

## Performance of nested PCR in the specific detection of *Mycobacterium tuberculosis* complex in blood samples of pediatric patients\*

Desempenho da técnica *nested* PCR na detecção específica do complexo *Mycobacterium tuberculosis* em amostras sanguíneas de pacientes pediátricos

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### Abstract

**Objective:** To evaluate the performance of nested PCR (nPCR) in detecting the *Mycobacterium tuberculosis* complex in blood samples of patients suspected of having TB, in order to determine its potential for use as an auxiliary tool in the laboratory diagnosis of TB in children. **Methods:** Detection of the *M. tuberculosis* complex in blood samples using as a target the insertion sequence IS6110 of the genomic DNA of the bacillus. Blood samples of 120 patients were evaluated. All of the patients were under 15 years of age at the time of their treatment at public hospitals in the city of Recife, Brazil (between January of 2003 and August of 2005). Attending physicians at the hospitals diagnosed TB based on the criteria recommended by the American Thoracic Society. The nPCR amplified a 123-bp fragment with outer oligonucleotides (IS1/IS2) and, in the subsequent reaction, using inner oligonucleotides (IS3/IS4), generating an 81-bp amplicon. **Results:** Active or latent TB was found in 65 patients, TB was ruled out in 28 suspected cases, and 27 patients were TB-free (controls). The sensitivity of nPCR was 26.15% and was significantly higher for the extrapulmonary form of the disease (55.56%) than for the pulmonary form (18.18%). The specificity was 92.73%. **Conclusions:** Despite the difficulties in diagnosing TB in children and the low number of cases evaluated in the present study, nPCR in blood samples proved to be a rapid and specific technique, albeit one with low sensitivity. In order to establish its true usefulness in the diagnosis of paucibacillary forms, especially extrapulmonary TB, further studies need to be carried out with a larger sample of children and analyzing biological specimens other than blood.

**Keywords:** Tuberculosis; Diagnosis; Blood; Polymerase chain reaction.

### Resumo

**Objetivo:** Avaliar o desempenho da técnica *nested* PCR (nPCR) para detectar o complexo *Mycobacterium tuberculosis* em amostras de sangue de pacientes com suspeita de TB para sua possível utilização como uma ferramenta auxiliar no diagnóstico laboratorial da doença em crianças. **Métodos:** Detecção do complexo *M. tuberculosis* em amostras de sangue usando como alvo a sequência de inserção IS6110 do DNA genômico do bacilo. Foram avaliados 120 pacientes, menores de 15 anos de idade, de ambos os sexos, provenientes de hospitais públicos do Recife (PE), no período entre janeiro de 2003 e agosto de 2005. O diagnóstico de TB foi realizado pelo médico assistente do serviço de saúde de acordo com os critérios da Sociedade Torácica Americana. A nPCR amplificou um fragmento de 123 pb com oligonucleotídeos externos (IS1/IS2) e, na reação subsequente, com oligonucleotídeos internos (IS3/IS4), gerando um amplicon de 81 pb. **Resultados:** A TB ativa ou latente esteve presente em 65 pacientes, foi descartada em 28 suspeitos e 27 não tinham a doença (controles). A sensibilidade da nPCR foi de 26,15%, sendo significativamente maior na forma extrapulmonar (55,56%) em relação à pulmonar (18,18%), e a especificidade foi de 92,73%. **Conclusões:** Diante das dificuldades diagnósticas da TB infantil e do baixo número de casos estudados, a nPCR em sangue demonstrou ser uma técnica rápida e específica, mas com baixa sensibilidade. Para saber a sua real utilidade no diagnóstico de formas paucibacilares, sobretudo as extrapulmonares, novas pesquisas devem ser desenvolvidas com uma casuística maior de crianças e com outros espécimes biológicos além do sangue.

**Descritores:** Tuberculose; Diagnóstico; Sangue; Reação em cadeia da polimerase.

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## Introduction

It is estimated that there are approximately 42 million people infected with TB in Brazil, and 10% of the cases reported annually occur in patients under 15 years of age.<sup>(1)</sup> In the state of Pernambuco, 184 new cases of TB in children ( $\leq 14$  years of age) were reported in 2006, and 74.46% of those were cases of pulmonary TB.<sup>(2)</sup> Data on pediatric TB (which is generally paucibacillary) are scarce in the literature, mostly due to the great difficulty in diagnosis, since there is rarely bacteriological confirmation. Such difficulty is related to the following factors: lack of a specific clinical profile; absence of pathognomonic pulmonary radiological imaging of the disease; low sensitivity of smear microscopy; and the fact that the reactivity of the Mantoux tuberculin skin testing is impaired by the recent BCG vaccination.<sup>(3)</sup> In this age bracket, the collection of sputum samples for *Mycobacterium tuberculosis* testing is restricted due to the difficulty in inducing expectoration, especially in children under 5 years of age. The identification of *M. tuberculosis* through smear microscopy or culture is the gold standard for the confirmation of the disease, although it takes 3 to 8 weeks to obtain the result of the latter,<sup>(4)</sup> and the time necessary for the identification of Koch's bacillus is an important factor for the early initiation of specific treatment.<sup>(5)</sup> In children, in addition to the delay, positivity in multiple cultures is seen in less than 20% of the cases suspected of primary TB.<sup>(6)</sup> The *M. tuberculosis* complex is considerably infectious. Therefore, rapid diagnosis is fundamental in order to avoid dissemination of the disease, principally in high-risk groups.

With the advent of molecular biology, new tools have become available to facilitate the diagnosis of various contagious infectious diseases. A sensitive method for the detection of the mycobacteria DNA directly from clinical specimen is PCR. It allows the *in vitro* enzymatic synthesis in specific sequences of the genome through the use of two primers that hybridize opposite DNA strands. Numerous PCR assays, using conserved sequences of DNA as the target of amplification, have been described for the detection of the *M. tuberculosis* complex in clinical specimen of adults,<sup>(7,8)</sup> showing its usefulness for early diagnosis.<sup>(9,10)</sup>

A variation of PCR, known as nested PCR (nPCR), uses two sets of oligonucleotide primers

in subsequent reactions, in which the amplification product of the first reaction is used as a template for the second reaction. This technique has been proposed for the detection of *M. tuberculosis* in cases requiring high specificity and sensitivity.<sup>(9,11)</sup> The purpose of the present study was to evaluate the performance of nPCR in blood samples of paucibacillary children, as well as its clinical applicability as a diagnostic tool for pediatric TB.

## Methods

This was a prospective study of patients under 15 years of age, with initial suspicion of TB. All of the patients evaluated were treated between January of 2003 and August of 2005 at the outpatient clinics or infirmaries of the Pernambuco Federal University *Hospital das Clínicas*, the Barão de Lucena Hospital or the Fernando Figueira Institute of Comprehensive Medicine, all of which are located in the city of Recife, Brazil. The attending physician of the health care facility diagnosed TB, in accordance with the American Thoracic Society criteria,<sup>(12)</sup> in a double-blind fashion. We considered as gold standard the routine conventional methods based on epidemiological, clinical and laboratory criteria (Mantoux tuberculin skin testing and chest X-ray), as well as on patient evolution and treatment response. Mantoux tuberculin skin testing was performed according to the norms of the Brazilian National Ministry of Health.<sup>(13)</sup> All patients diagnosed with active or latent TB were monitored and treated in the health care facilities participating in the study, all of which specialize in treating pediatric TB. The groups were classified as follows:

- 1) Suspected TB: clinical or radiological evidence, history of contact with adult TB patient or positive tuberculin skin test results ( $\geq 10$  mm in patients vaccinated with BCG in over 2 years and  $> 15$  mm patients vaccinated with BCG in less than 2 years)<sup>(13)</sup>:
  - a) Active TB: clinical or radiological evidence consistent with active TB, isolation of *M. tuberculosis* in a biological sample or clinical improvement after specific treatment.
  - b) Latent TB: without clinical or radiological symptoms of the disease; negative bacteriological tests (when possible);

positive tuberculin skin test result<sup>(13)</sup> and history of contact with adults with infectious TB.

c) Ruled out: history of contact with adult TB patient, absence of symptoms or alterations suggestive of TB and negative tuberculin skin test result.

2) TB-free: absence of contact with adult TB patient, clinical and laboratory status inconsistent with latent or active TB; presence of BCG vaccination scar.

The project was approved by the Ethics Committee of the Oswaldo Cruz Foundation Aggeu Magalhães Research Center, in Recife, Brazil. Parents or legal guardians of the minors gave written informed consent for their participation in the study as well as for blood sample collection. Clinical and epidemiological data related to each patient were recorded on a form previously developed by one of the researchers and were entered into a database for statistical analysis.

The following individuals were excluded: those with chronic diseases; those using corticosteroids or immunosuppressants for more than 15 days; those known to be HIV-infected; and those with other chronic lung diseases.

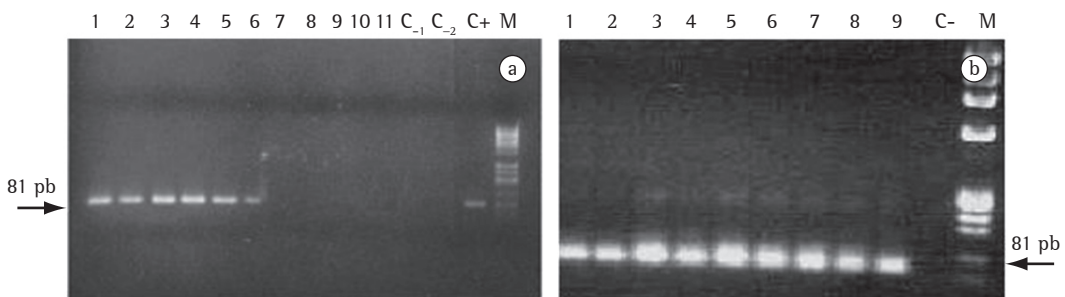
For the sake of standardization and more efficacious results, we used purified genomic DNA of the *M. tuberculosis* reference strain (H37Rv), using an extraction kit (Tissue and Cells GenomicPrep; Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer instructions. In order to determine the detection threshold through nPCR, we used a serial dilution curve factor 10, ranging from 10 ng to 0.01 fg. Subsequently, purified DNA

was added to the blood of a healthy individual. We used the DNA of this strain as positive control in the amplification reactions.

We collected 2-4.5 mL of blood from each patient through venous puncture using tubes (Vacutainer; Becton and Dickson, Oxford, England), containing ethylenediaminetetraacetic acid (EDTA) and having been stored at 4°C.

The extraction of the DNA was carried as follows: a 500- $\mu$ L aliquot of the sample was centrifuged at 13,000 rpm for 10 min and submitted to three lavages with Tris-EDTA (TE) buffer solution. The sediment was resuspended in 100  $\mu$ L of TE and heated in thermoblock at 100°C for 10 min. The supernatant was transferred to a new micro-tube and 5  $\mu$ L of resin (Sephaglas BandPrep Kit; Amersham-Pharmacia Biotech, Uppsala, Sweden) was added and to the double of the final volume was added to it with a solution of sodium iodide (0.9 g/mL). Subsequently, the tube was agitated for 5 min and incubated at room temperature for an additional 5 min. The tubes were centrifuged for 1 min, and the supernatant was discarded; 200  $\mu$ L of cold 70% ethanol was added, followed by new agitation and centrifugation for 1 min. The sediment was allowed to sit at room temperature for 60 min for complete drying and was then resuspended with 40  $\mu$ L of TE and incubated in a 50°C water bath for 10 min. After having been centrifuged for 1 min, the supernatant was transferred to another micro-tube and stored at -20°C until use in the PCR.<sup>(14)</sup> The insertion sequence IS6110, found in *M. tuberculosis* complex strains, was the target used for amplification.<sup>(15)</sup>

The DNA of *M. tuberculosis* in the biological samples was amplified in an automatic thermo-



**Figure 1** – In a, dilution curve using whole blood of a healthy individual mixed with *Mycobacterium tuberculosis* DNA. In b, dilution curve of *M. tuberculosis* DNA in water in order to establish the detection threshold of DNA through nested PCR.

**Table 1** – Clinical and demographic characteristics of the patients studied.

Characteristic	n	%
Age		
≤ 5 years	48	39.7
> 5 years	72	60.2
Place of initial treatment		
Outpatient clinic	105	87.5
Infirmatory	15	12.5
Gender		
Male	61	50.5
Female	59	49.5
Final diagnosis		
Active TB	42	35.0
Latent TB	23	19.2
TB-free <sup>a</sup>	55	45.8
Clinical form		
Pulmonary TB	33	79.0
Extrapulmonary TB	9	21.0

<sup>a</sup>Without TB (original controls) + TB suspected but ruled out (added late to control group).

cycler (model 4800; Perkin Elmer, Foster City, CA, USA). The mixture of the first amplification reaction consisted of 50 mM of KCl, 10 mM of Tris-HCl, pH 8.3, 2.5 mM of MgCl<sub>2</sub>, 200 μM of each dNTP, 25 pmol of each oligonucleotide (IS1-5'CCTGCGAGCGTAGGCGTCGG3' and IS2-5'CTCGTCCAGCGCCGCTTCGG3')<sup>(16)</sup> and 2.5 U of Taq DNA polymerase, in a final volume of 50 μL. The denaturing step was carried out at 94°C for 30 s, the annealing step was carried out at 68°C for 1 min, and the extension step was carried out at 72°C for 1 min, totaling 30 cycles. In the second reaction (nPCR), 2 μL of the product of the first reaction were added, at the same concentrations used in the previous reaction. The amplification conditions, in the steps described above, were, respectively, 94°C for 30 s, 57°C for 30 s, and

72°C for 30 s, using the oligonucleotides IS3 and IS4 (5'GGTGACAAAGGCCACGTAGG3' and 5'CCAGCACCTAACCGGCTGT3', respectively)<sup>(16)</sup> for 30 cycles.

Of the amplified products, 10 μL were analyzed on a 2.0% agarose gel and stained with ethidium bromide. The DNA bands separated through electrophoresis were visualized in a UV transilluminator and photographed with a camera (Polaroid MP4+ Instant Camera System; Polaroid, Minnetonka, MN, USA).

A descriptive analysis was carried out to show the results obtained, and the measured variables are presented in tables. The chi-square test was applied for the analysis of the variables, and Fisher's exact test was used when necessary. In order to validate the tests, sensitivity, specificity, positive predictive value and negative predictive value, together with the respective confidence intervals, were calculated. All conclusions were made at a level of significance of 5%. The programs used were Epi Info, version 6.04d, and the Statistical Package for the Social Sciences, version 8.0 (SPSS Inc., Chicago, IL, USA).

## Results

The results show the specific amplification of the *M. tuberculosis* genomic DNA, with no random amplification of the human genome. The first reaction produced a 123-pb amplicon, and the second produced an 81-pb amplicon (data not shown). The minimal quantity detected using *M. tuberculosis* purified genomic DNA and purified genomic DNA added to healthy donor blood was 0.1 fg and 100.0 fg, respectively (Figure 1).

In the present study, 120 patients were evaluated; 93 with initial suspicion of the disease and 27 control individuals. The median age was 7.00 ± 0.42 years. There was no significant differ-

**Table 2** – Performance of nested PCR in patients with active TB, patients with latent TB and controls.

Nested PCR	Active TB	Controls	Latent TB	Controls
Result				
Positive, n (%)	11 (26.2)	4 (7.3)	6 (26.1)	4 (7.3)
Negative, n (%)	31 (73.8)	51 (92.7)	17 (73.9)	51 (92.7)
Sensitivity, % (95% CI)	26.2 (14.4-42.3)		26.1 (11.1-48.7)	
Specificity, % (95% CI)	92.7 (81.6-97.6)		92.7 (81.6-97.6)	
Positive predictive value, % (95% CI)	73.3 (44.8-91.1)		60 (27.4-86.3)	
Negative predictive value, % (95% CI)	62.2 (50.8-72.5)		75 (62.8-84.4)	
p		0.1		0.033

**Table 3** – Performance of nested PCR in patients with pulmonary TB, extrapulmonary TB and controls.

Nested PCR	Pulmonary TB	Controls	Extrapulmonary TB	Controls
Result				
Positive, n (%)	6 (18.2)	4 (7.3)	5 (55.6)	4 (7.8)
Negative, n (%)	27 (81.8)	51 (92.7)	4 (44.4)	51 (92.2)
Sensitivity, % (95% CI)	18.2 (7.6-36.1)		55.6 (22.7-84.7)	
Specificity, % (95% CI)	92.7 (81.6-97.6)		92.7 (81.6-97.6)	
Positive predictive value, % (95% CI)	60.0 (27.4-86.3)		55.6 (22.7-84.7)	
Negative predictive value, % (95% CI)	65.4 (53.7-75.6)		92.7 (81.6-97.6)	
p	0.114		0.002	

ence in relation to the gender of the individuals. According to the final diagnosis, the individuals were characterized as follows (Table 1): active TB; latent TB; ruled out TB; and control (uninfected individuals).

Among the 93 individuals who presented initial suspicion, most diagnosed cases (69.89%) were of active TB or latent TB. In the group of infected patients, the pulmonary form was prevalent (78.57%). The extrapulmonary TB forms detected were peripheral lymph node (n = 3), bone (n = 2), abdominal (n = 1), pleural (n = 1), miliary (n = 1) and meningitis (n = 1). There were no significant age-related differences in terms of the presence or clinical form of the disease. In the initial suspicion group, nPCR was positive in 22.58% of the cases. The concordance between nPCR and the final diagnosis was 52.38% among the cases of active TB and 28.57% among the cases of latent TB. In 19.05% of the patients in whom TB was ruled out by the physician, nPCR was positive (Table 2).

For the evaluation of the true performance of nPCR, considering the limitation of the gold standard inherent to the confirmation of the disease in the pediatric age bracket, the patients in whom TB was initially suspected but later ruled out were also included in the control group. The sensitivity and specificity of nPCR were 26.15% and 92.73%, respectively. For extrapulmonary and pulmonary TB, respectively, nPCR sensitivity was 55.56% and 18.18% (Table 3).

## Discussion

Pediatric TB is a marker of the quality of the health care system, being an indication that infectious cases in adults are not detected early, thereby allowing dissemination of the disease. One third of all adult cases of active TB are diagnosed after the confirmation of the disease in a

pediatric contact. This shows that a considerable number of adults can be asymptomatic for a prolonged period and infect other groups, in addition to the children, leading to the development of the disease.<sup>(17)</sup> Due to the difficulty in confirming pediatric TB through smear microscopy, the implementation of PCR as an auxiliary tool for the detection of the disease, especially in difficult or paucibacillary cases, will allow the use of less traumatic measures for the collection of biological samples, which can be collected at outpatient clinics, whereas the other alternative, gastric lavage, requires hospitalization and sedation.

The clinical and demographic characteristics of the individuals evaluated in the present study are very similar to those of individuals evaluated in other studies.<sup>(3,18)</sup> It is possible that the variations in relation to gender and median age are due to differences in the number of patients included. Being in the lower age brackets was not directly correlated with developing the extrapulmonary forms,<sup>(19)</sup> probably due to the greater number of children concentrated in the under 5 years of age bracket, in which TB seems to be more common.

In the analysis of the performance of nPCR in amplifying the target (*IS6110*), the lower limit of genomic DNA detection of the bacillus was equivalent to less DNA than is contained in one mycobacterium (5 fg).<sup>(5)</sup> The sensitivity of the detection was more efficient than that observed in other studies using the same system.<sup>(19,20)</sup> Most patients with initial suspicion of TB were diagnosed as an active or latent case, probably due to the fact that the diagnoses were made at hospitals or outpatient clinics that were referral centers for TB.

The performance of a diagnostic test depends on the efficiency of the gold standard

in detecting the existence of the disease. Therefore, molecular tests based on PCR can present low sensitivity due to the limitation of the gold standard in detecting *M. tuberculosis* in childhood,<sup>(18,21)</sup> leading to the questioning of many of the cases classified as active TB or latent TB that were included in this study, in which the definition was based on clinical and subjective criteria.<sup>(19)</sup>

Although the sensitivity of nPCR for the diagnosis of TB, using total peripheral blood, in individuals under 15 years of age was low, its specificity was excellent. Another group of authors used PCR in blood samples collected from adults, most of whom had pulmonary TB, and obtained similar results (20% sensitivity and 94.44% specificity).<sup>(22)</sup> However, 26.7% of the patients who initiate specific pulmonary TB treatment do so without bacteriological confirmation. The decision to treat is made based only on the clinical and radiological profile.<sup>(23)</sup> Therefore, nPCR could play a relevant role in this group of patients with suspicion of TB who were undiagnosed or who initiated treatment without confirmation of the disease.

The performance of the PCR system has proven to be more promising for the diagnosis of TB in adults, especially in the pulmonary form, when sputum is used as biological sample.<sup>(24,25)</sup> In contrast, the use of nPCR in blood samples is important in the attempt to elucidate cases of the disease in the pulmonary or paucibacillary forms, in which the sputum smear microscopy is negative or when there is no expectoration.

The inadequate performance of nPCR found in the present study was probably due to the inaccuracy of the clinical diagnosis of pediatric TB, to the low bacterial load and to the difficulty in identifying *M. tuberculosis* in the routine tests, as well as to the low number of patients studied. The scarcity of studies in the literature using the PCR system in biological specimens for the diagnosis of pediatric TB, together with the presence of inhibitors in the blood that interfere in the DNA amplification, also contributed to this result.

In relation to the forms of TB and the nPCR results, the positivity rate of the test among the cases of extrapulmonary TB was significantly greater than that obtained among the cases of pulmonary TB. This finding corroborates those of another group of authors,<sup>(26)</sup> who stated that

the circulation of bacilli in the peripheral blood is more likely in the extrapulmonary form of the disease. Another group of authors<sup>(20)</sup> indicated the difficulty in finding *M. tuberculosis* in extrapulmonary specimens, in which there are few bacilli, leading to low sensitivity of smear microscopy and of culture. The authors also implicated the presence of inhibiting factors that can interfere in the DNA amplification using PCR. However, even when using the blood of paucibacillary patients, we found the sensitivity of PCR to be higher in children with extrapulmonary TB than in those with pulmonary TB. The extraction methods and the nPCR system used in our study should also be considered.

In the present study, there were 4 cases in which the clinical diagnosis was inconsistent with TB and the nPCR was positive. However, all 4 of those cases had, in some manner, been in contact with infected adults. In 1 of those 4 cases, the tuberculin skin test was negative, although the patient was a household contact for more than 2 years of an adult in poor compliance with TB treatment and with a history of pneumonia. Another presented a 9-mm induration on the tuberculin skin test and had been diagnosed with acute pneumonia. The third patient presented multiple cervical adenopathy. The fourth was tuberculin skin test reactive and had been in contact with a TB patient for more than 1 year. After the possibility of active TB had been ruled out, those children did not return to the health care facility for monitoring. Since PCR can detect viable or unviable bacillus DNA,<sup>(9)</sup> these patients might have been in the initial phase of the infection/disease or might have undergone the process of spontaneous cure.

Although negative controls were added in all DNA extraction/amplification steps and samples presenting inconclusive results were retested, a greater number of samples would be needed in order to decrease the possibility of false-negative results.

In contrast with those of other studies,<sup>(10,27)</sup> our results show that DNA amplification through PCR is a quite specific method, with reasonable sensitivity for detection of *M. tuberculosis* in whole blood. The performance of in-house PCR testing for TB in adult respiratory samples with negative smear microscopy test results has been described as promising,<sup>(28)</sup> although studies

involving blood samples collected from children are scarce.

Methods based on DNA amplification will facilitate the early diagnosis of pediatric TB and of paucibacillary individuals, in addition to reducing the period of definition of the disease to 1-2 days,<sup>(8,9)</sup> especially if there is the possibility of detection of *M. tuberculosis* DNA in blood samples, the collection of which is minimally invasive and feasible in this age bracket.

In difficult-to-diagnose cases in which a diagnosis is urgently needed due to the severity of the patient,<sup>(28)</sup> as well as in disseminated, extrapulmonary forms of TB, especially in HIV-infected patients,<sup>(3,25)</sup> PCR could play an important role in the clinical practice. In the view of these findings, nPCR can be recommended as an auxiliary method of elucidating the diagnosis. However, due to its low sensitivity, it should not be dissociated from the clinical, epidemiological or routine laboratory criteria. Although it presented high specificity, nPCR cannot be considered in itself an exclusion method in the group of pediatric patients with paucibacillary TB. In addition, blood can contain PCR inhibiting factors.<sup>(29)</sup> Early diagnosis in patients under 15 years of age and the surveillance for infectious adults will result in greater detection of index cases<sup>(30)</sup> and have a positive impact on TB control programs.

Due to the inaccuracy in identifying infected children or TB pediatric patients, to the fact that little attention is given to pediatric TB in Brazilian government programs and to the scarcity of studies of molecular techniques for detection of Koch's bacillus in the literature, it is fundamental that new studies be conducted in order to identify methods that are more sensitive and specific, using various biological samples from patients with the paucibacillary form of the disease. In view of our findings, we suggest that future molecular studies of blood sample-based diagnosis of paucibacillary TB, involving representative samples of the population, be conducted in order to improve the evaluation of our results.

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