THE REPUBLIC OF UGANDA
MINISTRY OF HEALTH

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Timely Accurate Diagonostics for a TB-Free Africa

Training on *Mycobacterium tuberculosis* drug susceptibility testing (first and second line LJ DST)

Module 7: Preparation of Löwenstein-Jensen Drug Susceptibility testing (LJ DST) media

Venue:

Presenter:

Date:

Introduction and Objective

Introduction

- This module details the procedures involved in preparation LJ DST media and the quality control processes required.

Objectives

By the end of training participates should;

- Be able to prepare LJ DST media and know all the materials, reagents and equipment required for this process.
- Be able to carry out quality Control on the prepared LJ DST media, its subsequent storage
- Will be able to complete all the required documentation for the media batch prepared.

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Course Outline

- Rationale for use of LJ DST media
- Requirements and Equipment
- Steps involved in LJ DST media preparation
- Quality Control
- Labelling and Storage of LJ DST media
- Documentation
- Summary

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Rationale for use of LJ DST media

- LJ DST Media is used because it is able to:
  - Support growth of the 1% *Mtb* resistant critical population.
  - Inhibit the growth of contaminants.
  - Economical and simple to prepare from ingredients that are readily available
  - No need for a CO_2 incubator

Requirements for LJ DST Media preparation

- Reagents/Chemicals:

- Magnesium citrate
- First and second line drug stock solutions
- Magnesium sulphate heptahydrate ($MgSO_4$. $7H_2O$)
- Malachite green dye
- 70% Ethanol
- Malachite green dye
- Asparagine
- Glycerol
- Reference strains (rapid grower like *M. fortitum* and sensistive strains like H37Rv strain and also resistant strains)
- Sterile distilled water

- Equipment:
- Analytical balance sensitive to 0.1 g
- Weighing masses of 10g, 20g and 100g
- Inspissator
- Racks
- Dispenser device
- Bunsen burner
- Water bath
- Homogenization equipment
- Autoclve
- Hand brush
- Biosafety hood
- Automatic pippete

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Requirements for LJ DST Media preparation

- Materials:
- 1000 mL sterile graduated cylinder
- 2000 mL sterile graduated cylinder
- 2000 mL sterile wide mouth conical flask
- 500 mL sterile conical flask
- 100 mL sterile graduated cylinderVolumetric Flask
- Sterile Funnel
- Weighing paper
- Clean glass bottles
- Spatula

- Materials:
  - 50ml falcon tubes
  - Gloves
  - Laboratory coats
  - Safety glasses
  - Reagent preparation logs
  - Paper towels
  - Autoclave tape
  - Sterile gauze/sieve

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Steps For preparing LJ DST medium

1. Prepare mineral salt solution and malachite green solution
2. Autoclave solutions or base
3. Prepare drug working solutions.
2. Clean eggs
3. Homogenize whole eggs
4. Combine mineral solutions, homogenized eggs and malachite green according to respective volumes in your procedure and add drugs.
5. Dispense and cap tightly
6. Slant the LJ DST media tubes and inspissate at 85°C for 45 minutes.
7. Allow the media to cool overnight and Quality control

Preparation of Malachite green solution

- Weigh Malachite green dye 2.0 g
- Measure Sterile distilled water 100.0 mL
- Dissolve the dye in sterile distilled water by gently swirling the cornical flask.
- Transfer the dye to the universal bottles, appropratelly label them and autoclave at 121°C for 20 minutes to sterilze.
- Store at room temperature for 6 months

Preparation of mineral salts

Distilled water	600
L-Asparagine	3.6g
KH_2PO_4	2.4g
Magnesium citrate	0.6g
$MgSO_4.7H_20$	0.24g
Glycerol	12ml

- Weigh and measure reagents as indicated in table
- Dissolve in distilled water
- Label container
- Autoclave at 121°C for 20 minutes
- Incubate overnight before use
- Incase not needed for use store at 2-8°C

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Preparation of Drug working solutions.

LJ DST drug working solutions can be prepared basing on the table below:

	1st line drugs (concentrations of working solutions)				2nd line drugs (working concentrations)			PNB
	INH	EMB	Rif	SM	Lev	Mox	Amk	PNB
Stock Concentration (µg/ml)	10,000	10,000	10,000	10,000	10,000	10,000	10,000	50,000
Vol. of Stock into 9ml (9.9ml for INH)	0.1ml	1ml	-	1ml	1ml	1 ml	-	
Working conc'n (µg/ml)	100	1000	10,000	1000	1,000	1,000	10,000	50,000

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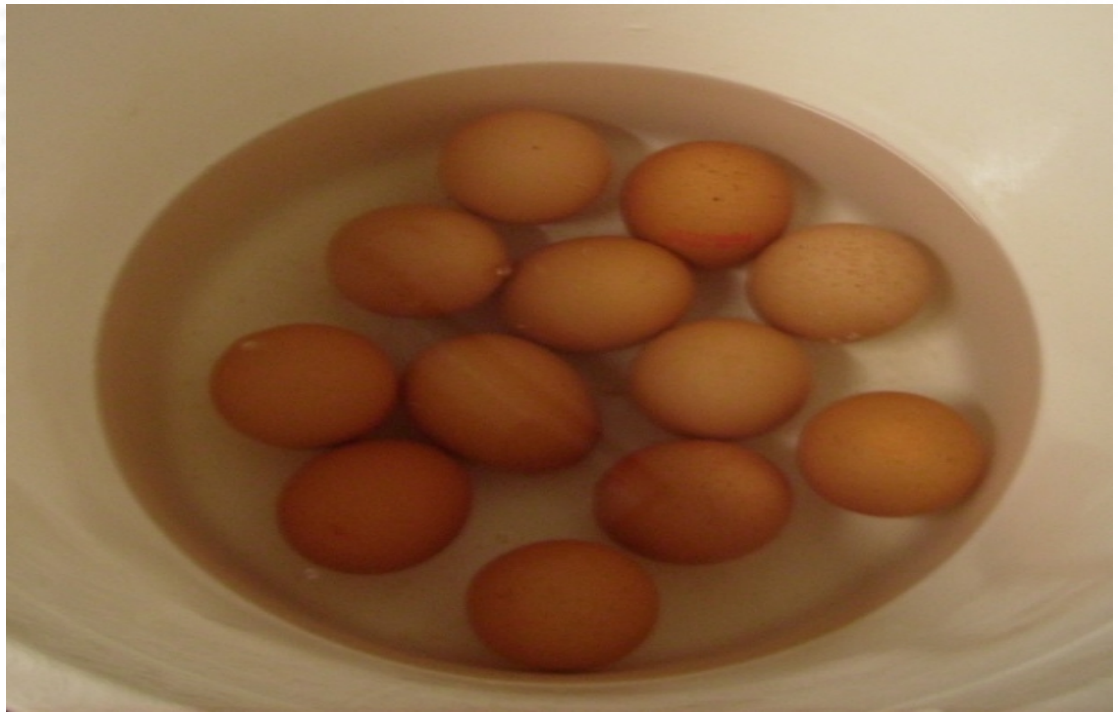
Egg preparation

- Inspect fresh hen's eggs for cracks (<2 days old)
- Soak eggs in soap solution for 30 minutes
- Scrub thoroughly with brush.
- Rinse with clean water.
- Soak in 70% ethanol for 15 minutes
- Crack eggs and homogenize using a blender.
- Measure desired volume of homogenized eggs.



- **Cracked and homogenized eggs filtered through a sterile gauze**

WORK PROCEDURE

- To 1000ml homogenized eggs add 600ml mineral salts and 25ml malachite green
- Mix thoroughly
- Divide equally the prepared LJ media into individual conical flasks.

Work procedure for LJ DST media preparation

- Label the conical flasks with individual drugs to be prepared leaving behind one for plain LJ media and PNB control.

- Aliquot the respective volume of working drug concentration into the individual conical flasks in order to achieve the final critical concentration required in the LJ media.

Dispensing of LJ DST media

- Connect the media dispenser machine in the laminar flow hood into the flask containing LJ DST media
- Adjust the media dispenser to dispense 5ml for universal containers
- Dispense Individual DST media into the universal bottles and cap immediately

Inspissation

- The inspissator should be switched on prior such that it attains a temperature of 85 °C
- Place the tubes containing media in the inspissator in a slanted position
- Cover the bottles with inspissator blanket
- Coagulate the media for 45 minutes at 85 °C.
- Check quality by beating one slant against a table.
- Let slopes cool down at room temperature over night.
- Incubate the sterile LJ DST media for 48 hours.

QC FOR LJ DST MEDIA

Inspect all tubes

- After incubation, Discard tubes that are discoloured, have poor texture or bubbles following inspissation

Color:

- Different shades of green colour: poor homogenization or presence of material residues in the tubes
- Dark green: low pH (acidic)
- Light green/yellow: high pH (alkaline)

QC FOR LJ DST MEDIA-2

Texture:

- If the medium disintegrates easily, the inspissation temperature might have been too low; not suitable for culture inoculation

Homogeneity:

- If bubbles: excessive temperature
- If clumps: poor homogenization

QC FOR LJ DST MEDIA-3

- **Sterility check:**
  - Place the media batch prepared at 37 °C for 48hrs
  - Evaluate each slant for growth
  - Calculate contamination percentage
    - % contamination = $\frac{\text{Number of slants contaminated}}{\text{Total number of slants prepared}} \times 100$

- If batch % contamination exceeds 5% notify Technical supervisor and an occurrence should be documented.

QC FOR LJ DST MEDIA-4

- **Quality check:**

  Confirm the performance characteristics of each lot by using reference strains such as *M. fortuitum, H37Rv and resistance strains if available.*

  - ***M. fortuitum*** *-expect growth on PNB and plain LJ media in 72hrs, supports fast growers (contaminants). 1st set fails set another*
  - ***H37Rv****- All drugs should be sensitive to the H37RV strain and results are expected within 4 weeks from the day of incubation*
  - ***Resistance strains****: Respective Resistant patterns for the individual drugs should be interpreted within 4 weeks from the day of incubation.*

NB: Incase of QC failure for a given batch of LJ DST media, that media should not be used to set patient samples and an occurrence should be documented.

Labelling and Storage

- Label the LJ DST media tray with batch number, date of preparation, date of expiry, person who prepared and identity of media
- Store plain LJ DST media up to 3 months at 2-8 ^{o}C.

Documentation;

- Document the Lot numbers of reagents used, LJ DST media batch number, date of preparation and expiry date on reagent preparation logs/worksheets.
- Document the quality controls set on a QC log/work sheet.

Unsatisfactory performance

- If culture media has unsatisfactory performance in two consecutive controls:
  - The laboratory should not use or distribute the culture media until the deficiencies are resolved
  - Culture media should be purchased or otherwise secured so that the laboratory can continue its activity
  - Retraining in culture media preparation procedures should be offered, if needed

Key factors for successful LJ DST medium preparation and trouble-shooting

- Eggs used to prepare LJ medium must be fresh (<2 days old) and from hens that are not treated with antibiotics as these may interfere with drug interpretation

- Time and temperature of inspissation are crucial and must be controlled:
  - Quality of egg medium deteriorates when coagulation is done at too high a temperature or for too long
  - Discoloration of coagulated medium may be due to excessive temperature.
  - Drug potency may be reduced due to excessive temperatures.

- Little holes or bubbles on the surface of the medium indicate faulty coagulation and poor dispensing technique

Assessment

- List some of the merits of using LJ DST media?
- What are the requirements needed for preparation of LJ DST media?
- How is inspissating time and temperature critical in LJ DST media preparation?
- What is the shelf life and storage conditions of prepared LJ DST media?
- What materials are required for running QC on prepared LJ DST media?

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Summary points

- Use freshly prepared drug working solutions
- Use only clean sterile glassware
- Ensure eggs are fresh and antibiotic-free
- Use good aseptic technique when preparing LJ DST media
- Pay close attention to time and temperature during inspissating.
- Inspect all prepared LJ DST media carefully; always

References

GLI TB training package

- http://www.stoptb.org/wg/gli/trainingpackages.asp

Acknowledgments

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