TEST EVALUATION
Steve Gerard, M.D.

TEST

Acid-Fast Stains (AFB Stains)

METHOD

Both the Auramine-Rhodamine (AR) (1) and the Kinyoun staining methods rely on the nearly unique property of mycobacterial cell wall to resist destaining by acid-alcohol. Destaining is followed by application of an appropriate counterstain. Although the AR stain employs fluorescence, the staining procedure does not involve the use of a labelled antibody.

Sputum specimens, which account for the vast majority of all specimens submitted, are first "decontaminated" with 2% NaOH containing 0.5% N-acetyl-L-cysteine. The alkali destroys non-mycobacterial, primarily gram-negative bacterial contaminants, the sulfhydryl reagent, reducing specimen viscosity to facilitate decontamination and subsequent concentration of organisms by centrifugation. Other specimens to which AFB staining may be applicable include CSF, urine, bronchial biopsies, bone marrow, gastric aspirates, and other "sterile" body fluids or tissues. Heat-fixed smears are stained initially with AR and examined by fluorescent microscopy. Acid-fast bacilli (AFB) appear as bright, yellow-orange, thin small rods against a black background. Scanning AR-stained smears can conveniently be performed using a 25X objective, without the need for high-power, oil-immersion magnification. AR-positive smears are subjected to Kinyoun staining of either the fluorescence-positive smear or of another smear prepared from the same specimen. By light microscopy using the oil-immersion objective, Kinyoun-stained AFB appear as small red rods ("red snappers") against a blue background. Morphology of the organisms by either staining method will vary with the species of mycobacteria present (see below under "Clinical and Technical Limitations").

Smears are reported as positive only if confirmed by Kinyoun staining, along with an estimation of the number of organisms seen. Reports of an AR-positive but Kinyoun-negative specimen are verbally communicated to the clinician by the Laboratory Medicine resident.

CLINICAL APPLICATIONS

Laboratory confirmation of mycobacterial disease ultimately requires the isolation of the organism. Since mycobacteria require 2-6 weeks to grow in culture, preliminary visualization of AFB by acid-fast staining is potentially of great value. A positive result in an appropriate clinical setting assists in making a tentative diagnosis and in deciding to initiate treatment with antimycobacterial drugs prior to confirmation by culture.

In pulmonary mycobacterial disease, effective drug therapy is followed by a variably prolonged phase during which non-viable but AFB-stainable mycobacteria are shed; ultimately the organisms gradually decrease and disappear from the sputum. Thus, staining of serial sputa over the course of drug therapy may assist in confirming the effectiveness of the antimicrobial regimen employed.
When suggestive colonies grow in AFB cultures, they are stained to reconfirm their probable mycobacterial nature.

**CLINICAL AND TECHNICAL LIMITATIONS**

There are several sources of technical error in specimen processing which can give rise to erroneous results:

1. Overly harsh decontamination of sputum specimens by prolonged exposure to NaOH can render AFB-stainable mycobacteria non-viable, and therefore non-cultivable.

2. Prolonged storage of gastric aspirates prior to neutralization of acidity can likewise compromise the viability of AFB-stainable organisms.

3. Non-pathogenic mycobacteria indigenous to water supplies may contaminate staining solutions (2,3). Some laboratories routinely use Millipore-filtered water for all staining solutions, since autoclaving alone does not necessarily destroy the stainability of non-viable contaminants.

4. Cross-contamination of specimens may occur during decontamination, e.g., a common pipette used to dispense the reagents or with the use of a common slide-staining chamber.

5. Acid-fast artifacts, which tend to be more of a problem in the AR stain, may be misinterpreted. The ability to accurately differentiate these artifacts from more representative AFB depends heavily on the skill and experience of the observer. It is to minimize this source of error that Kinyoun stains are done on AR-positive specimens, and it is generally recommended that a repeat specimen be obtained when particularly questionable or ambiguous smears are observed.

In addition to the technical considerations above, clinical variables may contribute to an incorrect laboratory result:

1. Specimens obtained from partially-treated patients may yield stainable, non-viable organisms, as suggested above under "Clinical Applications."

2. Clinical specimens, especially gastric aspirates and urine specimens, may contain non-pathogenic mycobacteria as part of the normal flora. These organisms may not be morphologically distinguishable from pathogenic species.

AFB stains are highly specific for mycobacteria, the literature usually reporting results of greater than 98%. While Nocardia and some species of Actinomyces are also acid-fast to a variable extent, false-positive results attributable to these organisms rarely cause clinical problems.

The most significant potential limitation of AFB staining is its low sensitivity compared to culture. In 1975, Boyd and Marr (4) presented their experience with AFB staining and indicated a sensitivity of only 22% and a positive predictive value
of only 45%. On this basis, they suggested that routine acid-fast staining of specimens submitted for AFB culture should be discontinued. In contrast to the data of Boyd and Marr, however, numerous other workers have reported estimates of 45–75% for sensitivity and predictive values of 80–90% (5–16). The observations of Boyd and Marr have been criticized for poor control over some of the technical factors noted above as well as to a low prevalence of infection in the patient and/or sample population in their survey.

The intermittent shedding of mycobacteria in the sputum of patients with untreated active pulmonary disease tends to compromise test sensitivity. In addition, it has been estimated that at least 5,000–10,000 AFB per ml of sputum are required to permit detection by staining. Therefore multiple sputum specimens should always be submitted for AFB staining and culture from patients with newly suspected pulmonary mycobacterial disease. Since only one of these serial specimens may contain AFB in sufficient numbers to permit detection, some investigators believe that it is more appropriate to calculate test sensitivity on a "per patient" rather than a "per specimen" basis. In this case, as would be predicted, estimates of sensitivity are markedly improved.

Ineffective concentration of AFB by centrifugation prior to staining can also decrease sensitivity. The decontamination procedure for sputum helps to decrease this problem by reducing specimen viscosity. Moreover, a higher centrifugal force is apparently required to sediment mycobacteria than is necessary for other bacterial species, possibly because of the high lipid content of the mycobacterial cell wall which contributes to buoyancy in suspension. There is a correlation between the sensitivity of AFB stain and culture and the relative centrifugal force (RCF) of specimen centrifugation (15). When RCF was increased from 1260xg to 3,000xg, and then to 3,800xg, AFB stain sensitivity with respect to culture positivity increased from 25% to 40%, and then from 40% to 82%, respectively. A correspondingly increased yield of positive cultures was also reported. While other uncontrolled parameters have biased these results, increasing RCF during processing merits consideration if staining sensitivities in a given laboratory are low.

In some instances, the non-tuberculous ("atypical") nature of mycobacteria is suggested by AFB morphology, e.g., the presence of very short rods, of heterogeneity of size, or—in particular—the cross-banding seen in M. kansasii. In one recent test (17) of the ability of observers to distinguish between tuberculous and non-tuberculous mycobacteria, 100 slides of each were examined in random order. Unfortunately, the results were unimpressive, one observer scoring as low as 60%.

The results of AFB staining must be considered in their clinical context, and cannot be used to rule either in or out a diagnosis of mycobacterial disease without regard for the clinical picture.

COMMENT

The continuing use of the AR stain merely as a screen for the Kinyoun stain merits reevaluation. The AR stain facilitates examination of the specimen, because the organisms fluoresce brightly and are found much more readily than by Kinyoun stain.
This feature also permits a more rapid scanning of a larger area of the AR smear using the lower-power 25x objective, versus the 100x oil-immersion objective required for the Kinyoun. With the exception of one report that 10/15 strains of M. fortuitum were AR-negative and Ziehl-Neelsen positive (18), the literature documents acceptable performance of the AR stain when used independently (9,11-13,15,16). The degree of AR-positivity, estimated by the number of organisms seen on the smear, correlates with the frequency of positive cultures (9,11,19). Nonetheless, the most recent CDC guidelines still recommend confirmation of all positive AR stains with a positive Kinyoun stain (20). The main factors which contribute to the continued reliance on the Kinyoun stain are probably the difficulty of the technologist discriminating between fluorescent artifacts and representative organisms, and reluctance to part with time-honored methods. All UC hospitals should accurately and independently record AR and Kinyoun stain results, including the number of organisms seen. We would then at some time in the future be able to evaluate the performance of the AR and Kinyoun stains in our laboratories.

REFERENCES


19. Unpublished data from SFGH, compiled by Dr. Ted Kurtz.