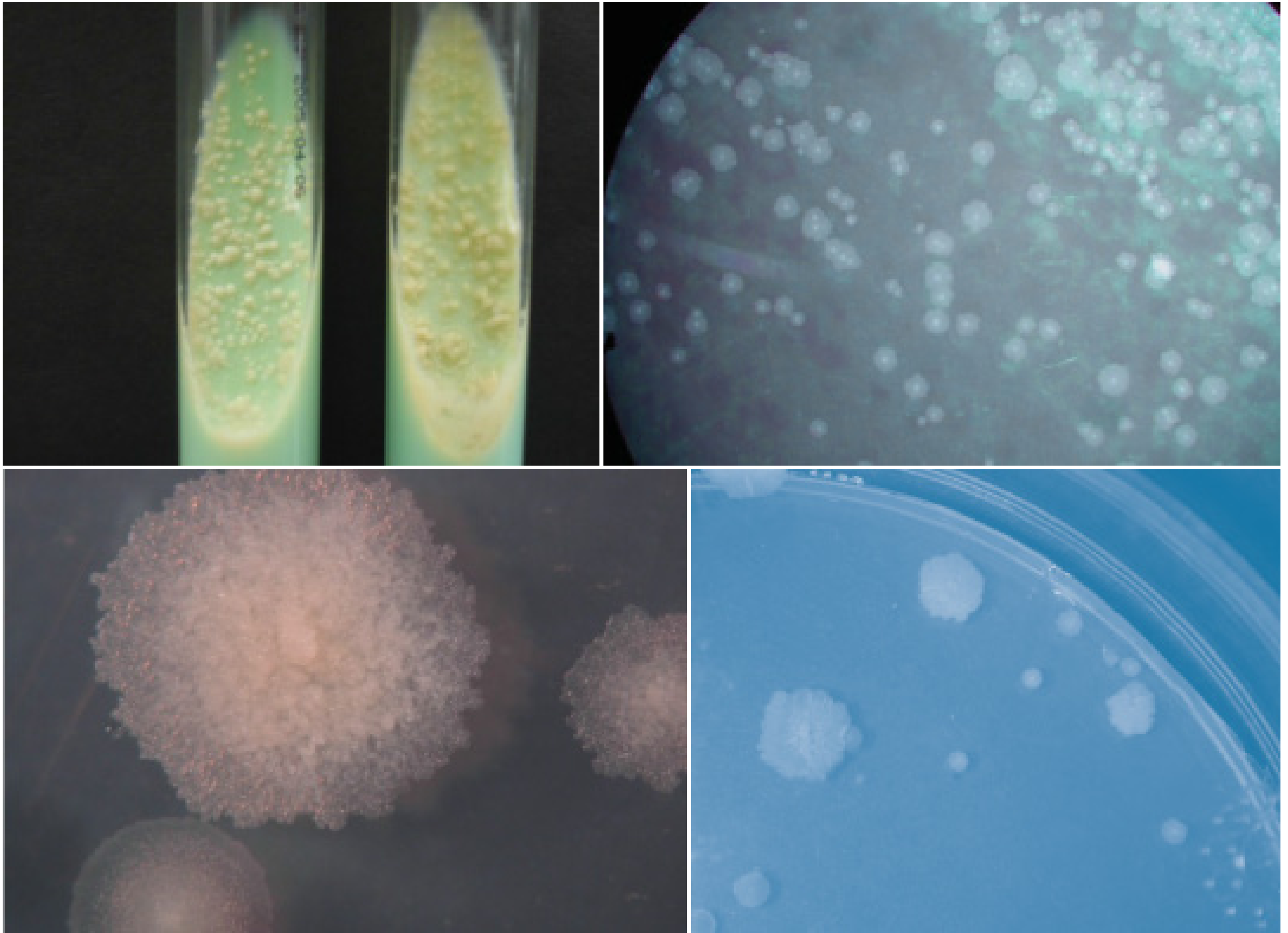

A Clinician's Guide to the TB Laboratory

HEARTLAND
NATIONAL TB CENTER
THE UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT TYLER

EXCELLENCE • EXPERTISE • INNOVATION

Dear Healthcare Professional,

This book provides basic information on the use of the public health and clinical laboratories in the diagnosis and monitoring of patients with tuberculosis (TB) infection and TB disease. The information provided is based on recommendations by the Centers for Disease Control and Prevention (CDC), American Thoracic Society (ATS), Infectious Disease Society of America (IDSA), The Clinical and Laboratory Standards Institute (CLSI), and the Association of Public Health Laboratories (APHL). There are variations in the services provided by individual laboratories; therefore we recommend that you work closely with your state or local public health laboratory staff in implementing the best care for your patients.

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Acronyms and Abbreviations

ADA	adenosine deaminase
AFB	acid-fast bacilli
AIDS	acquired immune deficiency syndrome
AII	airborne infection isolation
ALT	alanine aminotransferase
AST	aspartate aminotransferase
APHL	American Public Health Laboratories
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BCG	bacille Calmette-Guérin
CDC	Centers for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
CSF	cerebrospinal fluid
CXR	chest x-ray
DNA	deoxyribonucleic acid
DST	drug susceptibility testing
EDTA	ethylenediaminetetraacetic acid
EMB	ethambutol
FDA	Food and Drug Administration
HNTC	Heartland National TB Center
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IGRA	interferon gamma release assay
INF-y	interferon gamma
INH	isoniazid
MDR TB	multidrug resistant tuberculosis
MDDR	molecular detection of drug resistance
MIC	minimum inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MTBC	*Mycobaterium tuberculosis* complex
MTD	*Mycobacterium tuberculosis* direct test
NAA	nucleic acid amplification
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NTM	non-tuberculous mycobacteria
PCR	polymerase chain reaction
PZA	pyrazinamide
QFT	quantiFERON
RFLP	restriction fragment link polymorphisms
RNA	ribonucleic acid
RTMCC	regional training and medical consultation center
SNP	single nucleotide polymorphism
TB	tuberculosis
TDM	therapeutic drug monitoring
TLC	thin layer chromatography
TTP	time to positive
TST	tuberculin skin test
VRE	vancomycin resistant enterococus
WBC	white blood cell
WGS	whole genome sequencing
WHO	World Health Organization
XDR TB	extensively drug resistant tuberculosis

The Role of the Laboratory in TB Control

What Providers Should Know

Chapter 1

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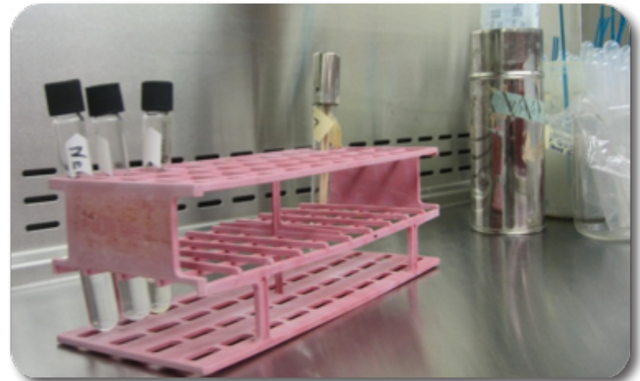
Chapter 1

What Providers Should Know

Introduction



Tuberculosis (TB) is a serious but curable disease that continues to cause unacceptable morbidity and mortality in the United States and the world. The purpose of this book is to increase the knowledge of physicians and other private and public healthcare providers about the laboratory testing services that, when utilized fully, can be powerful tools for the diagnosis and treatment of TB patients.

The goal of the laboratory is to provide rapid and reliable results that support the ability of physicians and public health professionals to diagnose TB infection and TB disease, to implement appropriate treatment, and to prevent transmission. Achieving this goal depends on a partnership between medical professionals and public health providers and the laboratory. Early clinical recognition of TB disease and rapid laboratory identification of *Mycobacterium tuberculosis* complex (MTBC) must be regarded as top priorities. It is critical that good quality specimens be collected, handled appropriately, and sent to the laboratory quickly. It is the responsibility of the healthcare worker charged with collecting specimens to follow established guidelines (See Appendix Table A1).

This book provides information regarding laboratory tests for TB - when requesting a test, appropriate collection and handling of specimens, and interpretation of test results. We hope this will assist in minimizing the delays in patient diagnosis that are linked to poorer outcomes.

What services does my laboratory provide?

It is important to be aware of the services provided by your laboratories and to understand referral patterns and acceptable timelines for reporting specimen results. In the event that a laboratory cannot provide a service, an established mechanism for expedient referral to another laboratory is recommended. When timely results are not available, the laboratory should be contacted to identify the problem and assess available options to support diagnosis of the patient. **Table 1** indicates which services TB laboratories provide, the purpose of the tests, and the expected turnaround times for reporting test results. **Figure 1** on page 5 graphically depicts these tests and important turnaround times.

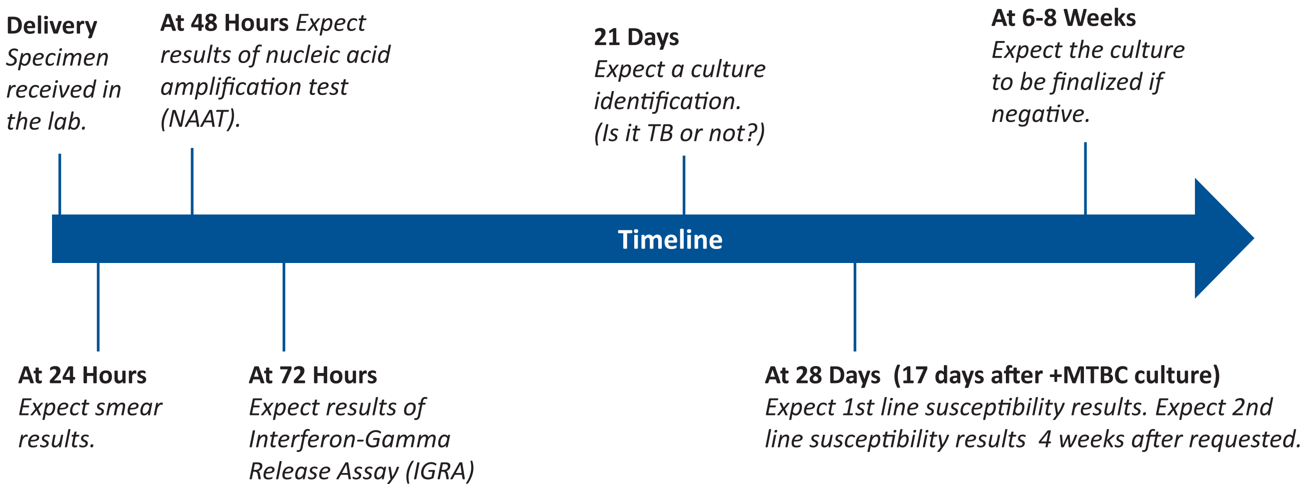
Table 1 – TB Laboratory Services

Testing Method	Purpose	Time Frame for Results
Acid-fast bacilli (AFB) Smear	• Detect AFB in a person suspected to have TB or non-tuberculosis mycobacterium (NTM) disease • If AFB smear positive, may predict likely source case and aid in prioritizing the need for contact investigation • Monitor response to treatment	• Within 24 hours of specimen receipt by laboratory • Positive result on initial specimen should be communicated to provider as a critical value
Nucleic Acid Amplification (NAA) Test	• Detect MTBC DNA or RNA in patient specimen	• Within 48 hours of specimen receipt by laboratory • Positive result on initial specimen should be communicated to provider as a critical value
AFB Culture and Identification	• Identify viable MTBC organisms • Monitor response to treatment • Recover isolates for drug susceptibility testing • Recover isolates for genotyping	• 80% of initial specimens are reported as culture positive by day 21 following receipt by laboratory • Positive result on initial specimen should be communicated to provider as a critical value • A negative culture is reported after 6 to 8 weeks
Drug Susceptibility Testing (DST)	• Determine if MTBC organism is susceptible or resistant to specific drugs	• First-line DST should be reported within 17 days after positive MTBC culture report (CDC guidance for public health laboratories)
Molecular Detection of Drug Resistance (MDDR)	• Identify mutations that predict drug resistance from smear positive/NAAT positive clinical specimens or from an MTBC isolate. Generally, the absence of a mutation decreases the likelihood of resistance. This varies from drug to drug.	• 48 – 72 hours after receipt by laboratory
Genotyping	• Identify genetic fingerprint of an isolate • May be used to assist with an epidemiological investigation • May identify a false-positive result • Distinguish *Mycobacterium bovis* or *M. bovis* bacilli Calmette-Guérin (BCG) from other members of the MTBC	• Turnaround time varies due to differences in individual state or jurisdictional TB Program genotyping project policies and due to varied degrees of compliance from the laboratories submitting MTBC isolates to their public health laboratory (PHL) for genotyping. In addition, the public health laboratory has to ship MTBC isolates to a reference laboratory for genotyping services.
Interferon-Gamma Release Assay (IGRA)	• Detect immune response to *Mycobacterium tuberculosis* complex (MTBC) antigens to identify infection	• Within 48 - 72 hours of specimen receipt by laboratory

Optimal isolation of mycobacteria is dependent on proper methods of collection, storage, and transport of specimens. Information regarding the best way to collect and store various specimens can be found in Appendix A1.

When can I expect the results from the laboratory?

Figure 1– Timeline of Results



Is *Mycobacterium tuberculosis* complex always a pathogen?

Mycobacterium tuberculosis complex should always be regarded as pathogenic. When the microbiology laboratory isolates it, health care providers should regard it as significant and initiate treatment unless a false positive is suspected. For more information on culture conversion see Chapter 5.

Identification of *Mycobacterium tuberculosis* is reported as MTBC. MTBC includes the species *M. tuberculosis, M. africanum, M. canetti, M caprae, M. microti, M. pinnipedii, M. bovis, and M. bovis Bacillus Calmette Guérin* (BCG). Further identification of the MTBC to the species level is not necessary except in the case of *M. bovis* because *M. bovis* is always resistant to pyrazinamide, has a propensity for disseminated infection and has generally poorer outcomes. *M. bovis* can be identified through genotyping though this is not the purpose or intent of this process. Although genotyping results are available relatively quickly and therefore clinically useful, they are primarily a surveillance tool and not for medical management.

M. bovis BCG can be recovered from immunocompromised individuals who have received BCG either as a vaccination or treatment for bladder carcinoma. It may represent disease in some individuals; however, *M. bovis* as BCG can routinely be cultured from urine following intravesicular instillation of BCG and, in this situation, generally, does not represent disease.

Notes:

Laboratory Diagnosis of TB infection: Interferon Gamma Release Assays

Chapter 2

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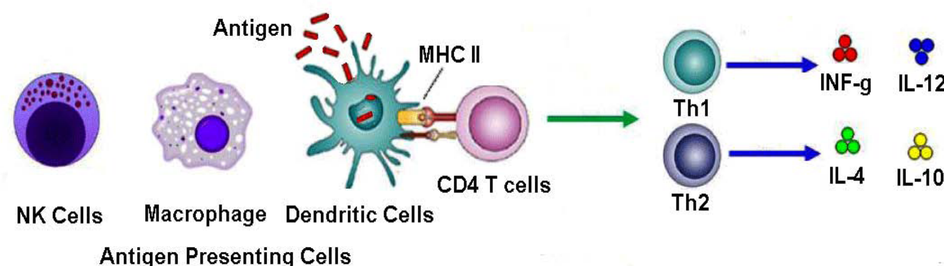
Chapter 2

Interferon-Gamma Release Assays

Background

The commercially-available generation of Interferon-Gamma Release Assays (IGRAs) include the QuantiFERON®-TB Gold In-Tube assay (QFT-GIT), and the T-SPOT®.TB assay (T-Spot). The Quantiferon TB Gold Plus was recently approved by the FDA and will replace the QFT-GIT. Currently available IGRAs measure immune responses related to CD4+ lymphocytes while the Quantiferon Gold Plus will measure both CD4+ and CD8+ lymphocyte responses. IGRAs are indirect tests developed to enhance the accuracy and simplicity of diagnosing TB infection (including disease). IGRAs assess the *in vitro* immune response following stimulation of lymphocytes by synthetic peptides representing proteins that are highly specific for MTBC. The Food and Drug Administration (FDA) approved the QFT-GIT in October 2007 and the T-Spot in 2008. These tests should be used in conjunction with a risk assessment, medical evaluation, symptom screen, and chest radiograph.

Figure 2 - IGRA Process for CD4+ T Cells



Created by Dr. Ed Graviss, Director, HMRI Molecular TB Laboratory, Department of Pathology and Genomic Medicine, Houston Methodist Research Institute.

Principles of the Test

Proteins used in these tests include the early secretory antigen target-6 (ESAT-6), the culture filtrate protein-10 (CFP-10) and, in the case of QFT-GIT, protein TB7.7. These proteins are specific for members of the MTBC except for BCG vaccine strains. They are absent in most non-tuberculous mycobacteria (NTM); exceptions include *M. kansasii, M. marinum, and M. szulgai.* Figure 2 depicts the process of IGRA testing. The QFT-GIT detects *in vitro* production of interferon gamma (INF-γ) following stimulation of whole blood samples and measures the quantity of INF-γ released by an individual's peripheral blood monocytes. The T-Spot reports the actual number of interferon gamma-releasing T-lymphocytes detected.

Clinical Considerations

Testing for *Mycobacterium tuberculosis* complex (MTBC) infection should focus on those individuals who are at high risk for being infected with MTBC and/or who have an increased risk for developing TB disease once infected **(See Table 2)**. IGRAs are preferred to the tuberculin skin test (TST) for all circumstances in which the TST is currently used, except in children <5 years of age. The tests are especially useful in the evaluation of BCG vaccinated individuals (including recent immigrants) and individuals with a poor rate of return for TST reading.

Table 2 – Persons at High Risk of TB Infection and Developing TB Disease

HIGH RISK OF	MTBC Infection	• Persons who have spent time with someone who has TB disease. • Persons from a country in which TB is common (most countries in Latin America, Africa, Asia, Eastern Europe, Russia and the Caribbean). • Live or work in high-risk settings (correctional facilities, nursing homes, homeless shelters). • Healthcare workers who care for patients at increased risk for TB disease. • Infants, children and adolescents exposed to adults who are at increased risk for TB infection or TB disease. • Mycobacteriology lab personnel.
	Developing TB Disease, if infected[1]	• Persons with HIV infection. • Pesons who became infected with TB bacteria in the last 2 years. • Babies and young children. • Persons who inject illicit drugs. • Persons who are sick with other diseases that weaken the immune system. • Persons with a past history of inadequately treated TB. • Elderly, renal failure, diabetes, silicosis.

[1]Not necessarily at high risk of MTBC infection

Recent research has identified a high rate of false-positive results when IGRAs are used for serial screening of low risk persons such as healthcare workers. IGRAs should be used with caution for serial testing of populations with the recognition that reversions of a positive to a negative result occur frequently. If it becomes necessary to test low risk individuals, an IGRA and a second diagnostic test are suggested if the initial test is positive. (Table 3).

Table 3 – Testing for TB Infection

Criteria (when testing for TB infection is warranted)	Test[1]	Recommendation and Quality Level	Interpretation
• >5 years old. • **Likely to be infected** with Mtb. • **Low or intermediate risk** of disease progression. • History of BCG vaccination or are unlikely to return to have the TST read.	IGRA[1]	**Strong** recommendation Moderate-quality evidence	IGRA preferred, TST acceptable
• >5 years old. • **Likely to be infected** with Mtb. • **Low or intermediate risk** of disease progression.	IGRA[1]	**Conditional, suggested** recommendation Moderate-quality evidence	IGRA preferred, TST acceptable
• >5 years old. • **Likely to be infected** with Mtb. • **High risk** of disease progression.	IGRA[1] TST	Insufficient data for a recommendation	IGRA preferred, TST acceptable
• >5 years old. • **Unlikely to be infected** with Mtb. • **Low risk** of disease progression.	Do **NOT** test; *however,* such testing may be obliged by law or credentialing bodies		Considered infected only if **BOTH** diagnostic tests are positive
	IGRA[1]	**Conditional, suggested** recommendation Low-quality evidence	
	2nd diagnostic test[2]	**Conditional, suggested** recommendation Very-low quality evidence	
• <5 years old. • Considered generally healthy.	TST	**Conditional, suggested** recommendation Very-low quality evidence	Considered infected if either test is positive

[1]A TST is an acceptable alternative, especially in situations in which an IGRA is not available, too costly, or too burdensome
[2]A second diagnostic test is suggested if the initial test is positive. The confirmatory test may be either an IGRA or TST.

Table 4 – Strengths and Limitations of the Interferon-Gamma Release Assays

IGRAs	
Strengths	**Limitations**
• A single patient visit is needed and the patient does not need to return for a reading. • IGRAs are as sensitive as and more specific than the TST. • Proteins used in the IGRAs are not found in NTM except *M. kansasii, M. szulgai and M. marinum.* • Proteins used in the IGRAs are not found in BCG vaccines. • IGRAs are less affected by subjective error such as incorrect placement or reading of the TST.	• IGRAs require a blood draw. This may be a challenge in some field situations and with populations in whom phlebotomy is difficult. • Potential for laboratory error such as mislabeling, broken tubes, delays in processing, and improperly handled specimens. • Indeterminate results may occur in some populations and there are no guidelines for interpretation. • Positive results close to the cut point for the QFT-GIT may revert to a negative result on a repeat TST. • Reversions are common in low risk healthcare workers undergoing serial testing.

Table 5 – Possible Indications for Use of Both a TST and IGRA

Indication	Clinical Group	Rationale
Initial test is negative and risk of infection, progression to disease or poor outcome is high.	• Persons infected with HIV. • Persons currently taking a TNF Alpha inhibitor or other immunosuppressive drug. • Children < 5 years of age exposed to TB or suspected of TB disease. • Persons in whom there is clinical suspicion for TB disease such as those with symptoms and/or an abnormal chest radiograph consistent with TB disease.	• Additional testing enhances the sensitivity and adds supportive evidence to a diagnosis of TB infection or disease. For patients with an initial test that is negative, acceptance of a positive result from a second test increases sensitivity but may decrease specificity.
Initial test is positive and additional evidence of infection is needed to encourage acceptance and adherence.	• Foreign-born healthcare workers who believe their positive TSTs are due to BCG. • Healthy persons with low risk for either TB infection or progression to TB disease.	• A positive IGRA might prompt greater acceptance of the diagnosis and treatment for TB infection. • A positive result from a second test increases the likelihood that the test result reflects a true infection. • An alternative approach is to assume the initial test was a false-positive or that risk for disease does not warrant additional evaluation or treatment.
IGRA result is indeterminate or borderline.	• Persons infected with HIV. • Persons currently taking a TNF Alpha inhibitor or other immunossuppresive drug. • Children <5 years of age exposed to TB or suspected of TB disease. • Persons in whom there is clinical suspicion for TB disease such as those with symptoms and/or abdominal chest radiograph consistent with TB disease.	• A positive result from a second test increases the likelihood that the test result reflects a true infection. • An alternative approach is to assume that the risk for disease does not warrant additional evaluation or treatment.

Laboratory Considerations

Collecting Blood for QuantiFERON®-TB Gold In-Tube

1. Arrange IGRA testing in advance to ensure that the proper tubes are available for collection of blood and that testing can be performed within the required time frame.

2. For the assay, blood is drawn directly into the tubes provided with the kit (3 individual tubes per patient). Do not remove the stopper to add blood to the tube. The order of tubes during blood draw is important for quality control and to prevent cross-contamination. The tubes should be filled in the order specified below.

   First - Nil Tube (grey cap)
   Second - Antigen Tube (red cap)
   Third - Mitogen Tube (purple cap)

3. Blood should be filled to the black mark on the side of the tubes (1mL). It is important to fill the tube adequately, per manufacturer's instructions. At high altitudes (HA), the standard QuantiFERON®-TB Gold In-Tube (QFT-GIT) tubes may not fill appropriately. QFT-HA tubes should be requested from the manufacturer when drawing blood at altitudes between 1,020 meters (3,350 ft.) and 1,875 meters (6,150 ft.).

4. Blood should be obtained through a venipuncture and **not from a peripherally inserted central catheter (PICC)** or other heparinized line. If a butterfly needle is used, the tubing should be purged to allow accurate filling of the tube. Adequate mixing is important; tubes should be mixed 10 times to allow the side of the tube to be coated evenly with blood. Mixing of the tube should not be so vigorous that the plug at the bottom of the tube is dislodged as this can negatively impact the results.

5. Tubes should be labeled appropriately and transferred to a $37^{o}C \pm 1^{o}C$ incubator as soon as possible and within 16 hours of blood collection. Prior to incubation, tubes should be kept at room temperature ($22^{o}C \pm 5^{o}C$). Alternately, the tubes may be incubated at the collection site standing upright at $37^{o}C$ for 16-24 hours before shipping to the laboratory at room temperature (or refrigerated). Incubation and transport should be reviewed with the laboratory before drawing blood samples. Do not refrigerate or freeze the blood samples prior to incubation.

Collecting Blood for T-SPOT®.TB

1. The T-SPOT®.TB requires 8 mL of blood for adults or 4 mL for children. Blood should be drawn into heparinized (green top) tubes or CPT tubes©.

2. For infants and children up to 2 years of age, draw one 2 mL pediatric tube.

Note: In some situations, (e.g., HIV seropositivity with lymphocytopenia) more blood may be needed, as 1,000,000 lymphocytes are needed for the assay. Drawing a CBC and assessing lymphocyte count may help in determining the volume of blood needed.

3. Blood samples for the T-SPOT®.TB assay must be stored at room temperature and tested within 8 hours on the day of draw or stored at room temperature for up to 32 hours with the use of a T-cell extend in the laboratory prior to processing. It is important to communicate with the laboratory about whether this is an option for the samples prior to storing them for an extended time. Blood samples should not be refrigerated or frozen.

Turnaround Time

IGRA results should be available within 48 - 72 hours. Low volume laboratories may batch the QFT-GIT.

Results for T-SPOT® assays should always be available within 48 - 72 hours.

It is best to contact the laboratory performing the test to identify when results can be expected.

General Interpretation of IGRA Results

IGRA results should always be interpreted as part of a complete assessment that includes epidemiologic risk factors, radiographs and a medical evaluation. CDC guidelines *(Updated guidelines for Using Interferon Gamma Release Assays to Detect Mycobacterium tuberculosis Infection - United States, 2010* - *http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5905a1.htm?s_cid=rr5905a1_w*) recommend both the qualitative and quantitative results of the IGRA assays be reported.

Neither the QFT-GIT or T-SPOT® can diagnose TB disease, but when positive, can support a diagnosis of either TB infection or TB disease. A negative TST or IGRA **NEVER** excludes the diagnosis of TB.

Table 6 - Interpretation of QuantiFERON®-TB Gold In-Tube

Nil (UI/ml)	TB Antigen minus Nil (IU/ml)	QFT-GIT Result	Mitogen	Interpretation
≤ 8.0	< 0.35 or ≥0.35 and < 25% of Nil value	Negative	≥ 5.0	MTBC infection unlikely
≤ 8.0	≥ 0.35 and ≥ 25% of Nil value	Positive	Any	MTBC infection likely
≥ 8.0	ANY	Indeterminate	Any	Indeterminate
≤ 8.0	< 0.35 or ≥ 0.35 and < 25% of Nil value	Indeterminate	< 5.0	Indeterminate

Interpretation of T-SPOT®.TB

The T-SPOT®.TB assay is read using 4 circles which depict the Nil Control, Panel A (ESAT-6), Panel B (CFP 10 antigen) and a positive control. The number of spots in panels A and B are determined by subtracting by Nil.

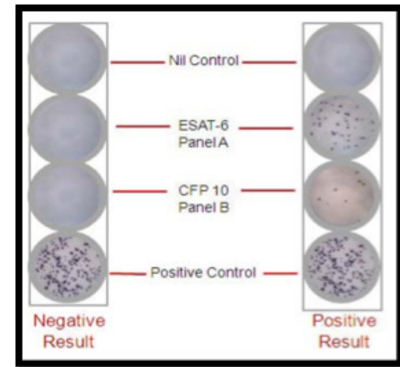


Figure 3. Image from Oxford Immunotec Website: http://www.oxfordimmunotec.com

Table 7 – Interpretation of T-SPOT®.TB

Result	Nil*	TB Response##	Mitogen++	Interpretation+
Positive	≤ 10 spots	≥ 8 spots	Any	*Mycobacterium tuberculosis* complex (MTBC) infection likely
Borderline	≤ 10 spots	5, 6, or 7 spots	Any	Uncertain likelihood of MTBC infection (repeat test)
Negative	≤ 10 spots	≤ 4 spots	Any	MTBC infection unlikely
Invalid	> 10 ≤ 10	Any Both panels < 5 spots	Any < 20 spots	Uncertain likelihood of MTBC infection (repeat test)

Source: *Based on Oxford Immunotec Limited. T-Spot.TB [Package insert]. Available at http://www.oxfordimmunotec.com/USpageInsert.*
** The number of spots resulting from incubation of peripheral blood mononuclear cells (PBMC) in culture media without antigens.*
##The greater number of spots resulting from stimulation of peripheral blood mononuclear cells (PBMCs) with two separate cocktails of peptides representing early secretory antigenic target-6 (ESAT-6) or culture filtrate protein-10 (CFP-10) minus Nil.
++The number of spots resulting from stimulation of PBMCs with mitogen without adjustment for the number of spots resulting from incubation of PBMCs without antigens.

Approach to a Borderline or Invalid T-SPOT®.TB or an Indeterminate QFT-GIT Result

Potential causes of indeterminate results include host factors such as high intrinsic gamma interferon secretion, recent vaccination, an indiscriminate lymphocyte response leading to a high nil result, helminth infections, and immunosuppression leading to a low mitogen response. Technical factors such as improper storage of tubes, incorrect handling of the blood sample with inadequate mixing, drawing blood through a PICC line, delayed (or prolonged) incubation or inappropriate filling of tubes leading to low mitogen response can also cause an indeterminate result.

When a borderline or indeterminate result is obtained, the test should be repeated on a new patient specimen. If a repeat test is read as borderline or indeterminate, another diagnostic assay, such as a TST or a different IGRA, and epidemiologic information should be used to determine the patient's TB infection status. Recent evidence suggests that a positive repeat test is predictive of a stable positive test in individuals who undergo serial testing.

Boosting

Repeated testing using the IGRA does not cause boosting of the IGRA result. However, limited observational studies suggest a prior TST may increase the quantity of interferon measured by a subsequent IGRA assay. Although relatively uncommon, boosting has been shown to occur as early as 3 days after a TST and as late as 3 months after. Boosting of an initially negative IGRA by a TST is less likely, but can occur. In general, the testing should be performed within 3 days of the TST or the test should be delayed by 3 months.

Follow-up Evaluation

In a person with a positive IGRA, in whom active TB is excluded, treatment of TB infection should be considered. No subsequent skin tests, IGRA tests, or chest x-rays (CXRs) need be performed.

A follow up IGRA may be performed when the test is used for serial screening of healthcare workers. Research is ongoing to define the significance of a positive IGRA test in low risk healthcare settings.

A negative IGRA does not exclude either TB infection or TB disease. If TB is a diagnostic possibility, further evaluation should be performed.

In a number of states, TB infection is a reportable condition and the positive result should be reported.

A negative IGRA DOES NOT EXCLUDE either TB infection
or TB disease

Notes:

Nucleic Acid Amplification Tests - Direct Specimen Analysis

Chapter 3

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Chapter 3

Nucleic Acid Amplification Tests Direct Specimen Analysis

Background

Current CDC guidelines *(Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis - http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm)* for use of the nucleic acid amplification (NAA) test recommend: "NAA tests should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test results would alter case management or TB control activities."

Rapid detection of TB disease is critical for optimal patient and public health outcomes. Rapid detection helps to prevent delays in diagnosis and treatment, limits transmission, and facilitates timely contact investigations. An NAA test can detect *Mycobacterium tuberculosis* complex (MTBC) directly from clinical specimens. Results should be available 24 – 48 hours after the specimen arrives in the laboratory. Although a positive NAA test supports a diagnosis of TB disease in a patient with a clinical syndrome and/or epidemiological setting consistent with TB, it supplements but does not replace acid-fast bacillus (AFB) smear and culture. The main advantage of an NAA test is the ability to diagnose TB early in persons with suspected TB disease who have negative sputum smear results and exclude MTB when the smear is positive and the NAA is negative on two samples. The positive AFB smear in this situation usually indicates the positive result is due to nontuberculous mycobacteria (NTM).

Principles of the Test

An NAA test performed ***directly on a patient specimen*** is designed to amplify MTBC nucleic acids in the sample to quantities that can be detected. Currently, two Food and Drug Administration (FDA) approved NAA tests are available in the United States. Laboratory developed tests may also be validated by the laboratory using them.

The Cepheid GeneXpert® (Xpert®) MTB/RIF assay was FDA-authorized in 2013 for use with both AFB smear negative and smear positive sputum specimens. Worldwide, the Xpert® MTB/RIF is used for its ease of operation and in the United States is rapidly replacing older MTBC NAA tests. The amplified *Mycobacterium tuberculosis* Direct Test (MTD), developed by Hologic®**,** was FDA-approved in 1995 for use with AFB smear positive respiratory specimens. In 1999, an enhanced MTD test was approved for use with AFB smear positive and smear negative respiratory specimens from patients suspected of having TB disease.

Some laboratories offer non-FDA approved NAA tests that have been validated as laboratory developed tests and some laboratories offer an NAA test on non-respiratory specimens.

Table 8 – Strengths and Limitations of the Nucleic Acid Amplification Test

<table>
<tr><th colspan="2">Clinical Utility</th></tr>
<tr><td colspan="2">• NAA tests can support the diagnosis of TB disease.
• NAA tests can confirm the absence of MTBC in AFB smear positive specimens of patients with suspected NTM infections. A contact investigation is not necessary when an AFB smear positive specimen is NAA test negative and NAA inhibitors were not detected.
• NAA tests can detect the presence of MTBC in approximately 50-80% of AFB smear negative, culture positive respiratory specimens.
• Information is limited regarding NAA test performance for non-respiratory specimens but if the test is positive, it can add valuable information to support a diagnosis of TB.</td></tr>
<tr><th>Strengths</th><th>Limitations</th></tr>
<tr><td>• Compared with AFB smear microscopy alone, a positive NAA test increases the clinical confidence in the diagnosis of TB disease.
• NAA tests can detect MTBC weeks earlier in 80% – 90% of patients suspected of having pulmonary TB ultimately confirmed by culture.
• NAA tests can impact patient care and TB control efforts by reducing unnecessary contact investigations, and AII for patients whose AFB smear positive specimens are NAA test negative.
• The Cepheid GeneXpert® (Xpert®) MTB/RIF assay tests simultaneously for the presence of MTBC DNA and rifampin resistance.</td><td>• With the exception of the new Xpert® MTB/RIF assay, NAA tests require technical expertise and significant technician time.
• False-positive results may occur due to cross contamination. This is primarily a concern with NAA tests that are not performed in closed systems. The Xpert® MTB/RIF assay takes place in an enclosed cartridge that minimizes the possibility of cross contamination.
• The test is an “added responsibility and cost” for laboratories, it does not replace any laboratory test.
• The presence of nucleic acid amplification inhibitors in specimens can cause false negative results. The design of the Xpert® MTB/RIF assay automatically tests for inhibitors.
• A negative NAA test does not exclude TB.
• There are currently no FDA approved tests for non-respiratory specimens.
• A NAA test does not provide information on the infectiousness of a TB patient.
• A NAA test is not currently approved for evaluating response to treatment. Research on new generation NAA tests for evaluation of patient response show some promise for the future.
• DNA from dead bugs is not used to determine viability.</td></tr>
</table>

Laboratory Considerations

NAA tests do not replace AFB culture.

1. Respiratory specimens submitted for NAA tests should be collected as outlined in Chapter 4, *AFB Microscopy*. Once collected, specimens should be processed and tested by AFB smear microscopy and culture. Laboratories should ensure referral for a full panel of testing to include culture even when NAA tests are ordered.
2. At least one specimen, preferably the first diagnostic specimen, or the best quality specimen, from each patient should be processed and tested using a validated NAA test for MTBC. AFB smear should be performed on all specimens tested by NAA to aid interpretation of the NAA test result.
3. Ideally, specimens for NAA testing should be collected prior to treatment initiation. However, the FDA has approved the use of NAA tests on specimens in which the patient has been on treatment for fewer than three days for Xpert® MTB/RIF or seven days for the MTD. A positive NAA test even after weeks of treatment may provide important information to the clinician in situations in which a culture has not been ordered or fails to grow. A negative NAA test, however, is not helpful in these situations after the recommended time limit has been exceeded.

Interpretation of Results

NAA test results should be interpreted in correlation with the AFB smear results and clinical assessment of the patient. Communication with the laboratory is important when requesting and interpreting results. Table 9 provides information on how results should be interpreted.

Table 9 – Interpreting Nucleic Acid Amplification Test Results

Result	Interpretation
NAA test positive and AFB smear positive	• The patient should be presumed to have active TB disease, and anti-tuberculosis treatment should be started while awaiting culture results. The positive predictive value of an FDA-approved NAA test in this situation for TB is >95%.
NAA test positive and AFB smear negative	• Clinical judgment is required to determine whether to begin anti-tuberculosis treatment before the culture results are available. Many experts would consider starting treatment for TB unless another diagnosis is evident. A second specimen can be tested to confirm the NAA test result. If two specimens are NAA test positive, the patient can be presumed to have TB, and treatment for TB should be started while the culture is pending.
NAA test negative and AFB smear positive	• A second NAA test should be considered on a new specimen. If the second test is also negative, clinical judgment should be used to determine whether to begin anti-tuberculosis treatment until culture results are available and to determine if additional diagnostic testing is needed. Usually, however, a patient can be presumed to be infected with an NTM if a second specimen is also smear positive and the NAA test is negative. -- If the MTD or a laboratory developed NAA test were used and TB is suspected, contact the laboratory to discuss whether they have tested for the presence of inhibitors that can cause a false negative test in some situations.
NAA test negative and AFB smear negative	• TB cannot be excluded with the use of either the NAA test or AFB smear. Clinical judgment should be used to determine whether to begin anti-tuberculosis treatment until the results of the culture and additional diagnostic tests are available.

The February 27, 2015, MMWR, "Updated labeling for the Xpert® MTB/RIF Assay" (*http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6407a8.htm*), and the NTCA and APHL document "Consensus statement on the use of Cepheid Xpert® MTB/RIF assay in making a decision to discontinue airborne infection isolation in health care settings" (*http://www.tbcontrollers.org/docs/resources/NTCA_APHL_GeneXpert_Consensus_Statement_Final.pdf*) include the recommendation that the decision whether to test one or two sputum specimens in determining the need for continued airborne infection isolation (AII) should be based on specific clinical circumstances and institutional guidelines. Clinical decisions regarding the need for continued AII should always occur in conjunction with other clinical and laboratory evaluations, and negative Xpert® MTB/ RIF assay results should not be the sole basis for infection control practices.

Turnaround Time

CDC guidelines recommend that NAA test results be available within 48 hours of receipt of the specimen in the laboratory. Laboratory staff should treat an initial positive NAA test result as a critical test value and immediately report the result to the clinician and public health authorities.

Notes:

AFB Smear Microscopy

Chapter 4

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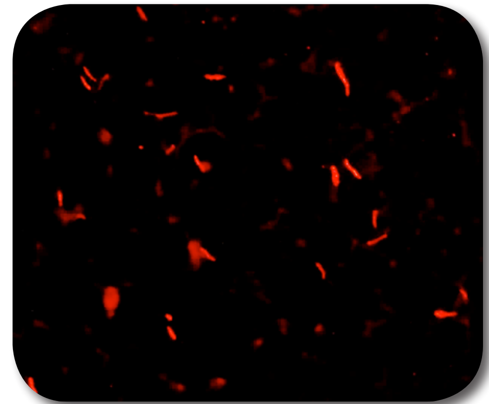
Chapter 4

AFB Smear Microscopy

Principles of the Test

Fluorochrome staining of a concentrated smear is the standard method for acid-fast bacilli (AFB) smear microscopy in U.S. laboratories.



Typical flourochrome stain (auramine-rhodamine)

Lipid-rich cell walls of mycobacteria prevent the penetration of traditional gram stain dyes but allow uptake of dyes such as basic fuchsin (Ziehl-Neelsen [ZN] or Kinyoun stains) or fluorochrome dyes such as Auramine-O (Truant's stain). Mycobacteria are not decolorized by acid-alcohol and retain the primary stain. All mycobacteria possess this characteristic and are referred to as "acid-fast". This property differentiates mycobacteria and closely related bacteria such as Nocardia species from all other bacteria that are decolorized by the acid-alcohol solvents.

Collection of Specimens for AFB Smears

AFB Smear Microscopy of Respiratory Specimens

Three sputa should be collected for AFB smear and culture to maximize the diagnostic yield. If the initial sputum smear is AFB positive from a patient who is not taking anti-tuberculosis drugs, the culture should be positive most of the time.

Table 10 – Essentials of Collecting Sputum Samples

1. Patients should be educated on how to produce a good quality sputum specimen. (Appendices B3/B4)
2. Each specimen should be 5 to 10 ml; the minimum volume is 3 ml. Smaller volumes reduce the probability of finding AFB. If the specimen is < 2ml, additional fluid added during the decontamination, centrifugation, and reconstitution may result in a diluted specimen. This can decrease the sensitivity of the smear. (Appendix A1)
3. Children as young as 5 years old have successfully produced sputum after induction. Assistance from an experienced and well-trained healthcare worker facilitates collection of an acceptable sample from young patients. (Appendix B2)
4. Specimens must be labeled with the exact name and identification number that are on the laboratory requisition form. Identification numbers may include date of birth, hospital accession number or other patient identifiers.
5. Sputum should be delivered to the laboratory as quickly as possible, ideally within 24 hours. During holding and transport, the specimen should be kept out of the heat and in a cool or refrigerated environment. The purpose of the cool environment is to minimize the risk of normal flora overgrowth and contamination of the specimen.

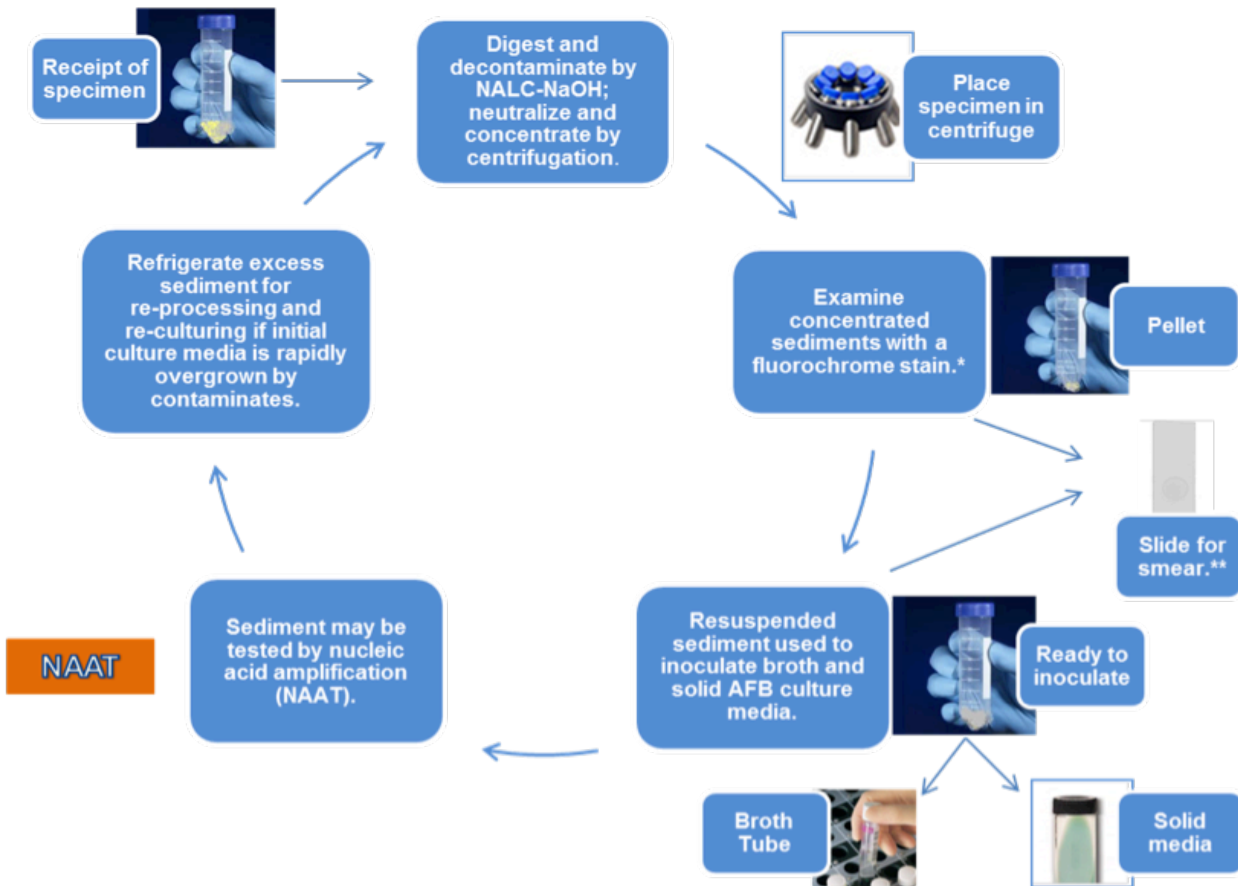
Steps during Laboratory Processing of the Specimen

Figure 4 shows steps in the processing of respiratory specimens in the laboratory. The standard microscopy specimen in the United States is a concentrated smear. The smear is stained using fluorescent staining techniques to maximize sensitivity for the detection of AFB.

At least 5,000 - 10,000 organisms / ml are necessary for a positive smear while approximately 10 - 100 mycobacteria / ml are needed to produce a positive culture.

A negative smear or culture does not exclude active TB!

Figure 4 – Laboratory Processing of Respiratory Specimens



*If a smear is made directly from an un-concentrated specimen to facilitate a rapid diagnosis, and the result is AFB smear negative, another smear must be prepared from a concentrated specimen and examined.

**Smear can be made from either a pellet or re-suspended sediment

Turnaround time for AFB Smear Microscopy

AFB smear results should be reported within 24 hours after the laboratory receives the specimen. The initial specimen should reach the laboratory as quickly as possible. If information regarding the specimen is not reported within 24 hours, follow up with the laboratory.

Interpretation of AFB Smear Microscopy Results

Laboratories report the presence or absence of AFB and most will report the quantity noted. Several reporting methods are employed by clinical laboratories to quantify the number of organisms present.

If the patient moves and case management is transferred to a new jurisdiction, the reporting scale used by the lab may be different. Correct interpretation of the bacteriological response based on smear requires the provider to be aware of the scale used. Table 11 shows the most commonly utilized AFB reporting scales.

A positive smear result should be treated as a critical value and must be reported ***immediately*** to the provider or TB program.

Table 11 – AFB Reporting Scales*

Reported	Number of AFB Seen in Smear at 250X Magnification:
No AFB Seen	0
Report Exact Count	1-2/30 Fields
1+ (Rare)	1-9/10 Fields
2+ (Few)	1-9/ Field
3+ (Moderate)	10-90/ Field
4+ (Numerous)	>90/ Field

*APHL "*Mycobacterium tuberculosis*: Assessing Your Laboratory", 2013 Edition

Table 12 – Strengths and Limitations of AFB Smear Microscopy

AFB Smears	
Strengths	**Limitations**
• AFB smear microscopy is universally available and easier to perform than a culture. • The clinician should expect a result within 24 hours after the laboratory received the specimen. • An AFB smear requires relatively simple laboratory technology.	• All mycobacteria are acid-fast. A positive smear does not specifically identify *Mycobacterium tuberculosis* complex (MTBC). In low TB incidence settings a positive AFB smear is more likely to represent a NTM. • AFB smear microscopy alone provides limited information; it must be paired with a culture to determine viability of the mycobacteria and drug susceptibility. • AFB positive smears may represent non-viable organisms in a patient who is on treatment, especially if the patient has extensive disease. • Accurate interpretation of an AFB smear requires a skilled laboratory scientist.

Clinical Considerations for AFB Microscopy

An AFB smear positive sputum, especially if confirmed as containing *Mycobacterium tuberculosis* complex (MTBC) by a nucleic acid amplification (NAA) test (Chapter 3), is critical for making an early diagnosis of TB disease. The AFB smear is positive in approximately 50% of persons with active pulmonary TB. TB can be presumptively diagnosed by a positive smear in most cases when the clinical and epidemiological setting suggests TB disease.

The AFB smear results are especially useful in the following situations:

Even three AFB negative sputum smears cannot exclude TB disease in a patient with a consistent clinical picture who has no other diagnosis.

1. AFB smear positive patients who are not on treatment are infectious. Information regarding smear status drives decisions made by the health department regarding contact investigation and release from AII. Smear status of the source case is a significant factor for prioritization of a contact investigation.
2. CDC guidelines allow persons with suspected TB to be released from AII once the laboratory confirms that three sputum specimens collected 8 to 24 hours apart are AFB smear negative, the patient is improving medically, has been on adequate treatment for 2 weeks (if initially smear positive) or one week (if all smears are negative) and there is no significant risk of drug resistant TB.
3. Sputum AFB smears are graded to reflect the number of mycobacteria. As a patient gets further into effective treatment, the quantity of AFB should decrease.
4. Conversion of a positive smear to negative generally indicates a good response to treatment. The time to smear negativity is an important measure of the response to treatment and varies depending on the presence of cavitary lesions, the extent of disease, and adherence to an appropriate anti-tuberculosis regimen. In general, with rifamycin containing treatment regimens, over 30% of patients with an initial smear positive are expected to be smear negative at 4 weeks, and 85% by 8 weeks.
5. Some patients with extensive cavitary disease may convert sputum cultures prior to sputum smears due to the presence of non-viable AFB present on smears.
6. Persistently positive smears may represent non-viable organisms if the patient is responding clinically and/or radiographically.
7. If the patient is NOT responding clinically and/or radiographically, persistently positive smears suggest a poor treatment response.
8. Persistently positive smears after several months of treatment signal the need for additional evaluation of the patient to determine if the response to treatment is adequate.

Tissue specimens from any site with possible TB disease should be evaluated with an AFB smear and culture. Extra-pulmonary specimens contain lower numbers of AFB than pulmonary specimens and are often AFB smear negative. Tissue specimens should also be submitted for pathology examination.

Sputum for AFB smear should generally be obtained with the following frequency:

1. For each patient with suspected pulmonary TB, three initial sputum samples should be collected 8 – 24 hours apart. One specimen should be a first morning specimen and, if possible, at least one collection should be observed.
2. During treatment for pulmonary TB, one sputum specimen should be obtained monthly for AFB smear and culture, until both smear and culture are negative for two consecutive months.
3. If sputum remains AFB smear positive, smears can be obtained more frequently to assess the patient's infectiousness and continued need for isolation. Culture may not be needed on these specimens.

Culture may not need to be performed when the sputum specimen is ordered for the purpose of determining when a patient can be removed from airborne infection isolation.

Notes:

Culture for Isolation of *Mycobacterium tuberculosis* complex

Chapter 5

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Chapter 5

Culture for Isolation of *Mycobacterium tuberculosis* complex

Background

The gold standard for laboratory confirmation of tuberculosis (TB) disease is isolation of *Mycobacterium tuberculosis* complex (MTBC) in culture. Growth of MTBC in the laboratory documents viable bacilli and allows for further testing (e.g., drug susceptibility tests and genotype). Once growth is detected in culture, an AFB smear is performed. Because the acid-fast bacilli (AFB) culture smear is not specific for MTBC, if the smear is positive, further testing is performed to confirm the identity of the organism. If MTBC is not identified, non-tuberculous mycobacteria (NTM) should be suspected and may be identified by another method. All mycobacteria are acid-fast and some can cause clinical syndromes that are similar to identification caused by *M. tuberculosis*. Rarely, weakly acid-fast organisms such as *Rhodococcus* and *Nocardia* can be mistaken for *M. tuberculosis* on staining but will be definitively identified by culture.

Principles of the Test

M. tuberculosis is a slow growing organism; up to 6 weeks may be needed before growth is detected on solid media. To reduce the time to MTBC growth and isolation, laboratories should use a commercial liquid culture media (broth). Use of selective broth media enhances the recovery and growth of MTBC. Use of solid media complements broth cultures by allowing for additional information such as colony counts and visualization of colony morphology. An experienced laboratorian can examine solid media directly to determine if the MTBC isolate is pure or is mixed with other organisms.

Laboratory Considerations

Unless the specimen is from a sterile site, it must be decontaminated by use of the N-acetyl L-cysteine-sodium hydroxide (NALC-NaOH) digestion decontamination method before it is inoculated to culture media. This is performed to prevent overgrowth of normal respiratory flora. Overly aggressive decontamination can prevent mycobacteria from growing. Sterile specimens can be inoculated directly to culture media.

> A culture should only be reported as positive for MTBC after the organism has been identified by a rapid definitive identification method.

Approximately 80% of initial diagnostic specimens collected from patients with pulmonary TB prior to start of anti-tuberculosis drug therapy exhibit growth of mycobacteria in broth by 21 days. After growth is detected, an aliquot is stained to determine purity and to look for AFB.

A culture should be reported as positive for MTBC only after the organism has been identified by a rapid definitive identification method. Many laboratories use a DNA probe (AccuProbe by Hologic) to identify MTBC, but laboratory developed tests such as a real-time polymerase chain reaction (PCR), DNA sequencing, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectometry, or high performance liquid chromatography (HPLC) may also be used.

If the processing laboratory cannot determine if an isolate is MTBC, any acid-fast isolate should be sent on the original media within one working day, preferably with overnight delivery, to a reference laboratory for definitive identification. (APHL, "*Mycobacterium tuberculosis*: Assessing Your Laboratory", 2013 ed., p. 55)

Clinical Considerations

Growth of mycobacteria identified as MTBC provides bacteriological confirmation of the diagnosis of TB disease.

A culture is needed to perform phenotypic drug susceptibility testing (DST). Other methods, such as rapid molecular detection of drug resistance (MDDR), complement but do not replace phenotypic methods.

The time it takes for a culture to become positive, time to positive (TTP), is a biomarker that correlates with the number of viable organisms present in the specimen. A longer TTP is an early marker of response to treatment as is conversion of the culture to negative. The time to culture conversion defines the overall length of treatment in those with a cavity who have drug-susceptible TB and in those with MDR TB. *(See Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis; https://www.cdc.gov/tb/publications/guidelines/pdf/clin-infect-dis.-2016-nahid-cid_ciw376.pdf)*

Table 13 – Strengths and Limitations of TB Cultures

TB Cultures	
Strengths	**Limitations**
• Culture is more sensitive than smear and confirms disease in 50% of TB patients with negative smears. • Culture allows for genotyping. • Any tissue or body fluid suspected of being infected with MTBC can be cultured. • Culture allows quantification of the number of viable organisms. • Culture based drug susceptibility testing is regarded as the gold standard for most drugs (see Chapter 6).	• Slow and expensive. • Laboratories need to process enough specimens to maintain expertise. • False negative results may occur even when a patient has active TB disease . • Specimens undergo harsh decontamination methods that also kill some of the MTBC. • The number of organisms present in the affected tissue or specimen may be below the limit of detection of the culture (usually 10 - 100 mycobacteria/ml). • Growth of MTBC can be suppressed if a patient is started on treatment for TB disease with a fluoroquinolone or other antibiotic, prior to sputum collection.

False-positive results may occur and usually represent mishandling or contamination at some point in the collection, processing, or reporting of the specimen. A false-positive culture should be considered when:

- A smear negative, culture positive specimen is processed adjacent to a positive culture, especially when the specimen in question was not expected to be culture positive.
- A single positive culture is obtained, especially in a patient who does not have a clinical or epidemiologic presentation suggestive of tuberculosis.
- A late report (6 weeks) of a single positive culture from a smear negative specimen is received.
- Multiple cultures from different patients isolated during the same time frame have the same unusual or unexpected drug resistance or genotype.

Time Frame for Obtaining a Culture for MTBC

- For each patient being evaluated for possible TB disease, three sputum samples should be collected 8-24 hours apart. One specimen should be a first morning specimen and, if possible, at least one should be observed during collection.
- It is especially important to obtain a culture at the end of the intensive phase (8 weeks). If culture conversion is not documented by the end of 8 weeks, treatment may need to be extended. Obtaining a specimen for culture at 2 months is very important as treatment decisions may be based on the result.
- During therapy, obtain one sputum specimen monthly from those with pulmonary TB until sputum smears and cultures are negative for 2 consecutive months.
- For patients with MDR or XDR TB, specimens for culture should be collected monthly throughout treatment.

Time Frame for Reporting Culture Results

- MTBC positive cultures should be reported for at least 80% of initial diagnostic specimens within 21 - 28 days. The average time for growth in broth is 10 - 14 days; solid media can take up to 6 weeks.
- Confirmation of MTBC should be reported within 24 hours of laboratory determination. Smear negative and extra-pulmonary specimens typically exhibit a longer time to culture positivity.
- All cultures should be incubated for at least 6 weeks prior to being reported as negative.

Table 14 – Interpretation of Culture Results for Respiratory Specimens

Result	Interpretation
Negative Culture	• The patient usually does not have TB. Negative cultures *may* be found in some persons with limited disease, an inability to produce a good sputum specimen, or who have had prior treatment. • Extrapulmonary TB may not be culture positive; a negative tissue culture does not exclude TB especially if the pathology and clinical presentation support a diagnosis of TB. • A smear positive, culture negative specimen can occur during the treatment follow-up phase due to the excretion of AFB that are no longer viable. Non-viable organisms may be due to the bactericidal activity of anti-tuberculosis drug. • A smear positive, culture negative specimen may be the result of excessively harsh decontamination procedures. Laboratories should routinely monitor their rates of contamination, MTBC, and NTM recovery. A contamination rate of 2 – 5% on solid medium cultures is expected; a slightly higher rate is expected for broth cultures. If a laboratory's contamination rate is zero, then over-decontamination may be occurring. • May be due to poor quality of specimen or mishandling.
Positive Culture	• The culture is recognized as the gold standard for diagnosis of TB disease. • A positive culture confirms the diagnosis of TB unless there is clinical or laboratory suspicion to suggest this is a false-positive result. • An estimated 3% of all MTBC cultures are false-positives. Errors can occur during any phase of collection or processing. a. It is important to communicate with the laboratory if a false-positive culture is suspected. • A persistent positive culture after 4 months of treatment represents treatment failure.

Laboratory Evaluation of Non-Respiratory Specimens for MTBC

Any body fluid or tissue can be submitted for AFB smear and culture if it is suspected of being infected with MTBC. Tissue can be collected in a specimen cup with or without sterile (not bacteriostatic) saline and transported to the laboratory. A portion of the specimen should be reserved for culture prior to the specimen's being placed in formalin. Because the smear may be negative, especially in non-pulmonary sites and the culture will take several weeks to become positive or may be negative, additional evaluation of non-respiratory specimens may help to establish a clinical diagnosis of TB disease.

Table 15 – Additional Evaluation of Non-Respiratory Specimens

Gastric Aspirates

- Gastric aspirates are interpreted the same way and have the same significance as sputum specimens.
- Gastric aspirates should be collected in infants and children; they can also be helpful in adults who cannot expectorate.

Cerebral Spinal Fluid (CSF)

- CSF is usually smear negative; culture is positive in < 30% of cases of TB meningitis.
- PCR is helpful when positive; this is not FDA approved but may be a laboratory developed test. Laboratories may note a positive PCR even when the AFB smear and culture are negative.
- The CSF cell count, glucose, and protein can be used to support a diagnosis.
    - The cell count usually has a predominance of lymphocytes, although early in TB meningitis, there may be mostly polymorphonuclear cells (PMNs).
    - A glucose of < 50% of the serum glucose along with an elevated protein suggests TB.
    - The cell count, glucose, and protein improve with treatment.

Pleural Fluid

- TB effusions are exudative (elevated protein and WBC count). The protein is usually >5 grams/dL.
- The cell count in TB effusions usually shows a lymphocytic predominance, although some effusions, especially TB empyema, may have a polymorphonuclear (PMN) predominance initially.
- AFB smears are positive in approximately 20-25% of TB effusions. This percentage is higher with TB empyema or in HIV co-infected patients.
- Sputum cultures are positive in as many as 40% of TB effusions, and higher with TB empyema and HIV co-infection.
- Adenosine deaminase (ADA) is found in high concentrations in TB pleural effusions. Values of ADA >70 U/L are virtually diagnostic of TB effusions, and values < 40 U/L virtually exclude the diagnosis of TB effusion.

Urine

- Sterile pyuria is the hallmark of TB infection of the urinary tract.
    - Urine should be cultured for mycobacteria in a patient with evidence of a urinary tract infection (elevated WBC) and no growth on routine culture.
    - First morning urine has the best return.

Ascitic Fluid

- TB ascitic fluid is exudative, generally with protein concentrations >3 grams/dL.
- The total cell count usually falls in the range of 150-4000 cells/uL and the differential cell count tends to be lymphocyte predominant (>70% lymphocytes), although in the first few weeks there may be a PMN cellular predominance that evolves into the classic lymphocyte predominance.
- AFB smears are rarely positive in ascitic fluid.
- Ascitic fluid cultures are positive for MTB in 20% of cases.

Notes:

Drug Susceptibility Testing for *Mycobacterium tuberculosis* complex

Chapter 6

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Chapter 6

Drug Susceptibility Testing for *Mycobacterium tuberculosis* complex

Background

Treatment of individuals with TB disease and their infected contacts is guided by the results of drug susceptibility tests (DSTs). The composition of treatment regimens as well as the duration of therapy are dictated by these results. The epidemiological surveillance of drug resistance is an important public health activity.

> "Susceptibility testing of *M. tuberculosis* is critical for appropriate patient management and should be performed on an initial isolate from all patients from whom *M. tuberculosis* is recovered." CDC ATS, IDSA Treatment of Tuberculosis, 2003.

Public health laboratories routinely perform DSTs on initial isolates of *Mycobacterium tuberculosis* complex (MTBC). This is not standard practice for many private laboratories; in some, susceptibility tests must be specifically ordered. Because some laboratories do not provide first- and/or second-line DSTs, slower turnaround times may occur due to fragmentation of services. A referral system for reflex testing (i.e., testing susceptibility on initial MTBC patient isolates) should be in place.

Drug resistance is defined by both the agar proportion and broth based methods as "growth of greater than or equal to 1% of an inoculum of bacterial cells in the presence of a critical concentration of anti-tuberculosis drug." This is based on the clinical observation that once 1% of the cells in a population of MTBC become resistant to a drug, that drug will soon lose, if it has not already, its therapeutic usefulness.

Principles of the Test

A standard inoculum of organisms must be used for accurate test results. Test results are influenced by inoculum age, number of bacilli, and the degree to which the bacilli are dispersed in the inoculum. It is important to prepare the inoculum from fresh growth. Details of and directions for preparation of the inoculum are beyond the scope of this document.

> Drug resistance is defined by both the agar proportion and broth based methods as "growth of greater than or equal to 1% of an inoculum of bacterial cells in the presence of a critical concentration of anti-tuberculosis drug."

Whenever possible, susceptibility testing should be performed using the initial or primary culture. Mycobacterial cultures usually contain a few bacilli with mutations that cause resistance to a particular drug. Mycobacteria with mutations causing drug resistance occur in small numbers in populations that are predominately composed of susceptible mycobacteria (referred to as "wild type"). Often, drug resistant TB bacilli grow more slowly in culture. For this reason the initial or primary culture, which contains all populations of bacilli in the specimen, is preferable to a subculture.

Often, drug resistant TB bacilli grow more slowly in culture. For this reason the initial or primary culture, which contains all populations of bacilli in the specimen, is preferable to a subculture.

A sample from the original culture, if grown in broth, i.e., a subculture, may not adequately represent the true number of resistant bacilli in the original specimen. It may contain too few slowly growing resistant TB bacilli to allow their detection. Use of a subculture may allow the drug susceptible population, which grows more rapidly to populate the broth. This could then give a *falsely susceptible* result.

When testing for resistance, it is important that the sensitivity of the test is sufficient to detect small populations of resistant organisms. This is especially important if a patient's isolate is in the process of acquiring drug resistance and drug susceptible mycobacteria are being replaced by drug resistant. Early identification of new drug resistance and proper adjustment of the drug regimen can help to prevent further drug resistance.

Critical Concentration

The critical concentration of a drug is the concentration that will inhibit 95% of wild type strains of MTBC never exposed to that drug and will not inhibit strains of MTBC grown from patients who are not responding to treatment (thus considered resistant to the drug). A critical concentration is not an MIC.

Agar proportion methodology is based on the definition of drug resistance developed by early TB clinicians who recognized treatment failure following use of a drug and a subsequent "*decrease in susceptibility of sufficient degree to be reasonably certain that the strain* ***concerned*** *is different from a sample of wild strains of human type that have never come into contact with the drug*." (Canetti G, et al). Susceptibility of MTBC is determined by evaluating the ability of an isolate to grow on agar or in broth containing a single "critical" concentration of a drug. The **critical concentration** of a drug is that concentration which will inhibit 95% of wild type strains of MTBC that have never been exposed to that drug and will not inhibit strains of MTBC that are grown from patients who are not responding to treatment and are thus considered resistant to the drug. A critical concentration is not a minimum inhibitory concentration (MIC).

Minimum Inhibitory Concentrations

Significant interest has developed recently regarding the use of the MIC to guide decisions on treatment of MTBC, especially in situations with drug resistance or intolerance to first line anti-tuberculosis drugs. The MIC has been used in development of the new anti-tuberculosis drugs and by clinicians to evaluate efficacy of repurposed drugs, including linezolid and the newer fluoroquinolones. Clinicians frequently use the MIC result for moxifloxacin combined with knowledge of usually achievable drug levels at the site of infection to guide treatment in patients with ofloxacin-resistant MTBC. Despite research interest and the increasing use of MICs by expert clinicians, no recommendations have been made regarding MICs by the Clinical and Laboratory Standards Institute (CLSI) for mycobacteriology laboratories.

Testing Methods

The CLSI and CDC recommend initial isolates of MTBC be tested against a single critical concentration of isoniazid, rifampin, ethambutol, and pyrazinamide using a rapid commercial broth-based system. The MGIT 960 and the VersaTrek systems are two FDA approved broth systems in the United States. Most laboratories in the United States now use the MGIT 960 broth method for testing initial isolates for susceptibility to first-line TB drugs. The agar proportion method (solid media) can be used to confirm results from the rapid broth systems and to test additional drugs and/or various concentrations of drugs when further information is needed. The agar proportion method is not commonly used in the United States except in reference laboratories that perform second-line drug testing. Some reference labs are also testing second-line drugs in MGIT or using sensititre (MIC). Results from the agar proportion method typically take longer than those from broth systems (21 days versus 10-12 days). However, a rapid (2 week) microcolony-based agar proportion method for isoniazid (INH) and rifampin susceptibility has been described.

Agar plates from XDR TB patient

General Guidance Regarding Susceptibility Tests

If drug resistance is suspected, this concern should be communicated to the laboratory.

The laboratory should automatically repeat susceptibility tests when cultures remain positive after three months of treatment.

The initial isolate from each individual diagnosed with TB should be submitted for susceptibility testing. Because results of the agar proportion method (solid media) may take up to 3 weeks, rapid broth-based methods should be used for initial susceptibility testing to first-line anti-tuberculosis drugs. When drug resistance is expected, when Cepheid GeneXpert® (Xpert®) MTB/RIF assay detects rifampin resistance, or when resistance to the first-line drugs is identified, a molecular test to identify mutations is also recommended (MDDR - Chapter 7).

Initial DST should include all first-line drugs at their critical concentrations. Knowledge of the susceptibility of each of the four drugs is essential to guide the provider in treatment of patients and their contacts.

When resistance to rifampin or two or more first-line TB drugs is discovered, all second-line drugs should be tested at the same time. This prevents a situation in which unpredicted drug resistance leads to a series of presumptive treatment adjustments that could result in weak or inadequate regimens and acquired drug resistance. Initial identification of the complete susceptibility profile allows the provider to design the best treatment regimen.

Second-line DSTs should always include a fluoroquinolone, traditional second-line drugs (ethionamide, amikacin, and capreomycin) and a higher concentration of isoniazid. In many situations, it may be appropriate to ask for testing against linezolid, newer generation fluoroquinolones, and other drugs.

Turnaround Times

CDC recommends that laboratories have a turnaround time goal to report rifampin growth-based DST from broth within 17 days of identification of MTBC.

When the laboratory notes a resistant result, even if this is ambiguous and requires confirmation, the resistant result should be reported to the provider, noting that repeat confirmatory testing is pending. The laboratory should not wait to notify the provider until confirmatory results are available. Second-line DST should be performed at the same time that the repeated first-line DST is initiated and molecular testing considered.

As soon as the laboratory suspects resistance it should be discussed with the provider, even if it is too early to officially report the result.

Clinical Use of Susceptibility Tests

Table 16– Timing of Susceptibility Testing

Time of Evaluation	What is Tested?
Initial isolation of MTBC.	• Susceptibility tests to all first-line drugs (isoniazid, rifampin, ethambutol, and pyrazinamide) on the initial isolate from each new case of TB. • If drug resistance is strongly suspected or documented by molecular testing, the complete panel of first and second-line tests should be set up.
Consider repeat susceptibility tests to all first-line drugs.	• If the patient is at risk of treatment failure due to extensive disease, has a slow response to therapy either clinically or radiographically, and is culture positive after 8 weeks of treatment. • If the patient is improving but is still culture positive at 8 weeks and there is a concern regarding adherence especially if treatment has not been by DOT. In this scenario even if the patient is doing well resistance could be developing.
12 weeks - all patients who remain culture positive.	• Susceptibility tests to all first-line anti-tuberculosis drugs should be repeated if the culture is positive after 12 weeks of treatment even if the patient is doing well. This is consistent with the CDC, ATS, and IDSA guidelines. If drug resistance is suspected, a complete panel of first and second line tests should be set up.
Any time.	• Susceptibility tests to all first-line anti-tuberculosis drugs should be repeated: a. Whenever there is evidence of clinical or radiographic deterioration. b. On all cases at time of identification of treatment failure. c. Whenever there is suspicion or evidence of drug resistance; in such cases, the complete panel of first and second-line tests should be set up.
Any time resistance is identified during treatment.	• A new specimen should be collected. • It is critical to know the susceptibility of the isolate on the day a new treatment regimen is started. • Susceptibility testing for first and second-line drugs (a minimum of ethionamide, injectables [amikacin and capreomycin] and a fluoroquinolone [ofloxacin, levofloxacin or moxifloxacin]) should be repeated when resistance is reported during treatment.
When a standard regimen cannot be used due to intolerance.	• All second-line anti-tuberculosis drugs should be tested

Table 17 – Strengths and Limitations of Drug Susceptibility Testing

Strengths and Limitations of DST	
Strengths	• Clinical correlation with outcomes is documented in the literature for isoniazid, rifampin, fluoroquinolones, and the injectable drugs, especially streptomycin. • Identifies emerging resistance. Molecular methods lack sensitivity when populations of resistant organisms comprise less than 20% of the mycobacterial population. • More than one concentration of an anti-tuberculosis drug may be tested. • Providers are familiar with the results of standard culture based susceptibility testing.
Limitations	• MGIT 960 may miss detection of resistance to rifampin in isolates with an MIC only slightly greater than the critical concentration. • Critical concentrations of some anti-tuberculosis drugs may be problematic. a. Laboratory performance testing suggests that testing with MGIT 960 misses a significant number of ethambutol resistant MTBC and reports them as falsely susceptible. Isolated ethambutol resistance is rare and usually is not clinically significant. However, when ethambutol resistance is missed in a patient with isoniazid resistance, acquired rifampin resistance may develop. If resistance to isoniazid or rifampin is noted, ethambutol susceptibility should be confirmed with molecular and agar based testing. b. Molecular methods have shown a relatively low sensitivity for detection of capreomycin resistance. This discrepancy is related to use of a critical concentration for capreomycin (10 mgc/ml) that incorrectly identifies some capreomycin resistant isolates as susceptible. The World Health Organization (WHO) is reviewing this information and will likely identify a lower critical concentration for detection of capreomycin resistance and likely result in better correlation with molecular methods and detect isolates that have been reported as falsely susceptible. c. Susceptibility testing uses a fluoroquinolone class drug at its clinical concentration. Therapeutic cross-resistance across all fluoroquinolone classes may not be complete. New studies suggest that if moxifloxacin has an MIC of ≤ 1.0 mcg/ml, it may have clinical activity. This correlates with the improved outcomes of XDR TB patients who are treated with moxifloxacin. d. The MGIT 960 may identify isolates as falsely resistant to pyrazinamide. Agar based testing is not useful. Molecular detection of pyrazinamide mutations may help. Most but not quite all non-synonymous mutations are associated with resistance. Silent mutations that are not significant also are noted (Chapter 7). • Testing can be delayed when a culture is contaminated or represents a mixed infection. • Discordant results even within the same laboratory and with the same specimen may occur between broth and agar testing. This is most commonly noted with ethambutol and ethionamide. • Even rapid broth based susceptibility testing requires 7 – 14 days before results are available, and may take as long as 21 days. Solid media results for second-line drugs usually take an additional 2 to 3 weeks.

Notes:

Molecular Drug Resistance Assays

Chapter 7

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Chapter 7

Molecular Drug Resistance Assays

Principles of the Test

Growth-based drug susceptibility (DST) of *Mycobacterium tuberculosis complex* (MTBC) requires 3 to 4 weeks of incubation time to produce results. Molecular methods for detecting drug resistance in MTBC provide information within 48 - 72 hours after receipt of the specimen by the laboratory about the susceptibility or resistance of the infecting organism.

Molecular drug resistance assays are based on the knowledge that mutations in the genome of MTBC associated with resistance to drugs are located in specific regions of a gene on the chromosome of the bacteria. These specific regions can be amplified an either hybridized to labeled probes (e.g., molecular beacons), an immobilized copy of the gene (such as line-probe assays), or sequenced to reveal mutations known to be associated with resistance. Whole genome sequencing can be performed to examine the entire genome.

DNA-based sequencing assays produce the actual DNA sequence of the organism. These DNA sequences can be compared to the wild type gene sequence (i.e., the gene sequence of an MTBC isolate that has not been exposed to a drug) to identify mutations known to be associated with drug resistance by DST.

Clinical Considerations

When resistance is suspected, molecular testing should be requested. Molecular detection of drug resistance (MDDR) testing identifies mutations associated with drug resistance to all first-line drugs and some second-line drugs, the most important being the injectable agents and fluoroquinolones. This is essential regarding patients who are acutely ill.

When resistance is suspected, molecular testing should be requested.

When extensive resistance is suspected and MDDR testing detects mutations to multiple groups of drugs, the clinician may wish to delay initiation of treatment until growth-based DST is completed.

Small children, persons living with HIV, and immunosuppressed persons are at high risk for progression from TB infection to active TB disease. When the source case for high-risk contacts is suspected of harboring a drug-resistant organism, early data regarding resistance will allow for the appropriate choice of TB infection therapy for these contacts.

MDDR testing can provide useful information to guide treatment of patients with drug susceptible TB who are intolerant of treatment with first-line anti-tuberculosis drugs. Prior exposure to fluoroquinolones, creates a risk for isolated fluoroquinolone resistance. Molecular testing detects over 80% of fluoroquinolone resistance as compared to growth-based testing and provides initial guidance regarding the treatment regimen.

Strengths

Unlike growth-based DST, molecular drug resistance testing can yield results in as few as 2 hours. This enhances the ability of the clinician to make decisions regarding patients and contacts when the choice of treatment must be made quickly. Knowledge that an organism is likely to be susceptible to key drugs in a regimen will prevent the addition of unnecessary drugs.

When molecular testing detects mutations associated with resistance to first-line anti-tuberculosis drugs, second-line DST and molecular testing to second-line drugs should be initiated. This give the TB clinician the opportunity to gather data rapidly on the efficacy of adding additional agents to a regimen.

A culture that is mixed (contains organisms in addition to MTBC) creates technical problems for the laboratory and can add significant delays to reporting DST results. Lengthy efforts may be required to produce a pure culture of MTBC needed for growth-based susceptibility testing. When the organism that is mixed with MTBC is a non-tuberculous mycobacteria (NTM), the culture may test resistant to several anti-tuberculosis drugs as most NTMs are inherently resistant. In the worst-case scenario, a pure culture of MTBC can never be isolated for growth-based DST. In this scenario, molecular drug resistance testing will offer valuable information regarding drug susceptibility of the MTBC.

Unlike growth-based DST, molecular drug resistance testing can yield results in as few as 2 hours.

Molecular drug resistance testing is not sufficient to replace growth-based DST.

In the case of drugs that, for various reasons, are difficult to test by growth-based susceptibility testing, such as ethambutol and pyrazinamide, molecular detection of drug resistance (MDDR) results gives additional information that can be used by an expert TB clinician to interpret discordant DST results.

Limitations

Not every mutation results in drug resistance. “Neutral polymorphisms” alter the DNA sequence and cause a change in the resultant protein structure, but the change does not affect the activity of the protein.

Molecular drug resistance testing is currently not sufficient to replace all conventional DST. It lacks the sensitivity to detect all instances of resistance in MTBC organisms. This is because not all mutations that represent antibiotic resistance are known or are tested for in these assays. Previous studies have identified resistance ‘hot spots’ in certain genes and molecular drug resistance assays have been developed to screen these areas of the genome for mutations. Recent studies have identified specific mutations associated withwith drug resistance, but not all mutations that represent antibiotic resistance are known. As scientific studies, including whole genome sequencing(WGS), continue, molecular tests will likely replace some conventional DSTs.

When resistance emerges in a population of mycobacteria, there will be some organisms in the population that are susceptible (no mutations) and some that have already developed resistance (with mutations). There must be a sufficient number of bacilli with mutations in the population for molecular drug resistance testing to detect them. If the number of emerging resistant organisms is lower than the detection limit of the assay, the assay will give a result of ‘no mutations detected’ when, in fact, resistance may be present. However, newer deep sequencing methods are able to detect smaller populations of resistant organisms. Generally, resistance is not detected until resistant organisms represent ≥30% of the total population.

Not every mutation results in drug resistance. The specificity of molecular drug resistance testing varies with each drug and mutation. Molecular drug resistance assays operate at the level of detecting changes in the DNA nucleotide sequence. "Silent" mutations alter the DNA sequence but do not cause a change in the resultant protein structure and are generally thought to not cause resistance. "Neutral polymorphisms" alter the DNA sequence and cause a change in the resultant protein structure, but the change does not affect the activity of the protein or result in resistance to a given drug.

Commercially Available Molecular Drug Resistance Assays

The only FDA market authorized molecular drug resistance assay available in the United States is the Cepheid GeneXpert® (Xpert®) ***MTB/RIF.*** Commercially available tests that are currently in use for research purposes or approved for clinical use outside the United States are discussed later in this chapter. These tests may have been locally validated as laboratory tests for use within some laboratories in the United States.

Cepheid GeneXpert® MTB/RIF

Xpert® MTB/RIF is a commercially available, FDA market authorized system, for detection of organisms such as methicillin-resistant Staphylococcus aureus (MRSA), vancomycin resistant enterococcus (VRE), Influenza A and B and others. The Xpert® MTB/RIF assay was approved by the FDA in 2013. This assay simultaneously tests for the presence of MTBC and rifampin resistance. The greatest strengths of this test are that it requires little technical training and has a rapid turnaround time.

Either a sputum collected directly from the patient or a processed sputum sediment is mixed with the reagent provided with the assay. The mixture is transferred to a test cartridge and placed in the Xpert® MTB/RIF instrument. All processing from this point is fully automated thus decreasing the chance of cross-contamination.

The assay produces a result in less than 2 hours and detects the presence of MTBC and the most common mutations for rifampin resistance. Rifampin resistance is the greatest predictor of multidrug resistant tuberculosis (MDR TB); therefore, knowledge about rifampin resistance early in the management of a patient has important implications. The assay tests most but not all rifampin-associated mutations. Those located outside the hotspot, although rare, would be missed. Silent or neutral mutations may be detected suggesting rifampin resistance when there is none.

The FDA has also approved the Xpert® MTB/RIF for use in the release of patients suspected of having tuberculosis from isolation. More information can be found at: *https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6407a8.htm*.

CDC Molecular Detection of Drug Resistance Service

In September 2009, the CDC implemented the MDDR testing service, a comprehensive, CLIA-compliant, clinical service made available to all domestic TB control programs and clinicians. MDDR testing is a DNA sequence-based assay that utilizes pyrosequencing and Sanger sequencing techniques. The CDC performs growth-based DST on all submitted samples in parallel with MDDR testing. Use of the MDDR service should be coordinated through the state public health laboratory.

Specimens or isolates submitted to the CDC for MDDR testing must be confirmed as positive for MTBC by the submitting laboratory. In general, the turnaround time of a CDC MDDR report is 2-3 business days.

Table 18 lists the drug testing that is available through this service as well as the sensitivity and specificity of the testing when compared to growth-based DST.

Table 18 – MDDR Performance Characteristics by Drug

Drug	Locus or loci examined	Sensitivity (%)	Specificity (%)
Rifampin	rpoB	97.1	97.4
Isoniazid	inhA + katG	86.0	99.1
Fluoroquinolones	gyrA	79.0	99.6
Kanamycin	rrs + eis	86.7	99.6
Amikacin	rrs	90.9	98.4
Capreomycin	rrs + tlyA	55.2	91.0
Ethambutol	embB	78.8	94.3
Pyrazinamide	pncA	86.0	95.9

Reference: Laboratory Division of TB Elimination Laboratory User Guide for U.S. Public Health Laboratories: Molecular Detection of Drug Resistance (MDDR) in Mycobacterium tuberculosis Complex by DNA Sequencing (Version2.0) June 2012

Other Molecular Drug Resistance Testing Methods

There are molecular drug resistance assays available through laboratories that represent 'laboratory-developed tests'. These laboratories have validated results and should be willing to provide validation data to the requester. These assays are generally based on DNA sequencing or real-time polymerase chain reaction (PCR) assays. For more information about the specifics of the testing in these laboratories, the requester should contact the laboratory directly.

Line Probe Assays

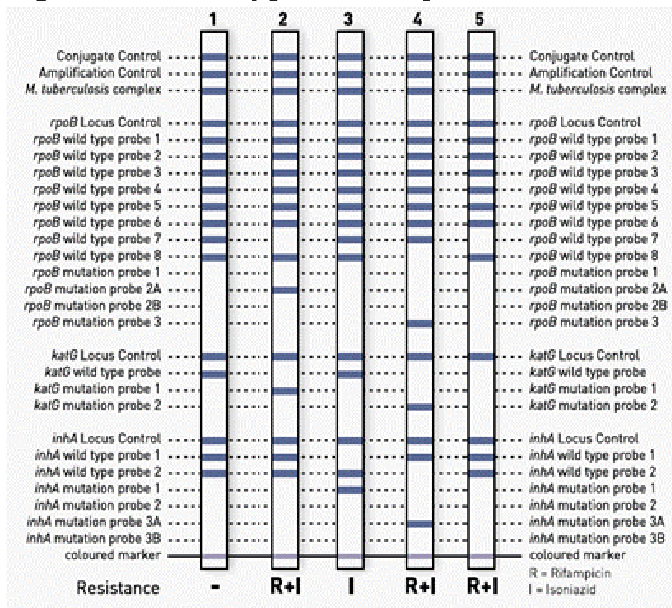
The **Hain Lifescience *Genotype® MTBDRplus and MTBDRsl*** assays can be used to test specimens that have been processed for acid fast bacilli (AFB) culture or isolates from positive cultures. They cannot be used with raw patient specimens.

The ***Genotype® MTBDRplus*** assay detects the presence of MTBC, isoniazid resistance and rifampin resistance while the ***Genotype® MTBDRsl*** assay detects the presence of MTBC as well as fluoroquinolone, ethambutol, and aminoglycoside resistance.

While line probe assays provide useful information about potential resistance, they suffer similar shortcomings as other probe-based molecular drug resistance assays. Target genes amplified for study do not identify all mutations associated with resistance, and silent or neutral mutations may suggest resistance when there is none. It should be noted that Hain Lifescience assays are not widely distributed in the United States and may be difficult to obtain.

In general, molecular drug resistance testing can be performed directly on processed sputum or processed tissue prior to culturing. Testing can also be performed on an organism growing in culture.

Figure 5 – GenoType MTBDR*plus* VER 2.0 Sample Results



https://www.hain-lifescience.de/en/products/microbiology/mycobacteria/tuberculosis/genotype-mtbdrplus.html

Laboratory Considerations

The most important consideration when requesting a molecular drug resistance assay is to contact the laboratory regarding which assays are available. In general, molecular drug resistance testing can be performed directly on processed sputum or processed tissue prior to culturing. Testing can also be performed on an MTBC positive culture.

Interpreting MDDR Results

Laboratory reports for molecular drug resistance testing will frequently list the genes tested and provide information as to the likelihood of resistance. Some reports, such as those using Xpert MTB/RIF, will simply state ‘Mutation detected’ or ‘No mutation detected’. When reviewing results, it is important to keep in mind that every gene associated with resistance is not tested in all molecular drug resistance assays.

‘No mutation detected’ suggests that it is likely the organism is susceptible to the listed drug; however, the sensitivity of the test varies for each drug and the mutation(s) associated with resistance to that drug (Table 17). Failure to detect a mutation favors susceptibility but is not a guarantee of susceptibility. It merely means that the mutations most commonly associated with resistance were not seen. Conversely, mutations that are detected can represent silent or neutral mutations and not true resistance.

Results from molecular resistance assays based on DNA sequencing of the target gene will report the specific mutation detected by the assay. For example, the report may show an *rpoB* mutation result of **TCG 531 TTG**. The first three letters represent the wild-type DNA sequence at position 531. The second three letters represent the mutant sequence. The mutated DNA sequence may also be reported as the actual change in the protein sequence; **Ser 531 Leu** indicates a change from the wild type amino acid serine at position 531 to leucine.

Genes that are associated with rifampin and isoniazid resistance are most commonly tested. Testing for rifampin resistance in all available assays has a high sensitivity (96-98%). When no mutation is detected, there is a very low probability that the organism is rifampin-resistant.

Tests for the detection of isoniazid resistance have lower sensitivity (80-88%) compared to growth-based DST and lower predictive value. Sensitivity and specificity for other genes tested by the CDC are listed in Table 17.

Rifamycins are extremely important to the successful treatment of TB patients. When there are mutations suggestive of rifampin-resistance, the question arises whether rifabutin may still have some activity. Growth-based DST shows rifabutin is still susceptible in ~15-25% of rifampin-resistant isolates. Accumulation of molecular drug resistance assay data correlated with growth-based DST, data reveals specific mutations are able to predict rifampin-resistant but rifabutin-susceptible strains. Two of these mutations are **GAC 516 GTC** and **CAC 526 CTC**.

Isoniazid, long considered a 'work horse' drug in the TB field, is important in the treatment of both active TB disease and TB infection. Two genes, *inhA* and *katG,* are generally tested to assess for INH resistance. While mutations in either gene can confer resistance, mutations in the *katG* gene are known to confer resistance at higher levels of drug resistance when tested by growth-based DST while mutations in *inhA* tend to be associated with resistance at lower drug concentrations. Understanding this may guide the decision to continue or discontinue isoniazid in a treatment regimen. Resistance to the fluoroquinolones is frequently associated with mutations in the *gyrA* gene of the MTBC. Various mutations may correlate with a range of moderate to high MIC values. These mutations may confer resistance to ofloxacin but suggest that levofloxacin or moxifloxacin may be active against the strain of TB tested. **GCG 90 GTG** (Ala90Val) is an example of a mutation which counters resistance to ofloxacin but not moxifloxacin.

Always seek expert consultation for the interpretation
of molecular drug resistance tests results.

Discordance between molecular and growth based test results should be discussed with experts regarding the specific drug in question and the method used to test both culture based drug susceptibility and to perform the molecular test. The science is changing, especially in relation to molecular testing and it is likely that newer molecular methods will give additional information regarding the susceptibility of an isolate to key drugs needed to treat tuberculosis. New methods with increased sensitivity will identify developing resistance and heteroresistance quickly and allow the clinician to treat using the most effective regimen for a particular patient.

Notes:

Genotyping

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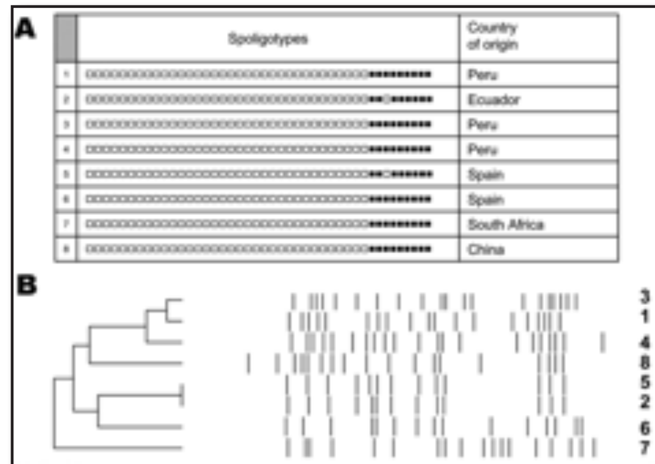
Chapter 8

Genotyping

What is Genotyping?

Genotyping is a general term that is synonymous with the term 'DNA fingerprinting'. The technique recognizes that there are regions of DNA that differ in different *Mycobacterium tuberculosis* complex (MTBC) isolates and allows for comparison of related of MTBC isolates based on the similarity of their genetic make-up. There are three methods of genotyping currently employed by the CDC:



- Spacer oligonucleotide typing (Spoligotyping)
- Variable-Number Tandem Repeat of Mycobacterial Interspersed Repetitive Units (VNTR-MIRU or MIRU)

The goal of the National TB Genotyping Service is to genotype at least one isolate for each new culture positive case of tuberculosis (TB). In March 2010, the TB Genotyping Information Management System (TB GIMS) was launched by the CDC as a secure Web-based system to support ongoing use of TB genotyping data in TB control activities. The centralized national database contains genotype and surveillance data that allow for detection of interstate transmission, monitoring of cluster growth at the regional and national levels, and tracking of special populations (outbreak strains, MDR and XDR TB strains, persons experiencing homelessness, and prison populations).

Clinical Considerations

Identifying transmission of MTBC in a community is one of the cornerstones of effective public health practice. Cases that are transmission-related, or epidemiologically linked, should have the same genotype. Data generated through genotyping allow TB control programs to examine the effectiveness of contact investigations.

When individuals are infected with the same organism and no epidemiologic link is found, the opportunity arises to re-examine potential gaps in the contact investigation. Perhaps there is an unsuspected relationship between patients or an unusual transmission setting.

Data generated through genotyping allows TB control programs to examine the effectiveness of contact investigations.

Following differences in strains can show patters and dynamics of TB transmission within communities.

State, regional, and local public health authorities can review genotyping data from patients in their jurisdiction. Following differences in strains can show patterns and dynamics of TB transmission in communities. This is especially relevant in transient populations such as jails and homeless settings.

While genotyping will provide valuable information about individuals who harbor the same strain of MTBC, it will not establish epidemiologic links. This technique is a complementary tool for use in conjunction with contact and outbreak investigations to determine the quality of these activities and to ensure all members of a cluster are identified using standard public health practice.

Genotyping does not establish epidemiologic links. It must be used in conjunction with contact investigations.

When there is a laboratory report of culture positive MTBC in the absence of a suggestive clinical presentation, genotyping can promptly help identify potential false-positive cultures. A single isolate that grows from a patient who has a clearly identified 'other cause' for his/her disease presentation or whose disease presentation does not suggest TB (such as cure without treatment) can be compared with isolates from specimens that were processed at the same time in the laboratory to look for possible cross-contamination. False-positive cultures can also be due to handling errors such as mislabeling containers with the wrong patient name at the time of specimen collection or reporting errors caused by data entry mistakes when patient records are created in an information system. It is important to be aware of and monitor for possible sources of false-positives, and genotyping is a valuable tool for confirming suspected false-positive reports.

Laboratory Considerations

Genotyping is performed reflexively on all initial MTBC isolates submitted through public health laboratories. To have this service performed on MTBC isolates tested in private laboratories, health departments may need to request the private laboratory transport an MTBC isolate to its state laboratory. Some states have legislation that requires laboratories to submit one MTBC isolate per patient to the state public health laboratory for genotyping. Compliance with this requirement can be inconsistent and locating the laboratory that possesses the MTBC isolate can be challenging.

Turnaround Time

Spoligotyping and MIRU results are available for health departments within 10-14 business days after receipt by the testing laboratory. It should be noted that genotyping cannot be performed directly on a clinical specimen and requires a certain amount of biomass to produce valid results. Therefore, the clinician should be aware that time is required for a clinical specimen to incubate and for the MTBC isolate to grow before it can be shipped to a laboratory that performs genotyping. This incubation time can vary from specimen to specimen.

Figure 6 - Sample Genotype Report

Last Name	First Name	DOB	Spoligotype	MIRU 1	MIRU 2	PCRType	GENType
Doe	Jane	8/1/1950	777777777760771	233326153321	733424423336	PCR00673	G00435
Doe	Sarah	12/12/1975	777777777760771	233326153321	733424423336	PCR00673	G00435
Smith	John	5/5/1975	000000000003771	223321153623	432331244234	PCR01325	G01965

Utilizing Genotyping Results

Genotyping reports list the name and date of birth of each individual patient tested, followed by a series of numbers representing the 'spoligotype' and MIRU-VNTR patterns. For an explanation of how these numbers are generated, see Appendix A3.

Each unique combination of spoligotype and 12-locus MIRU-VNTR result is assigned a national 'PCRType'. Each unique combination of spoligotype and 24-locus MIRU-VNTR is assigned a national 'GENType'. When reading these reports, strains that are identical by the two testing methods that are in the same CDC designated group can be considered to be the same strain of MTBC. Patients with the same pattern share the same organism, regardless of where they live in the United States.

Periodically, health departments should review the genotype data of isolates collected from patients in their jurisdictions. When the genotypes match previously identified epidemiologic links, this supports the finding of recent transmission. It also suggests the contact investigation has been effective.

When there is a genotype match between individuals but no epidemiologic link, this suggests that perhaps the contact investigation was not expanded widely enough and that perhaps a more thorough or wider investigation is warranted. If a careful contact investigation has been performed and a review of all available epidemiologic data still fails to reveal a link, other considerations would include the possibility of distant transmission or casual contact.

Genotype data is also helpful in the investigation of false-positive cultures. In some cases, MTBC is grown from a single specimen collected from a patient who does not fit the typical clinical picture of TB. These types of patients might include immunocompetent patients with normal imaging, patients with other causes identified that explain their clinical findings or patients with complete resolution of symptoms that prompted collection of the samples without any treatment. A request can be made to review all specimens collected and processed on the same day as the suspect specimen to see if there is a possibility of cross-contamination in the laboratory or incorrect labeling of a specimen from another patient known to have active TB disease.

Genotyping data is extremely helpful in the investigation of false-positive cultures.

Whole Genome Sequencing

Improved accuracy of outbreak detection systems should result in more targeted public health interventions

In 2018, the CDC will implement universal WGS for all isolates of *M. tuberculosis* from culture-confirmed TB cases in the U.S. As with conventional genotyping methods, the objective for using WGS as part of the National TB Genotyping Service is to provide molecular data that can be combined with traditional epidemiological data to aid public health programs in the detection and investigation of transmission. Unlike conventional PCR-based methods that examine less than 1% of the *M. tuberculosis* genome, WGS allows for examination of greater than 90% of the genome, increasing the accuracy of outbreak detection systems. This improved accuracy should result in more targeted public health interventions. CDC plans to continue conventional methods (e.g., MIRU and spoligotyping) for a 3 year transition period in addition to WGS as cluster detection methods and systems are modified for full reliance on WGS in 2021.

Two different analytic methods will be used to understand the genetic relatedness of *M. tuberculosis* isolates for the purposes of detecting clusters and possible transmission networks. These methods include whole genome multilocus sequence typing (wgMLST) and whole genome single nucleotide polymorphism analysis (wgSNP). Essentially, wgMLST will be used to analyze all isolates to identify clusters, and wgSNP will be used to provide greater specificity to aid in the identification of possible transmission networks. Although analytically complex, the CDC plans to convey the results from these analyses in a simple format that is useful for U.S. TB programs.

WGS allows not only for the examination of genetic relatedness for molecular epidemiologically purposes, but it also provides sequencing data useful for species identification and genetic prediction of drug resistance. The CDC plans to use data from the national service to develop TB drug resistance alerts for surveillance purposes but also with the intent of notifying TB programs when drug resistance is suspected. These alerts will allow notification to ensure that samples from the patient have been examined for molecular resistance in a CLIA-compliant laboratory, as the testing performed under the National TB Genotyping Service is not currently performed under a regulatory framework. However, U.S. and international laboratories are increasingly interested in evaluating how WGS performs in a clinical laboratory setting and how it might be used to replace other laboratory tests. Most certainly, this increasing interest will result in greater access to WGS services not only for surveillance but also for routine diagnostic work. Challenges will include interpretative criteria and reporting of results to healthcare providers using language that is clear and actionable.

Notes:

Serum Drug Levels

Chapter 9

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Chapter 9

Serum Drug Levels

Principles of the Test

Significant literature is available on the pharmacokinetic/pharmacodynamics of anti-tuberculosis drugs. By administering anti-tuberculosis drugs to normal volunteers, researchers have assembled a range of expected serum drug levels for each of the drugs in use. For therapeutic drug monitoring (TDM), TB patients are administered drugs under observation, and at the time at which the patients are expected to achieve the peak drug concentration in their blood, samples are taken by phlebotomy for analysis. Specialty laboratories analyze the serum from each blood sample by high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) to quantitate the amount of drug contained in the sample.

Clinical Considerations

Identification of the drug causing toxicity can be difficult to determine as patients generally receive a multidrug regimen. When a patient has limited drug options, especially due to resistance, determining which drugs can be safely retained in a regimen adds important information to clinical decision making. This becomes especially relevant when patients need to be on potentially toxic drugs for an extended period of time such as during treatment of multidrug resistant tuberculosis (MDR TB). Adjusting drug doses to produce therapeutic levels and to avoid the toxic range may buy more time for the drugs use.

Several anti-tuberculosis drugs are excreted by the kidneys. Most notably, ethambutol, pyrazinamide, levofloxacin, cycloserine, and injectable agents have a high potential for toxicity in patients with chronic kidney disease. In patients with reduced renal function, these drugs may worsen renal function and elevated serum levels may lead to toxicity. Documentation of serum levels may be especially helpful for patients who are on hemodialysis or peritoneal dialysis. Drugs can be dialyzed at varying rates and it may be impossible to adequately predict the dose of drug that can produce therapeutic but not toxic serum levels.

Adjusting drug doses to produce therapeutic levels and avoid the toxic range may buy more time for the drug's use.

In patients who are delayed in culture conversion, serum drug levels can be obtained to determine if the drug dose can be increased without putting the patient into a toxic drug range. While there are no published studies showing this to improve outcomes, it is generally considered safe and may add benefit.

Laboratory Considerations

The exact time the drugs were administered should be recorded.

The first step in obtaining serum drug levels is to contact the laboratory performing the assay to gather information about proper collection and transfer of the specimen to the laboratory. Pre-planning will ensure the entire collection procedure goes as smoothly as possible.

The patient should be asked to come to the clinic with the expectation of staying a minimum of two hours. Upon arrival at the clinic, the patient should be administered TB drugs at the doses he/she is currently receiving and monitored until the blood is drawn to assure the drugs are not spit out or vomited. The exact time the drugs were administered should be recorded.

The timing of the blood draw after administration of the drugs varies. This is why communication with the laboratory performing the assay is important. Most TB drugs peak around 2 hours after administration, and this time should be targeted for the first blood draw. A notable exception is rifabutin levels which should be drawn 3 hours after drug administration. ***Blood for serum drug levels must be drawn from a fresh stick and not from an existing IV, PICC line, or catheter.***

Patients may metabolize the drugs at different rates (some faster and some slower), so drawing blood at additional times (such as 6 hours after drug administration) may provide valuable information. Each time blood is drawn for analysis, the time should be carefully recorded.

Once blood has been drawn from the patient, it is important to transport the specimen to the laboratory as quickly as possible. The blood samples will likely need to be centrifuged and the serum removed and frozen soon after being collected from the patient.

Turnaround Time

Turnaround time for reporting of serum drug levels is laboratory dependent. It is best to contact the laboratory performing the assay to ask when reports can be expected. Should the assay need repeating for any reason, this will increase the time for reporting.

Notes:

Appendix

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Appendix Table A1

Collection, Transport and Evaluation of Specimens for Mycobacteria

Type/Source of Specimen	Evaluation of Specimen		Additional Information
Open abscess or lesion at any site, cellulitis and skin lesions, ear drainage, and eye exudates	AFB smear AFB culture Smears and Cultures for additional pathogens	**COLLECTION:** Use aseptic technique. Suction fluid with a syringe or remove tissue; for open lesions/ abscesses, try to aspirate from under the margin of the lesion rather than collecting necrotic or purulent tissue from the center; swabs are unacceptable. **HANDLING:** Use a sterile leak-proof container; submit in 2 to 3 mL sterile **(not bacteriostatic)** saline; refrigerate if transport to the lab is delayed more than one hour; do not freeze specimen..	NAAT may be helpful if positive
Blood	AFB culture	**COLLECTION:** 10 mL of whole blood in a 10-mL yellow-top collector tube containing sodium polyanethol sulfonate (SPS), or a green-top collector tube containing heparin; minimum volume is 5 mL; NO yellow-top ACD, red-top or EDTA (purple-top tubes). **HANDLING:** Do not refrigerate or freeze blood specimens.	Collect in patients with: sepsis syndrome risk factors for mycobacteremia (HIV/AIDS) Smears not recommended due to low number of mycobacteria in specimen
Body fluids Abdominal, amniotic, ascites, bile, gastric wash or lavage, joint, paracentesis, pericardial, peritoneal, pleural, synovial, thoracentesis	AFB smear AFB culture	**COLLECTION:** 10 mL or more of fluid in a sterile container; as much fluid as possible; swabs are unacceptable; bloody specimens can be collected and submitted in 10 mL yellow-top SPS blood collection tubes **HANDLING:** Sterile leak-proof container such as a 50 mL conical tube; minimum volume of 10 to 15 mL; refrigerate, but do not freeze specimen	Pleural and peritoneal fluid should be sent for cell count, protein and cytology if indicated. Pleural fluid - adenine deaminase (ADA), glucose with simultaneous serum glucose. NAAT may be helpful if positive
Bone marrow aspirate	AFB smear AFB culture	**COLLECTION:** Use yellow-top SPS (not ACD) collector tube and mix contents of tube after collection. **HANDLING:** Sterile leak-proof container such as a sterile 50 mL conical tube; do not refrigerate or freeze blood specimens.	Pathological exam to look for granuloma NAAT may be helpful if positive
Cerebrospinal Fluid (CSF)	AFB smear AFB culture	**COLLECTION:** 2 to 3 mL (optimal 10 mL) of cerebrospinal fluid; minimum volume required is 2 to 3 mL. **HANDLING:** Sterile leak-proof container such as a 50 mL conical tube or CSF tubes; *Do not refrigerate or freeze* the CSF specimen.	Cell count, protein and glucose with simultaneous serum glucose NAAT is usually regarded as significant if positive

Type/Source of Specimen	Evaluation of Specimen		Additional Information
Feces	AFB smear AFB culture	**COLLECTION:** Sterile leak-proof container; do not use holding or transport medium; minimum volume is 1 gram. **HANDLING:** Refrigerate if transportation to the lab is delayed > one hour; *Do not freeze* specimen	Prior arrangements must be made with the testing laboratory for submission of feces. Stool cultures for mycobacteria are not routinely encouraged. NAAT may be helpful if positive
Gastric wash or lavage	AFB smear AFB culture	**COLLECTION:** Patient NPO and still in bed; introduce 25 to 50 mL of chilled distilled water into the stomach and remove via NG aspiration. (See Appendix B1) Collect one specimen/ day on three consecutive days **HANDLING:** Sterile leak-proof container, such as a sterile 50 mL conical tube; neutralize sample with 100 mg sodium carbonate within one hour of collection if transport delayed > one hour, transport as soon as possible at room temperature	Mycobacteria die rapidly in gastric washings; collect as large a specimen as possible Non tuberculous organisms may cause a false-positive AFB See Appendix B1 Collection of Gastric Aspirates. NAAT helpful if positive
Respiratory, lower bronchoalveolar lavage (BAL), brush or wash, endotracheal aspirate, transtracheal aspirate	NAAT AFB smear AFB culture	**COLLECTION:** Sputum trap, place the brush in a sterile leak-proof container with up to 5 mL of sterile saline; minimum volume is 3 mL **HANDLING:** Sterile leak-proof container, such as a sterile 50 mL conical tube; refrigerate if delayed > one hour	AFB smear + should be regarded in the same way as a sputum with regard to isolation and contact investigation
Sputum, expectorated or induced	NAAT AFB smear AFB culture	**COLLECTION:** Cough deeply to produce a lower respiratory specimen; sterile container; minimum desired volume is 3 mL; **HANDLING:** Sterile leak-proof container, such as a sterile 50 mL conical tube; refrigerate specimen if transport delayed > one hour; *ship cold, not frozen*, in a triple container.	**Replica limits:** Three consecutive specimens collected at least 8 to 24 hour apart. One specimen should be a first a.m. and if possible one should be observed.
Tissue/lymph node	AFB smear AFB culture	**COLLECTION:** Always submit as much tissue as possible; add 2 to 3 mL sterile **(not bacteriostatic)** saline to tissue for transport. **HANDLING:** Sterile leak-proof container, such as a sterile 50 mL conical tube; transport at room temperature	Tissue is less likely to be AFB positive NAAT helpful if positive
Urine, including collections from a catheter	AFB smear AFB culture	**COLLECTION:** Approximately 40 mL (minimum 10 – 15); early a.m. specimen best; do not pool urine or use preservatives **HANDLING:** Sterile leak-proof container, such as a sterile 50 mL conical tube; transport at room temperature; Refrigerate specimen if transport to the lab delayed > one hour	AFB that are not *M. tuberculosis* may be in urine causing a false-positive NAAT may be helpful if positive

++Not FDA approved for these specimens. Lab developed NAAT may be used but may be less reliable

Appendix Table A2

Current Status of Drug Susceptibility Testing Methodology and Critical Concentrations for First- and Second-line DST

Drug group[a]	Drug	DST method available	DST critical concentration (mcg/ml)	
			Middlebrook 7H10[a]	MGIT960
Group 1 First-line oral anti-TB agents	Isoniazid	Solid, liquid	0.2	0.1
	Isoniazid (High)	Solid, liquid	1.0	0.4
	Rifampicin**	Solid, liquid	1.0	1.0
	Rifabutin	Solid, liquid	0.5	0.5
	Ethambutol	Solid, liquid	5.0	5.0
	Pyrazinamide	Liquid	-	100.0
Group 2 Injectable anti-TB agents	Streptomycin	Solid, liquid	2.0	1.0
	Kanamycin	Solid, liquid	5.0	2.5
	Amikacin	Solid, liquid	4.0-	1.0
	Capreomycin	Solid, liquid	10.0*	2.5
Group 3 Fluoroquinolones	Ofloxacin	Solid, liquid	2.0	2.0
	Levofloxacin	Solid, liquid	1.0	1.5
	Moxifloxacin	Solid, liquid	0.5	0.25
	Moxifloxacin (High)	Liquid	-	2.0
Group 4 Oral second-line anti-TB agents; all except ethionamide are bacteriostatic	Ethionamide	Solid, liquid	5.0	5.0
	Cycloserine	Solid	-[c]	-[c]
	P-aminosalicylic acid	Solid, liquid	2.0	-
Group 5 Anti-tuberculosis agents with unclear efficacy[b]	Clofazimine	Liquid	-	-
	Linezolid	Liquid	-	1.0

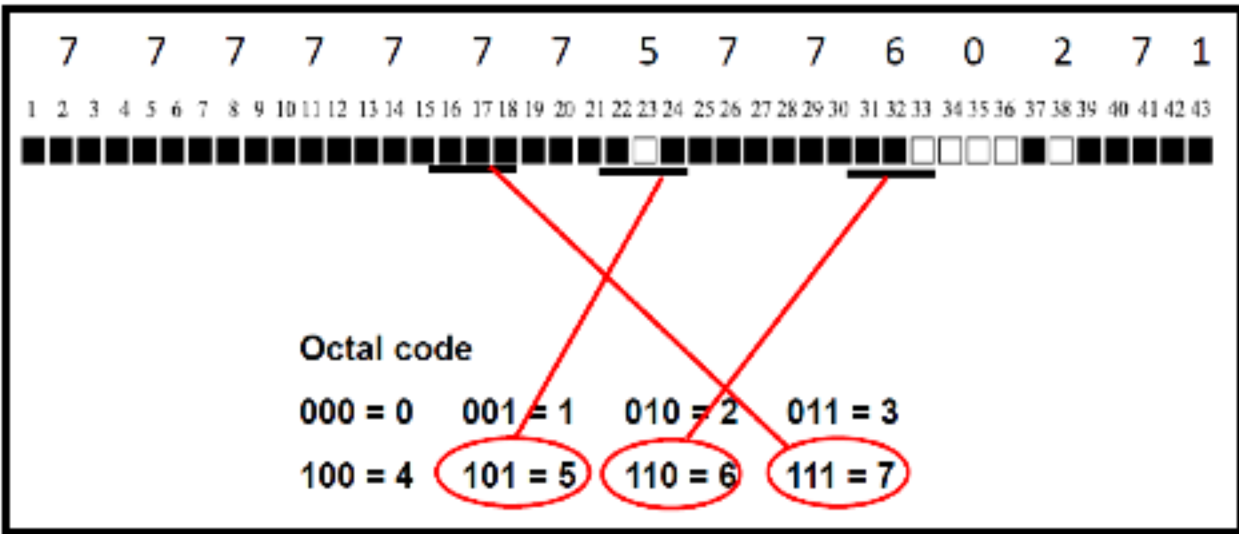
[a] Indirect proportion method recommended. Other solid media methods (resistance ratio, absolute concentration) have not been adequately validated for second-line drugs.
[b] Not recommended by WHO for routine use in MDR TB patients
[c] Cycloserine testing with 7H10 or MGIT960 is not recommended due to technical problems/reproducibility with the test
** Rifampin low level resistance is area for high priority research; Genetic mutations may suggest possible low level resistance.*
*** Capreomycin cut point may be too high leading to false susceptibility; Confirm with molecular testing.*

Appendix A3

Centers for Disease Control and Prevention Genotyping Methods

Spacer oligonucleotide typing (Spoligotyping)

There is a direct repeat locus on the chromosome of *Mycobacterium tuberculosis* complex (MTBC). This locus contains multiple, well-conserved DNA repeats with 43 variable spacers. Spoligotyping measures the variation in these spacers. Presence or absence of each of these spacers is translated into binary code, then octal code to produce a numeric value for each pattern that can be compared to the patterns of other organisms (see figure below).



This method is highly reproducible, has the most rapid turnaround time among the available methods, and can be performed on viable and non-viable organisms. Of note, all members of the Beijing lineage are identical by this method, which makes differentiation difficult unless paired with one of the other two methods.

Variable-Number Tandem Repeat of Mycobacterial Interspersed Repetitive Units (VNTR-MIRU or MIRU)

There are 41 different MIRU loci on the chromosome of MTBC. MIRU testing targets either 12 or 24 of these loci and determines the numbers of DNA repeats each contains. This method is paired with spoligotyping by the CDC genotyping service.

Each unique combination of spoligotype and 12-locus MIRU-VNTR results is assigned a national 'PCRType'. The PCRType is reported with the letters 'PCR' followed by five digits, such as PCR01974. All identical isolates, no matter where they are collected in the U.S. will share the same PCRType allowing for comparison of any isolates collected. Each unique combination of spoligotype and 24-locus MIRU-VNTR has been assigned a GENType, reported as "G" followed by five digits such as G00056.

Sample Genotype Report

There is a direct repeat locus on the chromosome of MTBC. This locus contains multiple, well-conserved DNA repeats with 43 variable spacers. Spoligotyping measures the variation in these spacers. Presence or absence of each of these spacers is translated into binary code, then octal code to produce a numeric value for each pattern that can be compared to the patterns of other organisms (see figure below).

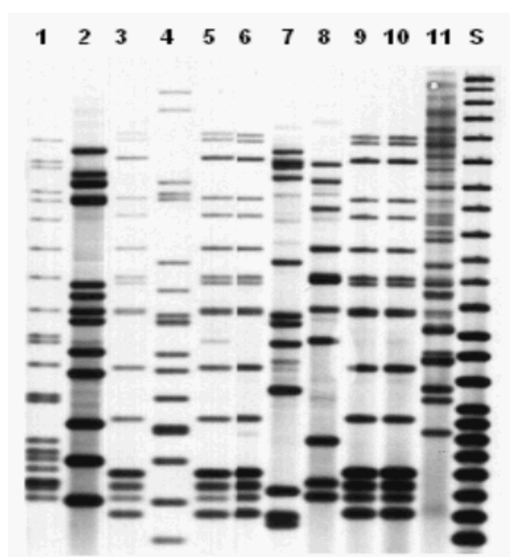
Last Name	First Name	DOB	Spoligotype	MIRU 1	MIRU 2	PCRType	GENType
Doe	Jane	8/1/1950	777777777760771	233326153321	733424423336	PCR00673	G00435
Doe	Sarah	12/12/1975	777777777760771	233326153321	733424423336	PCR00673	G00435
Smith	John	5/5/1975	000000000003771	223321153623	432331244234	PCR01325	G01965

Restriction Fragment Length Polymorphism (RFLP)

RFLP is based on the finding that there is a genetic element, IS6110 (Insertion Sequence 6110), randomly inserted in the chromosome of strains of MTBC. This insertion sequence can mobilize itself or 'jump' to random points on the MTBC chromosome, leaving a copy at the original insertion site. In general, MTBC strains contain 0-25 copies of IS6110 on their chromosome. Strain differences (or similarities) can be determined by counting the number of copies and the position of these fragments.

DNA from strains of MTBC is digested with enzymes called restriction endonucleases to generate 'restriction fragments'. These digested DNA fragments are separated by size using electrophoresis. The size separated DNA fragments are then hybridized with copies of IS6110 to show the number and position of the copies. A 'DNA fingerprint' pattern is generated that can be compared to other strains.

RFLP has the highest differentiating power of the 3 genotyping methods and is generally performed only if specifically requested. This technique requires a viable culture and is relatively labor intensive.



Representative IS*6110*-based RFLP image. Isolates represented by lanes 3, 5, 6, 9, and 10 have the same pattern and were epidemiologically links. Lane S shows the CDC molecular weight standard. (*Guide to the Application of Genotyping to TB Prevention and Control - https://www.cdc.gov/tb/programs/genotyping/chap3/3_cdclab_2description.htm*)

Appendix B1

Collecting Gastric Aspirates

Recommended equipment:

- 10 French or larger feeding tube
- 50 cc syringe to fit tube
- Specimen container with neutralizing agent (contact your laboratory)

Before Starting the Procedure

1. Remind the parent the night before that the child is to have nothing to eat after midnight. Multiple samples may need to be collected from babies who feed often (collected after naps and before feeding) during the course of the day. Also remind the parents that the child should have minimal activity before the procedure.

2. Prior to starting the actual procedure, confirm that the child has been NPO overnight.

3. Make sure you have the correct collection vessel. The stomach acid in the sample must be neutralized quickly to avoid killing the organisms. Contact the laboratory well in advance of starting the procedure to confirm a collection container that will neutralize the specimen.

4. Get everything ready before starting (e. g. the syringe needs to fit the feeding tube).

Starting the Procedure

5. If at all possible, remove a hospitalized child to a separate room for the procedure. This will reduce the child's anxiety while in the hospital room and create a 'safe zone'. Explain the procedure carefully to the child's caregiver and have him/her wait in the hospital room or waiting area until the child returns. The entire procedure takes only about 5 minutes.

6. Restrain the child well. Wrap well in a sheet and then place in a 'papoose board' if available.

7. Before placing the nasogastric (NG) tube, measure the estimated length carefully and mark your goal length with a pen rather than with a piece of tape.

8. As you insert the tube, stay away from the nasal septum and aim the tube straight down toward the bed (not up toward the nasal bridge).

9. If the child does not swallow as the tube passes into the throat, give a short puff into the child's face. This frequently elicits a swallow and allows the tube to pass without coiling in the mouth.

10. Attach the syringe to the end of the tube, inject air rapidly with the syringe and listen, checking the stomach placement with a stethoscope.

11. Once the tube is correctly placed in the stomach, apply steady suction with the syringe to collect mucus from the stomach.

12. If no mucus, try advancing and withdrawing the tube several inches or try turning the child to a different position (on the stomach, side, sitting up, etc.).

13. If no specimen is obtained despite the above attempts, inject 20-30 cc of water (not saline) into the stomach and aspirate rapidly.

14. Place the collected specimen into the collection vessel according to the laboratory's policy.

15. Label the specimen with the date and time of collection as well as required patient information (name, medical record number, etc.).

16. Work hard to get a good specimen the first day - it is the best yield.

17. Three consecutive morning gastric aspirates have the best yield. Only one may be necessary for a child with a good presumed source case.

Remember: A negative test does not rule out TB!

Appendix B2

Proper Sputum Collection

Materials Needed

- Clean leak proof specimen container (single use wide mouth 50ml preferred)
- Disposable gloves
- Patient identification labels
- Laboratory requisition
- Bio hazard bag
- Personal protection equipment

Prior to sputum collection

1. Clearly instruct the patient on:
   a. The importance of sputum examination.
   b. How to open and close collection containers.
   c. Not to touch the inside of collection container to avoid contamination of the container.
   d. The need for collecting sputum and not saliva or nasopharyngeal discharge.
   e. How to produce a good sputum.
   f. How to collect, store, and deliver the specimen to the laboratory or clinic.
   g. The need to collect 3 sputum samples to facilitate diagnosis.

2. Instruct the patient to choose a well-ventilated or open-air area distanced from others. Consider the following:
   a. **FIRST MORNING SPUTUM** will produce the best specimen
   b. Sputum should be collected **before brushing teeth**. Have the patient rinse his/her mouth with water before collecting the specimen to minimize contamination with food particles, mouth wash or oral drugs.
      - Tooth brushing with water is all right but avoid antiseptic solutions such as mouthwash.
      - If tap water in the patient's area has abundant non-tuberculous mycobacteria, sterile water is indicated.

3. Collect samples in a sterile, single use, plastic container without fixatives. The characteristics of a good collection container include:
   a. Strong and unbreakable
   b. Leak proof, screw-capped with water-tight seal
   c. Sterile
   d. Up to 50 ml capacity
   e. Translucent or clear material
   f. Single-use
   g. Easily-labeled walls

4. Clearly label the container with the patient's name, identification number, and date and fill out the specimen request form completely.

Collection of the first specimen should be observed!

---

5. Instruct patient on cough technique (see below) to limit throat irritation and possible contamination of sample with blood.

6. Instruct the patient to transport specimens in the biohazard bag with the requisition immediately or to refrigerate to prevent over-growth of other organisms.

Cough Techniques

Forceful Cough Technique - Instruct the patient to:

1. Take a deep breath, filling the lungs as completely as possible.
2. Contract the upper abdominal muscles.
3. Expel all of the air using one forceful cough.
4. Repeat the process one or two times until mucus is loosened

.

Huffing Technique - Instruct the patient to:

1. Take a deep breath, filling the lungs as completely as possible.
2. Blow the air out in forceful bursts through an open mouth while saying " huh or huff", as if blowing steam onto the mirror.
3. Repeat as many times as needed to clear the loosened mucus from the airway.

Deep Coughing Technique – Instruct the patient to:

1. Sit on a chair or on the edge of a bed with both feet on the floor.
2. Begin by taking in a DEEP breath.
3. Hold for 2-3 seconds.
4. Use stomach muscles to forcefully expel the air.
5. Avoid a hacking cough or merely clearing the throat.
6. A DEEP cough is less tiring and more effective in clearing mucus out of the lungs.

Sputum Collection Pearls

Adequate hydration is necessary to produce fluid like mucus.

- Steam from a hot shower can assist with loosening mucus in respiratory passages.

Characteristics of a good sputum specimen

- Mucoid or mucopurulent appearance.
- Minimum amounts of saliva.
- Optimal volume: 5 – 10ml.
- Minimum volume: 2 ml.

Appendix B3

Sputum Induction Protocol (Adults and Older Children)

Recommended equipment

- Nebulizer with mouthpiece
- Vial of 3% hypertonic saline
- Oxygen saturation monitor
- Personal protective equipment for healthcare worker performing the procedure (at least an N-95 mask)
- Bronchodilator
- Universal specimen containers

Setting

A well-ventilated or open-air area distanced from others, a sputum induction booth, or an airborne infection isolation (AII) room.

Procedure

1. Sputum induction has the potential to create dangerous aerosols. Infection control methods should be followed closely at all times during the procedure.

2. Before starting,

   a. The patient should be educated on what is to occur.
   b. A baseline O^2 saturation should be measured (a pulse-ox may be kept in place during the procedure).
   c. The patient should be seated comfortably in an upright position with support, such as in a chair.

3. Once the patient is prepared for the procedure, add the 3%-10% hypertonic saline to the nebulizer and start the nebulization of the solution.

4. If the patient normally uses a bronchodilator, it can be administered via metered-dose inhaler before starting the procedure.

5. Ask the patient to breathe normally through the mouthpiece or mask with occasional deep breaths of the nebulized solution until the patient begins to cough.

6. Once the patient starts coughing, ask him/her to sit erect, cough forcefully and expectorate into the specimen cup (instruct the patient never to touch the inside of the specimen cup).

7. Continue nebulising until a quality sputum specimen (not saliva) is obtained.

8. When assessing the collected specimen, remember:

    a. A quality sputum specimen will be mucoid or mucopurulent in appearance.
    b. There should be a minimum amount of saliva in the specimen.
    c. Optimal specimen volume is 5 – 10ml (2 ml minimum).

9. If the patient has bronchospasm or becomes distressed at any point, stop the procedure and re-assess the patient prior to resuming.

10. Carefully label all specimens with the appropriate identifying information for the patient as well as the time and date of collection.

11. Transfer samples to the laboratory in a timely manner.

Appendix B4

Sputum Induction Protocol (Small children)

Recommended equipment:

- Nebulizer
- Sputum trap **(Figure 1)**
- Suction machine
- Hypertonic saline solution
- Airway (nasopharyngeal preferred) **(Figure 2)**
- Bronchodilator
- Pulse oximeter
- Personal protective equipment

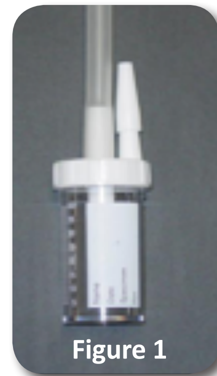


Figure 1

Figure 2

Prior to Starting the Procedure:

1. Though small children are generally not considered infectious, healthcare workers performing the procedure as well as anyone assisting should wear an N-95 mask.

2. It should be confirmed that at least 3 hours have elapsed since the child has had anything to eat or drink. This will decrease the chances of the child vomiting during the procedure.

3. All equipment, including the suction trap, should be assembled and tested prior to starting the procedure

4. The decision should be made whether the parent or medical personnel will hold the child during the procedure. The person conducting the procedure should be facing the child in the arms of the holder with full view of the nebulizer and monitoring equipment.

5. If the parent is to participate in the procedure, the entire procedure should be explained to him/her prior to starting.

6. The patient should be thoroughly assessed with baseline measurements such as respiratory rate and pulse at a minimum.

Starting the Procedure:

7. The child should be seated comfortably in the lap of the holder.

8. An appropriately dosed bronchodilator should be run in the nebulizer for several minutes before the hypertonic saline is added.

9. After the bronchodialor has been administered, add the 3% saline to the nebulizer.

10. Once the hypertonic saline is nebulising, a nasopharyngeal airway can be inserted into one of the child's nostril. This is to guide the suction catheter to the nasopharynx. (This step can be skipped and the suction catheter can be inserted directly into the nostril and guided to the nasopharynx.)

11. At this point, the person performing the procedure is listening for the child to cough and mobilize sputum to the nasopharnyx.

12. When there is an adequate yield of sputum in the oronasopharyngeal area, the catheter from the sputum trap should be inserted (either alone or through a nasopharyngeal airway) into the area and the vacuum should be applied.

13. At all times during the procedure, the child should be monitored for:

    a. Increased respiratory rate
    b. Increased breathing effort
    c. Nasal flaring
    d. Inappropriate chest wall movements such as intercostal and substernal retractions
    e. Profuse sweating
    f. Cyanosis
    g. Vomiting

14. Suction of sputum from the oronasopharynx should continue until at least 2 ml has been collected if possible. If the child shows signs of distress and the procedure needs to be discontinued, send whatever sample was collected to the laboratory for analysis.

15. The sample should be sealed in the collection trap for transfer to the laboratory.

16. All specimens should be labeled with the patient's name as well as the date, and time collected.

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Produced by Heartland National TB Center
Consultation to healthcare providers at 1-800-TEX-LUNG
www.heartlandntbc.org

This publication was supported by the Grant or Cooperative Agreement Number NU52PS910161-01 funded by the Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention or the Department of Health and Human Services.