

Immunodetection of *Pneumocystis carinii* in Bronchoalveolar Lavage Specimens Compared with Methenamine Silver Stain

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Immunodetection of *Pneumocystis carinii*, based on immunofluorescence and use of a monoclonal antibody specific for an antigen located within the cyst wall and detectable after trypsin digestion only, was compared with a methenamine silver stain in 553 bronchoalveolar lavage specimens from immunosuppressed patients. *P. carinii* was found by immunofluorescence in 72 (86%) and by silver stain in 68 (81%) of the total of 84 positive samples detected by either or both of these methods.

Pneumocystis carinii is an organism capable of causing fatal pneumonia in immunosuppressed individuals. Patients with AIDS and other groups of patients receiving immunosuppressive chemotherapy, such as transplant recipients and individuals with malignancies, are at increased risk of *P. carinii* pneumonia (6, 7, 11, 13, 15, 18).

Bronchoalveolar lavage (BAL) and transbronchial lung biopsy are the most common techniques for obtaining samples representative of lung infection, with both providing a high sensitivity in the diagnosis of *P. carinii* pneumonia (1, 16, 17). For a definitive diagnosis of *P. carinii* infection, a variety of staining methods, such as toluidine blue, methenamine silver, Gram-Weigert, and Giemsa stains, can be used. Giemsa staining is a rapid method commonly used for screening, and the results can be confirmed with either methenamine silver or toluidine blue staining. Both of the latter methods stain the cyst walls. All of these special stains are nonspecific, and *P. carinii* is identified on the basis of its morphology; it must be distinguished from yeasts and cell debris, always present in these specimens.

Immunological methods, especially immunofluorescence, have been found to be as sensitive as the conventional stains in detecting *P. carinii* (4, 9, 12). During recent years, several commercial monoclonal antibodies have become available, but their specificity and sensitivity vary widely (3, 8) and nonspecific reactions with cell debris and other material in the specimen are also common. To improve the specificity of the immunofluorescence assay, monoclonal antibodies have been developed to detect antigens which are not directly accessible on the surface of the microorganism but which can be exposed on the cyst wall by using digestive enzymes. In the present study with BAL material from an essentially non-human immunodeficiency virus-infected patient population, immunodetection with a monoclonal antibody against an epitope within the *P. carinii* cyst wall was compared with methenamine silver stain, considered the most reliable of the staining methods (14).

Five hundred fifty-three BAL specimens were obtained from organ transplant (heart, lung, heart-lung, liver, or kidney) or

bone marrow transplant recipients and from other immunosuppressed individuals, such as patients with hematologic or other malignancies, all presenting with clinical symptoms and signs of pneumonia. Only a few specimens were from human immunodeficiency virus-infected patients (9 of 553 specimens). In BAL, the bronchoscope was wedged into a segmental bronchus. Buffered saline solution, 20 ml, was infused into the lung and aspirated gently with a syringe; this was repeated five to ten times. The BAL specimens (10 to 20 ml) were centrifuged at 3,000 × g for 15 min and washed with distilled water, and the pellet was cytocentrifuged onto microscope slides; stainings were performed on the same material in parallel.

Immunofluorescence assay. Cytospin-prepared slides were fixed in cold acetone for 5 min and then allowed to dry at room temperature. The preparations were rinsed with a stream of distilled water to remove salts from the specimen. Air-dried preparations were first treated with the enzyme solution, containing 25 mg of trypsin per ml, at 37°C for 30 min to expose the antigen (located within the cyst wall) to the specific monoclonal antibody, which is reactive with both human and rodent *P. carinii* (Shield Diagnostics Ltd., Dundee, United Kingdom). This monoclonal antibody reacts with an antigen which is detectable after enzyme digestion only. The immunofluorescent stain was performed according to the manufacturer's instructions. The slides were incubated with the monoclonal antibody at 37°C for 5 to 15 min in a humidified chamber, rinsed with distilled water, and incubated with fluorescein isothiocyanate anti-mouse antibody at 37°C for 5 to 15 min. After the specimens were rinsed, they were air dried and examined at ×100 to ×1,000 magnification, using a fluorescent microscope. Positive and negative controls were stained in parallel. A positive finding was based on detection of fluorescent cysts with a characteristic morphology. The cysts appeared as round-to-elliptical structures in which the cyst wall was distinct.

Methenamine silver stain. Overnight air-dried Cytospin preparations were fixed with ethanol, and the Grocott modification of Gomori's methenamine silver nitrate technique was performed (5). The silver stain preparations were examined in a reference parasitology laboratory, and positive findings were confirmed by a professional parasitologist. Both the immunofluorescence and the silver stain preparations were examined blindly in different institutions by different physicians. If *P. carinii* cysts were found by either the methenamine silver method or immunofluorescence, the sample was considered

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TABLE 1. Comparison of *P. carinii* detection by immunodetection and methenamine silver stain

Results with silver stain	Results with immunodetection (no. of specimens)		
	Positive	Negative	Total
Positive	56	12	68
Negative	16	469	485
Total	72	481	553

positive. The specificity of both methods was considered to be 100% on the basis of typical cyst morphology of *P. carinii*. For statistical analysis, the χ^2 -test was used.

Among the 553 BAL specimens examined by both immunofluorescence and the methenamine silver method, *P. carinii* was found in 84 specimens (15%) by either or both of the methods. The immunofluorescence assay detected 72 (sensitivity, 86%) and the methenamine silver stain detected 68 (sensitivity, 81%) of the 84 positive specimens. The difference was not significant. The results are demonstrated in Table 1.

The positive findings of *P. carinii* cysts in BAL specimens demonstrated by either immunodetection or the methenamine silver technique are shown in Fig. 1. All positive particles

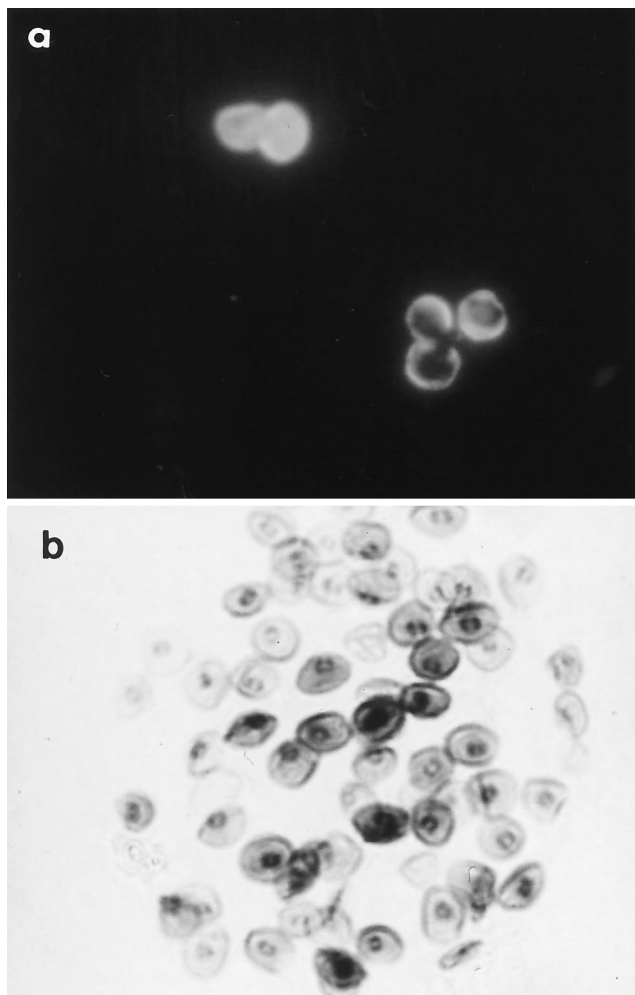


FIG. 1. *P. carinii* cysts in BAL specimens, demonstrated by either immunodetection (a) or methenamine silver stain (b).

detected by the specific antibody also had the characteristic morphology of *P. carinii* cysts. No such immunofluorescence with particles other than *P. carinii*, which could be considered a nonspecific reaction, was seen. The monoclonal antibody did not react with any cell debris or with yeasts which were present in nine specimens, all negative for *P. carinii*.

The false-negative results occurred in cases in which a very low number of *P. carinii* cysts were observed on one slide only, a single method giving a positive result consisting of one to two cysts only. Both methods gave positive findings in 56 (67%) BAL samples. Although the difference in overall sensitivity of each method was not significant, the sensitivity of *P. carinii* diagnosis could still be improved by using both methods in parallel. With a single method only, 12 (14%) positive specimens would have been missed by antigen detection and 16 (19%) positive findings would have been missed by silver stain.

Previous studies have demonstrated that immunofluorescence is a rapid and specific method to detect *P. carinii* in BAL specimens (4, 9, 12) and is even more sensitive than methenamine silver and other conventional staining methods (2, 4). In our study, the number of positive findings by immunofluorescence was slightly higher than that by silver stain, but the difference was not significant. As the method was based on enzyme pretreatment of the cysts to detect a *P. carinii*-specific antigen within the cyst wall, both methods demonstrated the same morphological structure of the microorganism, i.e., the cyst wall. In the specimens which contained only a few cysts, there was a tendency toward easier detection of *P. carinii* with immunofluorescence. This might be due to the bright fluorescent staining which is prominent against the dark background even if there is only one cyst on the slide and the difficulty in detecting one cyst by morphology only in silver stain preparations. False-negative findings could also be due to the low number of cysts in a specimen. In cases with only one or two cysts on a single slide, the positive result was usually given only by one of the methods; seldom were more numerous cysts detected by one method only.

In general, our material consisted of samples obtained from non-AIDS patients, and the positive *P. carinii* findings were less frequent than those described for the AIDS patient population (10). The number of cysts seen in lavages from AIDS patients, who often have fulminant *P. carinii* pneumonia, is frequently much higher, making the laboratory diagnosis easier. The low number of cysts seen in several of our specimens also may have been due to our prophylaxis policy for *P. carinii* with occasional breakthrough infections. An increase in the sensitivity of *P. carinii* diagnosis in cases with only a few cysts was seen by using both methods in parallel. The sensitivity probably could have been increased further by including a third specific method, e.g., PCR technology (2). However, the lack of a generally accepted gold standard for the diagnosis of *P. carinii* pneumonia makes the evaluation of the sensitivity of each test rather difficult.

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