

MYCOBACTERIAL CULTURE

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OVERVIEW AND PURPOSE

Mycobacterial Culture

- Gold standard method for TB diagnosis with respect to sensitivity and specificity
 - Use of culture increases the number of TB cases found by 30–50% over smear alone
 - ~10 viable bacilli/ml of sputum needed for culture compared to at least 5000 bacilli/ml of sputum for microscopy
- Culture is used for species identification, drug susceptibility testing (DST), and genotyping
- Culture also used to monitor patient response to treatment
- Culture is technically demanding & prone to contamination

Purpose of Mycobacterial Culture

Detection of *Mycobacterium tuberculosis* complex (MTBC)

- The most clinically significant mycobacterial species for public health
- Isolation almost always signifies disease, except in the case of laboratory cross-contamination
- MTBC organisms are not present in the environment

Detection of Non-tuberculous Mycobacteria (NTM)

- Are opportunistic pathogens in humans and may cause significant human disease
- Clinicians ultimately responsible for determining the importance of a NTM
- Almost all of these species can be found in environmental samples

MEDIA

Culture Media

Two major categories of media are routinely used:

- **Solid:** egg- and agar-based
- **Liquid:** also often referred to as broth media

An ideal media for isolation of mycobacteria will meet the following criteria:

- Supports rapid and robust growth of small numbers of mycobacteria
- Permits preliminary differentiation of species on the basis of pigment production and colony morphology
- Inhibits growth of contaminants

No single medium meets all these requirements, hence:

- the use of both liquid and solid media for initial culture recommended
- may be purchased commercially or prepared in-house
- Quality Control of media must be performed

Solid Media: Egg-based

Lowenstein-Jensen (LJ)

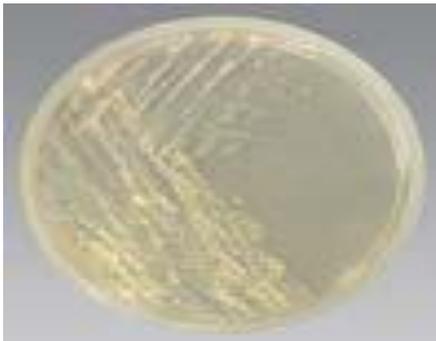
- Selective; malachite green-Inhibits growth of contaminating bacteria and fungi
- Supports growth of MTBC (with exception of *M. bovis*) better than NTM
- Shelf life of 6–12 months when refrigerated
- May have excess of water—should be removed before inoculation



Solid Media: Agar-based

Middlebrook 7H10 and Middlebrook 7H11

- Media is clear, thus allows easier colony observation and quantification
- Mycobacterial colonies can potentially be isolated if media is contaminated
- Can be selective if antibacterial and antifungal antimicrobials are added to inhibit contaminating bacteria
- The average time to detection of growth is earlier than with the egg-based media
- Available as plate or slant



*Middlebrook plate and slant:
Compared to plates, slants do not
dry out or expire as quickly but
growth may be more difficult to
detect*



Comparison of Solid Media

Conditions for Mycobacteria Growth and Recovery	Egg-based Media	Agar-based Media
Selective	Added malachite green	Added antibacterial and antifungal
Rate of growth	Slower	Faster and able to support INH Resistant and fastidious strains
Contamination	Usually less but if present, involves the entire surface	More frequent, but can still isolate colonies
Area of Inoculum	Relatively small	Large
Visual Examination	Difficult due to media opacity	Media is clear; allows easier colony quantification and morphology confirmation
CO₂	Not required	Required
DST	Not performed on LJ in United States	Preferred due to larger surface area and faster rate of growth
Shelf Life	Long (6–12 months)	Short (1–2 months)

Liquid Media

- Use is recommended standard practice for mycobacteriology laboratories
- Increased recovery of mycobacteria and decreased time to detection compared to solid media
- More easily contaminated than solid and the addition of antimicrobials is required
- Average time for growth detection of slowly growing mycobacteria is 12–16 days
- Some fastidious mycobacteria grow only in liquid media
- Shelf life is long; can be stored at room temperature
- Incubation with additional CO₂ is not required

AUTOMATED SYSTEMS

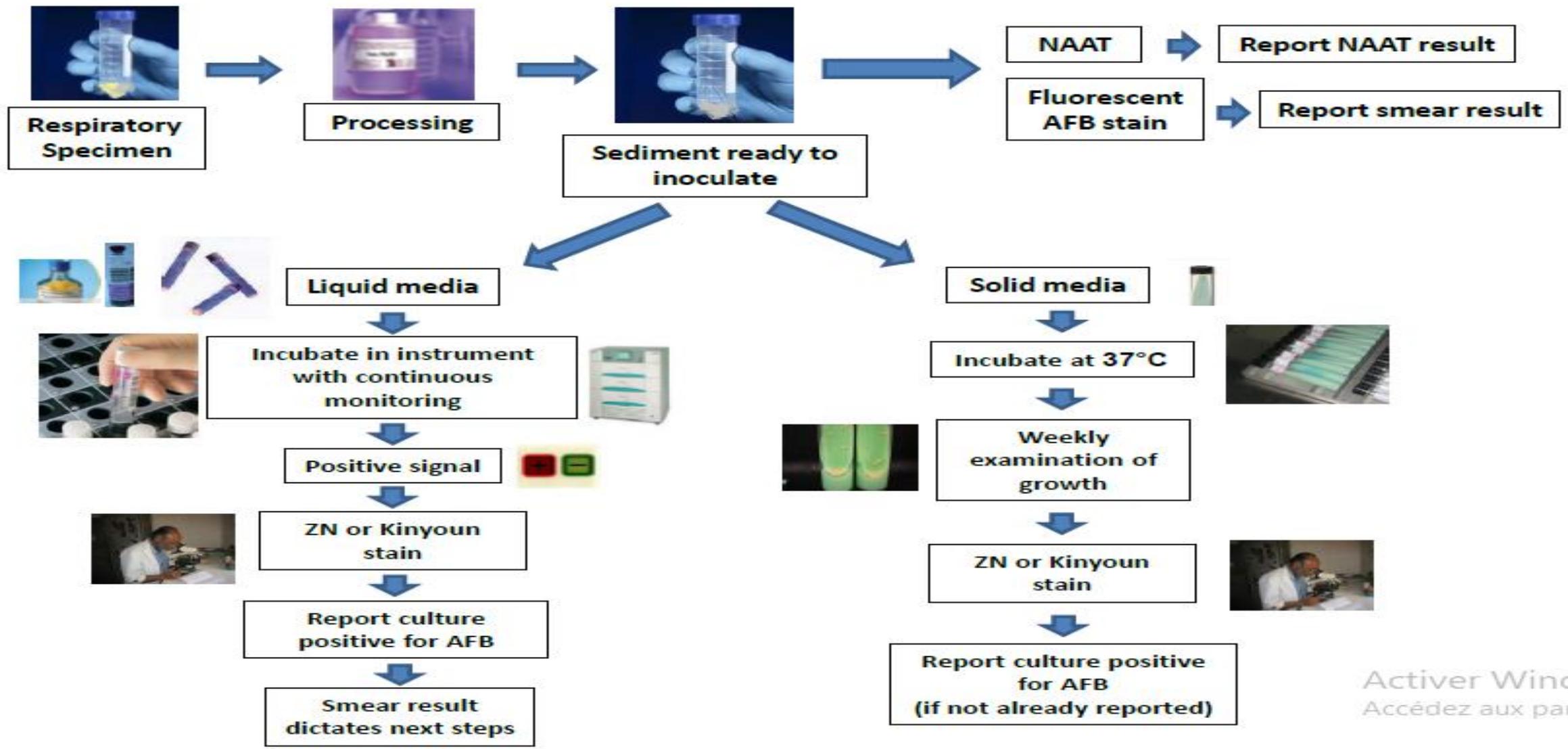
Automated Systems

- Continually monitor the media for detection of mycobacteria for 6 weeks
- Most widely used FDA-cleared automated systems for rapid detection of mycobacteria using liquid media
 - BiorMerieux BacT/ALERT® 3D
 - Becton Dickinson BACTEC MGIT™
 - Thermo Scientific VersaTREK™
- MGIT and VersaTrek are also FDA-cleared for susceptibility testing of MTBC

Comparison of Automated Systems

	BacT/ALERT	MGIT	VersaTREK
Means of Inoculation	Sharps or needleless devices	Disposable pipets	Sharps or disposable pipets
Specimen types not validated	Blood-must use MB bottles	Urine, Blood	N/A
Detection	Production of CO ₂ - color change	O ₂ consumption- fluorescence	O ₂ consumption - pressure change
DST (FDA Cleared)	No	Yes	Yes
Antibiotic Supplement	amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin	PANTA—polymyxin B, amphotericin B, naladixic acid, trimethoprim, and azlocillin	PVNA—polymyxin B, vancomycin, nalidixic acid, and amphotericin B

CULTURE PROCESS



Main processing methods for sputum

- Sodium hydroxide (modified Petroff) method.
- *N*-Acetyl-*L*-cysteine-sodium

Sodium hydroxide (modified Petroff) method – advantages/disadvantages

- Simple.
- Inexpensive reagents, easy to obtain.
- Effective control of contaminants.
- One hour.
- Sterilized NaOH solution can be kept for several weeks
- Specimen exposure times must be strictly followed.
- The procedure kills up to 60% of tubercle bacilli in clinical specimens.
- Not suitable for liquid media.

NALC–NaOH method – advantages/disadvantages

1. Kills only about 30% of tubercle bacilli, allowing more positive cultures than other methods.
 2. Time required to perform the procedure: single-specimen processing takes approximately 40 minutes; processing 20 specimens would take approximately 60 minutes.
 3. Suitable for liquid media
1. Acetyl-cysteine loses activity: it needs to be made fresh daily.
 2. Ready-to-use NALC-NAOH solutions are commercially available, but at higher cost

Inoculation

- Solid media

- Tubed—approximately 2-3 drops of processed specimen

Allow to spread over surface

- Liquid media

- Pipette against inside of tube/bottle to reduce splash

BacT/Alert 3D—0.5 ml

MGIT—0.5 ml

VersaTrek—up to 1.0 ml

Incubation

- Tubed solid media

Slanted position with screw caps loose for 5–7 days

After 7 days caps should be tightened and tubes may be positioned upright

- Temperature

Optimal temperature for MTBC is 35–37°C

Some species of NTM grow best at 25–33°C For skin, bone, and joint biopsies, inoculate two sets of media, one at 37°C and the other at the lower optimal temperature

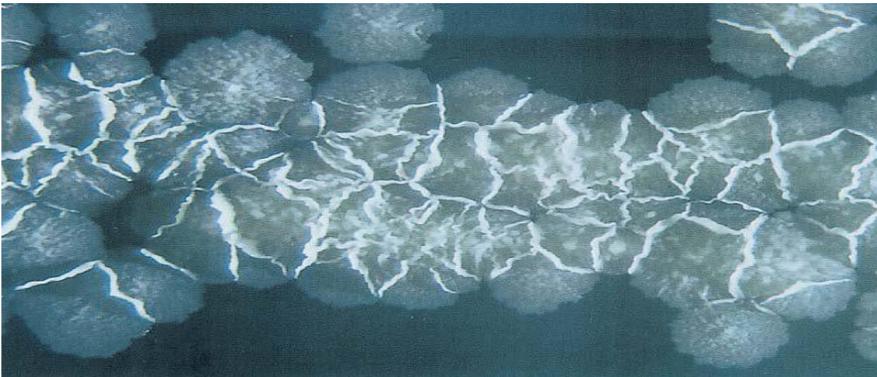
M. xenopi requires 40–42°C for optimal growth

Solid Media Examination Schedule

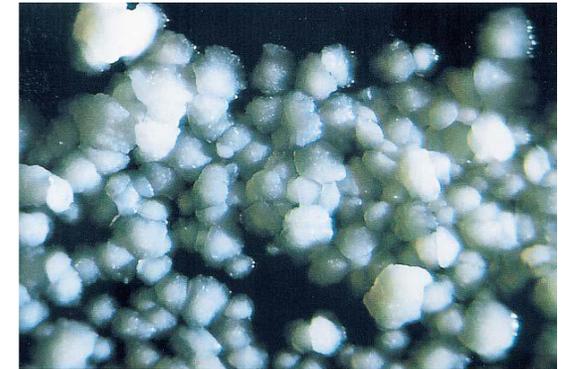
- Examine at end of week 1 to assess growth and possible contamination
- Continue to examine weekly for 6–8 weeks
- Corresponding liquid media is likely to become positive first but continue to incubate solid media until growth is observed or end of incubation time is reached
 - Growth should be identified as soon as possible regardless of media type

Visual Examination

Colony Morphology on solid media



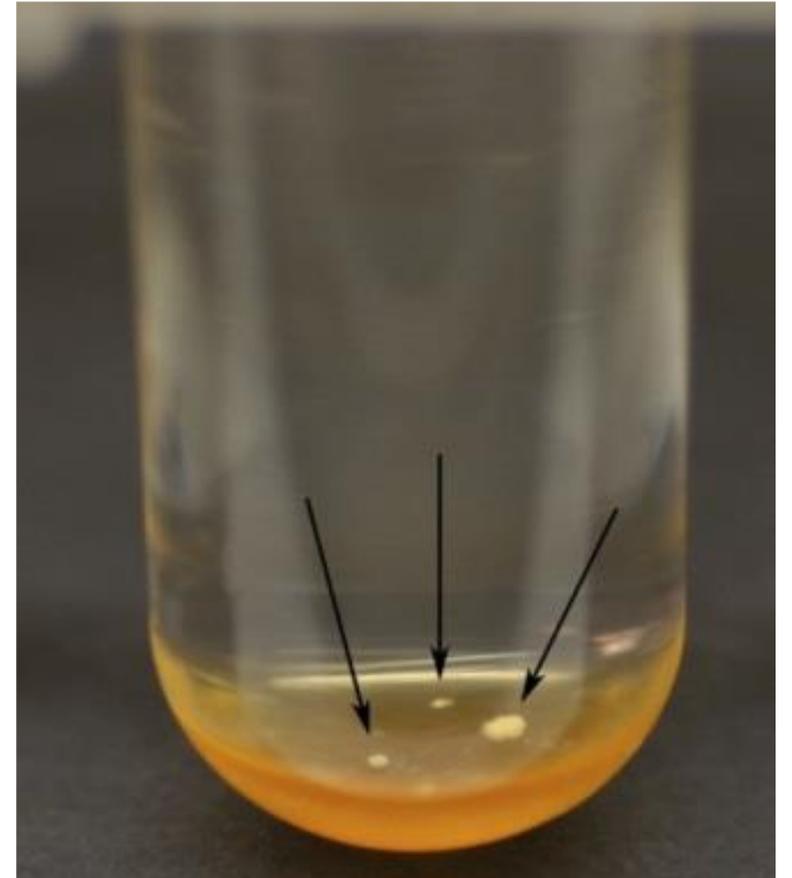
M. tuberculosis is rough and non-pigmented



M. bovis is flat, smooth and non-pigmented

Liquid Media Examination Schedule

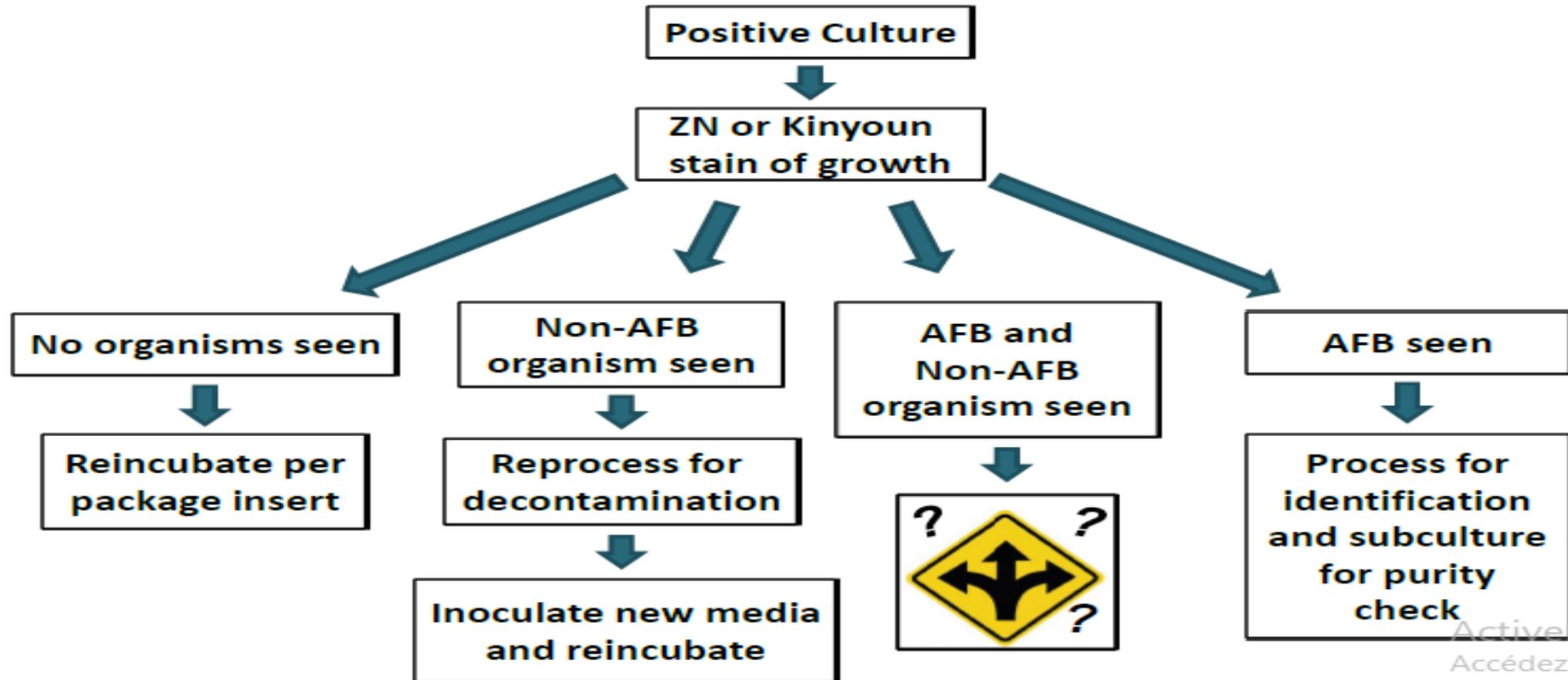
- In automated systems, tubes are read continuously and flagged when positive
 - Perform acid-fast bacteria (AFB) smear with Zeihl-Neelson (ZN) or Kinyoun
 - Smear result determines next steps
- All MGIT and VersaTREK negative tubes at end of incubation period should be visually checked for evidence of growth before being discarded



AFB Smear of Growth in Liquid Media

- ZN or Kinyoun staining should be performed on growth as soon as possible
- Deposit drop of culture on glass slide, let dry in biological safety cabinet, fix, and proceed with staining protocol
- After staining, culture should be handled according to the results

Algorithm for Growth in Liquid Media



REPORTING

Reporting Positive Cultures

- Provide interim report as soon as media turns positive and AFB are observed, indicating identification to follow
- Report should be updated when identification made
- Minimally, report should indicate either MTBC or NTM

Reporting Negative Result

- No clear guidance regarding when to issue a no growth (negative) report
- Final Report issued at 6–8 weeks (e.g., No growth of mycobacteria) with typically no interim report of no growth to date
 - Automated systems incubate liquid media for 6 weeks
 - Historically, solid media has been incubated for 8 weeks

CONTAMINATION

Contamination

Most specimens for AFB testing come from non-sterile sites, and despite the decontamination process, some contamination of culture media is to be expected.

Contamination – Solid media

- Most common contaminant is mold
 - Any culture showing the presence of mold contamination should be discarded
- Some bacterial species can liquefy or discolor the solid medium and those tubes should be discarded

Contamination – Solid media (2)

- If partial contamination present
 - Culture can be retained if not discolored or liquified
 - Smear can be prepared from surface of the medium
 - If smear shows AFB, growth may be streaked for isolation or re-decontaminated and re-inoculated
- Many laboratories discard contaminated solid media and continue the culture with only the corresponding liquid media

Contamination of Liquid Media

More susceptible to contamination than solid media

Needs to be supplemented with mixture of specific antibiotics to reduce contaminants

Contamination should be suspected if homogeneous turbidity is seen

Contamination – NTMs

Many NTM are found in the environment (e.g., *M. gordonae* in tap water)

The significance of the isolation of NTM may be difficult to assess since many species are opportunistic pathogens but may also be contaminants

Laboratories should communicate with healthcare provider to determine need for further identification of NTM based on clinical need

Monitoring Contamination

- Monitoring of contamination rates is a fundamental quality indicator
- *Specimen* contamination is defined as all media inoculated per specimen (liquid and solid) being completely compromised
- Important to monitor liquid and solid media contamination rates separately
 - Monitoring only specimen contamination rates may mask a problem with either liquid or solid media

Monitoring Contamination

	Numerator/Denominator	Recommended levels	Investigative actions*	
			Potential causes-increases	Potential causes-decreases
Specimen	# cultures reported out as "contaminated" in one month/Total # of cultures reported in one month	3-5%	May indicate insufficient processing, specimen delivery delays, contaminated reagents, media or equipment	May indicate use of harsh specimen processing reagents or excessive processing procedures
Liquid Media	# inoculated tubes discarded due to contamination in one month/Total # of tubes inoculated in one month**	5-8%	See above (>8% for upper threshold), PANTA volume insufficient or expired	See above
Solid Media	# solid media (slants or plates)*** discarded due to contamination in one month/Total # solid media (slants or plates) inoculated in one month	3-5%	See above. LJ media old/expired, increase in pseudomonas in cultures.	See above

*List is not all - inclusive

**This calculation should not include specimens that have been re-decontaminated from aliquots of previously processed or stored specimens

*** Slants or plates used in the initial set-up.

Monitoring Contamination

If contamination rate is increased, also consider these non-laboratory causes

- Specimens experiencing delays in transportation to laboratory
- Specimens not refrigerated during shipment
- Shifts in patient populations (e.g., more patients with cystic fibrosis)

Carefully examining your laboratory's data may reveal possible causes and solutions to contamination problems

Practices to Reduce Possibility of Cross Contamination

- Use daily aliquots of processing reagents and buffers
 - Any leftover should be discarded
 - Never use common beakers or flasks when processing
- Keep the specimen tubes tightly closed and clean the outside of the tube prior to vortexing or shaking
- Pour reagents slowly against the inside of the tube to minimize splashing
- Do not touch the container of reagents to the lip of the tube at any time

FALSE POSITIVES

False Positives

False positives are test results reported as positive for a *Mycobacterium* species not present in the patient specimen

- Not all false positives are due to laboratory cross contamination
- All laboratories are capable of producing false positive results

False Positives (2)

- Burman and Reves meta-analysis showed 3.1% of positive results for MTBC from laboratories are false positives (range of 2.2–10.5%)
 - False positive results may be generated at any step between specimen collection and reporting
 - May go unrecognized in previously confirmed patients
- In the study, 67% of patients with false positive cultures were treated for TB disease
- Previously confirmed TB patients were thought to have failed treatment due to false-positive culture

Determination of a False Positive Result

Determination cannot be made by the laboratory alone

If false positive culture is suspected, communication between the laboratory, clinician, and TB control is imperative to avoid unnecessary treatment

Consequences of False Positives

- Patients may be managed incorrectly
 - Unnecessary treatment and toxicity
 - Unnecessary isolation, hospitalization, and healthcare costs
 - Emotional repercussions to the patient
 - Unnecessary contact investigations
- Credibility of the laboratory, hospital, or clinician may be questioned
- May increase laboratory workload and testing costs

Thank you for your Attention