Cord factor detection and macroscopic evaluation of mycobacterial colonies: an efficient combined screening test for the presumptive identification of *Mycobacterium tuberculosis* complex on solid media*, **

Detecção do fator corda e avaliação do aspecto macroscópico das colônias de micobactérias: um eficiente teste de triagem combinado para a identificação presuntiva do complexo *Mycobacterium tuberculosis* em meios sólidos

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Abstract

Objective: The rapid differentiation between *Mycobacterium tuberculosis* and nontuberculous mycobacteria is fundamental for patients co-infected with tuberculosis and HIV. To that end, we use two methods in our laboratory: detection of cord factor and PCR-restriction enzyme analysis (PRA). The objective of this study was to evaluate the accuracy of a screening test on solid medium as a rapid method for the presumptive identification of *M. tuberculosis* complex, considering costs and turnover time. **Methods:** A total of 152 strains were submitted to a combined screening test, consisting of the detection of cord factor under microscopy (Ziehl-Neelsen staining) and evaluation of the macroscopic aspect of colonies, as well as to PRA, which was used as the gold standard. Costs were estimated by calculating the price of all of the materials needed for each test. **Results:** The overall accuracy of cord factor detection alone was 95.4% (95% CI: 90.7-98.1%), and that of the combined screening test was 99.3% (95% CI: 96.4-100%). Cord factor detection costs US$ 0.25, whereas the PRA costs US$ 7.00. Results from cord factor detection are ready in 2 days, whereas PRA requires 4 days to yield results. **Conclusions:** The presumptive identification of *M. tuberculosis* using the macroscopic evaluation of colonies combined with cord factor detection under microscopy is a simple, rapid and inexpensive test. We recommend the combined screening test to rapidly identify *M. tuberculosis* in resource-poor settings and in less well-equipped laboratories while awaiting a definite identification by molecular or biochemical methods.

Keywords: Tuberculosis; Mycobacterium/classification; Polymerase chain reaction; Diagnostic tests, routine.

Resumo

Objetivo: A diferenciação rápida entre *Mycobacterium tuberculosis* e micobactérias não-tuberculosas é fundamental para os pacientes coinfectados com tuberculose e HIV. Para tanto, utilizamos duas metodologias em nosso laboratório: detecção do fator corda e *PCR-restriction enzyme analysis* (PRA). O objetivo do estudo foi avaliar a acurácia desse teste de triagem em meio sólido como um método rápido para a identificação presuntiva do complexo *M. tuberculosis*, considerando custos e tempo de resultado. **Métodos:** Foram processadas 152 cepas pelo teste de triagem combinado, que consistiu da detecção do fator corda por microscopia (esfregaço corado por Ziehl-Neelsen) e avaliação do aspecto macroscópico das colônias, e PRA (padrão ouro). Os custos foram estimados através da obtenção dos preços dos insumos necessários para a realização de cada teste. **Resultados:** A acurácia da detecção do fator corda foi de 95,4% (IC95%: 90,7-98,1%) e a do teste de triagem combinado foi de 99,3% (IC95%: 96,4-100%). O custo da detecção do fator corda foi de R$ 0,60 e do PRA de R$ 16,00. Os resultados da detecção do fator corda estão prontos em 2 dias, ao passo que os de PRA necessitam de 4 dias. **Conclusões:** A identificação presuntiva de *M. tuberculosis* usando o aspecto macroscópico das colônias em conjunto com a detecção de fator corda por microscopia é um teste simples, rápido e de baixo custo. Recomendamos o teste de triagem combinado para rapidamente identificar *M. tuberculosis* em sítios com poucos recursos financeiros e em laboratórios menos equipados, enquanto se aguarda a identificação definitiva por métodos moleculares ou bioquímicos.

Descritores: Tuberculose; Mycobacterium/classificação; Reação em cadeia da polimerase; Testes diagnósticos de rotina.

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Introduction

Rapid differentiation between *Mycobacterium tuberculosis* and nontuberculous mycobacteria (NTM) is essential for the appropriate medical management of patients co-infected with tuberculosis (TB) and HIV or of patients with NTM and an underlying respiratory disease. Prompt specific treatment and patient isolation depend on the rapid and precise identification of the mycobacteria involved. This often represents a substantive challenge for the diagnostic laboratory. The identification of *M. tuberculosis* complex using traditional methods is time consuming. Such methods are based on bacterial growth on various substrates and require up to 15 days for the final identification. However, molecular methods are prohibitively expensive for use in developing countries, where the majority of cases of TB/HIV co-infection are concentrated.

Some studies have evaluated the utility of detecting cord formation, the so-called cord factor, present in *M. tuberculosis*, for the presumptive identification of TB, since the majority of NTM species lack this characteristic.[1-7] Cord factor is an effect caused by the major mycolic-acid containing molecule, trehalose-6,6'-dimycolate (TDM), a component of the mycobacterial cell wall, implicated in major immunomodulatory mechanisms that are responsible for mycobacterial virulence. It is also thought to play a fundamental role in the genesis and persistence of chronic and granulomatous lesions caused by mycobacteria.[8] The presence of TDM can be an important determinant for the successful infection and survival of *M. tuberculosis* within macrophages by inhibiting phagosome-lysosome fusion events during infection.[9,10] A few NTM species, such as *M. kansasii, M. terrae* and *M. phlei*, can occasionally present the cord factor.[11,12]

The morphological evaluation of mycobacterial colonies on solid medium is also useful for characterizing the species.[2,13] Whereas *M. tuberculosis* colonies are breadcrumb or cauliflower-shaped, dry, nonchromogenic and grayish-white or buff in color, NTM present a distinctive smooth, moist aspect. Some NTM isolates, especially the fast-growing ones, can, however, display morphological characteristics resembling those of *M. tuberculosis*. In a previous report, our mycobacteriology laboratory reported a screening test consisting of a combination of macroscopic morphological analysis of colonies on solid media and the detection of the cord factor by means of smear microscopy for the presumptive identification of *M. tuberculosis* complex. In a sample consisting of 2,601 *M. tuberculosis* isolates, the sensitivity and the specificity of cord factor detection for the diagnosis of TB were both 83%. Adding the microscopic evaluation of colonies (screening test) raised the sensitivity to 99%, with a specificity of 87%.[3] At the time of that study, molecular methods for confirming the species were not available in our laboratory.

The objective of the present study was to evaluate the accuracy of this screening test on solid medium as a rapid method for the presumptive identification of *M. tuberculosis* complex, considering PCR-restriction enzyme analysis (PRA) the gold standard.

Methods

The study was carried out in the Mycobacteriology Referral Laboratory at the Adolfo Lutz Institute, a public health laboratory in the city of São Paulo, Brazil. From April 2 to April 17 of 2007, we consecutively examined 152 strains of mycobacteria forwarded to the laboratory for susceptibility testing, species identification or both.

Strains were initially screened with Ziehl-Neelsen staining in order to detect the presence of the cord factor (Figure 1). Strains were also identified through the macroscopic observation of cultures by a different observer. Both observers were blinded to the gold standard result and to

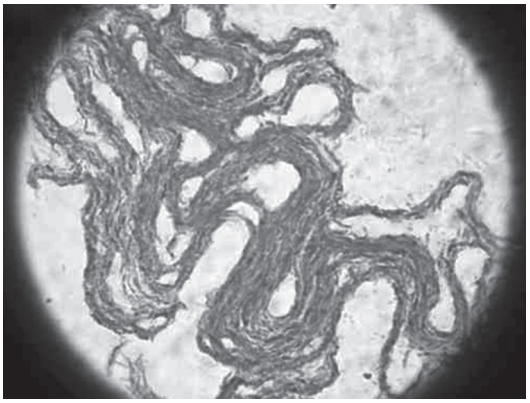

Figure 1 - Photomicrograph demonstrating cord formation (Ziehl-Neelsen staining).

the findings of the other. On the basis of our previous findings,[3] whenever there was discordance between the two tests, the morphological aspect was considered the final screening test result, except for cultures that were too dry or too humid and therefore unsuitable for evaluation. The PRA method[14,15] was used as the gold standard to confirm the identification. This molecular method consists of the amplification of a 441-bp fragment from the *hsp*65 gene by PCR followed by the enzymatic digestion with BstEII and HaeIII. Sensitivity, specificity and the corresponding 95% CIs were calculated.

Results

Of the 152 strains tested, 110 were identified as *M. tuberculosis* by PRA. Of those 110 strains, 106 demonstrated cord formation, corresponding to a sensitivity of 96.4% (95% CI: 90.9-99.0%; Table 1). Of the 42 NTM identified by PRA, 3 presented the cord factor, corresponding to a specificity of 92.9% (95% CI: 80.5-98.5%). When the macroscopic aspect of the colonies was also considered, the 4 *M. tuberculosis* strains yielding a false-negative result for cord factor had the typical rough aspect, increasing the sensitivity to 100% (95% CI: 97-100%). Among the 3 NTM strains yielding a false-positive result for cord factor, 2 had the typical aspect of NTM cultures, raising the specificity to 97.6% (95% CI: 87.4-99.9%). The remaining strains yielding a false-positive result for cord factor and identified as *M. peregrinum* by PRA were also suggestive of *M. tuberculosis* based on the macroscopic aspect. The overall accuracy of the detection of cord factor alone was 95.4% (95% CI: 90.7-98.1%), and that of the combined screening test was 99.3% (95% CI: 96.4-100%).

Table 1 - Comparison of the screening test and the PCR-restriction enzyme analysis method for the identification of *Mycobacterium tuberculosis*.

Screening test	PRA identification	
	Mtb	NMT
Mtb	110	1
NMT	-	41
Total	110	42

PRA: PCR-restriction enzyme analysis; Mtb: *Mycobacterium tuberculosis*; and NMT: nontuberculous mycobacteria.

Discussion

In the present study, the detection of cord factor by smear microscopy and the combined screening test consisting of cord factor detection and the morphological evaluation of the colony were both highly accurate in distinguishing *M. tuberculosis* from NTM. Although there was an overlapping between the CI for cord factor detection alone and that for the screening test, this was probably because of the high accuracy of cord factor detection alone. However, based on the simplicity of the macroscopic evaluation, we recommend adding this method to the detection of cord factor because of the clinical implications of false-positive and false-negative results.

In our sample, the accuracy of the screening test was entirely dependent on the macroscopic evaluation of the colony, since cord factor detection led to false results among the 7 discordant observations, 6 of which were corrected by the macroscopic evaluation of the colony. Nevertheless, in our previous study,[3] which involved a larger sample, both cord factor detection and the macroscopic evaluation yielded false results. In addition, the conditions of culture on a solid medium can interfere with the correct classification of the mycobacterium according to the morphological aspect of the culture. Water-saturated media will make rough colonies look smooth, whereas dry media will make smooth colonies look rough. These are the reasons why we recommend the screening test with both methods to rapidly distinguish *M. tuberculosis* from NTM species.

In order to accelerate the identification of species by means of culture in resource-poor countries, identification should be conducted in regional or local laboratories, rather than being restricted to the referral laboratory. However, such decentralization is difficult to achieve because the currently used identification method, biochemical characterization, is complex and requires several tests to achieve a conclusive result. Molecular methods are highly accurate and have become the gold standard for the identification of mycobacteria in many developed nations. Nevertheless, they represent a financial burden to developing countries: the cord factor detection costs US$ 0.25 per unit (observation of the macroscopic aspect will only add the cost of a few extra minutes spent by a

technician), whereas the PRA test costs US$ 7.00. Although an experienced technician is needed to correctly identify cord formation, this training is much faster and easier than is the training in molecular biology techniques, such as PRA. In addition, this screening test requires only a good microscope, whereas molecular biology methods require expensive equipment and complex maintenance, which are not available in less well-equipped laboratories in resource-poor countries. Furthermore, cord factor results are ready in 2 days, whereas the PRA test takes 4 days to yield results.

Other reports have shown the reliability of cord factor detection in liquid media.[5-7] Those studies were conducted in high-income countries, where laboratories employ modern methods of culture. In resource-poor countries with high incidence rates of TB, such as Brazil, solid medium, which provides similar results, is employed because of its lower cost.

In conclusion, although neither cord factor detection nor the morphological evaluation of colonies identifies NTM species, we recommend the combined screening test to rapidly identify *M. tuberculosis* colonies in resource-poor settings and in less well-equipped laboratories while awaiting definite identification by molecular or biochemical methods.

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