

Comparison of Four Methods for Rapid Detection of *Pneumocystis carinii* in Respiratory Specimens

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Four stains for the detection of *Pneumocystis carinii* in respiratory specimens were compared for sensitivity, specificity, preparation time, and ease of interpretation. One hundred specimens were collected. Of these, 50 were induced sputum specimens and 50 were bronchoalveolar lavage fluid. All specimens were stained with Diff-Quik (DQ) (a modified Giemsa stain), a quick silver stain, and direct and indirect immunofluorescence stains. A positive specimen was defined as any smear positive by two or more of the methods. Fifty-eight percent of specimens were positive. Seventy-four percent of the sputum specimens and 42% of the bronchoalveolar lavages were positive. The sensitivities for detection of *P. carinii* in sputum were 92% with silver stain, 97% with direct immunofluorescence assay (DFA), 97% with indirect immunofluorescence assay (IFA), and 92% with DQ. The sensitivities for detection in bronchoalveolar lavage were 86% with silver stain, 90% with DFA, 86% with IFA, and 81% with DQ. Preparation times varied from 90 min for the silver stain and IFA to 3 min for DQ. Costs of the tests varied from \$1.50 per slide for DQ to \$10.00 per slide for the silver stain and DFA. Reading times varied from 10 to 30 min for the silver stain and DQ to less than 5 min for the immunofluorescence assays. We conclude that all of these tests are viable options for the clinical laboratory, and the choice will be influenced by factors such as clinical volume, ability to batch specimens, and expertise of technological support. A reasonable option may be to use the quick and inexpensive DQ as a screening test and to confirm negative smears with a more sensitive assay.

Pneumocystis carinii pneumonia is the most common index diagnosis for acquired immunodeficiency syndrome. Current Public Health Service projections estimate that more than 100,000 cases of *P. carinii* pneumonia will occur by 1991 (4, 15). This dramatic increase in what was once a relatively rare infection has posed a diagnostic challenge for clinical microbiology laboratories and has increased the demand for a rapid, reliable, and cost-effective diagnostic test. Ideally, such a test should be capable of detecting *P. carinii* in an induced sputum (IS) specimen or bronchoalveolar lavage (BAL), thus minimizing the need for more-invasive procedures such as transbronchial biopsy.

A variety of staining techniques are available for the identification of *P. carinii*. Currently, the most commonly employed stains are the methenamine stain and modified Giemsa stain.

The silver stain has been the traditional method for detecting *P. carinii* and is usually considered the "gold standard" (1, 7, 9, 10). Modified "quick" silver stains are commercially available (13). Although quicker to prepare than the traditional Grocott modified Gomori methenamine silver stain, they involve multiple steps and over an hour of technologist time. Furthermore, the silver stain is a cyst wall stain and does not detect the trophozoite form of the organism (1, 7). It is conceivable that a very light infection or one with a high trophozoite-cyst ratio could be missed by this technique. Another disadvantage of this stain is the high background staining, including positive staining of yeast and fungal elements, which can be confused with *P. carinii*.

Diff-Quik (DQ), a modified Wright-Giemsa stain, has been used successfully by a number of laboratories (2, 5, 7, 14).

This stain has several advantages. It is inexpensive and takes only several minutes to prepare. It detects all stages of the life cycle of *P. carinii*, including trophozoites and sporozoites, and allows identification of cysts, which appear negatively stained (5, 7). The disadvantage of DQ is that there is significant staining of background material, particularly in IS specimens. The *P. carinii* organisms can be very subtle in appearance and difficult to distinguish from the background; thus, interpretation of this stain requires a high level of technical expertise. In a low-volume laboratory where *P. carinii* is not frequently seen, it may be difficult to maintain such expertise.

The modified toluidine blue O stain is similar to the silver stain, being specific for cyst walls and having a similar appearance. Although it is more rapid than the silver stain, the process requires several noxious chemicals which necessitate the use of a fume hood. There is also the problem of nonspecific staining, and yeasts may be mistaken for *P. carinii* (5, 10).

A variety of immunofluorescence (IF) assays has appeared following the development of mouse anti-*P. carinii* antibodies (3, 11, 12). Both direct and indirect IF assays (DFA and IFA) are available. These staining techniques offer the advantage of easy and rapid identification of the organism. Preparation time varies from 1 to 2 h. The appearance of the organism on the smear varies according to the specificity of the monoclonal antibody; some are specific for cysts only, and others are specific for cysts, trophozoites, and sporozoites. Because of the variability in appearance of the organism and the presence of background fluorescence, interpretation of these assays also requires a certain degree of technical expertise and familiarity with the morphology of the organism.

We report the results of a comparison of four stains: the

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modified silver stain, DQ, and direct and indirect IF stains. These stains were compared for sensitivity, specificity, and ease and rapidity of preparation and interpretation.

MATERIALS AND METHODS

Specimen collection and preparation. Respiratory specimens, including both IS and BAL, were obtained from the clinical microbiology laboratories of the Veterans Administration Medical Center, San Francisco, and San Francisco General Hospital. Both hospitals followed the same protocol for the collection of IS. Briefly, the patient cleansed his or her mouth thoroughly, inhaled a solution of 3% saline in water via an ultrasonic nebulizer, and collected the IS in a sterile cup. Such specimens were judged to be adequate in the microbiology laboratory if alveolar macrophages, alveolar epithelial cells, or ciliated columnar epithelial cells were present. Specimens which were judged to be inadequate or which were other than sputum or BAL were not included in this study.

All sputa were liquified by addition of Sputolysin mucolytic solution (dithiothreitol), which was neutralized by the addition of phosphate buffer after a 3-min incubation. The specimens were centrifuged, and slides were prepared from the sediment. All specimens obtained by BAL were centrifuged directly, without mucolytic treatment, and slides were prepared from the sediment.

The clinical laboratories of both San Francisco General Hospital and the Veterans Administration Medical Center routinely screen for *P. carinii* with the DQ stain. We collected the specimens after this screening was complete and prepared extra slides, which were fixed in acetone and frozen at -70°C . One hundred specimens were collected from 84 individual patients. Of these specimens, 47 were judged to be negative by DQ and 53 were judged to be positive. Fifty of these samples were IS, and 50 were BAL. The unstained slides were coded numerically so that once stained, they could be read blindly, without prior knowledge of the diagnosis. Each specimen was then stained with a modified silver stain, a direct IF stain, and an indirect IF stain according to the instructions of the manufacturers. These slides were read promptly after staining, by one of two pathologists (P.C. or A.Y.), and then stored at -70°C .

Staining procedures. (i) **DQ stain.** Slides for DQ were prepared according to the instructions of the manufacturer. Briefly, the smear was fixed for 5 s in 1.8 mg of triaryl-methane dye per liter of methyl alcohol, rinsed, immersed in 1 g of xanthene dye per liter for 10 s, and rinsed. The slide was then immersed in a 1.25-mg/liter thiazine dye mixture and buffer and subjected to a final rinse. Slides were examined under low power and under oil immersion.

(ii) ***P. carinii* IF test kit (Genetic Systems).** Smears were overlaid with 5 drops of a fluorescein isothiocyanate-conjugated murine anti-*P. carinii* monoclonal antibody, which is specific for an antigen found on cysts, trophozoites, and sporozoites. These smears were incubated for 30 min at 37°C in a humidified chamber, rinsed with deionized water, and read under a fluorescence microscope.

(iii) **Monofluo Kit *P. carinii* (Diagnostics Pasteur).** The dried smear was overlaid with 20 μl of trypsin and incubated at 37°C for 30 min in a humidified chamber. The smear was then rinsed with deionized water and overlaid with 20 μl of anti-*P. carinii* monoclonal antibody specific for antigen present on the cyst wall. The slides were then incubated at 37°C for 15 min, rinsed, overlaid with 20 μl of fluorescein isothiocyanate-conjugated anti-mouse antibody, and incubated again

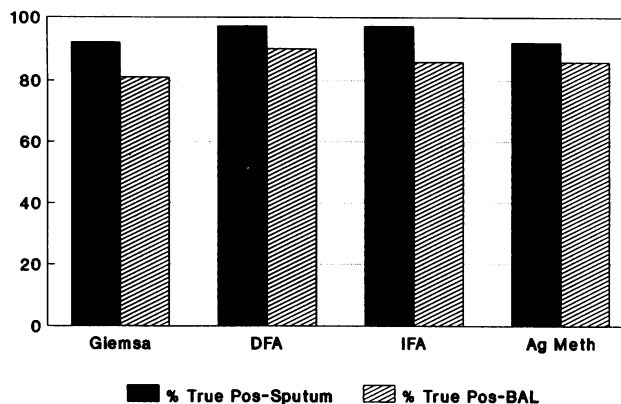


FIG. 1. Comparison of four stains for the detection of *P. carinii* in IS and BAL. Pos, Positive; Ag Meth, silver methenamine.

for 15 min. After a final rinse, the smears were read by fluorescence microscopy.

(iv) **Quick silver stain: Accustain (Sigma).** According to the instructions of the manufacturer, the dried smears were rehydrated in deionized water for 5 min, immersed in periodic acid solution for 5 min, rinsed, and stained with silver methenamine solution for 45 min. The slides were then rinsed, immersed in gold chloride solution for 30 s, rinsed, immersed in sodium thiosulfate solution for 2 min, rinsed, and counterstained with tartrazine solution for 5 min. The slides were examined under low power and under oil immersion.

Definition of positivity. The silver stain has traditionally been considered the "gold standard" for detection of *P. carinii*; however, we found that in a number of cases, the results of the silver stain were discordant with those of the other stains. To decrease the possibility of false-positives and -negatives, we defined a true-positive as a smear that was positive by two of the four stains. Criteria for positivity for the individual tests, based on the recommendations of the manufacturers, were as follows. Accustain silver stain was considered to be positive if one or more cysts were clearly visualized; the Monofluo indirect IF test was considered to be positive if five or more cysts were detected; the Genetic Systems direct fluorescence test was considered to be positive if two or more cysts or cysts and trophozoites were clearly visualized; DQ stain was considered to be positive if one or more clumps of trophozoites and cysts were detected.

Statistical analysis. Sensitivity and specificity were calculated as described previously (8). The statistical significance of differences in the sensitivities and specificities of the four stains was calculated by chi-square analysis as described previously (6).

RESULTS

Overall positivity was 58%. When broken down by sample type, 37 of 50 (74%) sputum specimens were positive, and 21 of 50 (42%) BAL were positive. Of the 37 positive sputum samples, 34 (92%) were detected by the silver stain, 36 (97%) were detected by DFA, 36 (97%) were detected by IFA, and 34 (94%) were detected by DQ (Fig. 1). Of the 21 positive BAL, 18 (86%) were correctly diagnosed by silver stain, 19 (90%) were detected by DFA, 18 (86%) were detected by IFA, and 17 (81%) were correctly diagnosed by DQ (Fig. 1).

Representative examples of each of the four stains are

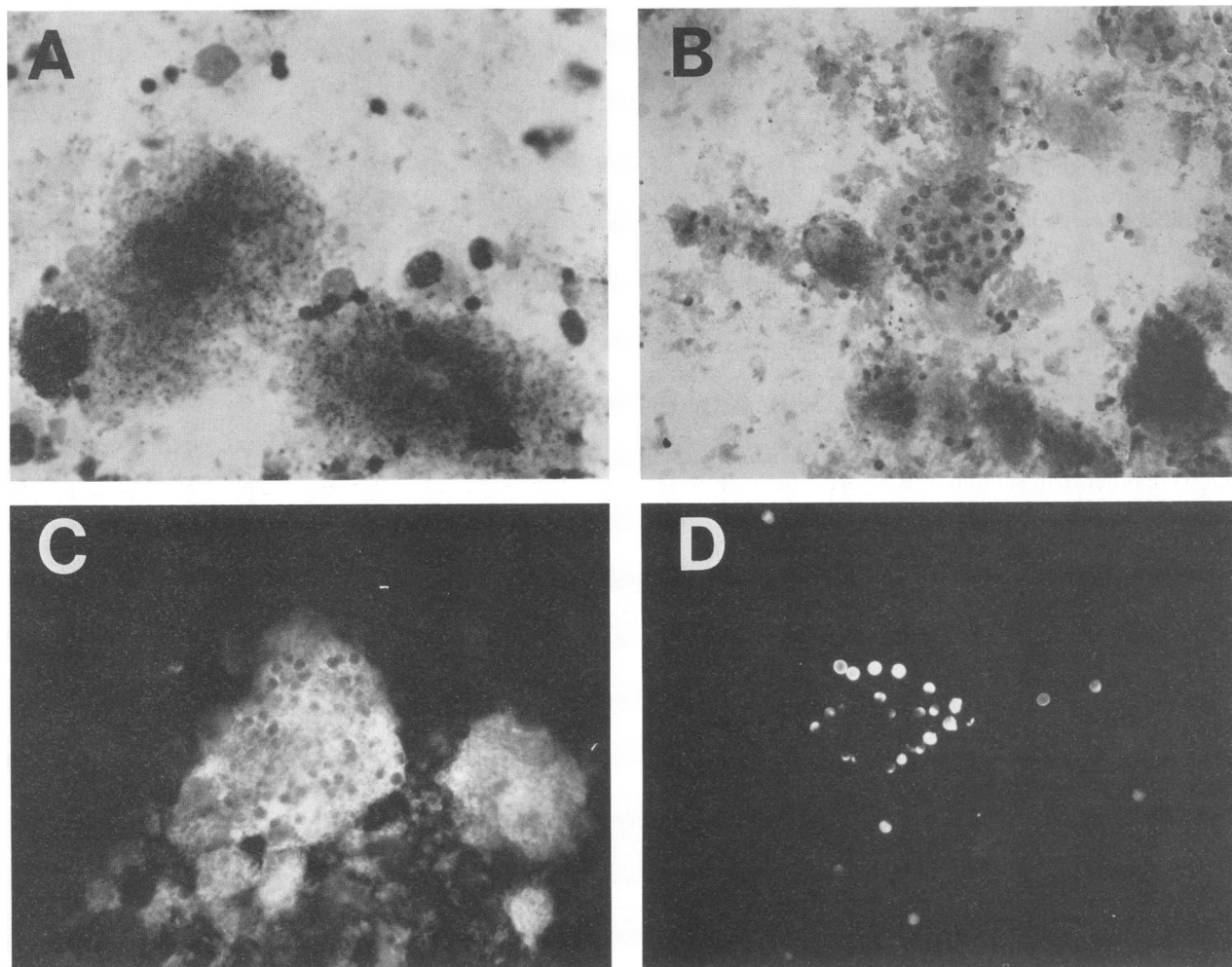


FIG. 2. Comparison of visual appearance of four staining methods for *P. carinii*. (A) DQ stain of *P. carinii* in IS at a magnification of $\times 98$. This foamy material is typical of the stained appearance of trophozoites and cysts. (B) Silver stain from the sputum of the same patient at a magnification of $\times 70$. Darkly stained round bodies are *P. carinii* cysts. (C) DFA of same sputum sample at a magnification of $\times 70$, made with a Nikon fluorescence microscope and with a 60-s exposure. Both cyst walls and trophozoites fluoresce. (D) IFA of same sample at a magnification of $\times 70$ with the same Nikon microscope and a 100-s exposure. The round, brightly fluorescent bodies are *P. carinii* cysts.

illustrated in Fig. 2. This series of stains was prepared from a single sample obtained by sputum induction. Panels A and C illustrate the characteristic morphological appearance of both cysts and trophozoites. The organism was frequently found in a frothy clump and was detected as such by DQ and DFA. Panels B and D illustrate the appearance of the cysts alone, as detected by the cyst-specific stains, silver and IFA.

With the exception of one smear, all 58 samples meeting our criteria for positivity were positive by either the silver or the DQ stain. One sample positive by DFA and IFA was negative by the other two methods. Chart review indicated that the clinical presentation of the patient was consistent with *P. carinii* infection. However, since infection was not documented cytologically or histologically, he was not treated with antibiotics, nor was he started on prophylaxis. His symptoms resolved gradually over a period of about 1 month with supportive care.

In a number of cases, there were false-positives which may have actually represented a highly sensitive test result or random sample variability in a patient with a light load of organisms. Among the sputa, there was one false-positive by silver stain (negative by all other methods). A specimen from

the same patient was positive by all four stains 5 weeks later. There were two false-positive sputum samples by DFA (negative by other tests). A subsequent BAL several days later from one of these patients was clearly positive by all four stains, strongly suggesting that one of these false-positives was in fact a true-positive. There were no false-positive IFAs or DQs among the sputum specimens, although in one case, the IFA stained fungal mycelial elements and budding yeast forms on the smear. This could have been mistakenly interpreted as a positive by an inexperienced technician, but for an experienced observer, this in no way resembled the morphology of *P. carinii*.

There was one false-positive BAL by silver stain. This was probably a true false-positive, as the patient was negative by all four stains of a prior sputum specimen and also of a subsequent BAL. There were three false-positives by DFA and one false-positive by DQ. No false-positives were detected by IFA.

Ten patients initially diagnosed as *P. carinii* negative by DQ had follow-up BAL. Of these 10, 3 were subsequently found to be positive for *P. carinii* by DQ. All three of the

TABLE 1. Comparison of four stains for *P. carinii* detection

Stain	Sensitivity (%)		Specificity (%)		Shelf life	Time (min) for:		Cost (\$) per:		No. of slides/kit
	IS	BAL	IS	BAL		Prepn	Reading	Kit	Slide	
Silver	92	86	92	97	1 yr	90	10-15	119	5-10	12-24
DFA	97	90	85	90	9 mo-1 yr	45	<5	225	~10	24
IFA	97	86	100	100	6 mo	90	<5	— ^a	—	45
DQ	92	81	100	97	9 mo-1 yr	3	10-30	70	~1.40	50

^a —, Not available in the United States.

original negative smears were positive by one or both of the IF assays, and two of them were also positive by silver stain.

The four stains were evaluated on cost-effectiveness as well as sensitivity (Table 1). Of the four, the DQ stain was the simplest to prepare, taking approximately 3 min. The reagents involved in this stain are very inexpensive and have a shelf life of more than 6 months. Only small amounts of reagent are required to stain a slide. We estimate the cost of reagents for preparation of a DQ-stained slide to be approximately \$1.40 to \$1.50. The interpretation of the stain is somewhat more difficult, however, and even in a laboratory with experienced personnel, reading these slides may take up to an hour of technologist time.

The silver stain kit, including counterstain, costs approximately \$120.00. One kit includes 12 vials of reagent, each capable of staining two slides. Thus, two patient specimens or a specimen and a control slide could be run simultaneously. The kit has a shelf life of 1 year. A slide takes about 90 min to prepare. The reading time averages 10 to 15 min. A high level of expertise is required to correctly interpret the stain, as fungal elements and certain other background materials also pick up the stain and could be falsely interpreted as positive.

The DFA kit costs \$225.00 and contains enough reagent for 24 tests. It has a shelf life of 9 months to 1 year. The slides take approximately 45 min to prepare and only several minutes to read. They can be scanned rapidly for fluorescence, and since the DFA detects both trophozoites and cysts, interpretation is facilitated. A small amount of non-specific background staining was noted with this test kit, but in most cases, this background material was easily distinguishable morphologically from *P. carinii*. A certain amount of familiarity with the typical morphology of the organism under light microscopy is helpful in correctly interpreting the background staining. Thus, a fairly high level of technical skill is still required for the successful application of this test method.

The IFA kit is currently being sold in Europe. The cost of a kit is approximately \$400.00. One kit contains enough reagent for 45 tests and has a shelf life of 6 months. A slide takes approximately 90 min to prepare and only several minutes to read. Background staining was often very faint on smears prepared according to the instructions of the manufacturer. This sometimes made interpretation of the smear difficult, as it was hard to determine the adequacy of the specimen or to be sure that the slide was in focus. There was very little background fluorescence, with the exception of the fungal elements detected on one slide. Cysts were morphologically unmistakable, making the level of technical expertise required for interpretation of this test somewhat lower than that required for the other stains; however, this stain does not detect trophozoites.

When calculating the cost of implementing any of the above staining methods, it must be noted that additional cost

and preparation time is involved in running controls. None of these kits contain positive controls for *P. carinii*.

DISCUSSION

Clearly, the final choice of a test must be based on multiple factors, including the patient population, the volume of specimens being processed by a laboratory, and the level of technical expertise available. In a high-volume laboratory, for instance, batching of specimens might be a practical solution to the problem of long preparation time. In a low-volume laboratory, shelf life may become an important factor.

The criteria used in this study to define a positive sample were somewhat unconventional, as we did not use the silver stain as the traditional "gold standard." However, the positive IF assays actually were corroborated, in every instance but one, by either the silver stain or DQ. Unfortunately, the clinical history of the patient who was positive only by IFA and DFA is ambiguous; it neither invalidates nor confirms these results. In our calculations of sensitivity and specificity, we have chosen to include this test as a true-positive, because of the clarity of morphology apparent on the immunofluorescent slides of this sample.

Of the four methods evaluated in this study, DQ was the least sensitive and the two IF assays were the most sensitive. DFA was the least specific; all of the other methods approached 100% specificity. None of the differences in sensitivity or specificity were found to be statistically significant by chi-square analysis. All four stains were more sensitive for detection of *P. carinii* in sputum than in BAL. This was probably an artifact. In our patient population, BAL is more often used to evaluate difficult cases, such as those of patients who have been previously diagnosed as negative by IS sampling or prior BAL. They may also be patients with a prior diagnosis of acquired immunodeficiency syndrome who are on prophylactic antibiotics. In many cases, we did not have access to patient records, and so we were unable to evaluate the effect of prophylactic antibiotic treatment on test sensitivity. There may be difficulty in detecting a light load of organisms in patients on prophylaxis. We postulate that there may be some advantage to utilizing a stain which detects trophozoites as well as cysts. The traditional silver stain and the IFA evaluated in this study were both cyst wall stains and did not detect trophozoites.

The DQ stain continues to be a very useful method, as it is inexpensive, quick, and easy to prepare. This stain is effective for recognizing trophozoites as well as cysts. In most cases, a skilled technologist can interpret DQ fairly quickly (we screened for 10 min), and in skilled hands, the sensitivity appears to be approximately 90%. However, in institutions where the positivity rate is low and/or the technologists are not skilled at reading DQ stains, this would not be a reasonable screen.

In cases in which the DQ smear is falsely interpreted as negative, infection is sometimes detected on a follow-up BAL. Of the 100 specimens evaluated in this study, 10 represented follow-up smears on patients initially diagnosed as negative. Three of these were subsequently found to be positive for *P. carinii*. All three of these specimens were positive by one of the alternate testing methods, DFA or IFA, at the time of the original sampling. Thus, if a more-sensitive screening method had been used, these three patients would have been spared a bronchoscopy.

We conclude that each individual laboratory must balance the cost and ease of a testing method against the sensitivity and the desirable goal of preventing unnecessary and invasive clinical procedures.

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