Modified Toluidine Blue O Stain for *Pneumocystis carinii*: Further Evaluation of Some Technical Factors

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A modified toluidine blue O (TBO) stain for *Pneumocystis carinii* cysts was evaluated with regard to the influence of (i) the age and extent of use of the sulfation reagent, (ii) the source of TBO, (iii) the TBO content of the staining solution, and (iv) the amount of TBO present in the alcohol wash solutions. All TBOs evaluated, except for a new TBO obtained from Roboz Surgical Instrument Co., Inc., Washington, D.C., produced satisfactory results. Each lot of TBO should be quality controlled before use to ensure that the *P. carinii* cysts stain lavender against a blue background. We have ourselves decided to use only certified TBO with a high dye content. As extensively used sulfation reagent provided less satisfactory results than did either freshly prepared or 1-week-old unused sulfation reagent, we have decided to prepare fresh sulfation reagent at least weekly and to discard used sulfation reagent after 10 slides have been processed.

We previously described the advantages of a modified toluidine blue O (TBO) stain for the diagnosis of *Pneumocystis carinii* pneumonia (1). Since that report, we have noticed some variability in both the intensity and the color of staining of the *P. carinii* cysts. Therefore, we investigated the influence of the following variables on the staining results: (i) the age and extent of use of the sulfation reagent, (ii) the source of TBO, (iii) the TBO content of the staining solution, and (iv) the amount of TBO present in the alcohol wash solutions.

**Specimens.** Touch preparations of lung tissue of rats with *P. carinii* pneumonia were used.

**Staining reagents.** *(i) Sulfation reagent.* A 250-ml clear glass bottle containing 90 ml of glacial acetic acid (J. T. Baker Chemical Co., Phillipsburg, N.J.) was placed in a basin containing cold running tap water. Concentrated sulfuric acid (30 ml; Mallinckrodt, Inc., Paris, Ky.) was added slowly (over approximately 15 s) and then mixed by gentle swirling of the bottle. Approximately 40 ml of the mixture was transferred to a clean Coplin jar, and the remainder was stored in the glass bottle at room temperature for up to 1 week. The Coplin jar was kept covered and sealed with petrolatum, except when slides were added or removed or when the reagent was stirred. With the jar sealed, the petrolatum helps to prevent odors, may prolong the life of the stain, and, in our experience, does not produce any contamination problems.

(ii) **TBO solution.** TBO was obtained from the following suppliers: (i) Aldrich Chemical Co., Inc., Milwaukee, Wis. (certified dye; lot 91814BP; dye content, 94%; catalog number, 19816-1); (ii) Eastman Kodak Co., Rochester, N.Y. (certified dye; lot A14A; dye content, 50%; catalog number, 1133420); (iii) Fisher Scientific Co., Fair Lawn, N.J. (certified dye; lot 864819; dye content, 50%; catalog number, T161-25); (iv) Roboz Surgical Instrument Co., Inc., Washington, D.C. (an old lot of dye; labeled as "Certified for use in: Histology"; lot unknown; dye content, 52%; catalog number unknown); and a new lot of uncertified dye of unspecified dye content; lot 92-31-9; catalog number, 1B481); and (v) Sigma Chemical Co., St. Louis, Mo. (an uncertified dye; lot 66F-3689; dye content, approximately 90%; catalog number, T3260; and a certified dye; lot 56F-6181; dye content, 91%; catalog number, T0394). For each staining solution, 0.15 g of TBO was placed in a 250-ml Erlenmeyer flask, to which was added 30 ml of filter-sterilized distilled water. The flask was swirled, and 1.0 ml of concentrated hydrochloric acid (J. T. Baker Chemical Co.) was added, followed by 70 ml of 95% ethyl alcohol. (We previously used absolute ethyl alcohol; 95% ethyl alcohol produces identical results.) The solution was mixed by swirling until the TBO was completely dissolved and was stored in a clear glass bottle at room temperature. Staining was done in a covered Coplin jar. (We have found TBO solutions to be usable for at least 1 year.)

**Other reagents.** Other staining reagents used were 95% ethyl alcohol, absolute ethyl alcohol, and Xylene (Columbia Diagnostics, Springfield, Va.). All were used in Coplin jars.

**Staining procedure.** The staining procedure used was the more rapid technique described previously (1) in which only one wash in 95% ethyl alcohol and one wash in absolute ethyl alcohol were used. Xylene was used instead of xylene.

**Evaluation of slides.** The slides were evaluated jointly by two observers (F. G. W. and J. W. B. A.), who were aware of the staining procedures, and were then coded and re-reviewed independently by two other observers (V. J. G. and J. D. M.), who were not aware of how the slides had been stained. Only interpretations on which all observers agreed were considered significant.

**Evaluation of sulfation reagents and TBOs.** Three different sulfation reagents were evaluated. One was a solution that had been used for 1 week after it had been prepared; 34 slides of clinical material had been processed through this solution before it was used for this study. Another sulfation reagent was an unused portion of the same 1-week-old batch that had been used for the clinical specimens. The third sulfation reagent was prepared just before use. Separate solutions of each of the seven different TBOs were prepared as described above. In addition, a half-strength solution of the Aldrich dye was prepared with 0.07 g of dye dissolved in the same volumes of solvents used for the full-strength dyes. Three separate slides, each processed with one of the different sulfation reagents, were stained with each TBO.

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Evaluation of alcohol wash solutions. To simulate 95% and absolute ethyl alcohol solutions that had been used extensively, we added several drops of three different TBO solutions (Aldrich, Aldrich one-half strength, and Kodak) in combination to alcohol solutions through which approximately four slides had already been processed, until the solutions were a darker blue than is considered acceptable for use. These solutions were the “used” alcohols. A comparison was made with fresh alcohols. For this part of the study, the same freshly prepared sulfation reagent was used for all slides, and three different TBO solutions (the same ones added to the alcohols) were used to stain the slides.

There was no difference in the staining intensity of either P. carinii cysts or background material with the unused 1-week-old sulfation reagent as compared with the freshly made sulfation reagent. However, both organisms and background material retained noticeably less intensely with the used sulfation reagent.

The only definite difference noted in a comparison of the TBOs with one another was that the new material obtained from Roboz stained everything blue or blue-purple. All the other dyes stained the P. carinii cysts lavender, while the background stained blue. There was no difference in staining intensity with variations in the dye content or in the amount of dye (full strength versus half strength) used to prepare the staining solution. There was no difference in staining with certified and uncertified dyes.

For a given TBO, simulated used and fresh alcohol wash solutions produced no difference in the final staining intensity.

Since our original description of the modified TBO procedure for P. carinii cysts was published, we have received some comments about problems with the stain. We ourselves were surprised at the blue-purple rather than lavender of the P. carinii cysts when we first used a new lot from the same supplier who had provided the dye we used originally. The color of the new dye solution was distinctly different from that of the old dye solution. The difference in cyst color led us to evaluate TBOs obtained from several other sources; all these other TBOs produced tinctorial characteristics identical to those we had observed originally. We have no explanation for the problems encountered with the new dye obtained from Roboz. With all the dyes, including the one from Roboz, the P. carinii cysts stained, but the organisms were more easily discriminated from background material when they stained lavender in contrast to the blue of the background. This was particularly the case when staining at a lower power. We therefore recommend that TBO from Roboz not be used for this staining procedure until material is available which stains P. carinii cysts the same lavender as that produced by TBOs from other suppliers. Also, we recommend that every new lot of TBO be evaluated before use by comparing it with a lot already known to produce satisfactory results, with cysts and background both staining with moderate intensity but different tinctorial characteristics.

We would like to emphasize that yeast cells, especially in sputum specimens, may present a major interpretive problem; the morphology of some yeast cells can be identical to the morphology of P. carinii cysts, and single yeast or P. carinii organisms are often indistinguishable from each other.

Given the number of specimens we routinely process, to maintain adequate staining intensity we now routinely replace our sulfation reagent twice a week. We prepare one batch of this reagent a week; the first half is put out on Tuesday, and the second half is put out on Friday. We have arbitrarily decided not to process more than 10 slides without replacing the sulfation reagent; when such a high workload occurs, we may need to prepare additional sulfation reagent. The working solution of TBO in a Coplin jar is replenished when necessary; it is completely replaced monthly. The used alcohols are replaced when they become moderately blue; generally, this means they are replaced after two to four slides have been processed through them. Xylene is replaced monthly or more often if it becomes cloudy. A control slide is stained weekly with each new batch of sulfation reagent to ensure that all the reagents are functioning properly. Other laboratories may want to determine for themselves the optimal frequency for replacing sulfation reagent and for staining control slides.

Although we were not able to discern any definite differences among the different dyes we tested as a function of dye content or certification, we have arbitrarily elected to use certified material of high dye content and therefore have been using the TBO obtained from Aldrich. Staining results obtained with this material on a variety of clinical specimens have been entirely satisfactory.

LITERATURE CITED