Published in final edited form as:

Tuberculosis (Edinb). 2020 January ; 120: 101893. doi:10.1016/j.tube.2019.101893.

Serum anti-Mce1A immunoglobulin detection as a tool for differential diagnosis of tuberculosis and latent tuberculosis infection in children and adolescents

Christiane M. Schmidt^{a,b,*}, Kathryn L. Lovero^c, Fabiana R. Carvalho^b, Daniele C. M. dos Santos^{b,d}, Ana Cláudia M.W. Barros^e, Ana Paula Quintanilha^f, Ana Paula Barbosa^g, Marcos V.S. Pone^e, Sheila M. Pone^e, Julienne Martins Araujo^h, Camila de Paula Martins^b, Solange G.D. Macedoⁱ, Ana Lúcia Miceli^j, Maria Luíza Vieira^j, Selma M.A. Sias^a, Adriano Queiroz^k, Luis Guillermo Coca Velarde^l, Afranio L. Kritski^m, Andrea A. Silva^{b,d}, Clemex C. Sant'Anna^m, Lee W. Rileyⁿ, Claudete A. Araújo Cardoso^{a,b}

^aDepartamento Materno-Infantil, Faculdade de Medicina, Universidade Federal Fluminense, Niterói, Brazil

^bLaboratório Multiusuário de Apoio à Pesquisa em Nefrologia e Ciências Médicas, Faculdade de Medicina, Universidade Federal Fluminense, Niterói, Brazil

^cNew York State Psychiatric Institute, Department of Psychiatry, Columbia University Vagelos College of Physicians and Surgeons, New York, NY, USA

^dDepartamento de Patologia, Faculdade de Medicina, Universidade Federal Fluminense, Niterói, Brazil

^eInstituto Nacional de Saúde da Mulher, da Criança e do Adolescente Fernandes Figueira, Fiocruz, Rio de Janeiro, Brazil

^fPrograma de Controle de Tuberculose da Secretária de Saúde de Itaboraí, RJ, Brazil

^gPrograma de Controle de Tuberculose da Secretária de Saúde de São Gonçalo, RJ, Brazil

^hHospital Municipal Getúlio Vargas Filho, Niterói, RJ, Brazil

ⁱHospital Municipal Jesus, Rio de Janeiro, Brazil

^jPrograma de Controle de Tuberculose da Secretária de Saúde de Duque de Caxias, RJ, Brazil

^kInstituto Gonçalo Moniz, Fiocruz, Salvador, Bahia, Brazil

^lInstituto de Matemática e Estatística da Universidade Federal Fluminense, Niterói, RJ, Brazil

^mFaculdade de Medicina da Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

*Corresponding author. Universidade Federal Fluminense Faculdade de Medicina Avenida Marquês do Paraná, 303 Centro, Niterói, RJ, 24033900, Brazil. christianeschmidt@id.uff.br, christianes850@gmail.com (C.M. Schmidt).

Declaration of competing interest
None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tube.2019.101893>.

ⁿDivision of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, United States

Keywords

Pulmonary tuberculosis; Serodiagnosis; Mce 1A; Children; Latent infection; Antibodies

1. Introduction

Children account for approximately 10% of TB cases worldwide, and those under five years of age are particularly susceptible to severe forms of disease [1]. Diagnosis remains a significant challenge in the pediatric population, as many children cannot expectorate and commonly present with paucibacillary form of pulmonary TB (PTB), resulting in low sensitivity of bacteriological tests (sputum smear and culture). Even in adolescents, who most often present with pulmonary TB, these tests may remain falsely negative [2,3].

In recent years, new diagnostic methods for TB have been developed, such as Xpert MTB/RIF® and, more recently, XpertUltra®, that have a higher sensitivity than smear microscopy and culture; however, in children, the sensitivity of both of these methods is lower than in adults [4,5]. The sensitivity of Xpert MTB/RIF® is estimated to range from 39% to 71.4%, according to a recent meta-analysis [6]. Additionally, the tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), both used as diagnostic methods for latent tuberculosis infection (LTBI) and active TB, are not able to reliably distinguish the two [7]. Thus, in order to improve the diagnosis of TB and to achieve the goal of the End TB strategy of the World Health Organization (WHO), the development of new point-of-care tests is necessary. These tests should be performed on samples that are easy to access and analyze, have low cost, and provide rapid results for the diagnosis of suspected TB cases, especially those with paucibacillary forms of the disease [8]. Serological tests for TB fit these criteria. However, to date, the WHO has not recommended the use of commercial serological kits because of low accuracy [9]. A review of studies on serological tests for TB in children emphasized the importance of considering certain factors in the evaluation of their sensitivity and specificity, among them: age-related immune system development in children; isotype of antibody and antigens studied; previous history of vaccination; definition of TB cases and use of in-house or commercial tests. Such differences make it difficult to compare the different studies [10].

Cellular immunity plays a key role in the pathogenesis and control of *Mycobacterium tuberculosis* (*Mtb*) infection, but research has also demonstrated the role immunoglobulins may play in regulating the immune response to intracellular bacteria [11,12]. The complex cell wall products of *Mtb* also play an important role in TB pathogenesis and in the induction of host immune response [13]. Among them, the Mce1A protein (mammalian cell entry) was initially shown to mediate entry of the bacterium into cells [14]. Mice infected with an *Mtb* strain unable to synthesize Mce1A showed a weak Th1 type response with the formation of disorganized granuloma architecture in the lungs [15]. Mce1A is a member of a putative mycolic acid importer encoded by the *mceI* operon [13,16,17]. Previous research

has indicated that anti-Mce1A IgG levels could be used to distinguish TB from LTBI in adults as a complementary diagnostic test [18].

In the present study, we evaluated serum levels of anti-Mce1A IgG and IgM in children and adolescents with PTB, LTBI and exposure to TB to assess the potential use of this biomarker to differentiate PTB from LTBI in the pediatric population.

2. Study population and methods

This study was approved by the Research Ethics Committee of the Universidade Federal Fluminense, in Niterói, Rio de Janeiro, Brazil (#45470115.0.0000.5243 and #26380513.3.0000.5243), as well as by all partnering health units. Parents or guardians of patients under 18 years of age provided written informed consent. Patients ages 18 years and over provided written informed consent; patients ages 7–17 years provided assent to participate.

2.1. Setting and study population

This prospective study was conducted in eight pediatric TB reference centers (five outpatient clinics and three hospitals) in five cities of Rio de Janeiro State, Brazil. From September of 2014 through June of 2017, we included children and adolescents (ages 0–19 years) with PTB (n = 43) or LTBI (n = 44), or who were exposed to TB (TBE) patients but remained TST negative (n = 20).

2.1.1. PTB group—Children and adolescents diagnosed with TB by their attending physician were invited to participate in the study. PTB was diagnosed according to signs and/or symptoms compatible with TB and one or more of the following data: chest X-ray suggestive of TB, positive tuberculin skin test (TST), positive acid-fast bacilli (AFB) sputum smear, *Mtb* positive sputum culture, positive GeneXpert® PCR test, and clinical improvement with the use of anti-TB drugs (MS Brazil, 2011). Patients who tested positive by culture and/or PCR were classified as confirmed TB [19].

2.1.2. LTBI group—Children and adolescents diagnosed with LTBI by their attending physician were also invited to participate in the present study. LTBI was diagnosed in patient who had contact with a smear-positive TB case, a reactive TST, and normal chest X-ray [20,21].

2.1.3. TBE group—We also included children and adolescents who had been exposed to a TB patient as members of a family with a case of TB included in the present study. TBE patients were defined as asymptomatic youths who had contact with a smear-positive TB case, non-reactive TST, and a normal chest X-ray.

2.1.4. Clinical diagnostic procedures—All patients were examined, diagnosed, and followed up by physicians at participating health units without interference from the research team. Completion of bacteriological tests such as sputum smear, culture, and Xpert MTB/RIF® for *Mtb* in patients with suspected PTB was determined by the attending

physician, considering the age of the patients and their ability to expectorate, as well as the availability of these tests at each health unit.

TST was performed by intradermal injection of 2TU of PPD RT23 (Statens Serum Institute, Copenhagen, Denmark) and read after 72–96 h by a trained technician. Brazilian Ministry of Health recommendations for interpretation of TST were used. Indurations of 10 mm in BCG- vaccinated patients with less than 2 years of age and 5 mm in BCG- vaccinated children more than 2 years of age or with immunosuppression were considered positive [20]. Culture, when indicated by the attending physician, was conducted by the Ogawa-Kudoh, Lowenstein-Jensen, or MGIT 90 methods, according to availability and standard procedures in participating health units.

2.2. Serum collection and ELISA

Following case definition (PTB, LTBI, or TBE) by the attending physician and informed consent procedures, 2 ml of venous blood were collected from patients in a serum separator tubes (BD Vacutainer®, SST® II Advance®, USA) by a trained health professional. Samples were sent to the Multi-user Research Support Laboratory of the Hospital Universitário Antônio Pedro of the Universidade Federal Fluminense (Laboratório Multiusuário de Apoio à Pesquisa em Nefrologia e Ciências Médicas of the Universidade Federal Fluminense - LAMAP) for processing.

Serum was obtained by centrifugation of blood (3500 rpm for 5 min) and stored at 20 °C until tested. The enzyme-linked immunosorbent assay (ELISA) protocol was performed following previously published procedures (Takenami et al., 2016), with minor modifications. Mce1A protein (10 µg/ml) (provided by L.W. Riley University of California, Berkeley, CA, USA) was diluted to 1:1000 in ethanol and 50 µl of it was dried overnight in each well of polystyrene ELISA plates. Plates were blocked with 3% low fatty-acid bovine serum albumin (BSA) (US Biologicals®, USA) for 1 h at room temperature and washed twice with phosphate buffered saline (PBS) pH 7.4. Serum samples were diluted 1:100 in 3% BSA and 100 µL were added to wells (triplicates for each participant sample) to incubate for 1 h at room temperature. After three washes with PBS, 100 µl of horseradish peroxidase (HRP) goat anti- human IgM (1: 10,000) or IgG (1: 50,000) (Sigma-Aldrich®, Germany) were added to wells and incubated at room temperature for 1 h. Subsequently, 100 µl of tetramethylbenzidine substrate (Invitrogen Life Technologies®, USA) were added, and after a 30 min incubation, the reaction was stopped with 100 µl of 1–2 M sulfuric acid (Pershing Chemicals®, Brazil). Reactions were read on a plate reader at optical density (OD) at 450 nm (SpectraMax® Molecular device, USA). Results were reported as the mean OD values of triplicate analyses; the coefficient of variation of all triplicates was less than 10%. Serum sample from patients (non-study related) with confirmed TB were used as a positive control; serum samples from patients with absence of TB signs, symptoms, or contacts of TB served as a negative control.

2.3. Statistical analysis

Data were analyzed by the program GraphPad Prism® v.5.0 (GraphPad Inc., San Diego, CA). Median values and interquartile ranges (IQR) were used to describe immunoglobulin

levels measured by OD. Spearman's correlation was used to assess the linear correlation between biomarkers levels and clinical and laboratory data. Student's t-test was used to compare the mean of biomarker levels in groups with normal data distributions. For comparison of medians between two or three study populations, the Mann-Whitney and Kruskal-Wallis test, respectively, were used followed by the Dunn's multiple comparison test. Chi-square tests were used to assess categorical variables. The ability of immunoglobulins to discriminate PTB patients from those with LTBI and TBE was assessed by measuring the area under the receiver operating characteristic curve (AUC). As children have a developing immune system and children under five are at increased risk of developing severe forms of TB, we stratified analysis by children under five years of age and children between five and 19 years of age. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characteristics of the study population

We included 43 patients with PTB, 44 with LTBI, and 20 with TBE. Table 1 outlines the sociodemographic, clinical, and laboratory characteristics of patients in the PTB, LTBI, and TBE groups. The median age of the entire study population was 8 years (IQR: 3.3–12); the median age was 11 years (IQR: 1.9–15) in the PTB group, 8.5 (IQR: 4–11) in the LTBI group, and 7 (IQR: 4.3–9) in the TBE group. All study patients had BCG (Bacillus Calmette-Guerin) scar greater than 3 mm in size. The mean TST response size was 14 mm (SD = 4) in the PTB group and 13 mm (SD = 4) in the LTBI group [$p = 0.289$]. There was no relationship between anti-Mce1A IgM ($r = 0.12$, $p = 0.16$) or anti-Mce1A IgG levels ($r = 0.13$, $p = 0.31$) with the size of TST induration. Ten (23%) PTB cases were confirmed by culture or Xpert®.

3.2. Serum anti-Mce1A IgM and IgG levels and diagnostic value of IgG in discriminating PTB from LTBI in patients under 5 years of age

Fig. 1 shows levels of anti-Mce1A IgM (Fig. 1A) and IgG (Fig. 1B) across groups under 5 years of age. Median IgM level was 2.447 (IQR: 1.771–3.339) in the PTB group, 2.250 (IQR: 1.254–3.704) in the LTBI group, and 3.843 (IQR: 2.581–3.882) in the TBE group. There were no significant differences in IgM levels between groups in this age range. Median IgG level was 0.401 (IQR: 0.280–0.534) in the PTB group, 0.259 (IQR: 0.187–0.355) in the LTBI group, and 0.253 (IQR: 0.172–0.476) in the TBE group. IgG levels were significantly higher in the PTB than in LTBI group ($p = 0.006$). There was no difference in IgG levels between the PTB and TBE nor between LTBI and TBE groups. Fig. 1C shows the ROC curve comparing PTB and LTBI.

3.3. Serum anti-Mce1A IgM and IgG levels and diagnostic value of IgG in discriminating PTB from LTBI and PTB from TBE in patients between 5 and 19 years of age

Fig. 2 shows levels of anti-Mce1A IgM (Fig. 2A) and IgG (Fig. 2B) in patients ages 5–19 years. Median IgM level was 1.947 (IQR: 1.265–3.013) in the PTB group, 1.945 (IQR: 1.265–2.826) in the LTBI group, and 2.578 (IQR: 2.097–3.886) in the TBE group. There was a significant difference between the TBE and PTB groups ($p = 0.022$) and between TBE and LTBI ($p = 0.024$) groups. There was no difference between LTBI and PTB.

Median IgG level was 0.331 (IQR: 0.215–0.443) in the PTB group, 0.198 (IQR: 0.146–0.331) in the LTBI group, 0.196 (IQR: 0.114–0.333) in the TBE group. There was a significant difference in IgG levels between the PTB and LTBI groups ($p = 0.009$) and between the PTB and TBE groups ($p = 0.020$).

3.4. Serum anti-Mce1A IgG levels and diagnostic value of IgG in discriminating bacteriologically positive PTB (PTBBACT+) from LTBI and TBE in patients between 0 and 19 years of age

Ten patients had culture or PCR-confirmed PTB. Of these, the anti-Mce1A IgG ELISA test was positive in 7 cases (70%). When comparing the confirmed PTB group with LTBI and TBE groups, there was a significant difference in the level of the IgG ($p = 0.043$ and $p = 0.05$, respectively). As we had only 10 cases of confirmed PTB and because TB is endemic in Brazil, we extended this analysis to include the nine additional PTB patients, who had positive sputum smear, but were not confirmed by culture or PCR. This group, referred to here as bacteriologically positive PTB (PTB BACT+), included 19 patients. The median age of this group was 13 years (IQR: 8–15). The median level of anti-Mce1A IgG was 0.334 (IQR: 0.249–0.412) in the PTB + group, 0.22 (IQR: 0.161–0.344) in LTBI and 0.224 (IQR: 0.131–0.33) in TBE. We found a significant difference between the IgG levels in the PTB BACT + versus LTBI groups ($p = 0.005$) and the PTB BACT + versus TBE groups ($p = 0.025$) (Fig. 3A). In the ROC analysis, using the 0.26 OD cut-off, we found the sensitivity to be 73.7% and the specificity to be 63.6% when comparing PTB BACT+ and LTBI groups (Figs. 3B), and 73.7% and 70%, respectively, comparing the PTB BACT + vs TBE groups (Fig. 3C). In the PTB BACT + group, the IgG test was positive in 14 (73.7%) patients based on the 0.26 OD cut-off.

4. Discussion

In this study, whose main objective was to evaluate whether serum levels of anti-Mce1A IgM and IgG were able to differentiate PTB and LTBI in children and adolescents, significantly higher levels of anti-Mce1A IgG were detected in patients with PTB. In the overall group, the best result for anti-Mce1A IgG was obtained with the ELISA cut-off set at 0.26 OD for comparison of both PTB vs LTBI and PTB vs TBE groups.

Previous research evaluating the potential use of anti-Mce1A IgG biomarker for the diagnosis of active TB in adults found higher levels of this antibody in patients with PTB compared to LTBI as well as PTB compared to TBE [18]. In children and adolescents, we show here that anti-Mce1A IgG is able to distinguish PTB BACT + vs TBE with 74% sensitivity and 70% specificity and PTB BACT + vs LTBI with 74% sensitivity and 64% specificity. These results demonstrate that anti-Mce1A IgG has similar or better test accuracy than previously evaluated serological tests assessing various Mtb antigens [10,22].

In children, whose immune system is under development, IgM, IgG and IgA levels similar to those of adults are reached on average at two, six and ten years of age, respectively [23]. In our age-stratified analysis, anti-Mce1A IgG was able to discriminate PTB from LTBI in children under 5 years of age. In patients ages 5–19, anti-Mce1A IgG also discriminated between PTB and LTBI, as well as PTB from TBE. Children, especially those under 10,

often develop paucibacillary form of PTB and also have difficulty expectorating [2]. In the present study, we found a small number of cases of confirmed PTB (23%), according to criteria of Graham et al., 2015 [19]. AFB sputum smear and Xpert® MTB/RIF, which offer results more quickly, were used for the diagnosis of 40% of patients, who had positive test results. In the remaining 60%, the initial diagnosis was based on the Brazilian scoring system for diagnosing pediatric TB [20]. In the overall group, based on the 0.26 OD cut-off, the anti-Mce1A IgG ELISA tested positive in 31 (72%) of all cases of PTB, indicating that there was a 32% increase in case-detection using the ELISA test compared to smear microscopy and Xpert® MTB/RIF. When we added PTB cases with positive AFB sputum test to those with confirmed PTB (PTB BACT + group), the ELISA also discriminated PTB BACT+ from LTBI and TBE, a finding also found by Takenami et al. (2016) in adults [18].

In Brazil, the BCG (*Mycobacterium bovis*) vaccination is recommended in the first month of life, with coverage higher than 90% of the target population [24]. The Mce1A protein is present in both the cell wall of *Mycobacterium bovis* and *Mtb*. Although some studies have found higher levels of immunoglobulins for certain antigens when compared to vaccinated and non-vaccinated children [25], others have generally shown that prior BCG vaccination did not influence antibody levels against *Mtb* proteins. In our study population, 100% of the children and adolescents were vaccinated, so it was not possible to compare anti-Mce1A IgM and IgG levels between vaccinated and unvaccinated patients. However, we did not find a significant association between anti-Mce1A IgM or IgG levels and participant age, indicating the time period from of vaccination did not impact biomarker levels.

After infection, the *Mtb* bacillus can be eliminated by the immune system, remain in the latent state, or proliferate to cause active disease. The bacillus may be eradicated at all stages [26]. In our study, 6 (30%) TBE and 17 (39%) LTBI patients had positive ELISA tests for anti-Mce1A IgG. It is possible that these individuals were newly infected, had a higher bacillary load, or had a higher risk of progression to subclinical or TB. Future studies in which LTBI and PTB patients are followed over time are required to determine if their higher anti-Mce1A IgG levels are associated with progression to active disease.

This study has several limitations, including a small sample size and the small number of confirmed PTB. Therefore, further studies with larger sample sizes are needed. Additionally, we were unable to assess the association of ELISA results and clinical progression outcomes, although we intend to follow the patients to further evaluate this question. Finally, we were not able to include and compare antibody levels in serum from patients infected with non-tuberculous mycobacteria. Such infections, are rare in our geographic region.

The WHO recommends that large-scale diagnostic tests for TB should have 90% sensitivity and 70% specificity [22]. Although anti-Mce1A IgG did not meet these targets, it has achieved a level of sensitivity comparable to or better than that reported by Xpert, which requires sputum samples or extrapulmonary samples that are not readily obtained from children. Thus, the anti-Mce1A IgG ELISA could potentially be used to screen suspected cases of pediatric and adolescent PTB during the initial evaluation phase.

Acknowledgements

The authors would like to thank Dr. Iukary Takenami for the support in implementing the ELISA protocol and Estela M. Cosme, Thaís R.D. Oliveira, Fernanda P. Barra, and Halana S.A.T. Sias for assistance with data collection and organization.

Funding

The present study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) #001, the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil. CAAC was supported by the CAPES (grant number 17862/12-4) and the FAPERJ (grant number E-26.202.777.2015). KLL was supported by the National Institutes of Health Fogarty International Center [grant number R25 TW009338] and by a Fulbright Postdoctoral Scholarship and CAPES. CCS was supported by the CNPq [grant number 305044/2012-5].

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Fig. 1.

Levels of igM (A) and igG (B) anti-Mce 1A, in patients (age 0–59 months) with pulmonary tuberculosis (PTB), latent tuberculosis infection (LTBI) and exposed to TB (TBE). Receiver operator characteristic (ROC) curve (C) comparing igG anti-Mce 1A level in PTB versus (vs) LTBI. AUC = area under the curve, Se = sensitivity, sp = specificity CI = confidence interval, ns = not significant and LR = likelihood ratio.

Fig. 2.

Levels of igM (A) and igG (B) anti-Mce 1A, in patients (age 5–19 years) with pulmonary tuberculosis (PTB), latent tuberculosis infection (LTBI) and exposed to TB (TBE). Receiver operator characteristics (ROC) curve (C) comparing igG anti-Mce 1A level in PTB versus (vs) LTBI. (ROC) curve (D) comparing igG anti-Mce 1A level in PTB vs TBE. AUC = area under the curve, Se = sensitivity, sp = specificity CI = confidence interval, ns = not significant and LR = likelihood ratio.

Fig. 3.

Levels of IgG anti-Mce 1A (A) in patients (age 0–19 years) with pulmonary TB positive bacteriologically (PTB BACT+), latent tuberculosis infection (LTBI) and exposed to TB (TBE). Receiver operator characteristic (ROC) curve (B) Comparing IgG anti-Mce 1A level in PTB BACT + versus (vs) LTBI and (C) ROC curve comparing IgG anti-Mce 1A level in PTB BACT + versus (vs) TBE. AUC = area under the curve, Se = sensitivity, sp = specificity CI = confidence interval, ns = not significant and LR = likelihood ratio.

Table 1

Demographic, clinical, and laboratory characteristics of the study population.

	All patients (n = 107)	PTB (n = 43)	LTBI (n = 44)	TBE (n = 20)	P
Age					
0–4y	37 (35%)	18 (42%)	14 (32%)	5 (25%)	0.37
5–19y	70 (65%)	25 (58%)	30 (68%)	15 (75%)	
Gender					
Male, n (%)	48 (45%)	20 (46%)	18 (41%)	10 (50%)	0.76
Female, n (%)	59 (55%)	23 (54%)	26 (59%)	10 (50%)	
Household contact					
Yes	90 (84%)	26 (61%)	44 (100%)	20 (100%)	–
No	17 (16%)	17 (39%)	0	0	–
TST *					
Positive		23 (79%)	44 (100%)	–	–
Negative	–	6 (21%)	–	20 (100%)	–
AFB *					
Positive	–	14 (42%)	–	–	–
Negative	–	19 (58%)	–	–	–
PCR *					
Positive	–	4(29%)	–	–	–
Negative	–	10 (71%)	–	–	–
Culture *					
Positive	–	8 (38%)	–	–	–
Negative	–	13 (62%)	–	–	–

PTB = pulmonary tuberculosis; LTBI = latent tuberculosis infection; TBE = tuberculosis exposed; TST = tuberculin skin test; AFB = acid fast bacilli; PCR = polymerase chain reaction.

* Percent positive and negative based on total number of performed tests.