**Implementation of the thin layer agar for the diagnosis of smear-negative**

**pulmonary tuberculosis in a high HIV prevalence setting in Homa Bay, Kenya**

**Authors:**

Anandi MARTIN*[1,2], Peter MUNGA WAWERU[†3], Fred BABU OKATCH[3], Naureen AMONDI OUMA[3], Laurence BONTE[2], Francis VARAINE[2], and Françoise PORTAELS[1]

[1]*Institute of Tropical Medicine, Antwerp, Belgium;* [2]*Médecins Sans Frontières, Paris, France;* [3]*Homa Bay District Hospital, Kenya*

**Running title:**

TLA for diagnosis of smear-negative pulmonary tuberculosis

**\*Corresponding author:**

Dr. Anandi MARTIN

Mycobacteriology Unit, Institute of Tropical Medicine

Nationalestraat, 155, Antwerp, B-2000-BELGIUM

Phone: 32-3 2476334, Fax: 32-3 2476333

E-mail: amartin@itg.be

[†]This work is dedicated to the memory of Peter MUNGA WAWERU

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**Abstract**

The objective of this study was to evaluate the performance of a low-cost method, the Thin Layer Agar (TLA), for the diagnosis of smear-negative patients. This prospective study was performed in Homa Bay district Hospital in Kenya. Out of 1584 smear-negative sputum samples, 212 were positive by Löwenstein-Jensen (LJ) (13.5%) and 220 positive by TLA (14%). The sensitivity of LJ and TLA was 71% and 74 % respectively. TLA could become an affordable method for the diagnosis of smear-negative tuberculosis in resource-limited settings with results available within 2 weeks.

Prevalence of smear-negative pulmonary tuberculosis (TB) has been increasing in countries with high HIV incidence especially in low-income countries where many patients are HIV/TB co-infected and where culture of *M. tuberculosis* is often not available (3, 4, 6, 13, 15). Incidence of TB in Kenya is estimated at 384/100 000 inhabitants (23). Kenya ranks 13th on the World Health Organisation (WHO) list of the 22 high-burden countries for TB worldwide and HIV prevalence in new TB cases is estimated at 52% (23). Culture is more sensitive than smear microscopy (1, 3, 7), but Löwenstein-Jensen (LJ) medium is very slow. The Thin Layer Agar (TLA) has been described as a simple, rapid and inexpensive method (11, 12, 18) allowing initial identification of *M. tuberculosis* based on colony morphology visualized microscopically, and by incorporation of para-nitrobenzoic acid (PNB) in the medium (10, 17, 19). We performed a prospective study to evaluate the performance of the TLA method for detection of *M. tuberculosis* in smear-negative samples as compared to cultivation in LJ medium.

The study was conducted in Homa Bay district Hospital, Kenya. All smear-negative respiratory samples from suspect TB patients received between November 2007 and September 2008 were included. A total of 1584 smear-negative samples were analyzed. Sputum was digested and decontaminated using the sodium hydroxide–N-acetyl-L-cysteine (NaOH/NALC) method (9). LJ was examined twice weekly up to 8 weeks (22). Cultures were considered positive for *M. tuberculosis* according to their morphological characteristics, a positive acid-fast bacilli (AFB) staining, and inhibition of growth by PNB performed on TLA (8). A culture was considered negative if no growth was observed after 8 weeks, and was considered contaminated if there was growth but was negative for AFB. TLA plates were prepared as previously described (18) with small modifications. One hundred µl of decontaminated sample was inoculated on a bi-plate Petri dish of 100mm x 15mm (Becton Dickinson, Sparks, MD USA) containing 20 ml of 7H11 agar supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase) (Becton Dickinson) plus piperacillin, trimethoprim and amphotericin B (Sigma Aldrich) at 0.05 μg/ml, 0.02 μg/ml and 0.02 μg/ml respectively. PNB at 500 µg/ml was incorporated in one compartment. The plates were sealed with sterile parafilm leaving a space of 1-2 cm and incubated at 37°C in 5% $CO_2$. Plates were

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checked after 24 hours for contamination and examined twice weekly up to 6 weeks using a standard microscope. A positive culture was identified by the characteristic cord of *M. tuberculosis* growth. Non-tuberculous mycobacteria (NTM) were recognized by their lack of cording and growth on PNB. Fungal or bacterial contamination was recognized by rapid overgrowth of the plates.

The reference standard was considered when a sample was positive in any of the two culture media, TLA or LJ. Sensitivity was calculated as the number of positive cultures on LJ or TLA divided by the total number of positive cultures in any culture media. The Wilcoxon test for non-normal distribution was applied to compare differences in time to detection between the two culture media; paired comparisons were performed excluding contaminated specimens. A *P* value < 0.05 was considered to be statistically significant. Analysis was performed with MedCalc® version 9.6.4.0 (MedCalc Software, Mariakerke, Belgium). The time to detection of growth and contamination rates were recorded for each culture medium.

Out of 1584 smear-negative samples, 212 were positive by LJ (13.5%) and 220 positive by TLA (14%). All positive cultures were presumptively identified as *M. tuberculosis*. Eight NTM isolates were identified by growth on PNB, morphology and colony pigmentation and molecular identification was performed at the Reference TB laboratory in Antwerp, Belgium for final confirmation. The NTM found were *M. gordonae*, *M. flavescens* and *M. kubicae.* Out of the 1584 samples, 1099 (69.5 %) were culture negative by LJ and 951 (60%) by TLA. Two-hundred and five (17%) samples inoculated on LJ, and 325 (26%) of TLA were contaminated. The sensitivity of LJ was 71% and 74 % for TLA. The median time to growth was 23 days for LJ and 14 days for TLA (P<0.0001).

Among the positive cultures, the combination of both media increased the positivity. One hundred and thirty five positive cultures on LJ were also found positive on TLA. Forty seven positive cultures found on LJ were negative on TLA, and 30 positive cultures on LJ were found contaminated on TLA. For TLA, 47 positive cultures were found negative on LJ and another 38 positive cultures were found contaminated on LJ. Figure 1 shows the average time to obtain a positive culture on LJ and TLA. More than 70% of cultures were found positive between 8 and 14 days on TLA while the majority of cultures were found positive on LJ between 15 and 22 days and after 23 days. Figure 2 shows a typical cord

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formation characteristic for *M. tuberculosis* on TLA (picture A) and cords together with contaminants (picture B).

This is the first operational study performed in a low-income country, in a rural setting, that demonstrates that the TLA method performed as well as the LJ for the diagnosis of smear-negative samples, having a higher speed for results than culture on LJ. All reagents were available from local suppliers. TLA was able to differentiate presumptively between *M. tuberculosis* complex and NTM which is an advantage compared to LJ. The support of other facilities for complete species identification is still required. Another method called the microscopic observation drug susceptibility (MODS) assay (14) is similar to TLA, but the disadvantage is the use of liquid medium and the need of an inverted microscope. Contamination was a serious problem in our study and caused the loss of several samples. The rate of contamination was 17% for LJ and 26% for TLA. We have to point out that contamination was high in both media not only in TLA. The concentration of NaOH used in the decontamination was 2% but could be increased up to 4% to attempt reducing contamination. However, care should be taken to avoid the toxicity for mycobacteria that may cause negative culture results (21). Another important point could be the delay between sputum collection and processing, but in this study, it was only 2-3 days and samples were kept at 4°C. Concerning antibiotic mixtures, they are frequently used to inhibit the growth of contaminants and the concentration of piperacillin, trimethoprim and amphotericin B used in this study could be increased, since they were rather low. Preliminary results using a concentration of 4.0 µg/ml of the three antibiotics showed that the contamination rate in TLA decreased to less than 10% (Martin et al., unpublished). Piperacillin is active against many gram-positive and negative bacteria, trimethoprim is a bacteriostatic agent, and amphotericin B is an antifungal drug. The most common contaminants were *Candida albicans*, *coagulase-negative Staphylococcus, Staphyloccocus aureus, Pseudomonas species,* and *Aspergillus*. Since some contamination was due to *S. aureus*, one possibility is the addition of vancomycin to TLA to reduce this contamination. We noticed that the contamination rate decreased slowly during the study. Such improvement suggests the need to set up a training period during which technicians can become familiar with culture to easily recognize cord specific of *M. tuberculosis*. In the few published studies

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using TLA, the contamination rate was reported as 5-16% (2, 11, 12, 18). However, those studies were carried out in reference laboratories in middle-income countries with smear-positive contrary to the present study performed in rural laboratory in a low-income country on smear-negative samples. Concerning the workload, in Homa Bay, an average of ten samples per day is inoculated into both media in duplicate. At least two technicians should be dedicated full time to prepare the media, registration of data, decontamination, inoculation and reading of TLA plates and LJ tubes. A cost-effectiveness study is ongoing to evaluate the impact of the introduction of the TLA to diagnose TB in smear-negative patients. Despite the high contamination rate found in both media, we believe that TLA could be implemented in TB/HIV settings to increase the TB case detection in smear-negative. TLA is faster than conventional culture, practical and inexpensive and allows the presumptive identification of *M. tuberculosis* complex. Smooth variants of *M. tuberculosis* have been described and named *M. canettii* (20). These variants are however infrequent and have been found only until now in the horn of Africa (16). TLA provides an alternative method when more sophisticated techniques are not available or affordable to implement.

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**Figure 1.**

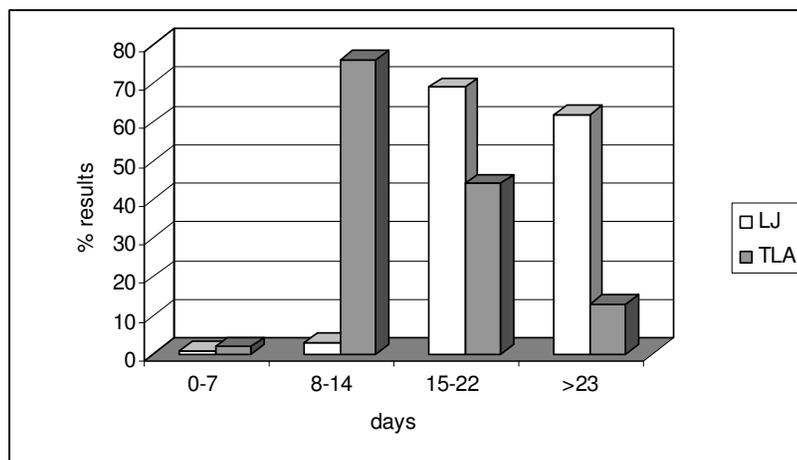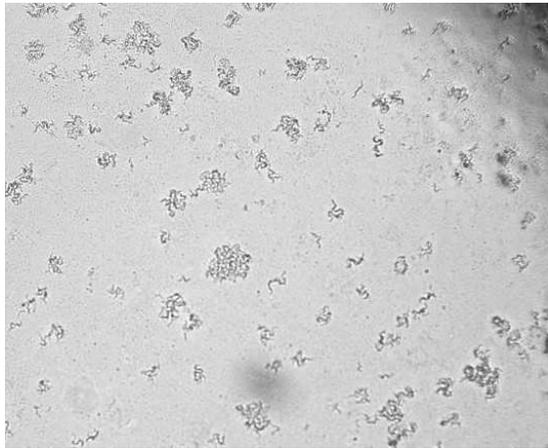Time to detection (days) of *M. tuberculosis* culture positive results in TLA and in LJ
medium

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A                                                            B

**Figure 2.**

Micro-colonies of *M. tuberculosis* in TLA seen under microscope (10x objective).

A: characteristic cord formation of *M. tuberculosis*. B: cord and contamination (filament)

together.

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