Destinatari: Coordinatore, Tecnici e Studenti del Settore Micobatteri - EBP

CONTENT

Rev.	Descrizione modifiche	Data
0	Prima emissione	05/08/2010
1	Cambio ragione sociale	07/01/2013

Compilazione	Sviluppo	Verifica	Approvazione
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1. SCOPE

This Instruction describes the auramine staining technique for detection of acid-fast bacilli by microscopy. The auramine staining technique applies to fluorescence microscopy and Light Emitting Diode microscopy.

2. APPLICATION

The auramine staining technique is used with fluorescence and Light-emitting Diode microscopes. The present instruction is applicable within the whole Advanced Mycobacterial Diagnosis (DMA) area.

3. DEFINITIONS AND ABBRAVIATIONS

Microscope magnification: individual objective magnification x eyepiece magnification

AFB: Acid-Fast Bacilli
QC: Quality Control
TB: Tuberculosis
USP: U.S. Pharmacopeia
HPF: High-power Fields
LED: Light-emitting Diode

4. RESPONSIBILITIES

Supervision and the correct application of the following instruction is the responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator, while the reading of the slides and the reporting are a coordinator's and technician's duties.

5. EQUIPMENT AND MATERIALS

5.1 General materials

- √ Quaternary ammonium compounds jar (only if a loop is used, not needed with disposable sticks).
- √ Diamond pencil or lead pencil (if frosted-end slides are available)
- √ Filter paper, small, appropriate for funnel size (when necessary)
- √ Funnels, small, for filtering solutions in use (when necessary)
- √ Forceps
- √ Lens paper or soft tissue paper
- √ Plastic containers for waste disposal
- √ Disposable loops or wooden sticks
- √ Fluorescence microscope or LED Microscope with objectives of 20x or 25x, 40x (ideally specific for fluorescence microscopy), 100x and eyepieces of 10x
- √ Immersion oil for microscopy
- √ Slide staining rack
- √ Slide boxes
- √ Slides supports
- √ New, clean slides
- √ Timer

- √ Absolute methanol; denatured alcohol
- √ Beaker for rinsing water
- √ Sink and water supply
- √ Oil-absorbing paper
- √ Disinfectant solutions

5.2 TB Fluorescent Stain Kit M (Becton and Dickinson – Code 212519)

a) 250 ml bottle TB Auramine M (Approximate formula per liter)

Auramine O	2.0	g
Phenol, USP	4.0	g
Glycerine, USP	100.0	ml
Isopropanol	250.0	ml
Distilled water	650.0	ml

b) 250 ml bottle TB Decolorizer TM (Approximate formula per liter)

Hydrochloridric Acid	5.0	ml
Isopropanol	700.0	ml
Distilled water	300.0	ml

c) 250 ml bottle TB Potassium Permanganate (Approximate formula per liter)

Potassium Permanganate	5.0	g
Distilled water	1000.0	ml

6. PROCEDURES

6.1 Principle

The property of acid-fastness is based on the presence of mycolic acids in the mycobacterial cell wall. Primary stain (Auramine) binds cell-wall mycolic acids. Intense decolourization (strong acids, alcohol) does not release primary stain from the cell wall and the mycobacteria retain the fluorescent bright yellow colour of auramine. Potassium permanganate is used to quench fluorescence in the background;

All mycobacteria are acid-fast, but very few other bacteria possess this property and then only weakly (e.g. *Nocardia*).

Fluorescence microscopy allows smears to be examined more rapidly than is possible with the basic fuchsin procedures and is particularly indicated for high-volume laboratories. It may also be more sensitive for paucibacillary specimens, since it allows examination of more fields with less effort. However, it requires a stable power supply, greater expertise in reading and microscope adjustment, and a regular supply of the costly and short-lived bulbs.

Newly developed of blue LED light sources adjusted to fluorescence microscopes overcomes these difficulties, because a 5-W lamp is sufficient, can be operated with simple batteries and has a life of at least 15 000 hours.

6.2 Specimen

Auramine staining is performed on the following biological samples:

6.2.1 Sputum

- Spontaneous sputa

  Sputa from suspects should be rejected only if they are liquid and clear as water, with no particles or streaks of mucous material. However, they should be accepted if the patient cannot produce a better specimen on a repeated attempt. Sputa from follow-up patients should be accepted and examined even if they look like saliva, since these patients often cannot produce mucoid specimens.

- Induced sputa

  These specimens resemble saliva but have to be processed as adequate specimens.

- Decontaminated sputa, concentrated by centrifugation.

6.2.2 Other specimens

- Laryngeal swabs, gastric lavages, bronchial washings, brushings and transtracheal aspirates.
- Urine and urine from sovra-pubic puncture.
- Body fluids (spinal, pleural, pericardial, synovial, fluids from ascites, blood, pus, bone marrow).
- Tissue biopsies.
- All the above mentioned specimen after decontamination (when necessary), and concentration by centrifugation.

6.3 Detailed Instructions

6.3.1 Preparation of smears

- Disinfect the working area with an adequate disinfectant solution.
- Label the slides properly with a lead pencil using the Country Database or any unique identification number.
- Place each slide in front of the corresponding 50ml-Falcon Tube used previously for the concentration step, including digestion and decontamination for non sterile biological material (see IOS EBP-DMA 004)
- Proceed to smearing, taking the labelled slides and opening containers one by one;

  - for a direct sputum smear, select a small portion of purulent or mucopurulent material with the stick/loop and transfer it to the slide;
  - if a smear is prepared after specimen decontamination, the concentrated material must be transferred to the slide with a sterilized loop to avoid splashing.

- Spread the material carefully over an area equal to about 2–3 cm x 1–2 cm using repeated circular movements, without touching the edge of the slide. Make the smear as even as possible by continuing this process until no thick parts remain. The thickness of the smear should be such that a newspaper held under the slide can barely be read through the dried smear.
- Disinfect the working area after smear preparation.
- Let the smears air-dry at room temperature under the safety cabinet; do not use heat to speed the drying.
- When dry, take the slides and put them on the staining rack 1 cm apart and place them under the chemical hood if the methanol fixing method is chosen
- Fix them by one of these options: cover the slides with absolute methanol and let them stand 10 minutes and then let them air-dry or pass them three times quickly through the flame of a Bunsen burner, smear upwards. Do not overheat or AFB will be poor. Do not use the burner under the chemical hood, but on the workbench nearby.

- Take the fixed slides on the staining rack and put them under a chemical hood in order to start staining
- Always keep smears out of direct sunlight.

6.3.2 Staining method

- Place the slides, smear upwards, on the staining rack over a basin, about 1 cm apart.
- Flood with TB Auramine M for 15 minutes
- Using forceps, tilt each slide to drain off the staining solution.
- Rinse the slides gently with distilled water or clean tap water from a beaker (not directly from the tap).
- Decolourize with TB Decolourizer TM for 30-60 seconds
- Wash slides gently in running water from a beaker
- Counterstain with TB Potassium Permanganate for 2 minutes
- Using forceps, tilt each slide to drain off the solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).
- Using forceps, take each slide from the rack and let the water drain off. Stand the slide on an absorbent paper and let them air dry.
- Once dried, remove the slides from the absorbent paper and put them on a slide support in order to be transported out to the microscopy room (Room n. 24)

Note: According to manufacturer's package insert, the staining protocol may vary in terms of time of exposure of the slides to staining solutions or decolourizers.

6.4 Quality Control

- Include positive and negative controls with each batch's reading. Read control slides before other smears.
- If results are unacceptable, re-stain smears of that day together with new controls, paying attention to correct technique; if these controls are also unacceptable, change new staining solutions, repeat the staining and detail all in the "Registro Non Conformità" (EBP 004)

Be aware! Among the possible reasons for *false-positive results* are:

- re-use of containers or positive slides;
- contaminated staining solutions;
- use of scratched slides;
- AFB floated off one slide and became attached to another during the staining procedure because there was no space between adjacent slides;
- inadequate decolourization;
- lack of experience, confusion with artefacts;
- microscope (lamp) in poor condition or poorly adjusted: interpreting glitter as AFB;
- poor quality of staining solutions.

Among the possible reasons for *false-negative results* are:

- poor quality of specimen;
- not taking proper portion of specimen for smear preparation;
- excessive decolourization;
- too little time staining with Auramine;
- overheating during fixing;
- reading less than one length;
- slide exposed to daylight for too long;
- too long an interval between staining and reading, particularly if slides were poorly stained or not kept in the dark.

7. RECORDING AND REPORTING

7.1 Reading

- Keep stained smears in the dark (in a box or folder) and read as soon as possible –fluorescence fades quickly when exposed to light.
- Switch on fluorescent or LED lamp 5 minutes before use; leave the lower ordinary lamp off.
- Rotate the nosepiece so that the 20x (or 25x) objective is in the light path.
- Load the positive control slide on the stage and move the stage to position the slide under the objective.
- Use the coarse adjustment first, and then the fine adjustment, to focus the objective. If this fails (i.e. in thin negative smears), turn the filter set to transmitted light, switch on the lower normal lamp and focus as with a light microscope. Then switch off the lower lamp and return to the required filter position. The field should now be in focus.

  Note: *Focusing and maintaining focus while moving the smears may prove quite difficult if the permanganate-quenched background is too dark.*
- Check that bright yellow fluorescent AFB are clearly seen. If not, adjust the lamp and/or the mirror position. Check that the whole field is evenly lit. If not, centre the diaphragm after partially closing it (see manufacturer's manual).
- Exchange the positive control for the first routine smear without changing focus or rotating the objective. Repeat the procedure with each smear to be examined.
- Using the 20x (or 25x) objective, scan the stained smear systematically from one side to the other and back again – at least one length must be scanned before reporting a negative. At 200x magnification, this corresponds to three lengths or 300 high-power fields (HPF) using the oil-immersion 100x objective; at 400x it equals two lengths or 200 HPF with the oil-immersion objective. The process will take 1–2 minutes.

  Acid-fast bacilli appear bright yellow against the dark background material.
  Tubercle bacilli are quite variable in shape, from very short fragments to elongated types, and may be uniformly stained or with one or many gaps, or even granular. The typical appearance is of bacilli that are rather long and slender, slightly curved rods. They occur singly or in small groups, and rarely in large clumps. With good staining (always check a freshly stained positive control first), there may also be fluorescing (sometimes green) artefacts, which do not have the typical shape. Non-fluorescing bacillary shapes must also be considered as artefacts.
- Use the 40x objective for confirmation of AFB
- Store the slides in a slide box.
- When finished, turn the power off. When work needs to be interrupted for just a few minutes only, block the light using the shutter but do not switch off the light source.

7.2 Recording

Because fluorochrome-stained smears are examined at magnifications of 200x to 400x, the number of AFB can roughly be divided by a factor 10 or 5, respectively (depending on the objective) to make them equivalent to fields seen on examination of fuchsin-stained smears at 1000x.

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system used		
	Bright-field (1000x magnification: 1 length = 2 cm = 100 HPF)	**Fluorescence** (200–250x magnification: 1 length = 30 fields = 300 HPF)	**Fluorescence** (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length	Zero AFB / 1 length
Scanty	1–9 AFB / 1 length or 100 HPF	1–29 AFB / 1 length	1–19 AFB / 1 length
1+	10–99 AFB / 1 length or 100 HPF	30–299 AFB / 1 length	20–199 AFB / 1 length
2+	1–10 AFB / 1 HPF on average	10–100 AFB / 1 field on average	5–50 AFB / 1 field on average
3+	>10 AFB / 1 HPF on average	>100 AFB / 1 field on average	>50 AFB / 1 field on average

If there is uncertainty about the presence of a bacillus because of the lower magnification, it is best to inspect this carefully with the 40x objective or, if unavoidable, with a 100x oil-immersion objective. This is more efficient than re-staining by the Ziehl-Neelsen technique (sometimes recommended), which may result in bacilli being washed off or simply not found again. Inexperienced personnel should seek advice from a supervisor.

7.3 Reporting

Results must be reported in the specific Country Database and in the Workflow Summary module (EBP-SM 004)

- For a positive result: report quantification of AFB seen. (It should not be assumed that AFB are tubercle bacilli.)
- Never report “No TB” (or equivalent wording).

8. RELATED DOCUMENTS

Basics of quality assurance for intermediate and peripheral laboratories, 2nd ed. Cairo, WHO Regional Office for the Eastern Mediterranean, 2002

Kent PT, Kubica GP. *Public health mycobacteriology: a guide for the level III laboratory*. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control, 1985.

Laboratory services in tuberculosis control. Part II: Microscopy. Geneva, World Health Organization, 1998.

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Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.

Rieder HL et al. *Priorities for tuberculosis bacteriology services in low-income countries*, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.