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Evaluation of a Dual-Staining Method for Acid-Fast Bacilli

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A dual-staining procedure for acid-fast bacilli was found to have poor correlation with the Ziehl-Neelsen and auramine-rhodamine staining techniques.

A method for the simultaneous staining of mycobacteria (2), similar to that of Fusillo and Burns (1), was compared with the conventional Ziehl-Neelsen and auramine-rhodamine staining techniques. This dual method incorporates auramine O, Kinyoun carbol fuchsin, and acid alcohol with methylene blue counterstaining. This procedure is reported to be simpler and faster to perform than either of the conventional techniques (2). Smears can be scanned for fluorescing bacilli and confirmed for aniline-dyed bacilli if so desired.

Two hundred and fifty sputums, randomly picked from clinical specimens, were examined in a double-blind study. All specimens were digested and concentrated by the acetyl-cysteine-alkali procedure (4), inoculated onto Lowenstein-Jensen agar, incubated at 37 C in 5% CO_2, and observed for growth over an 8-week period. Three slides were made from each concentrated sputum. All slides were heat fixed on an electrc slide warmer before being stained. One slide was stained by the Ziehl-Neelsen method (5) and examined for the presence of acid-fast bacilli under the ×100 oil immersion lens. The second slide was stained by Truant's auramine-rhodamine technique (3) and scanned for fluorescing mycobacteria with the ×10 objective and confirmed with the ×40 objective. The third slide was stained by the dual-staining technique. (Ingredients for this procedure are distributed by Bac-T-Reagents, N. Kingston, R.I. under the name of the Dual AFB Stain Kit.)

Procedure for dual staining. (i) Flood smear with auramine O solution for 3 min; (ii) drain slide; (iii) stain with Kinyoun carbol fuchsin solution for 2 min; (iv) rinse with tap water; (v) decolorize and counterstain with acid alcohol-methylene blue solution for 2 min; (vi) rinse with tap water and air dry.

The dual-stained slides were initially scanned with a ×10 objective and checked with a ×100 oil immersion lens under the fluorescence microscope. Bright field microscopy confirmation on each slide was done with the ×100 oil immersion objective. All slides were routinely scanned three times horizontally. For the fluorochrome stains, a Leitz Orthoplan microscope equipped with a Ploem incident light illuminator, a BG-12 excitor filter, and an OG-510 barrier filter was used.

Comparison of the staining results with each other and with growth are summarized in Table 1. All cultures were identified as *Mycobacteria tuberculosis* except one group III *M. avium* complex. Of the 250 specimens processed, 216 were negative by all staining techniques and did not produce growth of mycobacteria. Thirteen specimens grew mycobacteria and were detected by all stains, whereas six specimens were missed by both of the dual stains, five specimens were missed by the dual fluorochrome stain, and four by the dual acid-fast stain. It can also be seen that two sputums which produced growth of mycobacteria were detected only by fluorochrome stains and, one sputum was positive for growth and detected only by the Ziehl-Neelsen stain. The remaining three specimens had various staining reactions but were negative for growth. Patient history and final diagnosis in these three cases indicated previous positive mycobacteria culture, bronchiectasis with gram-negative bacilli suprainfection, and bronchogenic carcinoma. Statistical comparison of the different staining results was done using the binomial test. There was a significant disagreement, $P < 0.01$, between the dual auramine and the auramine-rhodamine stains with the former having fewer positive results. The acid-fast stains also disagreed significantly, $P < 0.005$, with the dual again having fewer positives. There was no statistical difference between the Ziehl-Neelsen and auramine-rhodamine results. From a practical point of view, the double-staining system completely missed seven positive sputums and, a total of 11 more were missed by one or the other of the dual stains using growth as the standard for comparison.

TABLE 1. *Results of the dual, Ziehl-Neelsen, and Auramine-rhodamine stains for detection of mycobacteria*

Number of specimens	Dual stain		Ziehl-Neelsen	Auramine-rhodamine	Growth
	Acid fast	Auramine			
216	−	−	−	−	−
13	+	+	+	+	+
6	−	−	+	+	+
5	+	−	+	+	+
4	−	+	+	+	+
2	−	+	−	+	+
1	−	−	+	−	+
1	−	+	−	+	−
1	−	−	−	+	−
1	−	+	−	−	−

Staining one smear by the combination technique was faster and easier than staining two smears by the Ziehl-Neelsen and auramine-rhodamine methods. Because background fluorescence wth the dual auramine stain was a problem however, mycobacteria were difficult to see under the ×10 or ×25 objectives and a ×100 oil immersion lens had to be used for scanning. This factor made scanning time for the dual auramine stain equivalent to that of an acid-fast stain, and also could be a reason for missing so many positive smears. The dual acid-fast method did not seem to sufficiently decolorize smears with a resultant difficulty in finding mycobacteria due to the darkly stained background. Perhaps this condition was due to the combination of acid alcohol and methylene blue.

Since fluorescence, in the case of mycobacteria, is equivalent to acid-fastness with the prime factor being retention of the dye after exposure to acid alcohol, we feel double staining is generally unnecessary. For those who wish to confirm fluorescing bacilli by the use of an acid-fast stain, we recommend restaining the smear by the Ziehl-Neelsen technique (5).

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