Laboratory Diagnosis of *Mycobacterium tuberculosis* Infection and Disease in Children

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Diagnosis of tuberculosis in children is challenging; even with advanced technologies, the diagnosis is often difficult to confirm microbiologically in part due to the paucibacillary nature of the disease. Clinical diagnosis lacks standardization, and traditional and molecular microbiologic methods lack sensitivity, particularly in children. Immunodiagnostic tests may improve sensitivity, but these tests cannot distinguish tuberculosis disease from latent infection and some lack specificity. While molecular tools like Xpert MTB/RIF have advanced our ability to detect *Mycobacterium tuberculosis* and to determine antimicrobial resistance, decades old technologies remain the standard in most locales. Today, the battle against this ancient disease still poses one of the primary diagnostic challenges in pediatric laboratory medicine.

Mycobacterium tuberculosis is a nonmotile, non-spore-forming, obligate aerobe, acid-fast bacillus that often appears beaded or unstained using Gram stain. Like all mycobacteria, it is distinguished by its ability to form stable mycolate complexes with arylmethane dyes (carbolfuchsin, auramine, and rhodamine). In 98% of cases, *M. tuberculosis* is transmitted through the air when a person with pulmonary disease coughs (1). Once the infected droplet nuclei are inhaled, *M. tuberculosis* bacilli land in the alveoli where they are consumed by alveolar macrophages. In some individuals, the immune system is able to clear the infection without treatment. In others, *M. tuberculosis* subverts the alveolar macrophages' attempts at its degradation and instead replicates inside the macrophages for several weeks (1). As the bacilli multiply, they are frequently carried into regional lymph nodes by alveolar macrophages and can spread hematogenously to other sites, including but not limited to the lung apices, vertebrae, peritoneum, meninges, liver, spleen, lymph nodes, and genitourinary tract. Most patients are asymptomatic during this time and usually have no radiologic evidence of disease, but around this time, they develop cell-mediated immunity, and tests of tuberculosis (TB) infection—the tuberculin skin test and the interferon gamma (IFN-γ) release assays (IGRAs)—become positive. In the majority of individuals, the pathogenesis ceases at this point, and the person remains asymptomatic and is said to have tuberculosis infection (1).

However, in some individuals, tuberculosis infection progresses to tuberculosis disease. While healthy adults infected with *M. tuberculosis* have a 5% to 10% chance of developing TB disease within their lifetime, and the majority who do so develop disease within the first 1 to 2 years after infection, infants and toddlers who are infected but untreated have a 40% to 50% chance of developing disease within 6 to 9 months; beyond these early years, the rate of progression to disease decreases significantly with increasing age (2). Any condition or treatment that depresses cell-mediated immunity (such as HIV infection, diabetes mellitus, poor nutritional status, or tumor-necrosis factor alpha inhibitors) increases the risk of progression from infection to disease in adults and children.

In young children, the organisms tend to spread from the original lung focus to the regional hilar and mediastinal lymph nodes, which then enlarge if inflammation is intense. The lymph nodes can compress or erode into the bronchi, which frequently results in a distal atelectasis or parenchymal infection, causing the so-called "collapse-consolidation" lung lesion. However, the hallmark of childhood TB is intrathoracic lymphadenopathy with or without subsequent parenchymal disease. The number of organisms involved in this process tends to be small; hence, childhood TB is often called paucibacillary. As a result, finding direct evidence of the organism in body fluids and tissues is difficult, and in most case series, fewer than 40% of childhood TB cases can be microbiologically confirmed (3–5). In the other ≥60% of cases, the diagnosis is made by the analysis of signs and symptoms, radiography, tests of infection, and epidemiology—knowing that the child has been exposed recently to a case of contagious tuberculosis. However, adolescents with pulmonary disease often have the hallmarks of adult-type disease (cavitary lung lesions or extensive infiltrates) with large numbers of organisms that can be detected by various means.

IMMUNODIAGNOSTIC TESTS OF TB INFECTION

Determining if a patient has immunologic evidence of TB infection, "germs in the body," also contributes to the diagnosis of tuberculosis disease, especially in those cases when organism cannot be detected directly. Two tests are available to determine if an individual is infected with *M. tuberculosis*: the tuberculin skin test (TST) and the IFN-γ release assays (IGRAs) (Table 1). These test results are interpreted the same for children as they are for adults. The two types of tests produce continuous results, but the tests are interpreted in a binary fashion with cutoff values used to interpret results as positive or negative (6). The definitive TST uses 5 tuberculin units of purified protein derivative (PPD) stabilized in Tween 80. A 26- or 27-gauge needle and a graduated syringe are

TABLE 1 Performance of immunodiagnostic assays for diagnosis of TB infection in children ([6])

Characteristic	TST	IGRA
Antigens used	Many; PPD[a]	3 (QFT) or 2 (T-SPOT)
Sample	Intradermal injection	Blood draw
Patient visits required	2	1
Distinguish between LTBI[b] and TB disease	No	No
Cross-reactivity with BCG	Yes	No
Cross-reactivity with NTM	Yes	Only rare species[c]
Differing positive values by risk (mm)	Yes (5/10/15)	No
Estimated specificity in BCG-unvaccinated children, %	95–100	90–95
Estimated specificity in BCG-vaccinated children, %	49–65	89–100
Estimated sensitivity (confirmed TB disease), %	75–85	80–85
Estimated sensitivity (clinical TB disease), %	50–70	60–80

[a] PPD, purified protein derivative.
[b] LTBI, latent tuberculosis infection.
[c] *M. marinum*, *M. kansasii*, *M. szulgai*, and *M. flavescens*.

used to inject 0.1 ml of PPD intradermally into the volar surface of the forearm; the immediate appearance of a wheal indicates correct technique. A delayed hypersensitivity reaction to TST usually peaks at 48 to 72 h. In some individuals, reaction occurs after 72 h and should be measured at that time. The diameter of induration, not erythema, is measured perpendicular to the axis of administration and is recorded in millimeters. TST results should not be recorded as simply positive or negative. Of note, it may take up to 10 weeks after infection occurs for an individual to react to the TST.

A nonreactive TST result does not exclude *M. tuberculosis* infection or disease, as a variety of factors can lower tuberculin reactivity. Approximately 20% of immunocompetent children with culture-confirmed TB disease do not react initially to the TST; the rate is even higher in individuals that are significantly immunocompromised as a result of disease or medication. Improper storage, dilution, placement, and interpretation of the TST can cause false-negative results.

The most significant causes of false-positive TST reactions are recent nontuberculous mycobacterial (NTM) infection and prior BCG vaccination. NTM infection, which occurs more frequently near the equator, usually causes a cross-reaction of ≤10 mm (but can be larger); cross-reactivity can last for several months. In studies of BCG-vaccinated newborns, only 50% have a positive TST result, and 80% to 90% lose such reactivity within 5 years ([7]). Older children or adults have higher initial and longer responses to BCG, but most lose tuberculin reactivity within 10 years of vaccination. The degree of reactivity is also affected by BCG product and nutritional status. Of note, countries that use the BCG vaccine frequently have high rates of TB endemicity, and studies have demonstrated that a positive TST in a previously BCG-vaccinated child who is in close contact with an active TB case likely indicates *M. tuberculosis* infection.

Three different cutoff values are used to interpret TST reactivity. These cutoff values represent a statistical attempt to minimize false-positive or false-negative readings and vary according to individual and epidemiologic factors, of which recent exposure to *M. tuberculosis* is the most heavily weighted. For children at high-est risk of infection progressing to disease, an induration diameter of ≥5 mm is classified as a positive result. For other high-risk groups, an induration diameter of ≥10 mm is a positive result. For low-risk children, an induration diameter of ≥15 mm is a positive result ([8]).

There are two commercially available IGRAs, Quanti FERON-TB Gold (QFT; Cellestis/Qiagen, Carnegie, Australia) and T-SPOT.TB (T-SPOT; Oxford Immunotec, Abingdon, United Kingdom). In terms of performance, neither test is preferred over the other. IGRAs measure IFN-γ secreted by the patient's T-lymphocytes (QFT) or the number of IFN-γ-secreting lymphocytes (T-SPOT) upon *ex vivo* stimulation with *M. tuberculosis*-specific antigens that are not found in BCG vaccine strains or most NTM species (except *Mycobacterium marinum*, *Mycobacterium kansasii*, *Mycobacterium szulgai*, and *Mycobacterium flavescens*). The two IGRAs utilize positive and negative controls; if either control fails, the result is deemed indeterminate (QFT) or invalid (T-SPOT). For T-SPOT only, an invalid result is classified as borderline. Unlike the TST, each IGRA has only one cutoff value regardless of the patient's exposure history or immune status. However, some experts have questioned this lack of risk stratification and suggest a need for further refinement of IGRA cutoff values.

Pediatric studies have demonstrated that IGRAs have higher specificities than that of the TST for tuberculosis infection, particularly in settings of low tuberculosis burden and among BCG-vaccinated children (Table 1). One meta-analysis estimated a specificity of 85% to 95% for IGRAs in BCG-vaccinated individuals, compared to 45% to 60% for the TST. IGRAs and the TST have comparable sensitivities in immunocompetent individuals ([9]). However, like the TST, IGRAs have poor sensitivity among immunocompromised hosts and children with severe tuberculosis disease and cannot differentiate TB infection from disease. A lack of data on IGRA performance in children of <5 years of age has led to hesitancy to use these assays in this age group ([6], [8]). In contrast, the TST is routinely used in children as young as 4 to 6 months of age.

SPECIMEN SELECTION, COLLECTION, AND TRANSPORT

One of the most important parameters affecting the performance of a microbiological diagnostic test is the quality of the specimen. Clinicians have tried to collect a broad variety of specimen types to improve the microbiological diagnosis of TB in children (Table 2). The classic specimen is the gastric aspirate (GA); fasting, early morning specimens are recommended in order to obtain sputum swallowed during sleep. Samples of 5 to 10 ml are collected on 3 consecutive days, and if not processed within 4 h of collection, they should be adjusted to neutral pH with sodium carbonate since long-term exposure to acid can be detrimental to mycobacteria ([10]). However, one recent study reported that culture yield of nonneutralized specimens was, in fact, superior to neutralized specimens ([11]). Additionally, many recent studies have demonstrated that sputum can be induced from children as young as 1 month of age and that the microbiologic yield from one well-collected induced sputum (IS) is similar to that from 3 gastric aspirates ([4]). The induction procedure may not require hospitalization, but precautions should be in place to reduce the risk of specimen aerosolization during collection. Cerebrospinal fluid (CSF) is collected in cases of suspected tuberculous meningitis, congenital or neonatal TB, and in infants with disseminated dis-

TABLE 2 Specimens collected in children for the diagnosis of TB

Specimen type	Description
Gastric aspirate/lavage	Specimen of choice in young children unable to produce sputum. Fasting, early morning specimens are recommended in order to obtain sputum swallowed during sleep (10). Has been performed on inpatients and outpatients.
Sputum (expectorated)	Collected in older cooperative children that can produce sputum.
Sputum (induced)	Collected in children as young as 1 mo of age by nebulization with hypertonic saline followed by nasopharyngeal suction (4). Has been performed on inpatients and outpatients. Caution should be used due to risk of aerosolization.
Fine-needle aspirate	In cases manifesting with peripheral tuberculous lymphadenopathy, culture of fine-needle aspirate can augment testing of other specimen types (13).
Cerebrospinal fluid	Collected in cases of suspected congenital or neonatal TB and in infants with disseminated disease.
Stool	Culture of stool may yield *M. tuberculosis* since young children swallow their sputum. However, the method is fairly insensitive (14). The need for stringent decontamination procedures to prevent overgrowth of normal bowel flora may also kill or inhibit growth of mycobacteria.
Blood	The yield of *M. tuberculosis* from culture of blood is low, even among ill, HIV-infected pediatric patients from an area of high *M. tuberculosis* endemicity.
Bone marrow	Not routinely recommended. May be of use for confirmation of disseminated disease, establishing an alternative diagnosis, or ruling out underlying malignancy.

ease. The yield of *M. tuberculosis* from culture using blood or bone marrow specimens is low but may be used for confirmation of disseminated disease, establishing an alternative diagnosis or ruling out underlying malignancy.

Specimens need to be representative of the site of infection, collected aseptically, and stored and transported rapidly to the laboratory to minimize multiplication of contaminating organisms. Ideally, specimens should arrive in the laboratory on the day of collection. If transport to the laboratory is delayed by >1 h, specimens should be refrigerated at 4°C as well as upon arrival in the laboratory until they are processed. One study in adults showed that mycobacterial load and culture time to positivity were not significantly affected by refrigerated storage for ≤3 days (12). If prolonged storage or transport is unavoidable, preservatives can be added to the specimens to inhibit growth of contaminant bacteria and thus improve the yield from culture. Examples of these preservatives include sodium carbonate, cetylpyridinium chloride, and sodium borate. There are concerns that some of these compounds may not be compatible with some of the newer liquid-based culture systems, such as the Bactec mycobacterial growth indicator tube (MGIT) system (Becton Dickinson Diagnostic Systems, Sparks, MD), and they may also reduce the sensitivity of microscopy. Fine-needle aspirates can be submitted in a culture medium (Middlebrook 7H9, glycerol, and Tween), which allows them to be stored for ≤7 days prior to inoculation with no significant reduction in culture yield (13).

CULTURE DETECTION METHODS

Culture is the World Health Organization (WHO)-recommended gold standard for the diagnosis of TB disease. Organism isolation is not only important for definitive diagnosis but also for determining phenotypic drug susceptibility testing (DST). However, the sensitivity of *M. tuberculosis* detection by culture isolation for children thought to have clinical disease is much lower than that for adults due to the paucibacillary nature of pediatric disease. The limited sensitivity of culture as well as the rapid progression to disease in children necessitates that the decision to initiate treatment for TB is usually made prior to microbiological confirmation. Bacteriologic confirmation of childhood TB disease typically occurs in <40% of cases (3–5). However, in areas where TB is highly endemic or in infants, culture positivity rates can be as high

as 70%. The sensitivity of smear microscopy for detection of childhood *M. tuberculosis* is quite low. The rate of positivity of direct smear from either GA or IS specimens is <20% in children with probable tuberculosis; importantly, GA specimens are more frequently positive than IS specimens (14, 15).

The process of digestion, decontamination, and concentration of pediatric specimens prior to culture set up is typically performed as that for specimens from adults (10). Due to the paucibacillary nature of specimens from children, it is possible that decontamination conditions that are too stringent may easily render the small concentration of organisms present nonviable for culture (16). Mycobacterial culture can be performed on either a solid or a liquid medium. The yield of *M. tuberculosis* isolated from a liquid medium (e.g., Middlebrook 7H9) is greater than that from a solid egg-based medium (e.g., Lowenstein-Jensen [LJ]) or a solid agar-based medium (e.g., Middlebrook 7H11) (17). Automated liquid culture systems, such as the Bactec MGIT system (BD) or the BacT/Alert (bioMérieux), provide continuous monitoring for mycobacterial growth and, in adult and pediatric studies, significantly improve the recovery of *M. tuberculosis* as well as reduce the time to detection compared to a solid medium culture (18, 19). Specimen type influences the sensitivity of culture; culture yield from GA is generally greater than that for other specimen types, such as induced sputum (IS), nasopharyngeal aspirates, bronchoalveolar lavage (BAL) specimens, and stool samples (20–22), except for one study comparing the yield of *M. tuberculosis* from repeated IS and GA specimens over 3 days from children who were <5 years of age from an area with a high rate of HIV and TB (4). The sensitivity of GA culture is often higher in children with advanced disease (15, 23) and in those who are <1 year of age (24). When paired GA and sputum specimens were compared in 191 culture-confirmed cases of tuberculosis, the yield of a single IS was similar to that of a single GA (38% versus 42%, respectively, of culture-confirmed cases), and the combined yield of same day IS and GA was equivalent to two consecutive GA specimens (67% versus 66%, respectively, of culture-confirmed cases) (22). In an area with a low prevalence of tuberculosis, the increase in diagnostic yields of the 2nd and 3rd day gastric aspirates were 25% and 8%, respectively (25). Once growth from a pediatric specimen is detected in culture, the procedures for identification of *M. tuberculosis* are identical to those used for adult samples.

TABLE 3 Assays that detect *M. tuberculosis* and/or drug resistance

Characteristic	Test (reference)						
	Direct microscopy (14, 15)	Culture (5)	AMTD (28, 29)	Xpert MTB/RIF (30, 33)	MODS assay (20, 26)	Proportion method (36)	LiPA (42)
Sample type	Any body fluid or tissue	Any body fluid or tissue	Various[a]	Sputum	Any body fluid or tissue	Any body fluid or tissue	Sputum
Time to result	<4 h	<6 weeks	<4 h	<2 h	7–14 days	2–4 weeks	1–2 days
Simultaneous DST	No	No	No	Yes	Yes	No	No
Phenotypic/genotypic	Phenotypic	Phenotypic	Genotypic	Genotypic	Phenotypic	Phenotypic	Genotypic
Sensitivity for TB disease in children, %	<20	<40	96–100 compared to culture[b]	62 (pooled) (95% CI, 44–80) compared to culture	87–91 compared to culture	NA[c]	NA
Specificity for TB disease in children, %	Variable	>99	85–100 compared to culture[b]	99 (pooled) (95% CI, 97–99) compared to culture	>99	NA	NA
Sensitivity for INH[d] resistance, %	NA	NA	NA	NA	82–100	Reference standard	57–100
Specificity for INH resistance, %	NA	NA	NA	NA	95–100	Reference standard	99
Sensitivity for RIF resistance, %	NA	NA	NA	95	95–100	Reference standard	97
Specificity for RIF resistance, %	NA	NA	NA	98	95–100	Reference standard	99

[a] Specimens included gastric aspirate, sputum, bronchoalveolar lavage, lymph node, and sterile body fluids.
[b] Data limited to two studies (28, 29).
[c] NA, not applicable.
[d] INH, isoniazid.

An alternative to conventional liquid and solid media culture available outside of the United States (not currently licensed) is the microscopic observation drug susceptibility (MODS) assay. This system involves direct inoculation of a decontaminated sample into the wells of a tissue culture plate containing a liquid growth medium. Some wells include rifampin (RIF) and isoniazid at critical concentrations, allowing simultaneous detection of drug resistance. Growth is determined by visual inspection using an inverted microscope to detect the presence of mycobacteria in cords. Performance of the MODS assay in children with a clinical diagnosis of pulmonary tuberculosis has shown a substantial improvement in microbiologic confirmation of *M. tuberculosis* compared to Lowenstein-Jensen (LJ) culture, and the median time to detection (7 to 14 days) was one-third of that of culture on a solid medium (20, 26) (Table 3). In children with clinically diagnosed TB meningitis, the yield from CSF using the MODS assay was equivalent to that of LJ culture (27).

MOLECULAR DETECTION METHODS

In 1995, the amplified mycobacterium direct test (AMTD) (Hologic, San Diego, CA) was the first nucleic acid-based amplification test (NAAT) to be cleared by the FDA for the detection and identification of *M. tuberculosis* from direct specimens. This assay utilizes transcription-mediated amplification of a portion of the 16S rRNA gene specific to the *M. tuberculosis* complex to identify the organism. The FDA-cleared sample types include smear-positive and smear-negative respiratory specimens from individuals suspected of having TB. Very little is published regarding pediatric-specific performance of the AMTD assay. In one study, 50 children were defined as having TB disease with 43 of these 50 having pulmonary TB; disease was defined as either culture positive or meeting specific clinical criteria. AMTD was positive in all culture-confirmed cases and was positive in an additional 13 of the culture-negative, clinically defined cases of pulmonary tuberculosis (28). The resulting sensitivity (100%) and specificity (85%)

compared to culture was similar to results reported in adult studies. In another small study, 30 of 50 children from families with a positive history of TB had culture-positive sputum samples. Of these, 29 were positive by AMTD (sensitivity, 96.7%) (29). When diagnostic test accuracy was assessed considering clinical diagnosis of TB as the reference standard, AMTD had 58% sensitivity and 96% specificity. The primary advantages of the AMTD are the increase in sensitivity of detection relative to smear microscopy and the rapid time to result compared to culture (<4 h compared to weeks). Although studies are extremely limited, analytical performance of this assay in pediatric specimens may be comparable to adult specimens.

The most recently FDA-cleared NAAT, Xpert MTB/RIF (Cepheid, Sunnyvale, CA), has also been recommended as the initial diagnostic test for children suspected of having multidrug-resistant TB (MDR-TB) or HIV-associated TB in a 2013 WHO policy update (30). This is the only fully automated *M. tuberculosis* NAAT. The GeneXpert platform is a self-contained cartridge-based system that utilizes microfluidics and automated nucleic acid extraction, amplification, and detection to provide detection and identification of *M. tuberculosis* directly from clinical samples in <2 h. The test procedure may be performed on either fresh sputum samples or on sputum sediments, which are obtained after decontaminating and concentrating the sputum. The Xpert MTB/RIF assay uses molecular beacons to detect *M. tuberculosis* sequences amplified in a hemi-nested real-time PCR assay. The assay is FDA cleared for use on smear-positive and smear-negative respiratory specimens, but there is no specific clearance for extrapulmonary specimens. In addition to the detection and identification of *M. tuberculosis*, the Xpert MTB/RIF assay detects rifampin resistance through detection of defined mutations within the core region of the RNA polymerase b (*rpoB*) gene.

Initial analytical studies showed that the limit of detection of the Xpert MTB/RIF assay was 131 CFU/ml of sputum (31). In adults with pulmonary TB, the MTB/RIF assay has been well characterized; for

smear-positive, culture-positive TB, the Xpert MTB/RIF pooled sensitivity was 98% with a pooled specificity of 99%. For smear-negative, culture-positive TB, the sensitivity was 68% (40). Importantly, as is true for all NAAT-based detection, the sensitivity of the MTB/RIF assay is less than that of liquid-based culture.

Multiple studies have been done utilizing the Xpert MTB/RIF in pediatric patients. In its 2013 policy update, the WHO reported a pooled sensitivity of 66% for Xpert compared to culture with a pooled specificity of 98% (30). A second comprehensive meta-analysis through December of 2014 reported a pooled sensitivity of 62% (95% confidence interval [CI], 44% to 80%) for Xpert compared to culture of sputum specimens and a pooled specificity of 99% (95% CI, 97% to 99%) (Table 3) (33). The same analysis reported that the odds of a positive Xpert result were four times greater in smear-positive pediatric samples than in smear-negative samples. Overall in this analysis, Xpert was 36% and 44% more sensitive than direct smear microscopy for sputum samples and GA, respectively.

Due to the difficulty in obtaining sputum samples from children, the need for Xpert performance data on additional specimens is high. Meta-analysis from 16 different studies showed that GA and sputum specimens (expectorated and induced) performed comparably, with pooled sensitivities compared with culture of 66% and 62%, respectively (33). Unfortunately, due to the paucibacillary nature of *M. tuberculosis* disease in children, the rate of culture-negative disease is high with <40% of clinically suspected cases resulting in culture positivity (3–5). As such, it is important to note that the clinical sensitivity of Xpert MTB/RIF is quite low. Compared with clinically diagnosed TB as the reference standard, the sensitivity of Xpert MTB/RIF in culture-negative samples from pediatric patients was 4% for samples of expectorated or induced sputum samples and 15% for gastric aspirates (30).

Despite the limitations associated with clinical sensitivity of Xpert MTB/RIF, the fact remains that the availability of the assay has changed the diagnostic capacity for detection of tuberculosis substantially. The improved sensitivity over smear microscopy and the relative sensitivity to culture, along with the rapid turnaround time and potential for detection of rifampin resistance, allow for appropriate treatment to be started weeks prior to standard culture-based methods.

DRUG SUSCEPTIBILITY TESTING

One of the most significant problems regarding *M. tuberculosis* control worldwide is the development of drug-resistant tuberculosis. Multidrug-resistant TB (MDR-TB) is defined as resistance to at least isoniazid and rifampin, two of the four first-line antituberculosis drugs that make up the backbone of any antituberculosis regimen. Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB that is also resistant to any fluoroquinolone and at least one of three injectable second-line agents (amikacin, kanamycin, or capreomycin). Accurate diagnosis of MDR- and XDR-TB is critical for successful patient management and control of transmission of resistant organisms. The estimated global burden of TB disease in 2014 was 9.6 million incident cases, of which the estimate for pediatric disease was 1,000,000 or about 9% of the total number of incident cases (34). An estimated 3.3% of new and 20% of previously treated TB cases were MDR-TB; 9% of MDR-TB is thought to be XDR-TB. However, there are no pediatric-specific estimates for *M. tuberculosis* resistance in the WHO

report. One recent meta-analysis estimated the regional and global incidence of MDR-TB in children (35). Using mathematical modeling, the authors were able to define the relationship between the treatment of naive adult MDR-TB and the proportion of child cases with MDR-TB. This study reported that an estimate of 3.1% of incident cases in children in 2010 would be MDR-TB.

There are two different approaches to determining drug susceptibility for *M. tuberculosis*, phenotypic methods and genotypic methods (Table 3). Phenotypic methods assess the inhibition of *M. tuberculosis* growth in the presence of antibiotics and define resistance based on the response of the organism when exposed to the drug. Genotypic methods are based on the detection of genes or mutations known to be associated with resistance. Conventional drug susceptibility testing (DST) utilizes phenotypic methods and depends on a variety of factors, the first of which is the definitive microbiologic diagnosis of *M. tuberculosis* with the isolation of the organism (36). Although slow, phenotypic methods provide the complete susceptibility profile of *M. tuberculosis*. The WHO and CDC consider the indirect proportion method to be the gold standard for defining resistance. This method requires weeks to complete due to the slow growing nature of *M. tuberculosis*. Growth of the organism in question on control medium is compared to growth on drug-containing media to determine susceptibility or resistance. By definition, if more than 1% of the organisms in a population are resistant to a drug, the strain is considered resistant. The lowest concentration of the drug that inhibits growth of 95% of wild-type strains that have not been exposed to the drug but does not inhibit strains considered resistant from patients not responding to therapy is called the critical concentration and varies depending on the drug being tested; this is the concentration of drug used in the comparator during the proportion method (37, 38). As is the case with *M. tuberculosis* organism detection, automated liquid culture systems reduce the turnaround time for detection of *M. tuberculosis* resistance. However, the time to result is still quite slow, taking 10 to 14 days.

Genotypic methods detect changes in the *M. tuberculosis* chromosome that have been shown to be associated with resistance. The most widely studied and least complex to perform genotypic method is the Xpert MTB/RIF assay. The assay detects rifampin (RIF) resistance, and results are available in hours as opposed to weeks or months. RIF resistance is frequently used as a predictor of MDR-TB; although the positive predictive value varies based on the prevalence of those organisms. In many settings, RIF resistance is associated with resistance to isoniazid and is, therefore, a reliable means of detecting MDR-TB. The Xpert MTB/RIF assay has been endorsed by the WHO (39), is FDA cleared, and can be used on direct respiratory specimens or concentrated sputum sediments. In adult studies, the Xpert MTB/RIF pooled sensitivity and specificity for detection of rifampin resistance are 95% and 98%, respectively (32). False-positive results may occur due to the detection of *rpoB* mutations that do not affect phenotypic susceptibility. Due to the possibility of false-positive results in settings with low prevalence of RIF resistance, confirmation of the result using phenotypic DST is advised (41).

The second type of genotypic test that is endorsed by the WHO for detection of resistance is the line probe assay (LiPA). There are two commercially available LiPAs, the INNO-LiPA Rif TB (Innogenetics NV) and the GenoType assays, which have multiple iterations including the WHO-endorsed GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) and, more recently, the

GenoType MTBDR*sl* (Hain Lifescience), which is not currently WHO endorsed. LiPA technology involves three steps: the extraction of nucleic acid from smear positive direct specimens or culture isolates, the amplification of target sequences, and finally hybridization of labeled PCR product with probes immobilized on a strip. Results appear as colored lines on the strip where the target sequence has bound. Although commercially available and widely used worldwide, LiPAs are not FDA cleared. The reported pooled sensitivity and specificity for detection of rifampin resistance are 97% and 99%, respectively (42). Importantly, the GenoType MTBDR*sl* is the only commercially available molecular assay capable of detecting resistance to fluoroquinolones and second-line injectable drugs.

A notable drawback to the aforementioned assays is that they are limited to detecting specific mutations in various genes, including *rpoB*, *katG*, and *inhA*, and cannot identify novel or less common mutations conferring resistance. Sequence-based technologies have the potential to overcome these limitations; while no sequence-based assays are commercially available today, as technologies improve, this approach will likely offer improvement in resistance detection particularly for second-line agents.

SUMMARY

Diagnosis of childhood tuberculosis remains a significant challenge even with the availability of advanced molecular technologies. The primary hurdle is the paucibacillary nature of pediatric disease. In comparison to adults, microbiologic detection of *M. tuberculosis* in pediatric patients lacks sensitivity regardless of the technology utilized. Importantly, however, the introduction of Xpert MTB/RIF has improved the sensitivity of direct detection compared to smear microscopy; overall, Xpert was as much as 40% more sensitive than direct smear microscopy. As such, WHO recommendations advise replacement of smear microscopy with this rapid molecular assay as the initial diagnostic test. Despite the increase in sensitivity relative to microscopy, there are significant barriers to implementing this assay in U.S. laboratories for this purpose, primarily the lack of FDA clearance of the most common and most appropriate pediatric specimen, the gastric aspirate.

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