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Acid-Fast Stain Protocols

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Information

History

In 1882 Robert Koch reported the discovery of the tubercle bacillus (4) and described the appearance of the bacilli resulting from a complex staining procedure. During the same time period several other researchers (Ehrlich, Ziehl, Rindfleisch, and Neelsen), intending to improve on Koch's method, introduced modifications to the reagents and the procedure. Franz Ziehl was the first to use carbolic acid (phenol) as the mordant. Friedrich Neelsen kept Ziehl's mordant, but changed the primary stain to the basic fuchsin (first used by Ehrlich in 1882). This method became known as the Ziehl-Neelsen method in the early to mid 1890s. In this method heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells. The use of heat in this method has been the reason that this technique is called the "hot staining" method.

The Ziehl-Neelsen method has endured as a reliable and effective way to demonstrate the acid-fast bacteria.

In 1915, Kinyoun published a method that has become known as the "cold staining" method because the heating step was removed in favor of using a higher concentration of the carbolfuchsin primary stain.

Purpose

The acid-fast stain is performed on samples to demonstrate the characteristic of acid fastness in certain bacteria and the cysts of *Cryptosporidium* and *Isospora*. Clinically, the most important application is to detect *Mycobacterium tuberculosis* in sputum samples to confirm or rule out a diagnosis of tuberculosis in patients.

Theory

There are three common acid-fast staining methods, Ziehl-Neelsen (hot), Kinyoun (cold), and Auramine-Rhodamine Fluorochrome (Truant method). The emphasis in this Atlas-Protocol project will be on the Ziehl-Neelsen and the Kinyoun methods because the slides produced by these methods can be visualized using a standard bright-field microscope. The fluorochrome method is used by large laboratories that have a fluorescent (ultraviolet) microscope. For comparison purposes, the recipe for the reagents and the protocol for all three methods are included below, but images for the fluorochrome method will not be a part

of the Atlas at this time.

Many bacterial cells are easily stained with simple stains or using the Gram stain. A few types of bacteria, such as the mycobacteria and *Nocardia* species, do not stain using these techniques or, if stained, they produce a variable reaction because their walls are not permeable to the rosaniline dyes in common staining regimens (12). The cell walls of the mycobacteria contain mycolic acids giving the cell walls a high lipid content. This characteristic is thought to be the reason (5, 10) these bacteria are difficult to stain. To view these cells in samples staining requires higher concentrations of the dye solution and/or a heating period (4). However, once a stain is introduced into the cell wall, removing it with a decolorizer is even more difficult. The expression "acid fast" is derived from the observation that even with the addition of hydrochloric acid to the alcohol decolorizer, some of the stained cells retain the primary stain (carbolfuchsin). Cells that release the primary stain (carbolfuchsin) with decolorizing will be visible after the counterstaining step is complete. Bacteria described as acid fast will appear red when examining specimens using bright-field microscopy. Non-acid-fast cells and field debris will appear blue.

Acid fastness is a characteristic that is shared by just a few organisms, so staining to determine if organisms possess this trait is useful in microbial identification schemes.

RECIPES (6, 7, 15)

A. Ziehl-Neelsen method for acid-fast staining (6, 7)

Carbolfuchsin stain:

Basic fuchsin, 0.3 g

Ethanol, 95% (vol/vol), 10 ml

Phenol, heat-melted crystals, 5 ml

Distilled water, 95 ml

Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water.

Mix and let stand for several days. Filter before use.

Decolorizing solvent:

Ethanol, 95% (vol/vol), 97 ml

Hydrochloric acid (concentrated), 3 ml

Counterstain:

Methylene blue chloride, 0.3 g

Distilled water, 100 ml

B. Kinyoun method for acid-fast staining (15)

Kinyoun carbolfuchsin solution:

Solution A. Dissolve 4 g of basic fuchsin in 20 ml of ethyl alcohol.

Solution B. Dissolve 8 g of phenol (melted) in 100 ml of distilled water.

Mix solutions A and B together and allow to stand for a few days.

Acid-alcohol decolorizing agent:

Ethanol, 95% (vol/vol), 97 ml

Hydrochloric acid (concentrated), 3 ml

Methylene blue counterstain:

Methylene blue chloride, 0.3 g

Distilled water, 100 ml

Dissolve by shaking.

C. Truant method for acid-fast staining (6, 7)

Fluorescent staining reagent:

Auramine O, CI 41000, 1.50 g

Rhodamine B, CI 749, 0.75 g

Glycerol, 75 ml

Phenol (heat melted crystals), 10 ml

Distilled water, 50 ml

Mix the two dyes well with 25 ml of the water and the phenol. Add the remaining water and glycerol and mix again.
Filter the resulting staining fluorescent reagent through glass wool and store at 4°C or room temperature.

Decolorizing solvent:

Ethanol, 70% (vol/vol), 99.5 ml

Hydrochloric acid (concentrated), 0.5 ml

Counterstain:

Potassium permanganate, 0.5 g

Distilled water, 99.5 g

PROTOCOLS (1, 6, 7)

Smear Preparation

Ordinarily, preparing a smear for staining involves applying a very small sample to the center of a carefully cleaned glass slide. The microbial sample is usually taken from a broth culture or a suspension of microorganisms produced by mixing a tiny amount of solid matter from colonies with water. The suspension can be made directly on the slide or it can be mixed in a tube and transferred to the slide.

Since many bacteria cling to each other in culture (both broth and colonial form), vigorous manual or mechanical mixing may be required to produce a proper distribution of the organisms for microscopic evaluation. Clumps of organisms make it difficult to observe the characteristics of individual cells.

The quality of the staining for acid-fast bacteria will be affected by the quality of the smear. A good quality smear will have a thin film of the specimen or culture, allowing individual cells to respond to the staining protocol. The waxy nature of the mycobacteria organisms causes them to repel water so fluids should be added to the slide after spreading the sample in a thin film over the slide.

Organisms grown in media containing complex lipids will stain better, and usually, grow better.

Basic Smear Preparation

1. Clean a glass slide (some labs will provide pre-cleaned slides; be sure to remove any dust or crushed glass debris) according to instructions provided by your instructor.

2. Prepare the sample according to instructions provided by your instructor. Make certain that an aerosol is not generated during this process.

3. Using a sterile pipet or microbiological loop, apply a small sample of the specimen to the slide by **slowly** spreading the liquid to make a thin film; If you are using solid matter from a colony, be sure to choose a very minute sample and spread it into a very thin film. Applying the cells before adding water (or other mixing fluid) will help the cells adhere to the slide. The size of the film should be about 1 cm in diameter. Avoid any actions that would splatter droplets of the sample in the surrounding area.

4. Allow the smear to dry completely.

5. Fix the smear at 80°C for 15 minutes or for 2 hours on a hot plate set for 65°C to 70°C.

6. Proceed to the staining protocol of your choice.

Note: Reports of the survival of mycobacteria at these temperatures have been made. Follow the proper handling precautions of your laboratory/institution.

A. Ziehl-Neelsen method for acid-fast staining (1, 6, 7)

1. Heat fix an air dried smear at 80°C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C – 70°C

2. Place a slide with an air-dried and heat-fixed smear on suitable staining device. Cut a piece of absorbent paper to fit the slide and saturate the paper with the carbolfuchsin stain.

3. Carefully heat the underside of the slide by passing a flame under the rack or by placing the slide on a hot plate until steam rises (without boiling!). Keep the preparation moist with stain and steaming for 5 minutes, repeating the heating as needed.

(CAUTION: overheating causes spattering of the stain and may crack the slide).

4. Wash the film in a gentle and indirect stream of tap water* until no color appears in the effluent.

5. Holding the slide with forceps, wash the slide with the decolorizing solvent. Immediately wash with tap water*, as above. Repeat the decolorizing and the washing until the stained smear appears faintly pink and the fluid washing off the slide runs clear.

6. Flood the smear with the methylene blue counterstain for 20 to 30 seconds, and wash with tap water*, as above.

7. Gently blot, or air dry the smear.

8. Examine under oil immersion.** Acid-fast bacteria appear red, and non-acid-fast bacteria (and other organisms and cellular materials) appear blue.

* NOTE: Most labs use deionized or distilled water for all lab procedures. See note in Tips and Comments section regarding the use of tap water.

** NOTE: For clinical samples, examination of at least 300 fields is required before declaring a specimen negative for acid-fast bacteria.

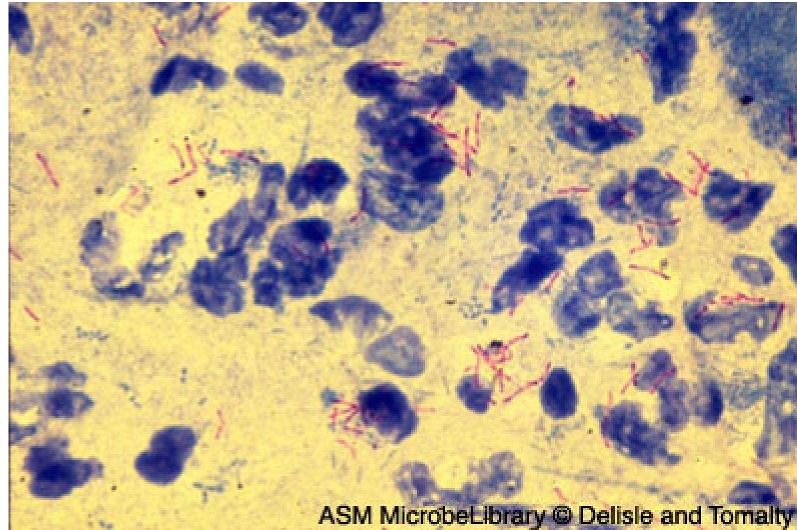

FIG. 1. *Mycobacterium tuberculosis* (5).

B. Kinyoun method for acid-fast staining (1)

1. Heat fix an air dried smear at 80°C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C – 70°C

2. Flood slides with Kinyoun's carbolfuchsin reagent and allow to stain for 5 minutes at room temperature.

3. Rinse with deionized water and tilt slide to drain.

4. Decolorize with acid-alcohol for 3 minutes and rinse again with deionized water.*

5. Redecolorize with acid-alcohol for 1-2 minutes or until no more red color runs from the smear.*

6. Rinse with deionized water and drain standing water from the slide surface by tipping the slide.

7. Flood slide with methylene blue counterstain and allow to stain for 4 minutes.

8. Rinse with distilled water and allow to air dry.

9. Examine under high dry (400X) magnification, and confirm acid-fast structures under oil immersion (1000X).

* NOTE: Most practitioners apply decolorizer until the fluid washing off the slide runs clear.

C. Truant method for acid-fast staining (1, 6, 7)

1. Heat fix an air dried smear at 80°C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C – 70°C

2. Wash the slide with a gentle and indirect stream of distilled water until no color appears in the effluent.

3. Flood the smear with the decolorizing agent for 2 to 3 minutes, and then wash with distilled water as above.

4. Flood the smear with the permanganate counterstain for 2 to 4 minutes.

5. Wash the slide with distilled water as above, blot with absorbent paper, and dry.

6. Examine the slide with a fluorescence microscope equipped with a BG-12 exciter filter and an OG-1barrier filter. Acid-fast bacteria appear as brightly fluorescent , yellow-orange cells in a dark field; non-acid-fast cells appear dark.

NOTE

This protocol is not intended to fully address the safe operations associated with this procedure.

Your institution must have in place instructions to guide your actions that comply with requirements unique to your state's laws and your organization's plan to ensure safety for all students and staff.

However, this protocol does warrant special notice regarding two hazards: the use of phenol in the carbolfuchsin reagent and the handling of samples and cultures containing mycobacteria.

1. Phenol is a component of the carbolfuchsin reagent for the Ziehl-Neelsen and Kinyoun methods for acid fast staining. Phenol is a dangerous chemical if not handled carefully. It is poisonous, corrosive and combustible. If you must handle the phenol crystals to prepare the carbofuchsin stain, you must be familiar with the safe handling requirements demanded by your institution's safety protocols. Use an MSDS (material safety data sheet) along with your departmental standard operating procedures to become familiar with the proper handling, disposal procedures, as well as the precautions to take to prevent hazards due to inhalation, skin or eye contact or ingestion of phenol.

At a minimum you should know that you should wear gloves when handling phenol and that heating the phenol for melting increases the risk of exposure through inhalation. Be sure that procedures are in place to prevent such exposure.

Phenol must be disposed of as a hazardous waste. Even small amounts on paper towels or disposable containers must be confined and processed. Consult your departmental operating procedures for specific instructions.

2. In courses for nonmajors nonpathogenic mycobacteria are typically selected to illustrate the acid-fast property attributed to a few groups of organisms. In advanced courses and in clinical laboratories however, cultures and specimens are likely to contain pathogens. Since

the mycobacteria can be spread in the air, creating an inhalation exposure hazard, care should be taken to confine cultures and specimens to areas in the lab designed to minimize the exposure risk. You should receive specific instructions for handling such specimens to protect yourself and to avoid generating aerosols with the specimens that would increase the risk of dispersing the mycobacteria in the air.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

Material Safety Data Sheet Links:

Basic fuchsin information:
www.jtbaker.com/msds/englishhtml/B0514.htm

Ethanol
information: www.emsdiasum.com/microscopy/technical/msds/15050.pdf

Phenol information: www.jtbaker.com/msds/englishhtml/P1949.htm

Hydrochloric acid
information: www.jtbaker.com/msds/englishhtml/H3880.htm

Methylene blue chloride
information: www.jtbaker.com/msds/englishhtml/M4381.htm

Kinyoun reagent information: see MSDS's for basic fuchsin, ethanol, phenol, and hydrochloric acid

Auramine O information: www.jtbaker.com/msds/englishhtml/A7750.htm

Rhodamine B
information: www.jtbaker.com/msds/englishhtml/R5400.htm

Glycerol information: www.jtbaker.com/msds/englishhtml/G4774.htm

Potassium permanganate
information: www.jtbaker.com/msds/englishhtml/P6005.htm

COMMENTS AND TIPS

Comments and tips come from discussions at the ASM Conference for Undergraduate Educators 2007.

1. A single acid-fast bacillus in the sputum sample of a symptomatic patient can be diagnostic, requiring treatment for tuberculosis.

2. For the Ziehl-Neelsen protocol: some practitioners prefer to apply heat by steaming the slide over a boiling water in a beaker.

3. For the Ziehl-Neelsen protocol: whether heating directly or steaming, stain is added to the paper covering the sample as it begins to dry. The specimen should constantly have contact with liquid stain during the heating step.

4. The use of tap water is not recommended when making the smears or when performing rinse steps in the staining protocol. *M. gordonae* has been found in tap water and may interfere with make an accurate assessment of the specimen to be stained. Deionized or distilled water is recommended.

5. Regarding the decolorizing step: many practitioners do not adhere to a fixed decolorizing time. Instead, students are taught to add the decolorizing agent drop by drop until the draining fluid runs clear.

Likewise, the counterstain doesn't require a 4 minute staining period. Typical modifications at this step call for leaving the counterstain on the slide from 30 seconds to 1 minute.

6. Organisms grown in media containing complex lipids will grow better and typically will stain better. Reviewers noted that growth on Blood Agar Plates provides only starvation level lipids and may limit the ability of the organisms to demonstrate the acid- fast property after staining.

7. In a controlled study reported in the journal *Chest* in 2001 (13) the fluorochrome staining method is highly sensitive because of the ease in detecting the acid-fast microbes. This allows the microscopist to examine more fields at a lower magnification resulting in faster processing of the samples. Consequently, they suggest this method would be the preferred method for detecting acid-fast bacilli.

The study also reported that the Kinyoun method was the only one to result in false negative reports. Based on their analysis and the results of other published studies, the authors concluded that the Kinyoun method is inferior to the Ziehl-Neelsen method. For more details, consult the *Chest* article (13).

8. Difficulties in interpreting results:

a. When cultures have not been sufficiently mixed to break up clumps of cells, the resulting smear can be difficult to read because individual cells are not discernible.

b. Artifacts from residual stain may occasionally be confused as acid-fast bacillus, although false positives are less common than false negative reports.

c. *Mycobacteria tuberculosis* often grow in cords or ropes making it difficult for the unfamiliar to confirm the morphology of individual cells or distinguish these forms from stain artifacts.

d. Partially acid-fast bacteria may also contribute to confusion during smear evaluation.

e. Since staining technique is one factor that affects results, analyzing

differences in applying the steps in the protocol might warrant analysis if problems in interpretation persist. Standardization of the protocol will minimize variation in results.

9. Issues that may affect results:

Somoskovi, et. al. (13) report that the specificity of acid-fast microscopy for the identification of acid-fast bacilli in stained smears is excellent, but that "the sensitivity is not optimal" Among the factors reported by the authors as influencing the results of the microscopic examination of slides are the following:

1. the type of specimen

2. the quality of the specimen

3. the number of mycobacteria present in the specimen

4. the method of processing (direct or concentrated)

5. the method of centrifugation

6. the staining technique

7. the quality of the examination

8. the prevalence and severity of the disease

If your experience suggests additional factors affecting results, you may want to forward these to Marise A. Hussey for inclusion in the next update of this protocol.

10. Trouble-shooting tips:

a. Inspect reagents for expiration dates

b. If making one's own reagents, confirm expiration dates on chemicals and confirm protocols to ensure proper reagent concentrations.

c. Confirm sample processing procedure. Was the sample concentrated or applied directly?

d. Post staining protocol steps for students to pay close attention to sequencing and time requirements. [Adherence to recommended procedure affects results.]

e. For beginners, confirm proper operation of the microscope and compare microscopic views with images from the ASM Acid-Fast Image Atlas or other suitable examples of acid-fast positive and acid-fast negative smears.

f. Examine the quality of the smear. Samples that are too thick will not be readable. Samples that are too thin may result in false negatives or to result in the need to repeat the procedure.

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11. Disclaimer:

While this protocol has been developed with the goal of accurately conveying important standards and background concerning the acid–fast staining procedure, it is not to be considered a comprehensive reference for application to clinical environments. The intended audience for this protocol is undergraduate students and faculty.

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