA).

Fungal culture specimens were planted on 2 Sabouraud dextrose Emmons agar plates, 1 Sabouraud dextrose agar tube, and 2 brain heart infusion agar tubes with chloramphenicol and gentamicin (Becton Dickinson Co., Sparks MD). Fungal cultures held for 28 days were evaluated according to standard criteria (8).

Data analysis. IPA cases with positive GMS staining results were compared with IPA cases with negative GMS staining results regarding baseline characteristics (underlying disease, clinical presentation, corticosteroid and immunosuppressant use, and azole exposure), *Aspergillus* galactomannan detection (in serum and BAL fluid), concurrent infections, and outcomes (42-day death). Quantitative variables were expressed as the mean $\pm$ standard deviation (SD) or as the median and interquartile range (IQR), as appropriate; qualitative variables were expressed as the number and percentage. Continuous variables were compared using the *t* test, and categorical variables were compared using the χ^2 test or the Fisher exact test, as appropriate. Stepwise logistic regression analysis was performed by including variables with *P* values of $<$0.05 in the univariate analyses. All statistical analyses were performed using IBM PASW Statistics for Windows v22.0 (SPSS Inc., Chicago, IL).

RESULTS

We identified 67 cases (proven in 2 cases and probable in 65 cases) among 66 patients (Table 1). The most common underlying disease was acute myeloblastic leukemia (39%). The majority of patients had active hematologic disease, and almost one-half had undergone prior stem cell transplantation (SCT). One-third had a history of prolonged corticosteroid use, and recent severe neutropenia was present in 22%.

TABLE 1 Characteristics of cases of IPA according to cytology results

Characteristic[a]	Total	Cytology positive	Cytology negative	*P*
No. (%) of patients	67	28 (41.8)	39 (58.0)	
Male (no. [%])	44 (65.7)	21 (75.0)	23 (59.0)	0.201
Age (mean ± SD) (yr)	55.7 ± 15.4	53.7 ± 14.6	57.2 ± 15.9	0.363
Race (no. [%])				0.174
White	51 (76.1)	21 (75.0)	30 (76.9)	
Nonwhite	16 (23.9)	7 (25.0)	9 (23.1)	
Hematological disease (no. [%])				0.898
Acute myeloblastic leukemia/myelodysplastic syndrome	28 (39.3)	13 46.4)	15 (38.5)	
Acute lymphoblastic leukemia	3 (4.5)	2 (7.1)	1 (2.6)	
Chronic myeloid leukemia	5 (7.5)	2 (7.1)	3 (7.7)	
Chronic lymphoid leukemia	9 (13.4)	4 (14.3)	5 (12.8)	
Multiple myeloma	5 (7.5)	2 (7.1)	3 (7.7)	
Lymphoma	17 (25.4)	5 (17.9)	12 (30.8)	
Status of malignancy (no. [%])				0.180
Active	42 (62.7)	14 (50)	28 (71.8)	
Remission	19 (28.4)	11 (39.3)	8 (20.5)	
Unknown	6 (8.9)	3 (10.7)	3 (7.7)	
SCT (no. [%])	31 (46.3)	18 (64.3)	13 (33)	0.015
SCT type				0.377
Autologous (no./total no. [%])	7/31 (22.6)	3 (16.7)	4 (30.8)	
MUD allogenic (no./total no. [%])	14/31 (45.2)	10 (55.6)	4 (30.8)	
MRD allogenic (no./total no. [%])	9/31 (29.0)	5 (27.8)	4 (30.8)	
Cord blood (no. [%])	1 (3.2)	0 (3.2)	1 (7.7)	
Comorbidity (no. [%])				
Diabetes mellitus	7 (10.4)	2 (7.1)	5 (12.8)	0.690
COPD or other lung disease	6 (8.9)	2 (7.1)	4 (10.3)	1 (NS)
ICU admission (no. [%])	8 (11.9)	2 (7.1)	6 (15.4)	0.419
Leukocyte count (mean ± SD) (leukocytes/ml)	210.7 ± 1,548.2	446.1 ± 2,303.7	16.9 ± 52.7	0.333
Neutrophil proportion (mean ± SD) (%)	69.6 ± 111.7	92.4 ± 158.6	49.9 ± 34.5	0.157
Lymphocyte proportion (mean ± SD) (%)	21.5 ± 26.1	17.1 ± 22.7	25.1 ± 28.5	0.255
Recent neutropenia (<500 neutrophils/ml for >10 days) (no. [%])	15 (22.4)	7 (25.0)	8 (20.5)	0.769
Serum *Aspergillus* galactomannan positive (no./total no. tested [%])	13/30 (43.3)	6 (66.7)	7 (33.3)	0.123
Prolonged use of corticosteroids (no. [%])	22 (32.8)	11 (39.3)	11 (28.2)	0.431
Prior azole exposure (no. [%])	30 (44.8)	21 (75.0)	16 (41.0)	0.007
Prior fluconazole treatment	28 (41.8)	15 (53.6)	13 (33.3)	0.133
Prior *Aspergillus*-active azole treatment	17 (25.4)	9 (32.1)	8 (20.5)	0.394
Chest CT imaging or X-ray findings (no. [%])				
Bilateral lesions	50 (74.6)	22 (78.6)	28 (71.8)	0.581
Central lesions	8 (11.9)	4 (14.3)	4 (10.3)	0.711
Cavitary lesions	11 (16.4)	9 (32.1)	2 (5.1)	0.006
BAL fluid analysis results				
Direct smear (calcofluor white staining) positive (no./total no. tested [%])	2/67 (2.9)	1 (3.6)	1 (2.6)	0.999
Proportion of macrophages with hemosiderin (mean ± SD [median [IQR]]) (%)	26.9 ± 33.5 (5 [0–50])	19.5 ± 28.4	32.1 ± 36.1	0.133
Aspergillus sp. (no. [%])				
A. fumigatus	34 (50.7)	16 (57.1)	18 (46.2)	0.460
A. flavus	6 (8.9)	3 (10.7)	3 (7.7)	0.688
A. terreus	17 (25.4)	4 (14.3)	13 (33.3)	0.094
A. niger	4 (6.0)	0 (0.0)	4 (10.3)	0.134
Other	2 (3.0)	1 (3.6)	1 (2.6)	0.999
Mixed	4 (6.0)	4 (14.3)	0 (0.0)	0.027
Galactomannan positive (no./total no. tested [%])	5/12 (41.7)	0 (0.0)	5 (45.5)	0.999

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TABLE 1 (Continued)

Characteristic[a]	Total	Cytology positive	Cytology negative	*P*
Culture results (no./total no. tested [%])				
BAL (aimed at lesion) fluid sample positive	53/64 (82.8)	26 (92.9)	27 (69.2)	0.007
Bronchial washing (bilateral) sample positive	27/39 (69.2)	9 (32.1)	18 (46.2)	0.456
Both BAL fluid and bronchial washing samples positive	15/36 (41.6)	9 (32.1)	6 (15.4)	0.006
Sampling of >1 lobe (no. [%])	12 (17)	6 (21.4)	6 (15.4)	0.374
Diagnosis of IPA (no. [%])				0.999
Proven	2 (3.0)	1 (3.6)	1 (2.6)	
Probable	65 (97)	27 (96.4)	38 (97.4)	
42-day death (no. [%])	19 (28.4)	9 (32.1)	10 (25.6)	0.354
Time from infection to death (mean ± SD [median [IQR]]) (days)	19 ± 12.9 (22 [5–28])	17.9 ± 16.4	20.2 ± 9.67	0.718

[a]MUD, matched unrelated donor; MRD, matched related donor; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; NS, not significant.

Prior use of *Aspergillus*-active antifungal agents was recorded in 25% of the cases. Serum galactomannan levels were available in 30 cases, and results were positive in 43%. Most patients (52 patients [78.7%]) underwent chest computed tomographic (CT) imaging within 1 week of the BAL procedure. In 14 cases, chest CT imaging data were not available and only chest X-ray findings were evaluated. Four patients underwent positron emission tomographic (PET) imaging in addition to CT imaging. Pulmonary involvement was most commonly (75%) bilateral, with cavitary lesions in 16% of cases, and the lesions were centrally located in at least 11% of cases.

Culture of BAL fluid obtained from the region of interest was performed in 64 cases (82.8% positive), culture of bilateral bronchial washing samples was performed in 39 cases (69.2% positive), and culture of both BAL fluid and bronchial washing samples was performed in 36 cases (41.6% both positive). Direct fungal visualization in BAL fluid cytology with GMS staining was positive in 28/67 cases (41.8%), in contrast to only 2/67 cases (3.6%) that were positive in direct smears with calcofluor white staining. Of note, 1 of the 2 cases with positive calcofluor white staining results had negative GMS staining results. Interestingly, BAL fluid cytology was diagnostic for coinfections in 7 cases, indicating 2 *Pneumocystis jirovecii* infections and 5 viral infections (cytopathic changes) (1 case had both). BAL fluid galactomannan assay results were available in only 12 cases (17.6%) and were positive in 41.7% (5/12 cases). Of note, GMS staining results were positive in 1 case with negative BAL fluid *Aspergillus* galactomannan assay results, and BAL fluid *Aspergillus* galactomannan assay results were positive in 5 cases with negative cytology results.

Comparisons between cases with positive and negative results for BAL fluid cytology with GMS staining showed that cases with cavitary lesions (9/28 cases [32.1%] versus 2/39 cases [5.1%]; $P = 0.006$), a history of SCT (18/28 cases [64.3%] versus 13/39 cases [33%]; $P = 0.015$), and IPA caused by >1 *Aspergillus* species (4/28 cases [14.3%] versus 0/39 cases [0%]; $P = 0.027$) more often had positive cytology results (Table 1). In addition, results for BAL fluid cytology with GMS staining were more often positive when the positive culture sample was from a BAL procedure aimed at the lesion, compared to bilateral bronchial washing (60.7% versus 7.1%; $P = 0.038$) or cases in which both cultures were positive (32.1% versus 15.4%; $P = 0.006$).

In contrast, there were no differences in the positivity rates of BAL fluid cytology with GMS staining according to the *Aspergillus* species causing IPA. Similarly, no other significant associations were found between cytology results and other variables; namely, no significant association was found between BAL fluid galactomannan assay results and cytology results or between serum galactomannan assay results and cytology results. Prior mold-active prophylaxis was not associated with the yield of cytology. In the multivariate analysis, only cavitary lesions were significantly associated with positive BAL fluid cytology (odds ratio, 6.21 [95% confidence interval, 1.04 to 37.11]; $P = 0.045$).

TABLE 2 Summary of studies of BAL fluid cytology findings for hematological patients with IPA

Reference	Year	No. of IPA cases	No. of BAL fluid samples	Cytology sensitivity (%)	Comments
Levy et al. (5)	1992	21 (16 proven)	343 from 300 patients	64	
Saito et al. (9)	1988	9	22	0	
Kahn et al. (3)	1986	17	82	53	Not all leukemia
Albelda et al. (10)	1984	16 (proven)	21 from 19 patients	43.75	

DISCUSSION

In the present study, we show that direct fungal visualization in BAL fluid cytology with GMS staining was positive in 42% of culture-documented IPA cases among patients with hematologic malignancies and it can be particularly useful in cases with cavitary lesions. This technique is fast and adds value, allowing the diagnosis of concurrent viral disease or pneumocystosis.

Information about ancillary techniques for the diagnosis of invasive aspergillosis, such as direct fungal staining of BAL fluid with GMS or calcofluor white, is scarce, with the latter being the recommended test for fresh specimens according to current guidelines (1). Previous series evaluating BAL fluid cytology with GMS staining for the diagnosis of IPA specifically in leukemia showed variable sensitivity between 0 and 63% (3, 5, 9, 10) (Table 2). Our data, which are similar to those of Levy et al. (5) and Albelda et al. (10), show a sensitivity of 41.8%, which is not good enough to exclude IPA but may contribute to establishing this diagnosis and may support the timely initiation of antifungal treatment. In addition, cytology with GMS staining typically allows for distinguishing between *Aspergillus*-like molds and Mucorales, which require different therapies, although definitive identification of *Aspergillus* cannot be made unless fruiting heads are seen (which is rare in direct human specimens), and specific diagnosis must be pursued. Regarding concomitant infections, GMS staining is more sensitive than Giemsa staining for identifying *Pneumocystis jirovecii*. In this context, the viral inclusions were identifiable only on the accompanying Giemsa stains.

GMS staining is considered a demanding and time-consuming technique and has been often replaced by fluorescence techniques such as the use of calcofluor white. In the present series, we found a notable disparity between calcofluor white direct smear staining and GMS staining of samples from the same bronchoscopy procedure. The low yield of filamentous fungal elements from calcofluor white stains versus GMS stains most likely results from the dramatically different sample sizes that are tested for fungal morphology. Only 150 μl of BAL fluid is used for calcofluor white staining, in contrast to the two 6-ml aliquots evaluated for fungal elements in cytopathology (an 80-fold difference in sample volumes). Traditionally, our microbiology department has emphasized organism recovery and identification by culture methods. The commitment to culture methods and the large battery of microbiologic tests ordered further affect the relative performance of calcofluor white and GMS stains in our practice setting. Our data suggest that high-volume cytocentrifugation of BAL fluid samples results in superior rates of identification of fungal elements and should always be performed, as recently recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines (1).

Previous studies did not show reliable associations between radiologic patterns and positive BAL fluid cytology results (3, 10). In the present study, however, we found that cases with cavitary lesions more often had positive results for cytology with GMS staining, probably as a result of direct communication with the bronchi accessible by the bronchoscope and the high *Aspergillus* burden in cavitary lesions. The availability of CT imaging findings in most of our cases might have provided an accurate evaluation of radiologic abnormalities, accounting for this result.

Among our cases, the best performance of cytology with GMS staining was obtained when the source of the positive culture was targeted BAL of the radiologic lesion, rather than bilateral washing. Other authors suggested that obtaining BAL fluid samples from more than one site could increase the yield of cytology with GMS staining (5). Finally,

it is unclear whether the yield of BAL fluid cytology for invasive aspergillosis is affected by previous antifungal treatment. We found that prior fluconazole or mold-active prophylaxis was not associated with cytology results.

Limitations of this study include its single-center, retrospective nature and the absence of a histologic gold standard. In current practice, it is difficult to obtain histologic confirmation, due to declining autopsy rates (11) and the infrequent performance of transbronchial or lung biopsies in this population, because of disease- and treatment-related thrombocytopenia (12). Because we included only culture-positive IPA cases, the rate of positivity might be an overestimation, as culture-positive cases likely have greater fungal burdens. Finally, because we did not include cases without invasive aspergillosis, we were not able to calculate the specificity, positive predictive value, or negative predictive value of the cytology. BAL fluid galactomannan assay results were available in only a few cases, which did not allow us to draw any conclusions. *Aspergillus* PCR was not performed at our center during the study period. In conclusion, our data support the usefulness of GMS staining for cytology of BAL fluid samples from patients with hematologic malignancies with IPA, as a widely available, rapid test that is complementary to other diagnostic tests and could also diagnose a variety of coinfections.

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