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Optimal Use of the Cytocentrifuge for Recovery and Diagnosis of *Pneumocystis carinii* in Bronchoalveolar Lavage and Sputum Specimens

VEE J. GILL,* NANCY A. NELSON, FRIDA STOCK, AND GLORIA EVANS

Microbiology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20892

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To facilitate the diagnosis of *Pneumocystis carinii* from bronchoalveolar lavage and sputum specimens, we have defined conditions for optimal use of the cytocentrifuge for this purpose. Centrifugation in the cytocentrifuge at 1,200 rpm for 10 min yielded the best recovery of *P. carinii*. To reliably ensure complete absorption of the fluid specimen from the cytocentrifuge chamber, it was necessary to use two absorption filters simultaneously. Different methods of treating induced sputum with mucolytic agents to process sputum with the cytocentrifuge were tried. Results of these studies and our current method for treating sputa are discussed. Comparisons of slides prepared by traditional centrifugation and by cytocentrifuge processing showed the latter to be equally effective for detecting *P. carinii*. The most prominent advantage of the cytocentrifuge was the much smaller area to review and consequently the shortened time required to read the slides.

Diagnosis of *Pneumocystis carinii* pneumonia, particularly in patients with the acquired immunodeficiency syndrome, is being made more frequently from bronachoalveolar lavage (BAL) specimens and from sputa (1, 6, 7). Microbiology laboratories have become increasingly involved with the prompt identification of this organism, which previously was identified primarily by pathology laboratories. This change was made possible by the availability of simple and rapid stains such as the toluidine blue O (TBO), Giemsa, and rapid silver stains, coupled with the profusion of *P. carinii* organisms usually present in BAL and sputa from patients with the acquired immunodeficiency syndrome.

The quantity of BAL fluid submitted for examination varies according to institutional protocols. In our hospital, we usually receive 20 to 30 ml of turbid, watery BAL fluid. To concentrate the *P. carinii*, we had been centrifuging 20 ml of BAL for 15 min at 3,000 × *g* in our laboratory. The pellet was then used to prepare a slide for examination. To simplify and perhaps improve on this procedure, we evaluated the potential usefulness of cytocentrifuge-prepared slides as an alternative to our standard centrifugation procedure. To establish an optimal cytocentrifuge procedure we studied the technical aspects involved and then compared the sensitivity of the cytocentrifuge slides with that of the standard slides for the detection of *P. carinii* in BAL specimens. The procedure was later modified to accomodate processing of induced sputa, which are usually more viscous than BALs. Cytocentrifuge-prepared slides of BAL and sputum specimens are easy to prepare and provide an effective means of concentrating *P. carinii*.

MATERIALS AND METHODS

Due to lack of information on the safety of the cytocentrifuge for infectious agents such as the human immunodeficiency virus we take the following safety precautions when using the cytocentrifuge. The rotor head is carried into a biological safety cabinet after centrifuging before the lid is removed. All handling of the specimen and processing steps through the cytospin are done with gloves on. Nondisposable chambers are disinfected after every use by soaking in 10% hypochlorite solution for 2 h, and the metal clips are soaked in 95% alcohol for 2 h. The bowl of the cytocentrifuge is also wiped out with disinfectant after each use. We feel that these safety guidelines must be adhered to since the majority of specimens for *P. carinii* are from human immunodeficiency virus-positive patients.

Cytocentrifuge and filters. The Cytospin 2 (C2; Shandon Southern, Sewickley, Pa.) was used throughout these studies. Two types of filters were available for this instrument; thin brown filters (catalog no. SCA-0005-1) designed for slower absorption and therefore used with smaller volumes of specimen (0.4 ml or less) and thick white filters (catalog no. SCA-0005) for extra absorption and suitable for 0.5-ml volumes. For these studies we used nondisposable specimen chambers with brown and white filters as described below.

Determination of optimal filters to use. A maximum volume of 0.5 ml can be used in the C2 specimen chambers. Preliminary experiments processing 0.4 to 0.5 ml of BAL with the white filter (recommended for these volumes) showed that on occasion not all of the BAL was absorbed from the chamber during centrifugation, particularly when the specimen was tinged with blood or thicker than usual due to either leukocytes or mucus. Experiments were done with multiple filters (various combinations of white and brown filters), either together or sequentially, to prevent this problem.

Determination of optimal spin speed for detection of *P. carinii*. The C2 can be adjusted between 0 and 2,000 rpm; the C2 handbook, however, suggests that speeds above 1,000 rpm may lead to filter compression and incomplete absorption of specimen as well as to some distortion of cellular elements. Using BALs known to be positive for *P. carinii*, we performed experiments with different speeds to compare recovery of *P. carinii* at these speeds. For these studies centrifugations were done for 10 min with one brown filter and one white filter simultaneously.

Preparation of BAL smears by standard centrifugation (SC). BAL (20 ml) was spun in an IEC centrifuge (Centra 8;

* Corresponding author.

International Equipment Co., Needham Heights, Mass.) at 3,000 × g for 15 min. Most of the supernatant fluid was decanted off, leaving 1 ml of fluid along with the pellet. The pellet and residual fluid were vortexed vigorously to suspend the pellet, resulting in a 20-fold concentration of the original BAL. A drop of this concentrate (approximately 0.05 ml) was placed on a glass slide and spread in an oval approximately 2 by 3 cm. Assuming perfect concentration, examination of this entire drop would be equivalent to examining 1 ml of the original BAL.

Preparation of BAL smears with the C2. C2 BAL smears were prepared by centrifuging 0.4 ml of BAL fluid at 1,200 rpm for 10 min with one white filter and one brown filter simultaneously. Theoretically, all particulate matter, including *P. carinii*, should be deposited in the C2 circle, which is 0.8 cm in diameter. Examining the entire circle is thus equivalent to examining 0.4 ml of the original specimen.

Comparison of C2- versus SC-prepared slides for recovery of *P. carinii*. Twenty-eight known positive BAL specimens were processed by both the SC and C2 procedures described above. Both smears were dried on a heating block and stained by a modified TBO procedure (3). Each slide was read either in its entirety if negative or until unequivocal *P. carinii* were seen. Each slide was read by two readers to confirm the positive or negative findings.

Processing of induced sputa for C2-prepared slides. Preliminary experiments were done with either KOH, *N*-acetylcysteine or dithiothreitol (DTT; Sputolysin Stat-Pak, Behring Diagnostics, San Diego, Calif.) to liquefy sputum so that it could be processed with the C2. Sputa positive for *P. carinii* were treated for up to 10 min with 5% KOH, 5% *N*-acetylcysteine (final concentrations), or DTT. DTT was eventually selected for routine use. DTT was diluted in 100 ml of sterile distilled water (final concentration, 6.5×10^{-3} mol/liter) according to the manufacturer's instructions and then stored in a refrigerator for up to 2 days. Sputum (2 ml) was placed into a 15-ml conical centrifuge tube. Then 4 ml of the diluted DTT was added, and the tube was capped securely and vortexed for 15 to 30 s. The tube was then incubated in a 37°C water bath for 3 to 5 min and vortexed again. Then 6 ml of phosphate-buffered saline (0.85% NaCl, pH 7.4) was added immediately, and the material was vortexed for 10 s. The tube was centrifuged at 2,500 × g for 5 min; the supernatant fluid was removed and discarded except for approximately 1.5 ml of residual supernatant fluid, which was left to suspend the pellet. This material was vortexed for 10 to 15 s, and 0.2 to 0.3 ml was used to prepare C2 slides. Two slides were prepared, one with 0.2 ml and one with 0.3 ml to obtain preparations of different density.

Efficacy of sputum concentration technique for detecting *P. carinii*. Sixty-eight induced sputum samples were processed for *P. carinii* as described above. In all but four instances the patient also underwent a BAL procedure within a week of induction of sputum (most were within 2 days). The BAL was processed as described above. In four instances a BAL procedure was considered contraindicated, and a second induced sputum sample was processed instead.

Final cytocentrifuge procedures based on above studies. Before cytocentrifugation, induced sputum samples should be processed with DTT as described above. BALs and processed induced sputa should then be handled as follows.

(i) Add 0.4 ml of BAL or 0.2 to 0.3 ml of processed induced sputum to the cytocentrifuge specimen chamber. Prepare at least two slides for each specimen. Volumes may need to be adjusted according to quality of the specimens (purulent, bloody, etc.).

TABLE 1. Recovery of *P. carinii* at different C2 speeds

Speed (rpm)	Total no. of *P. carinii* within C2 circle in BAL:		
	1	2	3
900	175	18	75
1,000	ND[a]	35	110
1,050	225	ND	ND
1,100	ND	39	130
1,200	265	69	179
1,300	ND	30	125
1,350	200	ND	ND
1,400	ND	13	110
1,500	100	ND	ND

[a] ND, Not done.

(ii) Use one brown and one white filter simultaneously, placing the brown filter next to the glass slide.

(iii) Centrifuge for 10 min at 1,200 rpm.

(iv) Remove the specimen chamber carefully and check to see that most or all of the specimen has been absorbed. If the specimen was too mucoid or bloody, there may be specimen left in the chambers. Smaller quantities or more dilute material should then be centrifuged.

(v) When removing filters, be careful not to pull the specimen off the slide; this may happen with a mucoid specimen.

RESULTS

Determination of optimal filters to use. For 0.4 to 0.5 ml of either BAL or processed sputum, a single white filter was not always sufficient to absorb all the fluid from the C2 specimen chamber. This occurred most frequently when the specimen was bloody, purulent, or mucoid. Attempts to recentrifuge these chambers by placing them back in the cytocentrifuge and spinning for an additional 10 min resulted in no further absorption of material, suggesting that the filter was clogged and could absorb no more specimen. Taking these partially emptied chambers, it was possible to carefully remove the first filter and insert a second white filter and respin for 10 min. This resulted in complete absorption of the residual specimen. Since this is technically difficult to do, we tried using two filters simultaneously to circumvent this problem. Two white filters were too thick, resulting in interference with proper swinging out of the slide holders during centrifugation. Two brown filters were not absorbent enough to absorb all the specimen. The use of one white and one brown filter was found to be satisfactory for most specimens; positioning the brown filter next to the glass slide produced better slide preparations.

Determination of optimal C2 speed for detection of *P. carinii*. In preliminary experiments with a BAL positive for *P. carinii* we compared speeds of 300, 600, 900, 1,200, 1,500, and 1,800 rpm to determine the setting for maximal recovery of *P. carinii*. These experiments showed a peak recovery at 1,200 rpm, with less recovery at speeds both above and below 1,200. Table 1 shows follow-up experiments looking at the range between 900 and 1,500 rpm in 100- to 150-rpm increments. The same pattern of recovery was found for three different BAL specimens, indicating an optimal speed of 1,200 rpm for *P. carinii* organisms.

Comparison of C2- versus SC-prepared slides for recovery of *P. carinii* from BALs. Using 28 different specimens positive for *P. carinii* by TBO and fluorescent antibody (2), we compared the sensitivity of the cytocentrifuge procedure to

our conventional procedure with a standard centrifuge. For the first 16 specimens, we concentrated the original specimen twofold by conventional centrifugation before inoculating the C2 chamber with 0.4 ml of specimen. By doing this concentration step first, the C2 slides were more closely comparable in the volume of the original specimen (approximately 0.8 ml) to the SC-prepared slides. All 16 of the C2 preparations were positive. For the subsequent 12 specimens, BAL fluid was used directly without prior concentration. All 12 of these specimens were also positive by the C2 procedure, resulting in 100% detection by the C2 procedure.

Processing of induced sputa for C2-prepared slides. The use of 5% KOH resulted in good liquefaction of the sputum specimens but produced distortion of the *P. carinii* cysts, which became both smaller and less clearly demarcated by TBO stain. Both *N*-acetylcysteine and DTT treatments were effective for sputum liquefaction with minimal distortion of organisms, but DTT was more practical to use due to the commercial availability of premeasured reagent. Treatment time studies with DTT showed that, depending on the viscosity of the specimen, incubation periods of 3 to 5 min were usually sufficient to liquefy the specimen and render it suitable for C2 processing. On occasion, a very purulent or mucoid specimen might have to be incubated longer. The effect of DTT on *P. carinii* morphology by TBO staining was minimal for incubation periods up to 10 min at 37°C. Beyond 10 min, the organisms looked smaller and less distinct. For this reason, it is important to add the phosphate-buffered saline promptly to dilute the DTT and stop any further deleterious action on the *P. carinii*. It is also important to check each lot of DTT to make sure that the morphology of *P. carinii* remains acceptable for up to 10 min of incubation.

Efficacy of sputum concentrating technique for detecting *P. carinii*. Among the 68 induced sputum samples that were treated by the DTT concentration technique, 18 (26%) were positive, whereas 50 (74%) were negative. In four instances an initial positive sputum sample was verified with a second positive sputum rather than by a BAL. One of the 68 sputum samples had an uninterpretable TBO result due to the presence of many yeast cells; the matching BAL was positive. On three occasions the induced sputum was negative and the matching BAL was positive. The false-negative sputum results may have been related to poor quality of the induced sputa rather than light infection, since two of the three patients had follow-up BALs that had moderate to many *P. carinii* organisms.

DISCUSSION

Use of the cytocentrifuge for microbiologic in additon to cytologic purposes has been suggested in recent years. Shanholtzer et al. (8) recommended cytocentrifuge-prepared slides for cerebrospinal and other body fluid Gram stains, whereas Kahn and Jones (4) have described their use of cytocentrifuge-prepared slides on BALs to detect bacteria, fungi, and *P. carinii* as well as herpesvirus and cytomegalovirus. Martin and Smith (5) likewise described the use of cytocentrifuge preparations for detection of cytomegalovirus in BAL specimens. The latter two papers do not provide information on the selection of the conditions chosen for use of the cytocentrifuge, whereas the paper of Shanholzer et al. defined conditions designed for optimal recovery of bacteria.

Proper use of the C2 to prepare slides for cytology entails attention to the number of tissue cells within the fluid to be examined, along with appropriate adjustment of the number of cells per milliliter to get a preparation optimal for staining and cell morphology (C2 handbook). Although this adjustment is ideal for obtaining good distribution and morphology of tissue cells, it is not pertinent for concentrating *P. carinii*. We therefore elected to prepare slides without regard to the number of tissue cells. Because of this, some preparations were fairly thick, particularly with sputum specimens, due to cellular, bacterial, or fungal elements in the background. In most instances, however, this was not found to interfere with the detection of *P. carinii*, although on occasion it was necessry to centrifuge smaller quantities of material to obtain readable slides.

Due to tissue cells, mucus, and other debris in BALs and sputa, it was not surprising that on occasion the filters became clogged and did not allow complete absorption of the specimen (particularly when only one filter was used). By using one brown filter and one white filter simultaneously we now only rarely encounter this difficulty. The brown filter should be placed adjacent to the glass slide since this seems to yield better preparations than when the white filter is positioned next to the glass slide. More recently we have changed to using disposable specimen chambers that have a white filter already attached to the chamber. These can be used in combination with a brown filter and also result in good C2 slides.

Finding a peak recovery of *P. carinii* at 1,200 rpm was unexpected, since we expected that recovery would reach a maximal level at a certain rotation speed and then remain constant at higher speeds as long as complete absorption of specimen was maintained. The observation that fewer *P. carinii* organisms were seen at speeds above 1,200 rpm, even though all of the specimen was absorbed out of the chamber, was a consistent finding. One possible explanation may be that the faster speeds tend to drag the *Pneumocystis* cysts out into the filter along with the specimen fluid due to the higher centrifugal force applied.

Our results comparing C2- and SC-prepared slides showed that the two preparations were equally sensitive for detecting *P. carinii*. Even without initially concentrating the BAL, we obtained comparable results between the two methods. C2 slides can be prepared quickly and easily. Since only small volumes need to be used (0.4 ml of BAL per slide), the remainder of the specimen is available to be concentrated for fungal, mycobacterial, or other culture purposes. The major advantage, however, is that the area of material to be looked at is confined to an 8-mm-diameter circle, as opposed to the significantly larger area on a SC prepared slide. On SC slides, the pellet must be spread out over the slide to yield readable smears. The time to completely review a C2 slide is very short, and two smears can usually be screened in less than 10 min. Our current procedure for BALs is to prepare two C2 slides, using 0.4 ml for each. Both slides are read in their entirety before the specimen is considered negative. On rare occasions when the BAL is either very purulent or bloody, it may be necessary to process the specimen with DTT, as described for sputum, before putting the material through the cytocentrifuge.

It is important to use a mucolytic agent to process sputum, even induced sputum, to improve the quality of C2-prepared slides. Mucus tends to interfere with good absorption of the sputum into the filter and may also form a thin film which lifts off from the glass slide (effectively removing the entire specimen) when the filter is lifted and removed from the glass slide. The DTT treatment described here is fast and simple and thus is suitable for practical laboratory use. Our results with C2-prepared sputum slides for diagnosing *P. carinii* pneumonia showed that the method was fairly sensi-

tive for detection of *P. carinii*. Due perhaps to variability in the quality of induced sputum, the possibility of low organism burden, and the occasional patient heavily colonized or infected with yeast cells, it is still prudent to consider a BAL procedure for all patients with a negative sputum smear. Extra caution must be used in interpreting TBO smears of sputa as compared to BAL smears because of the larger number of yeast cells frequently encountered. Expectorated sputum often contains heavier amounts of yeast cells than induced sputum, and for this reason we discourage the use of expectorated sputum. An experienced reader may be able to make the distinction between yeast cells and *P. carinii* with confidence, but on occasion it may be very difficult. For this reason, the use of a fluorescent antibody (6) or other specific antibody procedures would be more desirable than nonspecific stains such as TBO, silver, or Giemsa stains for accurate diagnoses to be made from sputum.

The cytocentrifuge is a useful, practical instrument for helping establish the diagnosis of *P. carinii* pneumonia. The studies reported here establish some guidelines that may be helpful to others seeking to simplify and optimize their current procedures for handling BALs or sputa for *P. carinii*. Our current procedures based on these studies are outlined in Materials and Methods.

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