Gomori Methenamine Silver Stain on Bronchoalveolar Lavage Fluid Is Poorly Sensitive for Diagnosis of *Pneumocystis jiroveci* Pneumonia in HIV-Negative Immunocompromised Patients and May Lead to Missed or Delayed Diagnoses

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**Context.**—Direct visualization of *Pneumocystis jiroveci* organisms, using Gomori methenamine silver (GMS) staining in bronchoalveolar lavage fluid (BAL), is a historical gold standard that has been widely used for the diagnosis of *P jiroveci* pneumonia (PJP). However, the stain may be less sensitive in human immunodeficiency virus (HIV)—negative immunocompromised patients owing to a lower burden of organisms.

**Objectives.**—To assess the sensitivity of the GMS stain on BAL fluid for the diagnosis of PJP in HIV-negative immunocompromised patients as compared to HIV-positive patients.

**Design.**—We conducted a retrospective review from 2012 to 2018 to identify immunocompromised patients (≥18 years old) who underwent bronchoscopy with BAL GMS staining for the diagnosis of PJP. To assess for sensitivity, we sought to identify BAL GMS-positive cases and BAL GMS-negative cases of PJP. The BAL GMS-negative cases were categorized into proven and probable PJP.

**Results.**—We identified 45 adult immunocompromised patients with proven and probable PJP, including 24 HIV-negative (11 BAL GMS-positive and 13 BAL GMS-negative) and 21 HIV-positive cases (all were BAL GMS-positive). The sensitivity of BAL GMS for the diagnosis of PJP in HIV-negative immunocompromised patients was 11 of 24 (46%) versus 21 of 21 (100%) in HIV-positive patients (CD4: median, 10 cells/mL; range, 3–300 cells/mL). Delayed or missed diagnoses were seen in 3 cases of BAL GMS-negative PJP. Re-examination of BAL GMS slides showed rare *P jiroveci* cysts in 1 case.

**Conclusions.**—BAL GMS has poor sensitivity for PJP in HIV-negative immunocompromised patients. Using BAL GMS as a sole method for PJP may result in missed or delayed diagnoses in this population.

(Pneumocystis jiroveci* is a unicellular yeastlike fungus that is tropic to human lung tissue and produces a potentially fatal interstitial pneumonia in immunocompromised individuals. *Pneumocystis jiroveci* pneumonia (PJP) has historically affected patients with human immunodeficiency virus (HIV)/AIDS who are particularly susceptible owing to progressive and severe HIV-induced lymphopenia. In the 1980s, an estimated 75% of people living with AIDS developed PJP, but the introduction of combination antiretroviral therapy and the institution of routine effective PJP prophylaxis led to decreased rates of PJP in the HIV population. In the current era, PJP is increasingly seen in HIV-negative patients with cell-mediated immune deficiencies, including recipients of solid organ and hematopoietic stem cell transplants and those receiving immunosuppressive therapies for autoimmune conditions and cytotoxic chemotherapy for malignancy. Direct visualization of *P jiroveci* organisms in bronchoalveolar lavage fluid (BAL), using cell wall, nuclear, or immunofluorescent stains, has been the mainstay of diagnosis for PJP. The Gomori methenamine silver (GMS) stain in particular has historically been considered a gold standard method, with studies from the early HIV/AIDS era reporting sensitivities on BAL of 90% to 95%. Though only cyst forms of *P jiroveci* (and not the 10-fold more abundant trophozoites) are visualized by cell wall stains like GMS, this method benefits from an ease of readability over nuclear stains like Giemsa and remains an important diagnostic method for PJP in many medical centers across the United States. However, the burden of *P jiroveci* organisms in BAL fluid has been demonstrated to be approximately 15 times...
lower in HIV-negative immunocompromised patients than in HIV-positive patients with PJP,5 raising concerns that the BAL GMS stain is less sensitive in this population and may lead to missed cases when used as a stand-alone diagnostic method.

To better characterize the sensitivity of GMS staining on BAL for the diagnosis of \textit{P. jiroveci} in HIV-negative immunocompromised patients, we performed a retrospective review of PJP cases at our institution.

**MATERIALS AND METHODS**

**Study Design and Location**

We conducted a retrospective observational study by using electronic medical record chart reviews from 2012 to 2018 at Yale-New Haven Hospital (YNHH) in New Haven, Connecticut, a 1521-bed tertiary care center with a large population of immunocompromised individuals, with 200 solid-organ transplants (SOTs) (kidney, liver, heart, lung, and pancreas) and 150 hematopoietic cell transplants performed annually, and serving more than 9000 newly diagnosed cancer patients annually. BAL GMS staining is the primary method for diagnosis of PJP at YNHH.

**Study Population and Inclusion/Exclusion Criteria**

Electronic medical record charts of all patients who underwent bronchoscopy with BAL GMS staining were reviewed to identify immunocompromised adult patients (≥18 years of age) with a diagnosis of PJP. Patients, including those with proven or presumed PJP, who did not undergo GMS staining on BAL fluid were excluded from the study. Immunocompromising conditions were defined as those associated with cell-mediated dysfunction including HIV infection, SOT, hematopoietic stem cell transplant (HSCT), and receipt of any T-cell suppressive agents (including corticosteroids at an equivalent of ≥20-mg prednisone daily).

**Study Definitions**

To assess the sensitivity of the BAL GMS stain for PJP, we sought to identify BAL GMS-positive cases and BAL GMS-negative cases of PJP. A case of BAL GMS-positive PJP was defined as \textit{P. jiroveci} organisms visualized on GMS staining of BAL fluid (and therefore a proven case). BAL GMS-negative PJP was categorized into proven and probable cases. These investigator-established definitions (Figure 1) were derived from European Organization for Research and Treatment of Cancer and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria for invasive fungal disease, which do not include PJP.6 A proven case was defined as (1) \textit{P. jiroveci} organisms visualized on GMS staining of a concurrent lung tissue specimen or (2) a positive \textit{P. jiroveci}–specific direct immunofluorescent antibody (DFA) on BAL or (3) a positive \textit{P. jiroveci} BAL polymerase chain reaction (PCR) finding and a host factor (immunosuppressed patient) and clinical criteria (symptoms of pneumonia and diffuse ground glass opacities or interstitial markings on chest imaging). A probable case was defined as having (1) an elevated serum (1-3)-β-D-glucan of 80 pg/mL or greater plus (2) a host factor and (3) clinical criteria and (4) no BAL \textit{P. jiroveci} PCR or DFA assay performed. If multiple (1-3)-β-D-glucan results were available, the one most proximate to the date of the bronchoscopy was included.

**Data Collection**

For study cases, we collected additional patient factors such as age, sex, race, HIV status, immunocompromising condition and immunosuppressive medications, duration of hospitalization, date of PJP diagnosis, use of antecedent PJP prophylaxis, highest acuity of care, APACHE score on admission, chest imaging, type and duration of PJP treatment, use of adjunctive steroids, laboratory...
testing (white blood cell counts, differentials, lactate dehydrogenase level), in-hospital and 30-day outcomes, among others. The Yale Institutional Review Board (IRB) approved this study (IRB protocol ID 2000021997).

**Laboratory Methods**

GMS staining on BAL fluid was performed per standard protocol by trained technicians within the cytology laboratory of YNHH. Briefly, a BAL specimen was centrifuged for 5 minutes then cytospun onto a slide with a ThinPrep 2000 instrument (Hologic, Marlborough, Massachusetts). The slide was fixed with 95% methanol, then oxidized by using chromic acid, rinsed with 1% sodium bisulfite, and then placed in a methenamine/silver solution. Slides were heated for 37 seconds at a power of 630 watts. With intervening steps of distilled water rinses, slides were placed in 0.4% gold chloride solution, then in 2% sodium thiosulfate followed by light green counterstain, dehydrated with absolute alcohol, cleared with 2 changes of xylene, and finally mounted with a synthetic mounting medium in preparation for reading under a light microscope.

*Pneumocystis jiroveci* DFA and qualitative PCR assays on BAL were performed upon clinician request via send-out testing to Quest Diagnostics (Chantilly, Virginia).

**Retrospective GMS Slide Review**

Slides for BAL GMS-negative cases of PJP were retrieved from the archives of the YNHH pathology laboratory and re-examined under light microscopy by a certified cytopathologist for evidence of organisms consistent with *P jiroveci*.

**RESULTS**

During the study period, a total of 1451 patients underwent bronchoscopy with GMS staining of BAL fluid. Of these, we identified 45 adult immunocompromised patients with PJP. These included 24 HIV-negative and 21 HIV-positive cases (Figure 2). Among the 24 HIV-negative cases, 11 had BAL GMS-positive PJP (Table 1) and 13 had BAL GMS-negative PJP including 9 proven and 4 probable cases, all of which met host and clinical criteria for PJP as per study definitions (Table 2).

The BAL GMS-negative PJP cases included 6 patients with hematologic malignancies, 2 with SOT, 2 with SOT plus hematologic malignancies, 2 with autoimmune disorders while taking high-dose corticosteroids, and 1 with HSCT. Of the 9 proven cases, 6 had a concurrent lung tissue specimen that was GMS positive for *P jiroveci*, 2 had positive *P jiroveci* DFA finding on BAL (both had negative GMS staining of lung tissue specimens), and 1 had a positive *P jiroveci* PCR finding on BAL of 5151 copies/mL (no concurrent lung tissue). As per study definitions, all probable cases had elevated serum (1-3)-β-D-glucan levels. GMS staining of lung tissue was negative in 1 case and not done in the other 3 cases.

All 21 HIV-positive PJP cases were BAL GMS-positive; no BAL GMS-negative cases of PJP were identified among HIV-positive patients. The median CD4 count in this group was 10 cells/mL, with a range of 3 to 300 cells/mL. Using these data, the sensitivity of BAL GMS PJP (on initial examination) for the diagnosis of PJP in HIV-negative immunocompromised patients was 11 of 24 (46%) versus 21 of 21 (100%) in HIV-positive patients (Figure 3, A through D).

Original slides of GMS-stained BAL were retrieved for 12 of 13 cases of BAL GMS-negative PJP. Re-examination of slides showed rare *P jiroveci* cysts in 1 case, whereas the remaining slides where negative on review.

We further examined the 13 cases that were BAL GMS-negative to determine if a falsely negative BAL GMS, the primary diagnostic method for *P jiroveci* at our institution, was associated with poorer outcomes (Table 3). All patients received treatment for PJP with a median duration of 21 days (10–35 days). Treatment regimens included trimethoprim-sulfamethoxazole, atovaquone, and clindamycin with primaquine. Adjunctive corticosteroids were used in 12 of 13 cases. In-hospital outcomes included PJP resolution (n = 11), partial improvement with worsening after premature treatment discontinuation (n = 1), and death due to PJP (n = 1). Though all 13 patients were ultimately diagnosed with PJP by alternative methods and received treatment, worse
Table 1. Admission Characteristics and Diagnostic Testing Results for Cases of HIV-Negative Immunocompromised Patients With Bronchoalveolar Lavage (BAL) Gomori Methenamine Silver (GMS)–Positive Pneumocystis jiroveci Pneumonia (PJP)

| Patient | Age, y, Sex, Race | Host Factor | PJP Prophylaxis | Level of Care (APACHE Score), Lowest SaO2 on Admission | Chest Imaging on Admission |
|---------|------------------|-------------|-----------------|------------------------------------------------------|---------------------------|
| Patient 1 | 66, male, white | Allogeneic HSCT for acute myeloid leukemia on tacrolimus and sirolimus | Yes (inhaled pentamidine) | MICU (12), 83% on room air | Bilateral patchy GGOs predominantly in upper lung zones |
| Patient 2 | 81, male, white | Lung SOT, metastatic renal cell and spindle cell carcinoma on tacrolimus and prednisone 7.5 mg daily | Yes (TMP-SMX) | MICU (23), 96% on FiO2 6 L/min | Bilateral patchy GGOs with RUL and LLL consolidations |
| Patient 3 | 63, male, white | Granulomatosis with polyangiitis on azathioprine and prednisone 80 mg daily | No | Ward (11), 93% on FiO2 2 L/min | Bilateral patchy GGOs |
| Patient 4 | 60, male, white | Autoimmune HSCT for T-cell non-Hodgkin lymphoma on prednisone | No | Ward (18), 97% on room air | Bilateral patchy GGOs predominantly in upper lung zones |
| Patient 5 | 49, male, white | Autoimmune HSCT for follicular lymphoma and eosinophilic pneumonia on prednisone 50 mg daily | No | Outpatient, 98% on room air | Bilateral patchy GGOs |
| Patient 6 | 47, male, white | SLE and rheumatoid arthritis on adalimumab, methotrexate, and azathioprine | No | MICU (23), 93% on room air | Bilateral patchy GGOs |
| Patient 7 | 78, male, white | Chronic lymphocytic leukemia on ibritumomab | No | Ward (20), 89% on room air | Bilateral patchy GGOs |
| Patient 8 | 80, male, white | Chronic lymphocytic leukemia and small lymphocytic lymphoma with HH on prednisone 20 mg daily | Yes (inhaled pentamidine) | MICU (19), 94% on FiO2 2 L/min | Bilateral patchy GGOs |
| Patient 9 | 51, male, white | CNS lymphoma on rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone | No | Ward (13), 85% on room air | Bilateral patchy GGOs |
| Patient 10 | 60, female, white | Granulomatosis with polyangiitis on azathioprine and prednisone 20 mg daily | No | Outpatient, 81% on room air | Bilateral patchy GGOs and diffuse nodular opacities |
| Patient 11 | 61, male, white | Small lymphocytic lymphoma on bendamustine | No | Ward (20), 90% on room air | Bilateral patchy GGOs predominantly in upper lung zones |

Abbreviations: ATRA, all-trans retinoic acid; CNS, central nervous system; DFA, direct fluorescent antibody; FiO2, fraction of inspired oxygen; GGO, ground glass opacities; HH, hemophagocytic lymphohistiocytosis; HSCT, hematopoietic stem cell transplant; LDH, lactate dehydrogenase; LLL, left lower lobe; MICU, medical intensive care unit; MMF, mycophenolate mofetil; MTX, methotrexate; PCR, polymerase chain reaction; SaO2, oxygen saturation; SLE, systemic lupus erythematosus; SOT, solid organ transplant; TMP-SMX, trimethoprim-sulfamethoxazole.

Table 2. Admission Characteristics and Diagnostic Testing Results for Cases of HIV-Negative Immunocompromised Patients With Bronchoalveolar Lavage (BAL) Gomori Methenamine Silver (GMS)–Negative Pneumocystis jiroveci Pneumonia (PJP)

| Patient 1 | Age, y, Sex, Race | Host Factor | PJP Prophylaxis | Level of Care (APACHE Score), Lowest SaO2 on Admission | Chest Imaging on Admission | Serum LDH Level on Admission (Baseline Level if Available), U/L |
|-----------|------------------|-------------|-----------------|------------------------------------------------------|---------------------------|-------------------------------------------------------------|
| Patient 1 | 66, female, white | CNS lymphoma on MTX and rituximab | No (TMP-SMX held owing to potential interference with methotrexate clearance) | MICU (16), 88% on room air | Bilateral patchy GGOs | 657 (217) |
| Patient 2 | 44, female, black | Acute promyelocytic leukemia on 6-MP, MTX, and ATRA | No | Ward (10), 85% on room air | Bilateral patchy GGOs and pulmonary nodules | 408 (126) |
| Patient 3 | 59, female, white | CNS lymphoma, combined kidney and pancreas SOT on prednizone, rituximab, and dexamethasone 4 mg daily | No | SDU (13), 89% on room air | Bilateral patchy GGOs | Not done |
| Patient 4 | 48, male, white | Allogeneic HSCT and GVHD on tacrolimus, prednizone (20 mg daily), and basiliximab | Yes (ataxiaquone) | MICU (11), 76% on room air | Bilateral patchy GGOs | 846 (442) |
| Patient 5 | 58, male, white | Primary CNS lymphoma on prednizone, rituximab, and 4-mg dexamethasone daily | No | MICU (22), 88% on room air | Bilateral patchy GGOs | 862 (203) |
| Patient 6 | 65, male, white | Multiple myeloma on bortezomib, cyclophosphamide, daratumumab, and dexamethasone 4 mg daily | No | MICU (23), 93% on FiO2 100% | Bilateral patchy GGOs | 1471 (678) |
| Patient 7 | 67, female, Hispanic | Renal SOT on sirolimus and prednizone 5 mg daily | No | Ward (18), 91% on room air | Bilateral patchy GGOs | 513 (286) |
| Patient 8 | 67, male, white | Rheumatoid arthritis on rituximab and prednizone 20 mg daily | No | Ward (16), 86% on room air | Bilateral patchy GGOs | 466 |
| Patient 9 | 60, male, white | CNS lymphoma, combined kidney and pancreas SOT on prednizone 5 mg daily, tacrolimus, and MMF | No | Ward (15), 89% on room air | Bilateral consolidations and patchy GGOs | Not done |
| Patient 10 | 79, male, white | Nephrotic syndrome on prednizone 40 mg daily | No | MICU (21), 80% on room air | Bilateral patchy GGOs and septal thickening | 685 |
| Patient 11 | 37, male, white | B-cell lymphoma on cyclophosphamide, vincristine, doxorubicin, and prednizone 20 mg daily | No | SDU (7), 80% on room air | Bilateral patchy GGOs | 653 |
| Patient 12 | 83, female, white | Multiple myeloma on bortezomib and dexamethasone 4 mg daily | No | Outpatient, 94% on room air | Few scattered GGOs | Not done |
| Patient 13 | 44, male, white | Combined kidney and pancreas SOT on tacrolimus and sirolimus | No | MICU (15), 79% on room air | Bilateral patchy GGOs | 244 (233) |

Abbreviations: ATRA, all-trans retinoic acid; CNS, central nervous system; DFA, direct fluorescent antibody; FiO2, fraction of inspired oxygen; GGO, ground glass opacities; HH, hemophagocytic lymphohistiocytosis; HSCT, hematopoietic stem cell transplant; LDH, lactate dehydrogenase; MICU, medical intensive care unit; MMF, mycophenolate mofetil; MTX, methotrexate; PCR, polymerase chain reaction; SaO2, oxygen saturation; SOT, solid organ transplant; TMP-SMX, trimethoprim-sulfamethoxazole; 6-MP, 6-mercaptopurine.
### Table 1. Extended

| Serum LDH Level on Admission (Baseline Level if Available), U/L | BAL GMS Stain Performed on Treatment? | GMS Stain of Lung Tissue | BAL Fungal Culture | P jiroveci BAL DFA | P jiroveci BAL PCR | Serum (1-3)-β-D-Glucan Level |
|---------------------------------------------------------------|---------------------------------------|--------------------------|-------------------|------------------|-------------------|-------------------------------|
| 442 (332)                                                    | 1 d of treatment                      | Positive for P jiroveci   | Negative          | Not done         | Positive (qualitative) | Not done                      |
| 321                                                          | No                                    | Not done                 | Aspergillus terreus| Not done         | Not done           | Negative                       |
| 312 (252)                                                    | 1 d of treatment                      | Not done                 | Negative          | Not done         | Not done           | Not done                      |
| 361 (258)                                                    | No                                    | Negative                 | Negative          | Not done         | Not done           | Not done                      |
| 297 (236)                                                    | No                                    | Negative                 | Negative          | Not done         | Not done           | Not done                      |
| 1093                                                         | No                                    | Not done                 | Negative          | Not done         | Not done           | Not done                      |
| 240 (161)                                                    | 2 d of treatment                      | Positive for P jiroveci   | Aspergillus species| Negative         | Not done           | Not done≥500 pg/mL           |
| 774                                                          | No                                    | Not done                 | Negative          | Not done         | Not done           | Not done                      |
| 286 (138)                                                    | 4 d of treatment                      | Positive for P jiroveci   | Negative          | Not done         | Not done           | Not done                      |
| Not done                                                     | No                                    | Positive for P jiroveci   | Negative          | Not done         | Not done           | Not done                      |
| 1286 (263)                                                   | 5 d of treatment                      | Not done                 | Negative          | Not done         | Not done           | Not done                      |

### Table 2. Extended

| BAL GMS Stain Performed on Treatment? | GMS Stain of Lung Tissue | BAL Fungal Culture | P jiroveci BAL DFA | P jiroveci BAL PCR | Serum (1-3)-β-D-Glucan Level | BAL GMS-Negative Classification | BAL GMS Slide Review |
|--------------------------------------|--------------------------|-------------------|-------------------|------------------|-------------------------------|---------------------------------|----------------------|
| No                                   | Positive for P jiroveci   | Negative          | Not done          | Not done         | Not done                      | Proven                          | Rare positive P jiroveci cysts |
| No                                   | Positive for P jiroveci   | Negative          | Not done          | Not done         | Not done                      | Proven                          | Negative             |
| No                                   | Positive for P jiroveci   | Negative          | Not done          | Not done         | Not done                      | Proven                          | Negative             |
| No                                   | Positive for P jiroveci   | Negative          | Not done          | Not done         | Not done                      | Proven                          | Negative             |
| 4 d of treatment                     | Not done                 | Negative          | Not done          | Not done         | ≥500 pg/mL                    | Probable                        | Negative             |
| No                                   | Not done                 | Negative          | Not done          | Positive (5151 copies/mL) | ≥500 pg/mL                    | Proven                          | Negative             |
| 6 d of treatment                     | Negative                 | Negative          | Positive (few organisms) | Not done         | Negative (39 pg/mL)          | Proven                          | Negative             |
| 1 d of treatment                     | Positive for P jiroveci   | Negative          | Not done          | Not done         | ≥500 pg/mL                    | Proven                          | Negative             |
| 4 d of treatment                     | Not done                 | Negative          | Not done          | Not done         | ≥500 pg/mL                    | Probable                        | Negative             |
| 2 d of treatment                     | Negative                 | Negative          | Not done          | Not done         | ≥500 pg/mL                    | Probable                        | Negative             |
| 4 d of treatment                     | Not done                 | Negative          | Not done          | Not done         | 109 pg/mL                     | Probable                        | Slide not available    |
| No                                   | Positive for P jiroveci   | Negative          | Not done          | Not done         | Not done                      | Proven                          | Negative             |
outcomes were seen in 3 cases, including 2 cases of treatment delays and 1 case of an initially missed diagnosis leading to discharge then readmission.

In terms of PJP prophylaxis, only 4 of 24 HIV-negative immunocompromised patients (1 of 13 cases of BAL GMS-negative PJP and 3 of 11 cases of BAL GMS-positive PJP) were receiving chemoprophylaxis at the time of PJP diagnosis. Two of these cases occurred in patients taking inhaled pentamidine: 1 taking atovaquone and 1 taking trimethoprim-sulfamethoxazole. Among patients not receiving PJP prophylaxis, 8 of 12 GMS BAL-negative and 4 of 8 GMS BAL-positive patients were receiving an equivalent of 20-mg prednisone daily or greater for at least 1 month, qualifying them for PJP prophylaxis.

Among BAL GMS-negative PJP cases, serum (1-3)-β-D-glucan assay was performed in 8 of 13 cases and was positive in 7 (>500 pg/mL in 6, 109 pg/mL in 1) but negative in 1 proven case (39 pg/mL).

**DISCUSSION**

Direct visualization of *Pneumocystis jiroveci* organisms in BAL fluid using the GMS stain is widely used for the diagnosis of fungal pneumonia including PJP and remains the primary diagnostic method at our institution. This study evaluated the sensitivity of BAL GMS for the diagnosis of PJP in HIV-negative immunocompromised patients as compared to HIV-positive patients with PJP. The staining method performed very well in the HIV-positive population (sensitivity of 100%), but was decidedly suboptimal for HIV-negative immunocompromised patients (sensitivity of 46%). Though the sensitivity of BAL GMS has been reported at 90% to 95% in the literature, most of these studies were performed in the HIV/AIDS era, reflecting the higher burden of infecting organisms found in HIV-infected patients with PJP. In an elegant study by Limper et al, the number of *P jiroveci* organisms (cysts/mL) was assessed in 75 patients with PJP, including 19 patients with HIV/AIDS and 56 HIV-negative immunocompromised patients. Patients with HIV/AIDS had a median number of organisms that was around 15 times that of HIV-negative patients (27.3 × 10^3 versus 1.9 × 10^3 cysts/mL; P < .001). Despite these concerns, there are few data directly comparing the sensitivity of BAL GMS for PJP in HIV-negative immunocompromised populations versus HIV-positive patients. In
one study of 84 immunosuppressed patients with SOT, hematologic malignancies, or other malignancies, GMS BAL was 81% sensitive in diagnosing PJP versus 86% for an immunofluorescent stain. In a study of HIV-positive and HIV-negative patients with suspected PJP, the diagnostic yield of BAL GMS was 31% and 1.5%, respectively. Outside of HIV infection, factors including BAL sampling technique, natural course of the disease, and intraoperator variability in stain interpretation may explain some of the variation in BAL GMS sensitivities encountered in the literature. Additionally, GMS staining may be particularly vulnerable to false negatives in the setting of a low burden of disease because P. jiroveci trophozoites, which are 10 times more abundant than cyst forms, are not visualized. This is in contrast to both DFA and PCR assays that detect some or all developmental stages of P. jiroveci organisms. Concerns of poor sensitivity in HIV-negative populations are reflected by consensus guidelines, including those by the American Society of Transplantation Infectious Disease Community of Practice (AST IDCOP), which recommend using immunofluorescent stains or PCR rather than routine stains alone.

To assess whether operator proficiency in reading GMS-stained slides contributed to low sensitivity in this study, slides for cases of BAL GMS-negative PJP were retrieved and reviewed by a certified cytopathologist. Re-examination of the slides demonstrated rare P. jiroveci cysts in only 1 sample, suggesting that intraoperator variability was not an important factor in suboptimal sensitivity in our study.

It is clear that using BAL GMS as a stand-alone diagnostic method for HIV-negative patients with suspected PJP carries the potential for missed diagnoses. In this study, we noted 3 instances in which a negative GMS BAL finding was detrimental to care, including premature discontinuation of treatment and delayed initiation of treatment for PJP. Appropriate diagnostic testing is especially important in the current era in which a greater proportion of PJP is encountered in HIV-negative immunocompromised patients, including recipients of SOT and HSCT and oncology patients. These include immunofluorescent testing and PCR, although false positives reflective of transient colonization are more likely with the latter.

In the absence of PCR and DFA testing or when turnaround time is prolonged as with send-out testing, an elevated serum (1-3)-β-D-glucan was very useful in making a diagnosis of PJP. This was consistent with data from a recent study of oncology patients that found that serum (1-3)-β-D-glucan values greater than 200 pg/mL and positive PJP PCR results were associated with clinically significant PJP.

In the current study, GMS staining of lung tissue specimens was helpful in establishing the diagnosis of PJP in some patients with negative BAL GMS findings but the method was overall suboptimal owing to false negatives (in 3 cases) and barriers to biopsy particularly in patients with hematologic malignancies and thrombocytopenia. The rate of appropriate PJP prophylaxis was low in this study, suggesting a need for better clinician education on PJP chemoprophylaxis in HIV-negative immunocompromised patients.

There are several limitations to this study. In addition to limitations associated with a retrospective design, our data were collected from a single academic center with a large population of immunocompromised individuals, limiting the generalizability of our findings to smaller centers that serve different populations of patients. Although we evaluated a large sample of 1451 patients who underwent BAL GMS, the final sample size of PJP cases for which a

| Duration From First Evaluation for Dyspnea to PJP Treatment Initiation, d | PJP Treatment | Adjunctive Corticosteroids | Duration of Treatment, d | In-hospital Outcome | Missed Diagnosis or Delayed Treatment |
|---|---|---|---|---|---|
| Patient 1’ | 3 | TMP-SMX followed by atovaquone | Yes | 21 | Resolution | No |
| Patient 2’ | 4 | TMP-SMX | No | 21 | Resolution | No |
| Patient 3’ | 5 | TMP-SMX | Yes | 21 | Resolution | No |
| Patient 4’ | 2 | TMP-SMX followed by clindamycin and primaquine | Yes | 35 | Death due to PJP | No |
| Patient 5’ | 4 | TMP-SMX | Yes | 10 | Partial improvement followed by clinical worsening after discontinuation of PJP treatment once BAL GMS resulted as negative | Missed diagnosis (readmitted 1 month later in respiratory failure with BAL GMS-positive PJP) |
| Patient 6’ | 6 | TMP-SMX | Yes | 14 | Resolution | Delayed treatment (clinical team did not initiate PJP treatment until results of send-out PJP PCR returned as positive) |
| Patient 7’ | 8 | TMP-SMX followed by atovaquone | Yes | 21 | Resolution | Delayed treatment (clinical team did not initiate PJP treatment until results of send-out PJP PCR returned as positive) |
| Patient 8’ | 0 | TMP-SMX followed by atovaquone | Yes | 21 | Resolution | No |
| Patient 9’ | 5 | Atovaquone | Yes | 21 | Resolution | No |
| Patient 10’ | 2 | Atovaquone followed by clindamycin and primaquine | Yes | 18 | Resolution | No |
| Patient 11’ | 0 | TMP-SMX | Yes | 21 | Resolution | No |
| Patient 12’ | 0 | TMP-SMX | Yes | 21 | Resolution | No |
| Patient 13’ | 21 | TMP-SMX followed by atovaquone | Yes | 21 | Resolution | No |

Abbreviations: DFA, direct fluorescent antibody; PCR, polymerase chain reaction; TMP-SMX, trimethoprim-sulfamethoxazole.
BAL GMS staining was performed was relatively small. Finally, owing to lack of accepted definitions for PJP, we used investigator-established criteria, although these were closely derived from EORTC/MSG criteria for other invasive fungal infections. Notably, serum (1-3)-β-D-glucan is not a specific marker for PJP and can show positivity in the setting of other invasive fungal infections. However, when taken in context of the clinical and radiographic picture as done in this study, this biomarker has a high positive predictive value for PJP.14

In conclusion, the BAL GMS stain is insensitive for the diagnosis of PJP in HIV-negative immunocompromised patients and should not be used as the stand-alone diagnostic method in this population. Clinicians and pathologists in institutions that use GMS BAL as the primary fungal stain on BAL should be aware of sensitivity limitations for PJP and the potential for missed diagnoses. In HIV-negative patients with a high pretest probability of PJP, a negative BAL GMS finding does not rule out the diagnosis of PJP and should be initiated once PJP is suspected. In this setting, an elevated serum (1-3)-β-D-glucan is a very useful adjunct diagnostic test for PJP.

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