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New specimens and laboratory diagnostics for childhood pulmonary TB: progress and prospects

Mark P Nicol[Professor]
Division of Medical Microbiology, Department of Clinical Laboratory Sciences, Institute for Infectious Diseases and Molecular Medicine, University of Cape Town and National Health Laboratory Service of South Africa IIDMM, Faculty of Health Sciences, Anzio Road, Observatory, Cape Town, 7925

Heather J Zar[Professor]
Department of Paediatrics and Child Health, University of Cape Town and Red Cross War Memorial Children's Hospital, Cape Town, South Africa 5th floor ICH Building, Klipfontein Road, Rondebosch, Cape Town, 8001 Heather.Zar@uct.ac.za

Summary

Childhood pulmonary TB (PTB) is under diagnosed, in part due to difficulties in obtaining microbiological confirmation. However, given the poor specificity of clinical diagnosis, microbiological confirmation and drug susceptibility testing is important in guiding appropriate therapy especially in the context of drug resistant TB. Confirmation is often possible, even in infants and young children, if adequate specimens are collected. Culture yield varies with the severity of illness, specimen type and culture method. Induced sputum is recognised as a safe procedure with a high diagnostic yield. Advances include optimised protocols for smear microscopy and modified culture techniques, such as the Microscopic Observation Drug Susceptibility Assay. Detection of *Mycobacterium tuberculosis* nucleic acid in respiratory specimens has high specificity but relatively poor sensitivity, particularly for smear negative disease. The recent development of an integrated specimen processing and real-time PCR testing platform for *M. tuberculosis* and rifampicin resistance is an important advance that requires evaluation in childhood TB.

Keywords

induced sputum; culture; nucleic acid amplification; pulmonary tuberculosis; child

Introduction

Globally, under diagnosis of childhood pulmonary tuberculosis (PTB) remains an obstacle to effective management. TB control programmes still focus predominantly on the diagnosis and cure of cases of smear-positive adult TB, as these are the major drivers of TB transmission. Whilst adult TB cases are often easily recognizable, due to typical radiological

corresponding author Tel: +27 21 406 6083 Fax: +27 21 406 6796 Mark.Nicol@uct.ac.za.

features and a positive sputum smear, childhood TB is frequently more difficult to diagnose. The clinical and radiological features of childhood TB are often non-specific and subject to variable interpretation[1]. Structured diagnostic scoring systems based on clinical and radiological findings and tuberculin skin testing (TST) show high variability in case yield and very poor agreement[2].

The diagnosis is even more problematic in HIV-infected children, since clinical and radiological features overlap with other infections and anergy to the TST is common[3]. Clinical scoring systems have not been adequately evaluated in HIV-infected children, but it is likely that their performance will be even poorer in this patient population.

Microbiologic confirmation of PTB is still rarely attempted in children, especially in primary care settings, in contrast to adults where this is the accepted standard of care. This is due to the incorrect perception that respiratory specimens are difficult or impossible to obtain in children, the lack of infrastructure or trained staff to obtain such specimens and the lack of policy regarding microbiologic confirmation in children. However, even when samples can be obtained, since disease is typically paucibacillary, the yield of direct acid-fast smear microscopy is very low[4] and prolonged mycobacterial culture is required. As a result, microbiological confirmation may be delayed by weeks. This has important implications for a disease that may progress rapidly in young children, with associated morbidity and mortality. Extra-pulmonary TB is common in young children and poses particular challenges for specimen collection and culture.

The major advantages of obtaining microbiological confirmation are the ability to make a definitive diagnosis and to perform drug susceptibility testing to exclude drug-resistant TB. In the era of increasing multidrug resistant (MDR) and extensively drug resistant (XDR) TB this information becomes critical in order to guide appropriate therapy.

However, mycobacterial culture is frequently negative in children with clinically diagnosed PTB, particularly amongst less ill patients in a primary care setting[5]. This may represent the poor specificity of clinical diagnosis or alternatively, the impaired sensitivity of culture for childhood TB. This presents a fundamental problem in assessing the performance of any novel diagnostic test or clinical algorithm as there is no reference standard which is both highly sensitive and specific to which results can be compared. A further implication is that clinicians are often wary to discontinue TB therapy when a negative culture result is obtained.

This review will address the progress made in recent years in obtaining laboratory confirmation of PTB in children. The diagnosis of latent TB infection and the use of serological tests and interferon-gamma release assays are considered elsewhere in this edition.

Obtaining representative specimens from the lower respiratory tract of children

Microbiological confirmation of TB in young children is not routinely attempted in many high burden settings due to the difficulty in obtaining samples and the poor performance of smear microscopy. However, if facilities for mycobacterial culture and drug susceptibility testing are available, such confirmation is invaluable.

Since young children are frequently unable to expectorate, additional procedures are often required to obtain samples from the lower respiratory tract. For many years the collection of three consecutive early morning gastric lavage (GL) or gastric aspirate (GA) samples has

been the accepted method for attempting microbiological confirmation. However GL is unpleasant, relatively invasive, requires trained staff and hospitalization for an overnight fast, although it may be performed in an outpatient setting[6]. This procedure is not feasible in many high burden countries; moreover the yield for *M. tuberculosis* has been disappointingly low. More recently, a number of less invasive alternative methods have been proposed, including induced sputum (IS), nasopharyngeal aspiration (NPA) and the string test. [Table 1].

Sputum induction

Sputum induction does not require overnight hospitalization and can be performed in an out-patient setting. The technique involves administration of an inhaled bronchodilator followed by nebulised hypertonic (3%–5%) saline and then nasopharyngeal aspiration or expectoration of mucus from the lower respiratory tract[4]. IS has been successfully used for diagnosis of childhood TB in several countries in the developing world (South Africa, Kenya, Uganda, Tanzania, India, Colombia). In an early study of 149 children (median age 9 months) hospitalised with acute pneumonia, samples were successfully obtained from 95% of enrolled patients, of whom 10% had a positive culture[7]. The yield from a single IS sample (10%) was greater than that of sequential GL samples (6%). These results were subsequently confirmed in a larger study of children with suspected PTB admitted to the same paediatric referral hospital (250 children, median age 13 months) which demonstrated a significantly higher cumulative yield for 3 IS samples (87%) compared with 3 GL samples (65%, $p=0.018$)[4,8]. The yield from one IS sample was equivalent to three GL samples[4]. This has shifted clinical practice to include induced sputum as a diagnostic procedure in young children and infants with suspected PTB.

The yield of mycobacterial culture is likely to vary with the patient population (primary care versus referral hospital) and severity of illness. Whilst positive cultures were obtained from 3 GL and 3 IS samples in 25% of children admitted to a paediatric referral hospital[4], only 10% of children admitted with suspected TB or with a TB contact in a community-based study had positive cultures (from 2 GL and 2 IS samples)[5]. In this community-based study, the yield of a single IS and GL sample were equivalent (38% and 42% of patients with a positive culture respectively), again highlighting that the severity of illness may impact on diagnostic yield. The safety of IS in infants and young children is now well established with thousands of procedures having been performed with no documented serious adverse events[4–8].

Nasopharyngeal aspiration

Nasopharyngeal aspiration is an attractive diagnostic procedure, requiring minimal facilities and training. Since passing a nasal cannula elicits a cough reflex in many children, NPA may be regarded as a form of sputum induction. Early data suggested that the culture yield from NPA (19/64; 30%) was similar to that of GA (24/64; 38%) amongst Peruvian children (mean age 5 years)[9]. This was confirmed by a small study of older children (median age 48 months) in Uganda[10], where the yield of mycobacterial culture from a single NPA sample (24%) was similar to that from an IS sample (22%)[10]. In contrast, a comparison of the culture yield from stool, NPA and gastric aspirate specimens amongst 165 Peruvian children with suspected TB (median age 4.6 years) demonstrated relatively poor recovery from stool (4/292; 1.4%) and NPA samples (12/314; 3.8%) compared with GA (22/321; 6.8%) by culture[11]. A similar pattern was observed in Yemen, where one NPA and three consecutive GA and IS or expectorated sputum samples were collected from 213 children with suspected TB (median age 5 years)[12]. Solid mycobacterial culture was positive in 7% of NPA samples, 9% of gastric aspirates, 8% of expectorated sputum samples and 14% of induced sputum samples. The total yield from culture was 13.6% and that of acid fast staining 8.5%.

Differences in the relative yield of NPA and GA in different studies may relate to the patient population, to specimen collection techniques and to methods for culturing or identifying *M. tuberculosis*.

Stool

Since young children tend to swallow rather than expectorate sputum, mycobacterial culture of stool may be expected to be useful. However, the major drawback of stool culture is the need for stringent decontamination procedures to prevent overgrowth of normal bowel flora. Such procedures are likely to kill or inhibit growth of most mycobacteria in the sample as well. It is therefore unsurprising that stool culture has proven insensitive[11].

String test

The string test was first evaluated as a sampling method for TB in HIV-infected adults with suspected TB. Patients were asked to swallow a gelatine capsule containing a coiled nylon string which unravelled as the capsule descended to the stomach. After 4 hours the string was withdrawn and used for mycobacterial culture. The culture yield from string test samples (9%) was better than that from IS (5%) in this adult population[13]. The test was subsequently proposed for use in children and shown to be well tolerated by older children (median age 8 years)[14]. A preliminary study demonstrated that the time the string remained in the stomach could be reduced to 1 hour without decreasing yield[15]. It is unlikely, however, that this test will be feasible in young children (<2 years) who account for a high proportion of TB cases in some settings.

Lymph node aspiration

In children with a palpable peripheral lymph node as well as respiratory symptoms, fine needle aspiration and culture is a very useful adjunct to culture of respiratory specimens, and may have a higher yield than such culture (sensitivity of 60.8 vs. 39.2% respectively)[16]. The procedure may be performed safely on an outpatient basis by appropriately trained staff in a resource-limited setting[17].

Improvements in smear microscopy

There have been incremental advances in the performance of smear microscopy for the rapid detection of MTB. It is clear that concentration of specimens by centrifugation[18] and the use of fluorescent microscopy[19] increase the yield of smear microscopy [Table 1]. Until recently, one of the drawbacks of fluorescent microscopy has been the need for a costly, short-lived mercury vapour light source. The recent availability of low cost, long-lived light emitting diode (LED) light sources for fluorescent microscopes is likely to increase access to fluorescent microscopy in low-resource settings[20]. However, even under optimal circumstances, the sensitivity of smear microscopy for the diagnosis of childhood TB remains less than 15%[4], except in older children with adult-type disease[21].

Improvements in culture techniques

There is no doubt that liquid culture systems with continuous monitoring for mycobacterial growth (such as MB/BacT [Biomerieux, Marcy l'Etoile, France], BACTEC 9000 [Becton Dickinson, NJ, USA] and the mycobacterial growth indicator tube [MGIT; Becton Dickinson]) are a significant advance over solid culture (typically Löwenstein Jensen, LJ) [Table 1]. In adult studies, mean time to detection is substantially lower for automated liquid culture (13.2 vs. 25.8 days for MGIT vs. LJ in a recent meta-analysis)[22]. Similarly, sensitivity of MGIT culture is higher in adult studies (88% vs. 76% for MGIT vs. solid

media). It is likely that these findings can be extrapolated to children with TB; however there is a paucity of paediatric data.

The Microscopic Observation Drug Susceptibility Assay (MODS) is a potentially low-cost alternative to MGIT culture, which remains relatively expensive and typically requires costly instrumentation (although bottles can be visually inspected for growth)[23]. Decontaminated sample is directly inoculated into wells of a tissue culture plate containing liquid growth media. Some wells include rifampicin 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 typical `cording' of mycobacteria. A small study compared MODS with MGIT culture in 96 children with suspected TB[24]. When using clinical diagnosis as the reference standard, sensitivity of MODS (39.7%) was similar to MGIT (42.3%) on per patient analysis but inferior to MGIT on per sample analysis (43.8% vs. 48.5% respectively, p=0.03). Median time to detection for MODS (8 days) was shorter than MGIT (13 days). The manual inspection of plates for growth in MODS is laborious and efforts are underway to automate this process.

Despite these advances, culture remains cumbersome, requires biosafety level 3 facilities and highly trained staff and is seldom available in a clinically relevant time frame. These limitations are particularly relevant for poorly resourced, high burden regions where, due to these constraints, culture facilities are seldom widely available.

Detection of M. tuberculosis nucleic acid

Whilst detection of live MTB is likely to remain the gold-standard for diagnosis in the immediate future, nucleic acid amplification assays (NAA) for the detection of MTB DNA or RNA are finally coming of age. There are obvious attractions to NAA. These assays are theoretically highly sensitive, able to detect very low copy numbers of nucleic acid, rapid (results typically available on the same day), may not require biosafety level 3 facilities and hence may be deployed closer to the point of care and are relatively easy to automate. Detection of the most common drug resistance-causing mutations for isoniazid and rifampicin may also permit rapid detection of the majority of MDR TB cases.

Commercial nucleic acid amplification assays

NAA, both in-house as well as commercial assays, have been extensively evaluated in adult patients. Commercial assays, (such as the GenProbe Amplified M. tuberculosis Direct test [AMTD; San Diego, CA, USA], the Roche Amplicor MTB test [Basel, Switzerland], the Cobas Amplicor test [Roche, Basel, Switzerland], the Abbott LCx test [Chicago, IL, USA; discontinued] and the BD-ProbeTec SDA [Becton Dickinson, MD, USA] test), offer high specificity (85–98%), high sensitivity for smear-positive TB (pooled estimate 96%) but poorer sensitivity for smear-negative TB (pooled estimate 66%)[25]. These assays are costly and often designed for processing of samples in large batches, which may defeat the goal of more rapid diagnosis. Their performance in paediatric TB has not been thoroughly evaluated; however limited studies to date suggest that their performance in children is likely to be similar to that in smear-negative adult patients [Table 1][26].

In house nucleic acid amplification assays

`In house' NAA show substantial heterogeneity in performance in adult patients, with sensitivity estimates ranging from 9.4%–100% and specificity estimates from 5.6%–100%[27]. These assays are highly operator dependent. Performance is also influenced by the choice of target sequence and DNA extraction method. Interpretation of the performance of these assays in paediatric TB suspects is confounded by the lack of a sensitive and specific reference standard. When compared with culture, the sensitivity of NAA for the diagnosis of

childhood TB is typically low (40–83%)[28]. However, it appears, at least from some reports, that NAA identify a group of children who are clinically diagnosed with TB but in whom mycobacterial culture is negative[26,29,30]. This may reflect poor specificity of both the NAA and clinical diagnosis or alternatively poor sensitivity of mycobacterial culture.

Non-respiratory specimens for nucleic acid amplification

There have been several recent evaluations of NAA performed on non-pulmonary samples for the diagnosis of pulmonary TB. Urine is easily obtained and theoretically might contain small fragments of DNA derived from lysed TB bacilli in the lung which are filtered by the glomeruli ("transrenal DNA")[31]. Studies in adult patients show wide variation in performance (7%–100% sensitivity) of NAA for transrenal DNA[31]. There is a need to evaluate the performance of NAA for detection of transrenal mycobacterial DNA in children with pulmonary TB.

Since young children swallow their sputum, MTB or MTB DNA may be detected in stool. A small study in 16 children using a hemi-nested PCR assay demonstrated relatively poor sensitivity for detection of culture-proven cases (31% and 38% for two different DNA extraction protocols)[32]. A larger, more recent, study of adult patients, using a similar extraction and PCR protocol showed higher sensitivity (86% of sputum culture-positive cases)[33]. This again highlights the relatively poor performance of TB diagnostic tests in children compared with adults.

PCR has also been performed with limited success on blood from children with TB. In a Brazilian study[34], the sensitivity of nested PCR on blood from children with clinically or microbiologically-defined active TB was 26.2%, however the test was also positive in 7.3% of children without TB and 26.2% of children characterized as having latent TB.

The bewildering and contradictory literature around the performance of NAA is likely a reflection of differences in sampling protocols, patient population, DNA concentration and extraction method, and operator experience. Evaluations of commercial assays are show less heterogeneity, probably because these assays are more standardized. A number of important developments in NAA technology in recent years promise to reduce operator-related variability and are likely to revolutionize this field.

Real-time PCR

Firstly, the development and widespread availability of real-time PCR is a significant advance. It is now possible to detect the presence of amplified nucleic acid target in a closed system without having to resort to cumbersome gel – or ELISA-based detection. This substantially reduces the risk of cross-contamination of samples by amplified DNA from previous samples and reduces operator dependence.

A second major advance is the development of integrated DNA extraction and amplification systems, such as the GeneXpert® MTB/RIF system, developed by Cepheid (CA, USA) together with the Foundation for Innovative New Diagnostics. This system is designed for use at, or close to, the point-of-care and requires minimal manipulation of sample and operator training. The test amplifies a region of the *rpoB* gene of MTB. Mutations of this region give rise to 95% of rifampicin resistance. The test is therefore able to simultaneously detect the presence of MTB and rifampicin resistance. Preliminary studies in adult patients have been promising demonstrating a sensitivity of 100% for smear-positive patients and 71.7% for smear-negative, culture-positive patients[35]. The results of a large, multi-centre evaluation are awaited. The system is currently under evaluation for the diagnosis of TB in children. Given the relatively good performance of GeneXpert in smear-negative cases this may prove to be a useful tool for rapid diagnosis of TB in children.

Other NAA are specifically designed to detect resistance to INH and rifampicin directly in smear-positive samples. They have a limited role to play in the diagnosis of TB (particularly in children, who have high rates of smear-negative disease), but are valuable for the rapid identification of drug resistance. These tests are typically in the format of a line probe assay (e.g. GenoType MTBDR*plus*, Hain Lifesciences, Nehren, Germany). Amplified DNA from regions of the genome involved in resistance to isoniazid and rifampicin is hybridized onto a strip containing probes for the wild type (sensitive) as well as the most common drug resistance mutations. This assay is able to reliably detect rifampicin resistance (pooled estimate 98.4%) as well as most isoniazid resistance (pooled estimate 88.7%)[36]. Performance in smear-positive adult samples is excellent[37]. Limited data exist on performance in smear-negative samples and these tests have not been specifically evaluated in childhood TB.

Other tests

There are two novel diagnostic modalities which have not, to our knowledge, been evaluated in children, but which are likely to be tested in this population in the near future; the urinary lipoarabinomannan (LAM) assay and tests for volatile organic compounds in the breath. The LAM assay is an ELISA-based test for a mycobacterial glycolipid. The sensitivity of urine LAM testing for adult disease has varied widely (44%-67%) [38,39]with the higher estimates in HIV co-infected patients with advanced immunosuppression, presumably due to higher bacterial burden and increased frequency of disseminated disease[40]. The performance of this assay in TB-HIV coinfected children, in whom disseminated disease is common, will therefore be of interest.

A number of studies have identified specific patterns of volatile organic compounds produced by MTB. These compounds were first detected in the headspace of cultures of MTB[41], but have more recently also been detected in the breath of adult TB patients. A recent study has demonstrated suboptimal sensitivity (84%) and specificity (65%) for one such assay[42], however this may improve with further development. Since breath sampling is simple and non-invasive, this would be an attractive assay system for paediatric TB.

Conclusion

Advances in the diagnosis of childhood TB in the past decade have included the identification of alternative specimen types such as induced sputum and nasopharyngeal aspirate as well as improvements in smear microscopy and liquid culture systems. A number of novel and exciting candidates have been identified for diagnosis of adult TB, such as integrated real-time PCR detection systems, urine LAM and testing for volatile organic compounds in breath. There is a clear need for a large, prospective evaluation of the relative performance of new culture methods and the newer nucleic acid amplification assays in different respiratory specimens against a well-defined reference standard in children. The evaluation of new diagnostic tests in children should be prioritized.

Educational Aims

- To describe current state-of-the-art for diagnosis of pulmonary tuberculosis in children
- To discuss the advantages and limitations of various specimen types for diagnosis of pulmonary tuberculosis in children
- To discuss the role and limitations of nucleic acid amplification assays in the diagnosis of pulmonary tuberculosis in children

Practice Points

For diagnosis of pulmonary tuberculosis in children:

- Microbiological confirmation should be sought
- Sputum induction is a safe procedure with a high diagnostic yield
- Samples should be processed by chemical lysis of sputum, centrifugation, fluorescent microscopy and liquid culture
- The results of `in-house' nucleic acid amplification assays should be interpreted with caution and such assays require rigorous clinical evaluation prior to use

Research Directions

- The performance of novel integrated nucleic acid amplification systems (such as GeneXpert) for the diagnosis of pulmonary tuberculosis in children
- The utility of nasopharyngeal aspiration for the diagnosis of pulmonary tuberculosis in children

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Table 1

Summary of new specimens and laboratory diagnostic tests for pulmonary TB in children

Diagnostic test	Description	Major findings
Specimen		
Induced sputum	Samples lower respiratory tract secretions; nebulisation with hypertonic saline followed by suction or expectoration	Obtainable in most children and safe 1 IS provides same yield as 3 GLs in hospitalised children Incremental yield with a 2nd IS specimen[4]
Nasopharyngeal aspirate	Suctioning of the nasopharynx to sample upper respiratory tract secretions; stimulation of cough reflex may include lower respiratory secretions	Relatively non-invasive procedure; variable performance, but some studies suggest similar yield to culture of IS[9]
Laboratory test		
Optimised smear microscopy	Chemical processing and centrifugation Fluorescent microscopy	Increases sensitivity by 18%[18] Increases sensitivity by 10%[19]
Automated liquid culture	Liquid culture medium with continuous monitoring for bacterial growth	Higher sensitivity than solid medium and shorter time to detection[22]
MODS	Inoculation into multiple wells of liquid culture with or without INH or rifampicin – growth determined by visualisation with inverted microscope	Simultaneous detection of drug resistance May be more rapid than automated liquid culture systems[24]
Nucleic acid amplification tests	Identification of gene sequences specific to *M. tuberculosis* and specific for drug resistance	High specificity of commercial assays[25] Lower sensitivity especially in smear negative samples
Urine LAM	Detection of *M. tuberculosis* glycolipid by ELISA	Low sensitivity, higher in disseminated disease in severely immunosuppressed adults[40] Not yet evaluated in children