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ORIGINAL ARTICLE

Laboratorial diagnosis of Childhood tuberculosis

Maria de Fátima B. Pombo March[1], Rafaela Baroni Aurílio[2]

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Abstract

In 1993, tuberculosis (TB) was recognized by the World Health Organization (WHO) as a global emergency, and in 2014, an estimated one million cases of TB were reported in the pediatric age group, and approximately 10,000 cases in the Americas. Diagnosis in the pediatric age group in Brazil is still based on the Ministry of Health's score system (particularly in children younger than 10 years of age). However, new bacteriological and immunological methods have been proposed for the diagnosis of TB. The Xpert MTB- Rif system (RMT-TB) is a fast method that is used for bacteriological proof. This real-time PCR-based system allows the detection of fragments of *M. tuberculosis* in respiratory secretions, ganglia, and cerebrospinal fluid, and the identification of resistance to rifampicin (RMP). In the pediatric age group, the use of this system in routine diagnostic is still controversial because of its low sensitivity. The main immunological methods used to determine the presence of *M. tuberculosis* infection are the tuberculin skin test and the interferon-gamma release assay (IGRA). Negative results from these tests do not discard TB whereas positive results do not confirm TB. This study reviews the role of the Xpert tests, tuberculin skin test, and IGRAs in children.

[1] Associated professor of Faculty of Medicine. Universidade Federal do Rio de Janeiro (UFRJ) and Universidade Federal Fluminense.
[2] MD. Pediatric pulmonologist. Instituto de Puericultura e Pediatria Martagão Gesteira. Universidade Federal do Rio de Janeiro (IPPMG-UFRJ).

Correspondence to:
Maria de Fátima B. Pombo March.
Departamento Materno Infantil da Faculdade de Medicina da Universidade Federal Fluminense (UFF). Av. Marquês do Paraná, nº 303, Centro.
Niterói - RJ, Brasil. Pais. CEP: 24020-071. E-mail: fmarch@uol.com.br

INTRODUCTION

In 1993, the World Health Organization (WHO) recognized tuberculosis (TB) as a global emergency and established the goal of reducing the incidence of TB from 1990 until 2015[1]. (Ministry of Health, Brazil 2016). In 2014, an estimated one million cases of TB were reported in the pediatric age group, 10,250 of which (1% of all cases)[2] occurred in the Americas (Volz 2016).

Data from the WHO revealed that, of the 22 countries that account for 80% of the cases of TB worldwide, Brazil ranks number 18 for TB burden, representing 0.9% of the estimated cases worldwide and 33% of the estimated cases in the Americas. The coefficients of mortality and incidence were reduced by 38.9% (from 3.6 to 2.2 per 100,000 inhabitants) and 34.1% (from 51.8 to 34.1 per 100,000 inhabitants), respectively, from 1990 to 2014. Despite the decrease in these coefficients, an analysis of epidemiological and operational indicators of new cases and retreatments published in 2016 in the Ministry of Health Epidemiological Bulletin [Boletim Epidemiológico do Ministério da Saúde (MS)] indicated that the control of TB continues to be a challenge in Brazil (MS, Brazil 2016)[1].

Most children develop primary TB and are either non-bacillary or paucibacillary (in 80% of cases, the diagnosis is made without bacteriological confirmation). Moreover, there are limitations inherent to the age of the patients, who in general, are unable to expectorate spontaneously; therefore, sputum bacilloscopy cannot be conducted (MS, Brazil 2011)[3].

Because of these limitations, in our practice, TB diagnosis in children and adolescents continues to be performed using the point system developed in Brazil and recommended in the national norms by the National Tuberculosis Control Program since 2002 (MS, Brazil 2002) This system disregards bacteriological positivity because this practice is common in the evaluation of childhood TB in endemic areas[4,5]. The system adopted by the Brazilian MS has been validated in non-HIV-infected children and has been successfully tested in HIV-infected children. This method is auxiliary for the diagnosis of pulmonary TB (PTB) in children treated in low-complexity health facilities, ideally with trained personnel (Orfaliais 2006, Pedrozo 2009). This system is based on the fact that the diagnosis of PTB in children is performed using clinical-radiological and epidemiological criteria and the tuberculin skin test. In children and adolescents with negative bacilloscopy, the diagnosis of PTB is considered very likely in cases in which the total score is greater than or equal to 40, possible when the total score is 30 to 35 points, and unlikely when the total score is equal to or less than 25 points. In cases in which the score is less than 30 points, child follow-up should continue[3].

In this case, differential diagnosis with other lung diseases should be conducted, and complementary diagnostic methods such as gastric lavage, bronchoscopy, sputum induction, punctures, and rapid methods can be used when pertinent, depending on the availability of services (MS, Brazil 2011).

The collection of specimens for bacteriological examination in nursing and preschool children is usually only possible with the use of invasive methods such as gastric lavage and sputum induction because children are usually unable to expectorate until the age of 5 or 6 years. Because PTB is paucibacillary in children, bacteriological confirmation occurs in less than 20% of the cases in routine practice. A *Mycobacterium tuberculosis* culture may be positive in 40% to 50% of cases but the results are delayed, which makes early diagnosis and treatment of TB unfeasible in children[3,6,7]. From this age, sputum smear bacilloscopy and culture can be attempted whenever possible (MS, Brazil 2011, WHO 2014, Sant´Anna 2013). On the other hand, the diagnosis of PTB in adolescents can be confirmed bacteriologically because of the predominance of bacillary forms, which are known as the adult type.

Sputum smear bacilloscopy can be a useful method when combined with clinical-radiological findings and should be requested in all suspected cases in adolescents and, if possible, the Xpert MTB-Rif molecular diagnostic system (RMT-TB)[3,7] should be indicated because of the fast results and the possibility of identifying resistance to rifampicin (RMP) (Brazil, MS 2011, Sant´Anna 2013). In the pediatric age group (younger than 10 years of age), the use of RMT-TB as a routine diagnostic method is still controversial because of its low sensitivity, and the application of the MS clinical scoring system with RMT-TB is recommended in all cases as a complementary test whenever possible.

The immunological methods used for the confirmation of *M. tuberculosis* infection can assist in the diagnosis of TB in children and adolescents. The main methods are the tuberculin skin test and the interferon-gamma release assay (IGRA). These two tests are used in children to assess the presence of infection with *M. tuberculosis*, whereas other factors, including the history of exposure to the agent and clinical and radiological findings, are used to confirm the diagnosis. Therefore, a negative result from these tests does not exclude TB and a positive result does not confirm TB.

THE RAPID MOLECULAR TEST (RMT-TB)

The RMT-TB test is based on the amplification of nucleic acids and is used for the detection of *M. tuberculosis* and screening for drug-resistant strains. This test uses the real-time polymerase chain reaction qPCR technique on the Gene Xpert platform, which permits the integration of three processes: purification, concentration, and nucleic acid amplification by PCR, and the nuclei acid detection in the genome of *M. tuberculosis*, specifically of the rpoβ gene. This technique does not require manipulation of mycobacterial DNA[8] following amplification, and therefore reduces the complexity and the

risk of cross-reaction by the amplification of DNA products (Nicol 2013). Moreover, this laboratory-based method allows the identification of *M. tuberculosis* and bacterial resistance to rifampicin (RMP) in less than 2 hours using PCR amplification of five overlapping probes that are homologous to the 81-bp region that confers RMP resistance in the *M. tuberculosis rpoβ* gene. This region has been examined for the identification of mutations associated with resistance (Brazilian Health Technology Assessment Bulletin [Boletim Brasileiro de Avaliação de Tecnologia em Saúde], MS 2011)[3].

RMT-TB is rapid, highly automated, and machine operator-independent. The only manual step involves the addition of the correct amount of reagent to the analyzed specimen, which is then homogenized for 15 minutes and transferred to the Xpert cartridge[8].

The system consists of the GeneXpert instrument, a computer, a barcode reader, and a pre-installed software, which performs tests on the collected samples and displays the results on the screen as either negative or positive for *M. tuberculosis* and either sensitive or not to RMP. However, a positive result does not necessarily indicate the presence of viable bacilli because the method can identify DNA of both live and dead microorganisms (Standard RMT-TB operating procedure, MS 2015)[9].

Different samples can be processed, including organic liquids and peripheral ganglia aspirates. However, the test is used primarily in sputum samples from adults with suspected PTB[10]. The results of the studies differ about the sensitivity and specificity for extra-pulmonary samples, depending on the material used, and about the cost effectiveness of these samples[11].

The following samples should not be processed: those containing either saliva, food particles, pus (without mucus and greenish in color), and blood, those with less than 1 mL of pulmonary or extrapulmonary samples (with the exception of cerebrospinal fluid (CSF)), and those with less than 0.1 mL of CSF samples (standard operating procedures for RMT-TB, MS 2015)[9].

The sensitivity and specificity of a single RMT-TB test in adults are approximately 88% (CI 95%: 83-92) and 98% (CI 95%: 97-99), respectively. The sensitivity was approximately 98% (CI 95%: 97-99) and 68% (CI 95%: 59-75) in subjects with a positive and negative bacilloscopy, respectively[12].

The use of RMT-TB in children is still limited because its performance is excellent in bacteriologically-confirmed cases of TB and a few such cases have been reported in children[10]. The sensitivity of RMT-TB in a single test in children younger than 15 year of age was 42.9-90.0% for sputum and induced sputum using culture as the gold standard[11,13,14]. The sensitivity was 68.8%[13] for gastric lavage (Bates 2013) and 48%[15] for nasopharyngeal aspirate (Zar 2012). The specificity was similar to that of adults (greater than 98%). A second test in children with negative bacilloscopy might increase the sensitivity of the test to 27.8%[11].

The prospect of expanding childhood TB diagnosis by using Gene Xpert or RMT-TB was the subject of several studies from South Africa and other African countries. Greater RMT-TB positivity was observed in *M. tuberculosis* cultures in cases of PTB in specimens such as gastric lavage, nasopharyngeal aspirate, and induced sputum. Furthermore, RMT-TB improved the diagnosis of PTB in bacteriologically confirmed cases of TB[11,15].

In the meta-analysis conducted by Detjen et al.[16] with studies that used RMT-TB for the diagnosis of PTB in children and adolescents, the sensitivity and specificity of RMT-TB were 62% (CI 95%: 51-73) and 98% (CI 95%: 97-99), respectively, in sputum and induced sputum, and 62% (CI 95%: 51-73) and 98% (CI 95%: 96-99), respectively, in gastric lavage compared with culture. The sensitivity of RMT-TB was higher in these samples compared with bacilloscopy (Detjen 2015).

RMT-TB was used in a study investigating pediatric cases of suspected TB in public services in New Delhi, India, and detected twice as many cases of pulmonary TB than bacilloscopy and improved the detection of resistance to RMP, making it a rapid and promising test for use in the pediatric age group[17].

IMMUNOLOGICAL DIAGNOSIS (PURIFIED PROTEIN DERIVATIVE AND IGRAS)

The tuberculin test with purified protein derivative (PPD) performed using the Mantoux technique introduced in 1907 consists of an intradermal injection of 5 tuberculin units (TU) obtained from PPD-S or 2 TU from PPD-RT23 (these two formulations are equivalent). A delayed-type hypersensitivity reaction may occur within 48 to 72 hours in cases of cellular immunity to these tuberculin antigens.

The reaction causes induration of the skin at the injection site, and the transversal diameter of the induration should be measured (millimeters) by a trained individual and interpreted using two stratified cutpoints: 0 to 4 mm indicates absence of reactivity whereas 5 or more mm indicates reactivity.

The reactivity measured by the tuberculin test may also indicate exposure to other environmental mycobacteria, vaccination by bacillus Calmette-Guérin (BCG) *Mycobacterium bovis*, or a previous TB infection. This type of reactivity can lead to false-positive results. False-negative results may also occur, particularly in immunosuppressed patients, including those with advanced HIV infection and those using immunosuppressant medications.

The IGRA is an *in vitro* test used for detecting the production of IFN-γ in peripheral blood by the T cells of the host infected with *M. tuberculosis.* The identification of the immunogenic mycobacterial protein antigens ESAT-6, CFP-10, and TB 7, which are expressed by pathogenic strains of the *M. tuberculosis* complex, enabled the development of the IGRAs. These antigens are codified in the region of difference 1 (RD1)

of the *M. tuberculosis* genome. RD1 genes codify the bacterial protein secretion system known as ESX-1. These antigens are highly specific for *M. tuberculosis*, despite the evidence of cross-reaction with *M. leprae*, which needs to be confirmed. Positive IGRA results have also been observed in individuals infected with *M. marinum* and *M. kansaii*.

There are two types of IGRA. The QuantiFERON TB Gold test allows the *in vitro* measurement of the levels of IFN-γ produced by T cells that have been stimulated by the antigens mentioned. The result is reported as the amount of IFN-γ in international units (IU) per milliliter. A patient is considered positive in cases in which the amount of IFN-γ is higher than the test cutpoint (considering the negative control).

Another available methodology is the T-SPOT.TB. This enzyme-linked immunospot assay involves counting individual mononuclear cells after the incubation of peripheral blood with ESAT-6 and CFP-10. The result is reported as the number of T cells that produce IFN-γ (spot-forming cells). A patient is considered positive in cases in which the number of spots in the samples containing the antigens exceeds the number of spots in the negative control. Indeterminate IGRA results may occur by a low IFN-γ response of the positive control (mitogen) or a high response of the negative control. This test still needs to be evaluated in children.

Both immunological tests have limitations inherent to the methodologies and rationale. In addition, the IGRAs are still little studied in children who live in geographical regions with a high prevalence of TB or in children infected with HIV. Several studies suggest that IGRAS may be preferred to the tuberculin test for children older than 5 years of age vaccinated with BCG, whereas IGRAs and the tuberculin skin test are equally indicated for individuals not vaccinated with BCG.

In Brazil, for confirmation of TB infection in children, a tuberculin test cutpoint greater than or equal to 5 mm was adopted for immunosuppressed children and those vaccinated with BCG more than 2 years earlier. For children vaccinated with BCG less than 2 years earlier, the cutpoint is greater than or equal to 10 mm. The rationale for the establishment of these cutpoints is that the reaction to the tuberculin test decreases gradually during the 2 years following the administration of the BCG vaccine.

IGRAs and the tuberculin test alone do not permit a diagnosis of TB, but they serve to indicate TB infection. The sensitivity of both tests for the detection of infection with *M. tuberculosis* is similar. More tests involving younger children are needed because the results of these two tests are still indeterminate, which is justified by the low capacity of production of immunoglobulins by children in early childhood. The sensitivity of both tests is low in patients infected with HIV.

IGRAs are significantly costlier and technically more complex than the tuberculin test. In addition, IGRAs involve blood collection, which is a limitation in the case of children. On the other hand, the results of the tuberculin test are ready in 48 to 72 hours and the test requires two visits to the health unit. The trend in international norms is to maintain the tuberculin test as the standard for the identification of cases of TB infection because of the advantages of this test regarding lower cost, reliable results for small children, and the need for fewer laboratory resources.

Table 1 shows the main differences between the IGRAs and the PPD.

Table 1. Comparison of the TST and IGRAS

Characteristic	TST	IGRA
Antigens used	Many; PPD	3 (QTF) or 2 (T-SPOT)
Sample	Itradernal injection	Blood draw
Patient visits required	2	1
Distinguish between LTBI and TB disease	No	No
Cross-reactivity with BCG	Yes	No
Cross-reactivity with NTM	Yes	Only rare species[a]
Differing positive values by risk	Yes (5-10-15)	No
Causes "boosting"	Yes	No
Subject to boosting by previous TST	Yes	Possible
Durability over time (stays positive with or without treatment)	Yes	Unknown
Difficulties with test reproducibility	Yes	Yes
Relative cost	Lower	Higher
Location of need for trained staff	"Bedside"	Laboratory
Estimated specificity in BCG-unvaccinated children	95% to 100%	90% to 95%
Estimated specificity in BCG-vaccinated children	49% to 65%	89% to 100%
Estimated sensitivity (confirmed TB disease)	75% to 85%	80% to 85%
Estimated sensitivity (clinical TB disease)	50% to 70%	60% to 80%

[a] M Marinum, M kansasii, M szulgai, and M flavescens.
Comparison between TST and IGRAS.
1. Fonte: Pai et al, (mod). Nat. Rev. Dis. Primers 2016; 2: 16º 76.

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