

## Diagnostic methods

### Contribution of conventional methods and PCR to the diagnosis of extrapulmonary Tuberculosis

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**INTRODUCTION:** Although lungs are usually the first organs to be affected by tuberculosis, other organs and systems may be endangered by a *Mycobacterium tuberculosis* infection. During 2006, Colombia registered 1700 extrapulmonary tuberculosis cases, i.e., 15 % of the total number of cases. Diagnosis is done through culture and PCR as diagnostic aid. **OBJECTIVE:** To determine the contribution of culture and PCR methods in the diagnosis of extrapulmonary tuberculosis. **METHODS:** Thirty six cases of suspected extrapulmonary tuberculosis referred back to the National Institute of Health Micobacterial Group during 2007 from different health institutions in Bogotá were studied. Samples were processed for culture, phenotype identification and PCR to amplify gene *hsp65*. **RESULTS:** Among the 36 samples under study, 30 were negative by PCR and culture and six were positive for *M. tuberculosis* complex; out of these, two were detected by culture, four through PCR and one using both methods. PCR results were reported in five days and culture results in three weeks. **CONCLUSIONS:** Culture is a sensitive and specific method for extrapulmonary tuberculosis diagnosis; however, its usefulness diminishes given the time needed to report results. Such findings allow us to conclude that PCR may be the diagnosis method to be used when the disease is highly suspected.

**Key Words:** Tuberculosis, extrapulmonary, PCR.

### Latency antigens as sensors of different stages of tuberculosis (TB)

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**INTRODUCTION:** Two thousands millions people may suffer latent Tb infections. Latently infected individuals provide a highly relevant population to study what human protective immune responses against *Mycobacterium tuberculosis* look like. **OBJECTIVE:** To test latency antigens and infection markers so as to identify latently infected individuals. **METHODOLOGY:** Whole blood was stimulated with: latency antigens (HspX, Rv2624c, Rv2626c), CFP10, PPD and Pokeweed mitogen as

controls. Interferon gamma (IFN- $\gamma$ ) was measured in stimulated plasmas belonging to seven cohorts: 1<sup>st</sup>) Mantoux negative\*; 2<sup>nd</sup>) Mantoux positive; 3<sup>rd</sup>) TB patients without antibiotic treatment; 4<sup>th</sup>) TB patients with antibiotic treatment; 5<sup>th</sup>) ex TB patients; 6<sup>th</sup>) relapse patients; 7<sup>th</sup>) patients with other pulmonary diseases. Four out of 8 individuals of cohort 1<sup>st</sup> are working at TB lab. The others are contacts of TB patient. RESULTS: HspX produced high levels of IFN- $\gamma$  (1<sup>st</sup> 2<sup>nd</sup> 4<sup>th</sup>). Middle levels of IFN- $\gamma$  were observed (5<sup>th</sup>) Low responses were shown (3<sup>rd</sup> 6<sup>th</sup> 7<sup>th</sup>) Rv2624c and Rv2626c produced very low levels of IFN-g in all cohorts. CFP10 showed variable values of stimulation (2<sup>nd</sup> 3<sup>rd</sup> 5<sup>th</sup> 6<sup>th</sup>) Low levels of IFN $\gamma$  were produced as expected (1<sup>st</sup>, 7<sup>th</sup>). CONCLUSIONS: HspX produces high levels of IFN- $\gamma$  in healthy individuals (1<sup>st</sup> 2<sup>nd</sup>) and in those with antibiotic treatment (4<sup>th</sup> 5<sup>th</sup>). First cohort is latently infected but couldn't be detected by Mantoux. Rv2624c and Rv2626c don't show a stimulation pattern similar to HspX. CFP10 doesn't show its condition of infection marker (small size of the sample) Immunodiagnostic can be improved by latency antigens as a contribution to detect latently infected individuals.

**Key Words:** Tuberculosis, Latency antigens, IFN-  $\gamma$ .

### **Pleural fluid adenosine deaminase activity for the diagnosis of pleural tuberculosis**

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Adenosine deaminase (ADA) activity has been shown as a useful marker for the diagnosis of pleural tuberculosis, however reported sensitivity and specificity differ between laboratories and settings. We determined the sensitivity and specificity of ADA activity for the diagnosis of pleural tuberculosis in HIV negative adult patients from Caracas, Venezuela. ADA activity in pleural liquid was determined with the method of Giusti and activity of  $\geq 40$  U/L was considered positive. To determine the sensitivity of the ADA assay we reviewed the ADA activity in exudative pleural fluids that were culture positive for *Mycobacterium tuberculosis*. To determine the specificity, the ADA activity was examined in exudative pleural fluids showing predominantly mononuclear cells from patients with a diagnosis other than tuberculosis. Of the 91 culture positive pleural fluids, 87 had an ADA level considered positive. Of the 30 lymphocytic pleural exudates from patients with a diagnosis other than tuberculosis, 3 had elevated ADA levels. The sensitivity and the specificity of the ADA assay for the diagnosis of tuberculous pleural effusions was calculated as 95.6 % and 90 % respectively. This is an ongoing study. Preliminary results show that ADA determination is highly sensitive and specific for the diagnosis of tuberculous pleural effusions. In addition, the determination of the ADA activity is fast, economic and has a better sensitivity than other available laboratory assays for the diagnosis of pleural tuberculosis, i.e. 80 % sensitivity for histopathology of pleural biopsies, and 40 % for culture of pleural fluid.

**Key Words:** Pleural tuberculosis, sensitivity, specificity.

### **A simple algorithm based on the adenosine deaminase activity in cerebrospinal fluid for the diagnosis of tuberculous meningitis in HIV positive patients**

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Tuberculous meningitis (TM) is one of the leading causes of opportunistic meningitis in AIDS patients. The diagnosis with smear from cerebrospinal fluid (CSF) has low sensitivity and culture takes at least 3 weeks before coming positive. Therefore, there is a need for alternative diagnostic methods. Adenosine deaminase (ADA) activity determination in CSF is considered a specific test for the diagnosis of tuberculous meningitis. However false positive results due to cryptococcal infection have been reported. We evaluated the utility of the determination of ADA activity in CSF of HIV positive patients for the diagnosis of TM. CSF from 19 HIV positive patients that contained predominantly mononuclear cells (>50 %) were processed for culture of *Mycobacterium tuberculosis*. ADA in CSF was determined with the method of Giusti (positive  $\geq 10$  U/L), and possible infection with *Cryptococcus neoformans* was evaluated with the latex agglutination test (LAT). Of the 19 patients, 12 had elevated ADA levels, of which 4 were positive for *C. neoformans* by LAT. In 7 of the 8 LAT negative patients with high ADA levels, tuberculosis (TB) treatment was started. All improved with TB treatment and in 4 cases culture confirmed TB 4 weeks later. In one patient with a high ADA level who died, post mortem examination diagnosed histoplasmosis, toxoplasmosis and a lymphoma. In the 7 patients with low ADA levels there was no evidence of TB, but a variability of other infections [toxoplasmosis (n=2), syphilis (n=1) and cryptococcal meningitis (n=4)]. We conclude that in our setting, in HIV positive patients with meningitis and CSF leukocytes less than 50 % polymorphs, and a negative LAT, an elevated ADA level ( $\geq 10$  U/L) indicates a sufficiently high probability of TM (P=0.875) to begin TB treatment. The determination of ADA in combination with the LAT is at least as accurate as or better than other available laboratory assays for the diagnosis of TM.

**Key Words:** Tuberculous meningitis, *Cryptococcus neoformans*, latex agglutination test.

### **Comparative performance of two in-house methods for drug resistance assessment in *Mycobacterium tuberculosis* isolates**

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Drug resistance evaluation in clinical isolates of *Mycobacterium tuberculosis* by the gold standard method (Proportion Method) requires up to 50 days. Novel methods such as REMA (Resazurin Microtiter Assay) and PhaB (Phage Replication Based Assay) shorten that time to one week or less. Since these methods are simple and do not require special equipment we tested the feasibility of their use at our low-complexity mycobacteriology laboratory, comparing their performance against the

Proportion Method. Sixty nine clinical isolates and two lab reference strains (*M. tuberculosis* H37Rv and *M. bovis* var. BCG) were tested for their susceptibility to Rifampicin, Isoniazid, Streptomycin and Fluoroquinolones. Our results showed for REMA and PhaB the following sensitivity and specificity: Rifampicin, 97.6 % and 100 %; Isoniazid 93.6 % and 100 %; Streptomycin, 94 % and 100 %. The turn-over time was between two and four days. Although REMA correctly evaluated the sensitivity of all the tested strains to Ofloxacin, PhaB failed on that task, misidentifying 20 out of a subset of 29 sensitive strains as resistant regardless of the fluoroquinolone used or the length of treatment. The replication of D29 -the mycobacteriophage used in the PhaB protocol- fell three logs upon Nalidixic acid treatment of *M. smegmatis* cultures suggesting that PhaB should be able to differentiate between Fluoroquinolone resistant and sensitive strains. Regardless of that failure, both REMA and PhaB proved to be simple to perform and reliable for drug resistance assessment even in clinical settings with little equipment, allowing prompt detection of drug and multi-drug resistant *M. tuberculosis* isolates.

**Key Words:** *M. tuberculosis* clinical isolates- drug susceptibility assessment - rapid methods.

### **Molecular markers of tuberculosis disease progression in patients with or without diabetes Mellitus Type 2**

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No molecular methods are available to identify tuberculosis disease progression in human risk groups, including diabetic patients. Transcriptome analysis has been used to identify differential gene expression associated to several human pathologies. Thus, microarrays represent a good strategy to identify molecular markers of disease progression in tuberculosis. **OBJECTIVES:** To identify the transcriptional profile associated to active human pulmonary tuberculosis using microarray technology. **METHODS:** Oligonucleotides custom microarrays (CombiMatrix 4X2K, USA) were designed and build with gene sequences related to active pulmonary tuberculosis (TBPA), inflammatory response, antimicrobial peptides and DM2 pathogenesis. Blood from individuals with TB latent infected (TBLT), TBPA and non-TB infected (Control), from DM2 y non-DM2 subgroups previously selected by clinical and laboratory criteria was collected (6 individual per group, 36 in total), in PAXgene tubes and total RNA extracted, enriched and concentrated. Globin transcripts reduction and biotinylated-cRNA synthesis was done. Fragmented labelled-cRNA transcripts were hybridized to microarrays according to manufacturer's instructions (CombiMatrix, USA). Statistical analysis was performed using SAM 1.15 software. Cluster analysis for gene expression levels was done with "Gene Cluster 2.11 and processed by TreeView 1.60 software. **RESULTS:** Specific transcriptome including 103 TBPA associated genes were identified in non-DM2 patients. Only 11 of them were associated to TBPA from both DM2 and non-DM2 patients, and have statistical significance according to SAM analysis.

**CONCLUSIONS:** We identified a specific transcriptional signature that distinguishes DM2 or non-DM2 patients with active TBPA from those with TBLT or non TB infected individuals.

**Key Words:** Microarrays Analysis, Diabetes mellitus 2, Tuberculosis progression.

### **Usefulness of new methodologies for the rapid diagnosis of Tuberculosis**

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**INTRODUCTION:** Actual TB situation including MDR or XDR-TB has pointed to a change in tuberculosis or micobacteriosis diagnosis. In the recent years multiple efforts to improve TB-diagnosis have been reported; those proposals with applications in laboratories with low income and high number of clinical samples are ideal. Among them, are methodologies like thin layer plate that in combination with "in house 7H9 tube" could reduce costs and time for bacterial recovery from clinical samples. **PURPOSE OF THE STUDY:** To determine the usefulness of thin layer agar and "in house 7H9" for the recovery of micobacteria from pulmonary samples. **METHODS:** To determine the usefulness of thin layer agar and "in house 7H9 tube", a total of 250 pulmonary samples from the same number of patients, were decontaminated and seeded on both culture mediums and Lowestein-Jensen. Plates and culture tubes were checked frequently for any mycobacterial growth evidence. Culture purity was determined and a multiplex PCR was performed to confirm that recovered bacteria belonged to *Mycobacterium* genus and to MTB complex, in case of non-tuberculosis micobacteria recovery, speciation was done by sequencing. Drug susceptibility of each isolate was performed by microplate alamar blue assays. **RESULTS:** A total of 50 clinical isolates were recovered, 49 of them were identified as MTB-complex, and the only one non-tuberculous micobacteria isolate was identified as *Mycobacterium abscessus*, the combination of thin layer plate and "in house 7H9 tube" allowed the maximum bacterial recovery in the shortest time period in comparison with Lowestein-Jensen medium. Only one of the MTB-complex clinical isolate was MDR and the *Mycobacterium abscessus* isolate was sensitive to clarithromycin and moxifloxacin. **CONCLUSIONS:** The combination of thin layer plate and "in house 7H9-tube" allowed rapid and efficient micobacteria recovery hence improving TB-diagnosis.

**Key Words:** MTB-complex, diagnosis, MDR, XDR-TB.

### **Contribution of spoligotyping to quality control in molecular biology procedures**

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**INTRODUCTION:** Cross contamination in tuberculosis diagnosis laboratories may result in false positives and mistaken molecular characterization. Molecular epidemiology tools have enabled a better knowledge of cross contamination as well as the implementation of quality control in lab procedures. **OBJECTIVE:** To implement quality control in DNA extraction procedures through spoligotyping. **METHODS:** Quality control was done to 220 DNA extraction procedures carried out from March 2007 to March 2008. Patterns obtained through spoligotyping were compared with those recorded in the National Institute of Health (INS) data base. **RESULTS:** No cross contamination was detected in 99.5 % of DNA extraction procedures. In 0.5 % of procedures cross contamination occurred as two different molecular patterns were obtained. **CONCLUSIONS:** Annually, the National Institute of Health Mycobacterium Group carries out a considerable number of molecular procedures; however, cross contamination does not exceed the 3 % limit reported in the literature, indicating that the lab working conditions comply with quality standards and that the results are, therefore, reliable and valid.

**Key Words:** Cross contamination, *Mycobacterium*, spoligotyping.

### **Utility of PCR (polymerase chain reaction) in extrapulmonary Tuberculosis diagnosis**

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Extrapulmonary tuberculosis cases have recently increased. In some instances, it is necessary to take a biopsy fixed in paraffin. The diagnosis is usually realized in pathology laboratories by microscopy analysis; however, unfortunately, specificity and sensitivity from this method is low.

Nucleic acid amplification (NAA) constitutes a rapidly evolving improvement in the detection and identification of *M. tuberculosis*; this test is usually highly specific for *M. tuberculosis* (close to 100 %). Polymerase chain reaction (PCR), based on repetitive elements such as IS6110, specific for members of *Mycobacterium tuberculosis* complex, has improved the diagnosis in these cases, showing better specificity and sensitivity. **OBJECTIVE:** To establish the potential use of PCR IS6110 in tissue samples embedded in paraffin.

**MATERIALS AND METHODS** Samples were included from tissues embedded in paraffin, collected in the Mycobacteria Laboratory of the Universidad Nacional de Colombia, between 2007 and 2008. A number from 5 cuts of tissue fixed samples were used for DNA extraction, based on the CHELEX100 method. The PCR IS6110 was obtained as previously described and clinical records from positive patients were evaluated. **RESULTS:** A total from 25 samples were collected and seven samples showed positive results for IS6110 amplification.

Biopsies were obtained from lymph node, bone spine, skin, esophagus, and adrenal mass. In most cases, results from pathological exams showed granulomatous lesions with caseating granulomas. Immunosuppression included two patients with HIV and metastatic CA.

**CONCLUSION:** PCR IS6110 is a simple and fast method for tuberculosis extrapulmonary diagnosis. The coordination of clinical evaluation, pathological exam, and PCR are necessary for accurate diagnosis.

**Key Words:** Tuberculosis, PCR IS6110, Paraffin Embedding, Tissue Embedding.

### ***Mycobacterium tuberculosis* DNA extraction from formalin-fixed paraffin-embedded tissues: a comparison of five different protocols**

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The detection of *M. tuberculosis* from formalin fixed paraffin embedded tissues (FFPET) by histochemical staining is low sensitivity; therefore, the PCR application in FFPET is used to confirm mycobacterial infections in suspected lesions. **OBJECTIVE:** To compare different protocols from DNA extraction to *M. tuberculosis* from FFPE tissues for use in PCR. **METHODS:** A biopsy from the lung was used, obtained from a patient with a clinical and histopathological diagnosis of Tuberculosis. Five different DNA extraction protocols were selected: Chelex 100 (BioRad); non-organic reagents; Triton-Chelex 100; Chelex 100 – phenol chloroform purification; and Spin column purification-based protocols (Quiagen). Cuts from different sizes were used (5, 10, and 15 µm). The quality and quantity from DNA obtained was estimated by both spectrophotometry (OD260/OD280) and electrophoresis on agarose gel methods. DNA presence from *M. tuberculosis* was evaluated through a PCR based on IS6110. **RESULTS:** Genetic material, with good quality and enough quantity, was obtained by the Chelex-100 method and Spin column purification-based protocols. No differences were observed for the different sizes that were evaluated, and DNA obtained through these two methods allowed for IS6110 amplification. **CONCLUSIONS:** The Spin columns purifications- based protocols provide DNA of good quantity and quality; and the Chelex-100 method is less expensive, rapid, and easy for *M. tuberculosis* DNA extraction from FFPET.

**Key Words:** DNA, *Mycobacterium tuberculosis*, Paraffin Embedding, Tissues Embedding.

### Identification of genomic and lipidic markers for *Mycobacterium colombiense* differentiation from other MAC strains

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*Mycobacterium colombiense* is an opportunistic non-tuberculous mycobacterial specie recently isolated from Colombian HIV patients. This novel specie is closely related to the *Mycobacterium avium* complex (MAC) according to biochemical and molecular analysis. Although of epidemiological interest, no genotypic or phenotypic markers for an easy differentiation of *M. colombiense* from other MAC strains have been defined.

In this work we use Randomly Amplified Polymorphic DNA (RAPD) analysis and thin layer chromatography (TLC) in order to identify genomic and lipidic markers for *M. colombiense* differentiation. Single fifteen primers with arbitrary sequence were tested to generate RAPD markers. Three decamers (OPA 03, OPA 04 and OPE 01) allowed differential genomic amplifications of six unique fragments that distinguished *M. colombiense* from *M. avium*. TLC analysis showed a complex glycopeptidolipid (GPL) pattern of *M. colombiense* as expected for MAC strains. In addition, neutral red staining of *M. colombiense* strains showed possible differences in virulence level that could be correlated with lipid profiling.

**Key Words:** non-tuberculous mycobacteria, RAPD, cell wall, neutral red.

### Improving bovine tuberculosis diagnostic

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The official test diagnostic in Argentina for *Mycobacterium bovis* in cows is the skin test of tuberculin or purified protein derivative (DPP) but it is questioned. AIMS: Evaluation of different diagnostic tests and identification of new specific proteins in *M. bovis* experimentally infected animals. METHOD: Ten 6 months old calves tuberculosis-free were challenged intratracheally with virulent *M. bovis*. Tuberculin skin test and IFN $\gamma$  was measured with specific recombinant proteins. Animals were killed and necropsied. RESULTS: There were no correlation between the skin test results and the illness advance as determined by lesions score. All the animals presented a high level of IFN $\gamma$  when stimulated with PPD, CFP10, ESAT6 and EsxI. Out of 5 of 10 animals responded to Rv2624 and two of them also responded to HspX. Rv2626 showed a low index of OD. Rv0138, Rv2524, Rv3740 and Rv1636 showed IFN $\gamma$  liberation levels in the 60 % of the animals. The necropsy showed different size of injuries and *M. bovis* was isolated. DISCUSSION The response to ESAT6, CFP10 and EsxI was high in experimentally infected

animals. Respect to latency antigens: Rv2626 was not recognized and this could be indicate that in none of the animals the *Mycobacterium* was in a latency state, HspX could be in an intermediate latency state indicated by the index obtained in two animals and Rv2624 showed a similar behavior compared with the infection markers (ESAT6 and CFP10). We cannot postulate if there is a transition from the active state to the latent one of the mycobacterium since they animals were analyzed at a single moment.

**Key Words:** *Mycobacterium bovis*, interferon-gamma, diagnosis.

### **Antigenic analysis of mycobacterial purified protein derivatives (PPDS)**

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**INTRODUCTION:** In the veterinary field, IFN $\gamma$  released in response to bovine PPD (PPDb) and avian PPD (PPDa) are considered good indicators of *M. bovis* and *M. avium* infection. However the sensitivity and specificity of the immunological assay is under discussion. The exact composition of PPDs is largely unknown, thus the characterization of these antigenic mixtures could represent a first step to a rational design of a recombinant defined mixture. **OBJECTIVE:** To evaluate the presence of a set of antigens in PPDs. **METHODS:** Two experimental approaches were performed. Polyclonal antibodies raised against the antigens were obtained in BALB-c mice and these sera were used to detect by immunoblotting the presence of the antigen in avian/bovine PPDs (Biocor) (*Direct* protocol). The *Indirect* approach used hyperimmune sera against each PPD to further detect the blotted antigen by Western blot. Some well characterized antigens and others under study in our lab were selected: Hsp70, LprG, Lpp34, Apa, LAM, Ag85, BfrA, DosR, Rv2626, Rv2628, Rv1636, L7/L12, Rv2524, HspX, CFP10. **RESULTS:** Preimmune sera did not show signal in the blots containing PPDs or individual antigens. Orthologous antigens as LprG, Hsp70, Ag85 and LAM were equally present in both PPDs. Apa antigen was more abundant in PPDa in comparison to PPDb. Antigens Rv1636 and L7/L12 were highly represented in PPDb while CFP10 and HspX could be under-represented in PPDb because they were no detected by the *Indirect* protocol however the *Direct* protocol rest to be assayed.

**CONCLUSIONS:** A differential representation of some orthologous antigens was found. The *Direct* protocol was more sensitive than the *Indirect* protocol.

**Key Words:** PPD, diagnosis, antigen.

**Direct detection of rifampin resistance in *Mycobacterium tuberculosis* by the nitrate reductase assay applied directly in sputum samples**

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A cost-effective and rapid drug susceptibility testing (DST) method is required to guide TB treatment. Commercially available systems such as BACTEC MGIT 960 and MB/BacT are faster but demand costly equipment and supplies, therefore, are not feasible in most resource-poor settings. Resistance to rifampin (RIF) is an important predictor for the early diagnosis of MDRTB. To compare the nitrate reductase assay (NRA) with the proportion method (PM) to detect RIF resistance in *M. tuberculosis* directly from sputum samples. The study was carried out by 4 regional laboratories from the state of São Paulo, Brazil. A total of 210 sputum samples tested smear positive from patients with pulmonary tuberculosis. The sputum was decontaminated by Petroff method and DST to RIF was carried out using the PM and the NRA. The results of the DST obtained directly from sputum were: 6 samples resistant to RIF and 204 samples susceptible. No discordance was observed between the two methods. The sensitivity and specificity of the NRA was 100 %. Results were available in 10 days for 75 (36 %) samples, 15 days for 107 (51 %) samples and 20 days for 28 (13 %) samples. The results of PM took 30 days to be available. The NRA proved to be a promising method for the screening of suspect MDRTB cases directly from sputum samples. The simplicity of the method, its low cost and celerity to give the results make it a good alternative method for laboratories in resource-poor settings.

**Key Words:** rifampin resistance, nitrate reductase assay, direct detection.

**Cord formation: a good tool for presumptive identification of *M. tuberculosis***

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Some studies have evaluated the utility of cord formation in liquid or solid medium for the presumptive identification of *M. tuberculosis* and to guide decisions regarding susceptibility testing or species identification. To evaluate the cord formation test in order to introduce it as a screening method, taking also into account the cost and the time it takes to get the result. A total of 152 strains were tested by the screening test consisting on microscopy examination (smear stained by Ziehl-

Neelsen) and the culture macroscopic observation. PCR restriction enzyme analysis (PRA) was used as gold standard to ascertain the confirmed identification result. There was disagreement between the two methods in only one strain (0.6 %). In the 6 cases where there was a preliminary disagreement between the cord formation test and the PRA, the evaluation of the macroscopic aspect resulted in agreement with the gold standard. The co-positivity of the screening test was 100 % and the co-negativity was 98 %. The cord formation costs US\$ 0.25 whereas the PRA test costs US\$ 7.00. The result of the cord formation test is ready in 2 days, whereas the PRA test needs 4 days. The presumptive identification of MT using macroscopic analysis of colony morphology associated with the presence of cording on microscopy is a simple, rapid and low-cost test. The results showed that this method can be introduced in the laboratory network as presumptive method in the tuberculosis diagnosis, before sending the culture to a reference center for confirmatory tests.

**Key Words:** cord formation, *M. tuberculosis* identification.