THE REPUBLIC OF UGANDA
MINISTRY OF HEALTH

UGANDA Supranational® Reference Laboratory
Timely Accurate Diagnostics for a TB-Free Africa

Training on LJ culture method

Module 8: Principles And Protocols Of Sample Processing

DATE:

VENUE: SRL, Uganda

FACILITATOR:

Content outline

- principle of NALC-NaOH method
- Materials, reagents and equipment used
- Preparation of NaOH/NALC-Na Citrate Solution
- Preparation of BSC and Special Microbiology Practices
- Specimen Digestion-Decontamination
- Sample innoculation and incubation

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Principle of NALC-NaOH method

- Sputum specimens are viscous and often contaminated with normal flora hence sample require pre-treatment;
  - **Digestion:** N-acetyl-L-cysteine (NALC), a mucolytic agent;frees the TB bacilli from the mucus, cells or tissue in which they may be embedded
  - **Decontamination:** done by NaOH, to eradicate normal flora that grow more rapidly than mycobacteria and would interfere with the ability to recover TB bacilli
  - **Homogenization** of the digested materials through vortexing.
  - **Concentration** of the TB bacilli by centrifugation before smear preparation and inoculation onto LJ culture medium

Most common processing methods

N-acetyl-L-cysteine/sodium hydroxide method

- Mildest decontamination method; though still kills about 33% of mycobacteria present
- Can be used with both liquid and solid media

Modified Petroff's sodium hydroxide (NaOH) method

- Harsher method; can kill up to 70% of mycobacteria in specimen
- Useful with highly contaminated specimens
- Not recommended for use with liquid media

Materials, reagents and equipment used

Reagents needed

- NALC powder
- 6% NaOH
- 2.9% Na citrate
- Phosphate buffered saline pH 6.8

materials and equipment used

- Weighing balance
- 50 ml falcon tubes
- Clean cylinder and beakers
- Biosafety cabinet
- Pasteur pipettes
- LJ slant tubes
- Slide warmer
- Vortex
- Refrigerated centrifuge
- Timer

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Preparation of NaOH/NALC-Na Citrate Solution

- Determine how much reagent will be needed for the day's work.
- Mix equal volumes of the NaOH and Na citrate solutions in a sterile flask
- Add NALC according to table below

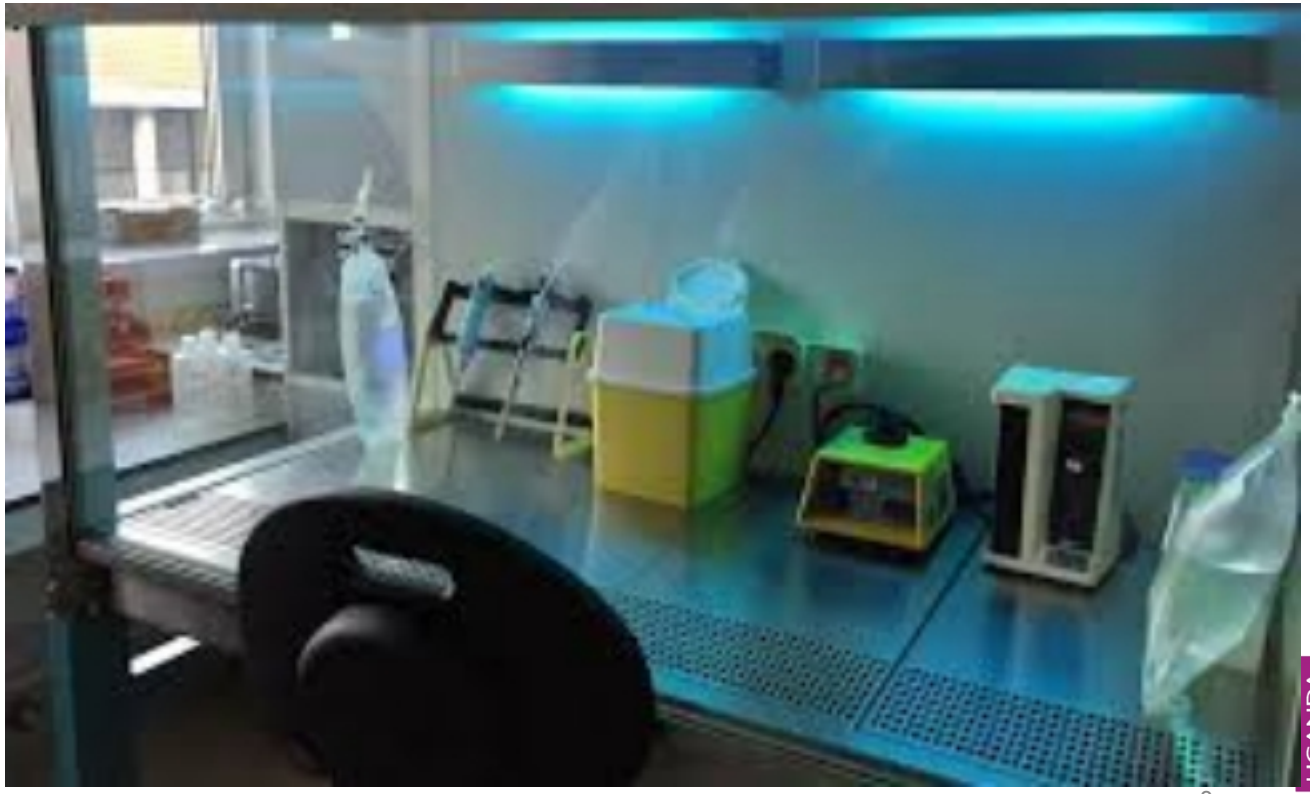
volume of digestion method needed	6% Sodium hydroxide	2.9% Sodium Citrate	NALC to be added(grams)
50	25	25	0.25
100	50	50	0.50
200	100	100	1.00
400	200	200	2.00

Preparation of BSC and Special Microbiology Practices

- Decontaminate the BSC with freshly diluted 1% bleach or 5% lysol (phenol), followed by 70% alcohol
- Cover the working area with paper towels and spray it with tuberclucidal disinfectant
- Use discard container and a biohazard bag with freshly prepared tuberclucidal disinfectant (1%bleach or 5% Lysol) for discarding liquid and solid wastes respectively.
- DO NOT block the grill

Preparation of BSC and Special Microbiology Practices cont'd

- Perform all operations at least 12 cm away from the front grill on the work surface
- Place all materials and aerosol-generating equipment away from the front grill



Specimen Digestion-Decontamination

- **Preparation of worksheets**
- Record all specimens to be processed in a batch using the Lab Processing Worksheet.
- Transfer all the same numbers to LJ culture register.
- Record the initials of personnel processing each batch
- Enter onto the worksheet the batch/lot numbers, preparation and expiry dates of the buffer, NALC, NaOH, Na-citrate.

Specimen Digestion-Decontamination

- **Preparation of solid media for inoculation and smear slides**

Remove Lowenstein-Jensen (LJ) slants from the refrigerator the day before use to allow them to adjust to room temperature

Aseptically remove any excess water from the slant since this may be a source of contamination

Label two LJ slant with the same specimen number

Label one clean new slide for each sample for microscopy

processing procedure

- Collect sputum in 50 ml sterile, plastic, screw-capped centrifuge tubes
- Note volume of specimen, add an equal volume of digestion solution and tighten cap
- Vortex each tube for 15-30 seconds and then invert several times to ensure that the digestion solution comes in contact with the inner surface of the tube and the cap.

processing procedure cont'd

- Start the timer immediately after adding digestant and leave mixture to stand for 20 minutes
- Vortex the samples at the fifth and at the twentieth minute of incubation.

- NB:DO NOT prolong exposure to digestion solution to avoid harsh decontamination
- Work only on manageable samples

- Dilute the specimen with pH 6.8 phosphate buffered solution to the 50 ml mark

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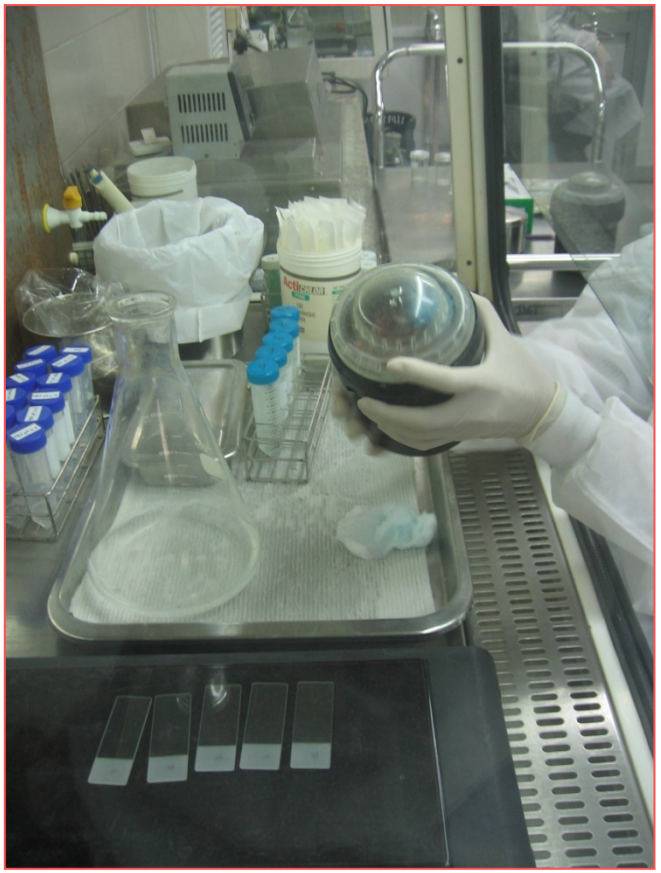
processing procedure cont'd

- Recap the tubes tightly and invert several times

In the BSC, load the diluted specimens into aerosol-free safety centrifuge buckets and tighten the caps

Centrifuge at 3000 x g for 15 min at 4 ºC

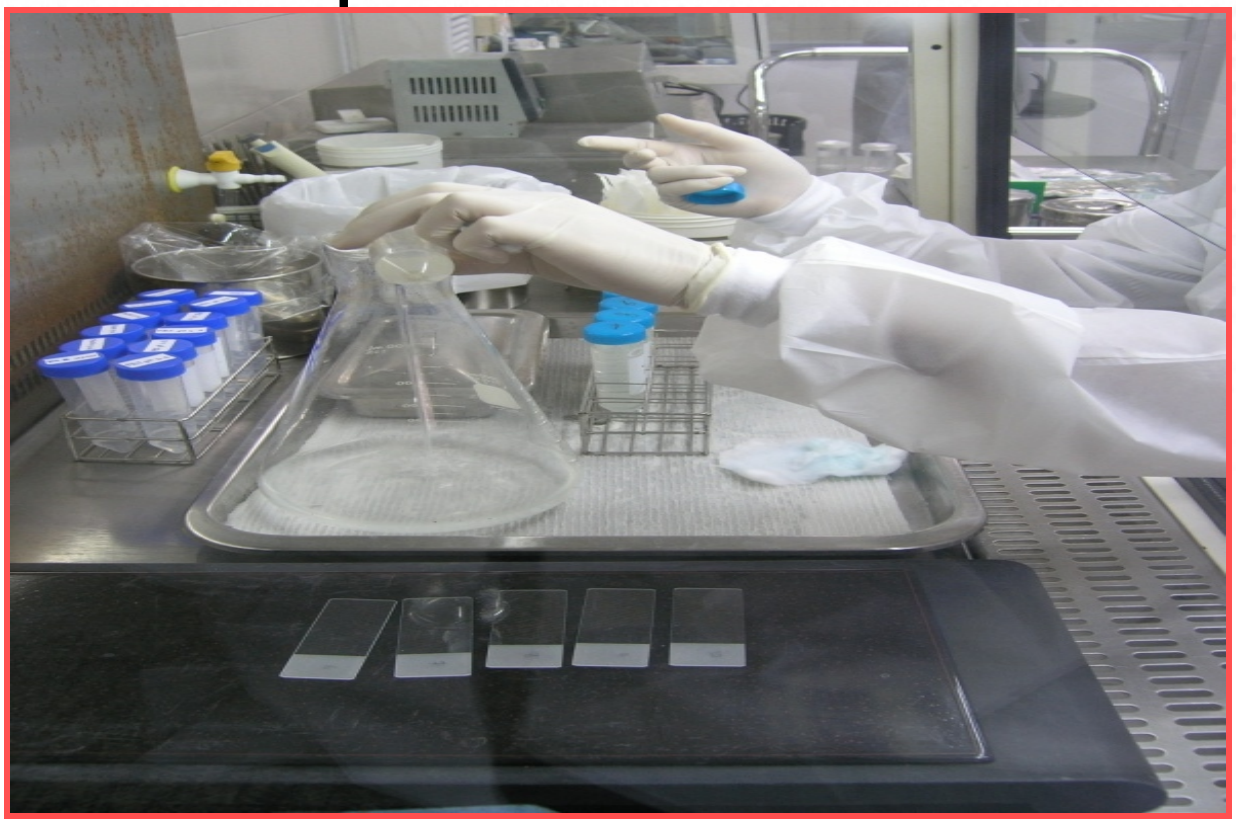


processing procedure cont'd

- After centrifugation, Carefully decant the supernatant into a splash-proof container containing a tuberculocidal disinfectant, taking care not to disturb the sediment at the bottom of the tube.

- Arrange processed sample batch onto a rack ready for inoculation

processing procedure cont'd



- NB: Incase all the supernatant is accidently poured off, add 4mls of PBS and re-suspended the pellet

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QUESTIONS (5mins)

1. Why should you pour off supernatant immediately after centrifugation?
2. Why should specimens be subjected to pre-treatment involving digestion, homogenization, decontamination?

Sample innoculation and incubation

- Add (0.1mls) of re-suspended sediment to each of two Lowenstein-Jensen (LJ) slants using a new sterile Pasteur pipette.
- Re-cap LJ media tube and tilt three times to allow homogenous spread of sediment onto the media.
- Using same inoculum remaining in the Pasteur pipette make a stand croscopy.

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Incubation of LJ slants

- Ensure caps are tightly closed and move slants to the incubator at 37◦C

- Avoid incubating slants adjacent to incubator door and ensure incubator door is always closed to avoid fluctuation in temperature which may cause slow or absolutely no growth with true positive samples

Incubation of LJ slants

- Examine and record results for the cultures weekly, up to a maximum of 8 weeks. Cultures can be read on the bench, as long as the caps are NOT loosened.
- Avoid slanting the samples to avoid condensation water contacting the media slant.
- Ensure adequate light e.g using culture reading lamp to allow for visibility of colonies

Decontamination of the Biosafety cabinet

- Decontaminate any materials (vials, tubes etc.) before removal from the BSC
- Clean pipettes, racks, instruments and the BSC with freshly diluted 5% lysol (20 min), followed by 70% alcohol
- Use area-dedicated spray bottles or beakers
- Discard all the generated waste into biohazard bag inside BSC and seal it with autoclave tape ready for autoclaving

Assessment

1. What is the relevancy of using two slants for each sample?
2. Mention the key equipment necessary for processing?
3. List three precautions taken during processing?
4. What is the importance of NALC-NaOH solution?

Summary

- Sputum specimens must be subjected to pre-treatment involving digestion, homogenization, decontamination to eliminate bacterial contaminants, and concentration by centrifugation

- Most common processing method:

  N-acetyl-L-cysteine/sodium hydroxide (NALC-NaOH) method (liquid and solid medium)

- Inoculation onto both solid and liquid media is the "Gold Standard" for sensitive growth detection

REFERENCES

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