Vol. 42, No. 7

Detection of *Pneumocystis jiroveci* in Respiratory Specimens by Four Staining Methods

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We examined four staining methods on replicate smears of 313 respiratory specimens submitted for *Pneumocystis jiroveci* examination. The sensitivity and specificity of Calcofluor white stain (CW) were 73.8 and 99.6%, respectively. The sensitivity and specificity of Grocott-Gomori methenamine silver stain (GMS) were 79.4 and 99.2%, respectively. The sensitivity and specificity of Diff-Quik stain were 49.2 and 99.6%, respectively. The sensitivity and specificity of Merifluor Pneumocystis stain were 90.8 and 81.9%, respectively. Only CW and GMS had positive and negative predictive values of >90%.

Pneumocystis jiroveci, previously known as *Pneumocystis carinii*, remains an important cause of pneumonia in immunocompromised patients, although infections with this agent have decreased after the widespread use of highly active antiretroviral therapy (HAART) for human immunodeficiency virus (HIV) infection (9, 12). Patients who are immunocompromised for reasons other than HIV infection are also at risk for pneumonia caused by *P. jiroveci*, including patients with hematologic malignancies and those who have received immunosuppressive agents for the treatment of autoimmune diseases (2, 11, 12).

Although a variety of nucleic acid amplification assays, including real-time PCR assays, have been developed for the detection of *P. jiroveci*, these assays are not commonplace in most clinical microbiology laboratories and are not available commercially (3, 5). Therefore, the principal laboratory method for the detection of *P. jiroveci*, an organism that cannot be cultivated by standard methods, is the direct visual examination of the clinical specimen after some type of staining method (12). A variety of histochemical stains have been used to detect *Pneumocystis* in clinical specimens. These histochemical stains include the Diff-Quik, Grocott-Gomori methenamine silver (GMS), and Calcofluor white stains. Diff-Quik stain is a modification of Wright stain (3). GMS stain is a silver precipitation stain commonly used to visualize fungi in histologic sections (10). Calcofluor white stain is a fluorescent stain, the active ingredient of which is cellufluor, which nonspecifically binds to beta-linked polysaccharides, such as chitin and cellulose, and is used for the direct visualization of fungi in clinical specimens (4). Immunofluorescent stains, which employ antibodies directed against *P. jiroveci*, are also available for the direct detection of this organism in clinical specimens

(3, 5). These stains are similar to the direct and indirect immunofluorescent stains used for the detection of viruses in clinical specimens. Each stain has its proponents; comparative data in the HAART era are limited.

Therefore, we performed a multi-institutional assessment of four commonly used staining methods for the direct detection of *P. jiroveci* in clinical respiratory specimens. The slides from respiratory specimens studied, the majority of which were bronchoalveolar lavage specimens, were prepared using cytocentrifugation. Replicate smears of 313 respiratory specimens submitted for *P. jiroveci* examination were examined for the presence of *P. jiroveci* with the Calcofluor white (Fungifluor; Polysciences, Inc., Warington, Pa.), GMS, Diff-Quik (Baxter Scientific, McGraw Park, Ill.), and Merifluor Pneumocystis (Meridian Bioscience, Inc., Cincinnati, Ohio) stains. Slides were stained with the Merifluor Pneumocystis and Diff-Quik stains according to the manufacturer's instructions. For Calcofluor white staining, 1 drop of Fungifluor was added to each slide, the coverslip was applied so that the reagent covered the entire specimen-containing area, and the slide was allowed to sit for at least 10 min in a dark humidity chamber at room temperature prior to interpretation with a fluorescence microscope. For GMS staining, the slides were microwaved for 40 s in a 10% chromic acid solution, washed with water, and then cleared with 1% sodium metabisulfite for 30 s. After the slides were washed with distilled water, they were placed in a Coplin jar containing 50.0 ml of methenamine working solution and microwaved for another 65 s. The slides were rinsed again with distilled water, treated with 1% gold chloride for 2 to 5 s, rinsed with distilled water, exposed to 5% sodium thiosulfate for 1 min, counterstained with a light green working solution, cleared in xylene, covered with cover slips, and examined by routine light microscopy.

Each of the laboratories from the four participating institutions performed one of the different stains and rendered their interpretations based on that staining method. The individuals at each institution had experience with the staining method

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TABLE 1. Statistical parameters of four stains used for *P. jiroveci*[a]

Stain	No. of positive slides	Total no. of slides examined	SEN (%)	SPEC (%)	PPV (%)	NPV (%)
CW	48	308	73.8	99.6	98.0	93.4
MF	59	310	90.8	94.7	81.9	97.5
DQ	31	307	48.4	99.6	96.9	88.0
GMS	50	310	76.9	99.2	96.2	94.2

[a] The sensitivity (SEN), specificity (SPEC), and positive and negative predictive values (PPV and NPV, respectively) are given for Calcofluor white (CW), Merifluor Pneumocystis (MF), Diff-Quik (DQ), and GMS stains.

performed there and the interpretation of the stain. The specimens were given unique identification codes, and each stain was examined independently, without knowledge of the result obtained by another staining method on the same specimen. The quantity of specimen was rarely insufficient to make all four slides; 308 slides were available for staining with Calcofluor white, 310 slides were available for staining with Merifluor Pneumocystis, 307 slides were available for staining with Diff-Quik, and 310 slides were available for staining with GMS. In none of the instances wherein a slide was unavailable due to an insufficient quantity of specimen did the categorization of that specimen as a true positive or true negative (see below) depend on the result of the absent slide.

The specimens were considered to contain *P. jiroveci* (i.e., a truly positive specimen) if this organism was detected by two or more of the staining methods. Conversely, specimens were considered to not contain the organism (i.e., a truly negative specimen) if all stains were negative or if only one stain was positive that could not be corroborated (8). Overall, 65 of 313 (20.8%) of specimens were considered true positives, whereas 248 of 313 (79.2%) were considered true negatives. Of the positive specimens, 48 of 65 (73.8%) were detected by the Calcofluor white stain, 59 of 65 (90.8%) were detected by the Merifluor Pneumocystis stain, 31 of 64 (48.4%) were detected by Diff-Quik stain, and 50 of 65 (76.9%) were detected by GMS. False-positive results were rare for Calcofluor white (1 of 243 [0.4%]), Diff-Quik (1 of 243 [0.4%]), and GMS (2 of 245 [0.8%]) but were more frequent for Merifluor Pneumocystis stain (13 of 245 [5.3%]).

The sensitivity, specificity, and positive and negative predictive values were calculated by standard methods (Table 1). The proportions of true-positive and false-positive results for paired tests were compared with the chi-square test using the EpiCalc 2000 statistical software (www.brixtonhealth.com /epicalc.html).

Although more true-positive specimens were detected by the Merifluor Pneumocystis stain, this number was not significantly different from those detected by the Calcofluor white ($P = 0.260$) and GMS ($P = 0.343$) stains. Similarly, the number of true positives detected by GMS and Calcofluor white stains were not significantly different ($P = 0.838$) from each other. The number of true positives detected by Diff-Quik stain, however, was significantly fewer than those detected by the Merifluor Pneumocystis ($P = 0.002$), GMS ($P = 0.027$), and Calcofluor white ($P = 0.042$) stains. The number of false positives was not significantly different between the Calcofluor white, GMS, and Diff-Quik stains (each comparison had a P value of

>0.05). The number of false positives reported after staining with Merifluor Pneumocystis stain, however, was significantly greater than those reported after staining with GMS ($P = 0.004$), Calcofluor white ($P = 0.001$), or Diff-Quik ($P = 0.001$).

In some instances, an induced sputum specimen may be the first specimen received for the assessment of the presence of *Pneumocystis* in the diagnostic workup of a patient with pneumonia suspected to be caused by this organism (6). If an induced sputum specimen fails to yield the etiologic agent of pneumonia, then a specimen from bronchoalveolar lavage, which is significantly more costly and does carry a small risk to the patient, may be needed to obtain diagnostic material. Because of the differences in the treatment of pneumonia caused by *Pneumocystis* versus pneumonia caused by other microorganisms and the possible need for bronchoscopy, which incurs additional cost and risk to the patient, in the event that a diagnosis is not established, it is important that the best possible staining method be used for the detection of this organism.

Moreover, the importance of a sensitive and specific staining method in the era of HAART is particularly important, since the lower prevalence of pneumonia caused by *Pneumocystis* adversely affects the positive predictive value of any assay that has a specificity less than 100% (9). Although the prevalence of pneumonia caused by *Pneumocystis* is different in the HAART era than in the pre-HAART era, the choice of the optimal staining method for the detection of *Pneumocystis* is also important for patients with other immunocompromising conditions who are at risk for infection. In fact, the choice of the optimal staining method may be more important for the detection of *Pneumocystis* in the non-HIV-infected, immunocompromised patients, since it has been shown that respiratory specimens from these patients have a lower burden of organisms than those from HIV-infected, immunocompromised patients (6).

In our experience, the Diff-Quik stain was not an effective means of screening for the presence of *P. jiroveci* (i.e., a primary staining method) because of the low sensitivity and negative predictive value of this stain. Significantly more specimens that contained *P. jiroveci* were missed by the Diff-Quik method than with Calcofluor white, Merifluor Pneumocystis, and GMS. Raab et al. also describe both a low sensitivity (68%) and a low specificity (88%) when the Diff-Quik stain alone was used for the detection of *Pneumocystis* and other fungi in bronchoalveolar lavage specimens (10). These findings, however, are in contrast to those of other groups, who have reported that the Diff-Quik method was comparable to the GMS staining method for the detection of *Pneumocystis* (7, 8). One group reported that the Diff-Quik method was more sensitive than Calcofluor white, direct immunofluorescent staining, and even PCR, but these results have not been corroborated (1).

Conversely, the Merifluor Pneumocystis stain was the most sensitive staining method and may prove useful as a screen to rule out the presence of *Pneumocystis*, but it was the least specific method in this study. However, the number of false-positive results with the Merifluor Pneumocystis stain was significantly greater than that reported for each of the other stains. The Merifluor Pneumocystis stain had a positive predictive value of only 81.9%, compared with the other three methods, all of which had positive predictive values greater

than 96%. Ng et al. have also described apparent false-positive results with this staining method (8). Therefore, we suggest that if Merifluor Pneumocystis is used as the primary staining method in a clinical microbiology laboratory, then confirmation with a second method should be performed to increase the specificity and positive predictive value of the final test result.

The sensitivities of both the Calcofluor white and GMS stains were intermediate between those of the Diff-Quik and Merifluor Pneumocystis stains, but they were highly specific and had positive predictive values above 96% and negative predictive values above 93%. Fraire et al. have also described the Calcofluor white and GMS stains as comparable for the detection of *Pneumocystis*, with 92.6% concordance (4). Baughman et al. (1a) also describe the sensitivity of an indirect immunofluorescent antibody stain that is directed toward *Pneumocystis* and superior in sensitivity compared with a modified Wright stain and a GMS stain, but they reported on a single false-positive result and therefore a much higher specificity than we describe. It is possible that with additional practice, one could more readily recognize the pathognomonic forms with the Diff-Quik stain, thereby raising the sensitivity of that assay. Similarly, perhaps with additional experience with the Merifluor Pneumocystis stain, one could learn to better discriminate between true-positive and false-positive staining and thereby diminish the number of false-positive results.

In our experience, the Calcofluor white and GMS stains have the best parameters for routine use in a clinical laboratory. Although these assays were less sensitive than the immunofluorescent assay, they were highly specific and had more than acceptable positive and negative predictive values.

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