

Evaluation of a rapid culture method for tuberculosis diagnosis: a Latin American multi-center study

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SUMMARY

SETTINGS: Tuberculosis (TB) diagnostic laboratories in Latin America.

OBJECTIVES: Evaluation of thin-layer agar (TLA) compared to Löwenstein-Jensen (LJ) culture for the diagnosis of TB.

DESIGN: Phase II prospective study in six laboratories. Samples included sputum and extra-pulmonary specimens from patients with a clinical diagnosis of TB. Respiratory samples were decontaminated using NaOH/NALC; all samples were centrifuged, stained with Ziehl-Neelsen for acid-fast bacilli (AFB), cultured on LJ and TLA and identified according to recommended procedures. Sensitivity and likelihood ratios (LR), growth detection time and contamination rate were calculated for both media.

RESULTS: A total of 1118 clinical specimens were studied. Cultures detected *Mycobacterium tuberculosis* in all

AFB-positive samples, whereas for AFB-negative specimens LJ detected 3.2% and TLA 4.4%. Sensitivity was 92.6% (95%CI 87.9–95.9) and 84.7% (95%CI 78.8–89.0) for TLA and LJ, respectively. Positive and negative LRs were similar. Contamination was 5.1% for TLA and 3.0% for LJ. Median time to detection of a positive culture was 11.5 days (95%CI 9.3–15.0) for TLA and 30.5 days (95%CI 26.9–39.0) for LJ ($P < 0.0001$).

CONCLUSION: Difference in the characteristics of the participating laboratories, the disease prevalence and the number and type of specimens processed did not affect the overall performance of TLA as compared to LJ, supporting the robustness of the method and its feasibility in different laboratory settings.

KEYWORDS: *M. tuberculosis*; isolation; tuberculosis; diagnosis

TUBERCULOSIS (TB) continues to be a life-threatening disease that predominantly affects developing countries. Global control efforts have focused on the implementation of the DOTS strategy (the World Health Organization [WHO] TB control strategy) in which at least 70% of smear-positive patients are identified and placed in a program of directly observed therapy.¹ Although the classical diagnostic method, employing microscopic examination of sputum smears, forms an integral part of the DOTS strategy, this technique can diagnose only about 50–60% of TB patients, and it has very limited sensitivity in patients with paucibacillary forms of pulmonary and extra-pulmonary disease.^{2,3}

Culture of clinical specimens is more sensitive than smear microscopy, as only 10–100 viable organisms will result in a positive culture while a minimum of 5000–10 000 acid-fast bacilli (AFB) per ml are required for detection by smear.⁴ In addition, isolates obtained from cultures can be used for mycobacterial species identification, determination of drug susceptibility and molecular epidemiology. Although the use of culture has been advocated for routine diagnosis,^{5,6} it requires 3–4 weeks to detect mycobacterial growth, plus an additional period for identification of *Mycobacterium tuberculosis*.⁷

Most recent innovations in diagnostic techniques have involved new technologies. Systems such as

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BACTEC®, which use radioactivity or fluorescence as indicators of bacterial growth in liquid media, have been extensively evaluated,^{8–10} and have become the standard for both primary culture and drug susceptibility testing in industrialized countries.^{6,11} While some rapid commercial molecular techniques are as sensitive as liquid cultures for diagnosing TB,^{12–14} their high technological level and direct and indirect costs have limited their use in resource-poor countries,^{15–17} where most TB occurs.

The bacteriophage replication assay can detect *M. tuberculosis* in 48 hours,¹⁸ but it is laborious, and its sensitivity is not better than that obtained with smear microscopy.¹⁹ There are other new and promising low-cost approaches to specimen cultures that can reduce the time to diagnose TB yet maintain the sensitivity and specificity of conventional culture methods. Two of these methods are based on the microscopic detection of early mycobacterial growth: MODS (microscopic observation broth drug susceptibility) uses liquid media,²⁰ while the thin-layer agar (TLA) technique^{21,22} uses solid media. Both methods are capable of detecting growth within 9–11 days while also permitting the initial identification of *M. tuberculosis* on the basis of its characteristic cording in liquid media (MODS) and colony morphology on solid medium (TLA), when viewed microscopically.

To evaluate the TLA technique for the routine diagnosis of TB, a phase II multi-center study was undertaken in TB laboratories in several Latin American countries, comparing the performance of this technique with conventional culture methods. The study was carried out in the context of the European Commission International Cooperation for Development Countries project 'Diagnosis, drug resistance detection, and control of tuberculosis in Latin America'.

MATERIALS AND METHODS

Participants

Six laboratories participated in this evaluation: three are national reference laboratories, one a regional reference laboratory and two are hospital-based laboratories. An additional laboratory acted as the coordinating center responsible for sending reagents to the other laboratories and collecting and analyzing their results.

Specimens

Specimens were collected prospectively in each laboratory from patients with a clinical presumptive diagnosis of TB; laboratories were asked to include only one specimen per patient.

Decontamination methods

All laboratories used the same protocol for digestion and decontamination: sodium hydroxide and *N*-acetyl-L-cysteine (NaOH/NALC) for respiratory specimens, followed by centrifugation under standard conditions.

Non-respiratory specimens were processed according to recommended procedures.²³ After centrifugation, part of the sediment was stained with Ziehl-Neelsen and interpreted according to WHO recommendations.²⁴

Culture techniques

For culture, 0.1 ml of sediment was placed onto Löwenstein-Jensen (LJ) medium prepared in each laboratory according to international standards, and another 0.1 ml of sediment was placed onto a 60 × 15 mm plastic Petri dish containing a thin layer (5 ml) of Middlebrook 7H11 agar medium (TLA) with added piperacillin 50 mg/l, amphotericin B and trimethoprim 20 mg/l, and oleic acid-albumin-dextrose-catalase (OADC) 100 ml/l.^{22,25} After inoculation, TLA plates were sealed and incubated at 36°C in a CO₂ atmosphere using a CO₂ incubator or a candle jar, while LJ medium was incubated at 36°C without CO₂. TLA medium was prepared in the coordinating laboratory and then distributed to the participating laboratories. Laboratories were asked to use the TLA medium within the first month of its arrival, to reduce the risk of contamination and inactivation of the antibiotics present in the medium.

LJ cultures were examined visually weekly for up to 8 weeks, according to standard recommendations.²⁴ TLA plates were examined twice a week with a conventional microscope at 100 × magnification for up to 6 weeks, identifying microcolonies as *M. tuberculosis* by their appearance and morphology.^{21,22,25,26} Time to detection of growth and contamination rate were recorded for each type of culture medium.

All participating laboratories were trained by at least one workshop in technical methods and microscopy for identification of mycobacterial microcolonies. Before the study, a pilot study was also conducted in each laboratory, which included the analysis of several dilutions of *M. tuberculosis* reference strains to familiarize the technicians with the appearance of *M. tuberculosis* microcolonies.

Table 1 Type of specimens and positivity of direct smear, LJ and TLA

Type of specimen (n)	Diagnostic method number of positive specimens		
	Smear	LJ*	TLA*
Sputum (754)	133	129	133
Bronchoalveolar lavage (14)	1	1	2
Bronchial wash (66)	4	11	10
Biopsy (56)	4	9	9
Pleural fluid (29)	2	2	3
CSF (56)	1	1	2
Gastric aspirate (28)	—	4	4
Urine (78)	—	2	4
Other (36)	2	0	1
Total (1118)†	146 (1115)	160 (1114)	168 (1116)

* Two non-tuberculous mycobacteria isolates and one *M. bovis* (BCG).

† One without data.

LJ = Löwenstein-Jensen; TLA = thin-layer agar; CSF = cerebrospinal fluid.

Table 2 Sensitivity and likelihood ratios of TLA and LJ in multibacillary and paucibacillary specimens, excluding specimens with contamination in either or both culture media

Culture media	AFB positive (multibacillary)			AFB negative (paucibacillary)		
	Sensitivity (95%CI)	+LR %	-LR %	Sensitivity (95%CI)	+LR %	-LR %
LJ	90.4 (84.1–94.8)	4.52	0.12	70.9 (57.1–82.4)	29.2	0.30
TLA	94.1 (88.7–97.4)	4.70	0.07	89.1 (77.7–95.9)	17.15	0.12
<i>P</i> *	0.155			0.788		

* Pairwise comparison of ROC curves for sensitivity

TLA = thin-layer agar; LJ = Löwenstein-Jensen; AFB = acid-fast bacilli; CI = confidence interval; LR = likelihood ratio; + = positive; - = negative.

Final identification of the isolates was performed according to standard recommendations,^{23,24} confirming the acid-fast conditions of the colonies obtained and running standard biochemical tests such as catalase at 68°C, nitrate reductase activity, and accumulation of niacin.²³ The concordance of *M. tuberculosis* microcolony with final identification was also assessed. A diagnosis of TB was considered as confirmed when a patient had a positive culture in any of the culture media used.

Statistical analysis

Both media were compared against a final diagnosis of *M. tuberculosis*, defined as growth in any culture medium, which was considered the gold standard for comparison purposes. Sensitivity and likelihood ratios (LRs) were calculated using receiver operating characteristic (ROC) curve analysis.²⁷ LRs were calculated instead of predictive values due to the difference in the prevalence of TB between the different sites. The Wilcoxon test for non-normal distribution was applied to compare differences in time of detection; paired comparisons were performed excluding specimen contamination in any of the culture media. A *P* value <0.05 was considered significant. All calculations were done using MedCalc® version 7.4.2.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Of a total of 1256 specimens studied in six sites, 1118 were considered for the analysis; the remaining specimens were excluded due to incomplete data. All specimens came from patients with a presumptive clinical diagnosis of TB. Clinical respiratory specimens were

the most frequently studied, with sputum representing 67.4% of the total (Table 1).

A total of 190 specimens were positive for *M. tuberculosis* by culture and 146 by AFB smear. Two isolates of non-tuberculous mycobacteria (NTM) and one of *M. bovis* were identified. Cultures identified the same positives as smear microscopy for sputum specimens, but detected more TB in respiratory tract specimens other than sputum, particularly bronchial washes, of which 4, 11 and 10 cases were positive by smear microscopy, LJ and TLA, respectively. No extrapulmonary specimens were positive by smear microscopy, while TLA detected *M. tuberculosis* in 7.45% and LJ in 5.49% of these samples (Table 1).

Cultures detected *M. tuberculosis* in all AFB-positive specimens, excluding the two containing NTM and the one with *M. bovis*, and specimens contaminated in either culture medium. All *M. tuberculosis* isolates were identified as *M. tuberculosis* microcolony-like on TLA and confirmed by biochemical identification. However, one microcolony identified initially as NTM gave a final identification of *M. tuberculosis* and two NTM were initially classified as *M. tuberculosis* microcolony type. In addition, LJ detected *M. tuberculosis* in 3.2% and TLA in 4.4% of AFB-negative specimens. Sensitivity values for TLA were greater than those observed for LJ (92.6%, 95% confidence interval [CI] 87.9–95.9 vs. 84.7%, 95%CI 78.8–89.0), after excluding contaminated specimens on either culture medium. The difference in sensitivity was also observed when AFB-positive and -negative specimens were compared. Nevertheless, pairwise comparison of ROC curves did not show a significant difference, and positive and negative LRs were similar for both culture media (Table 2).

Table 3 Percentage of recovery of *M. tuberculosis* and contamination on LJ and TLA by study site

Results	Culture media	Study sites (<i>n</i> = number of samples per site)						Overall %
		A (<i>n</i> = 94)	B (<i>n</i> = 272)	C (<i>n</i> = 81)	D (<i>n</i> = 201)	E (<i>n</i> = 321)	F (<i>n</i> = 148)	
<i>M. tuberculosis</i> positive	LJ	28.0	24.3	2.5	3.5	6.9	20.9	13.8
	TLA	38.7	20.2	3.7	4.0	9.4	21.6	14.7
Contamination	LJ	1.1	2.6	9.9	4.5	1.3	2.7	3.0
	TLA	6.5	4.8	4.9	9.5	3.4	2.7	5.1

LJ = Löwenstein-Jensen; TLA = thin-layer agar.

Table 4 Time to detection for both culture media used

Culture media	Smear-negative specimens		Smear-positive specimens	
	Paired median (95%CI) days	Unpaired median (95%CI) days	Paired median (95%CI) days	Unpaired median (95%CI) days
LJ	30.5 (26.9–39.0)	30.0 (21.3–39.0)	26.0 (24.5–30.0)	26.0 (25.0–30.0)
TLA	11.5 (9.3–15.0)	11.0 (8.3–12.0)	7.0 (6.0–8.0)	7.0 (6.0–8.0)
<i>P</i> *	< 0.0001	< 0.0001	< 0.0001	< 0.0001

* Wilcoxon rank test for non normal distributions (paired: excluding contaminated specimens in any of culture media; unpaired: not excluding contaminated specimens in any of culture media). CI = confidence interval; LJ = Löwenstein-Jensen; TLA = thin-layer agar.

Differences in *M. tuberculosis* detection and contamination of both culture media were observed when comparing the different study sites (Table 3). Detection of *M. tuberculosis* ranged from 2.5% to 28.0% for LJ and from 3.7% to 38.7% for TLA, with an overall positivity of 13.8% and 14.7% for LJ and TLA, respectively. A low rate of contamination was observed in both culture media, but was more frequent with TLA (5.1% vs. 3.0%). Contamination rates were not uniform, and varied according to the laboratory sites, ranging from 1.1% to 9.9% for LJ and from 2.7% to 9.5% for TLA.

The time to detection of a positive culture showed major differences between TLA and LJ. In smear-negative specimens, the time to detection of positive cultures in TLA was a median of 11.5 days (95%CI 9.3–15.0) while for LJ it was 30.5 days (95%CI 26.9–39.0) ($P < 0.0001$). Table 4 shows paired and unpaired comparisons for time to detection of positive cultures for both culture media in smear-negative and smear-positive specimens. When comparing time to growth detection among samples with different bacillary loads, we also found significant differences (Figure).

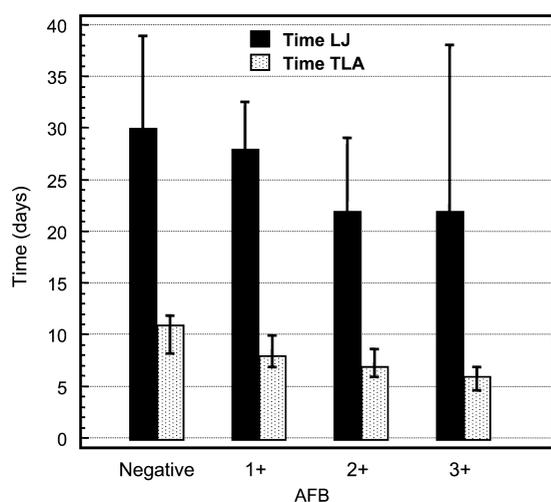


Figure Time to growth detection according to specimen bacillary load. Differences in time for TLA and LJ were significant in each AFB category ($P < 0.0001$). Medians (error bars: 95%CI for median). TLA = thin layer agar; LJ = Löwenstein-Jensen; AFB = acid-fast bacilli; CI = confidence interval.

DISCUSSION

Microbiological diagnosis of TB constitutes a fundamental step towards disease control. Smear microscopy is still essential for the detection of AFB in sputum, as it is a rapid, low-technology, low-cost technique widely used in countries where TB constitutes a major public health problem.

However, due to the lack of sensitivity of smear testing, especially in paucibacillary respiratory and non-respiratory specimens, there have been considerable efforts recently to foster the design, development and evaluation of new diagnostic techniques.²⁸ Conventional culture techniques are more sensitive than smear tests, but require several weeks to turn positive, while the more rapid, commercial liquid culture systems are costly.

This study, performed in six different laboratories in Latin America, demonstrates that the TLA technique is amenable for implementation in laboratories that use conventional culture techniques for diagnosis, and that it is considerably more rapid than conventional methods. The different characteristics of the participating laboratories, as well as the disease prevalence in each area and the number and type of specimens processed, did not affect the overall performance of TLA as compared to LJ, supporting the robustness of the method and its feasibility in different laboratory settings.

TLA cultures detected growth 19 days earlier than LJ medium in smear-negative specimens, and 21 days earlier in smear-positive specimens. The median time to detection of growth on TLA in this study (respectively 7 and 11.5 days for smear-positive and smear-negative specimens) was equivalent to or better than that reported for the Bactec 460, Bactec MGIT 960, MB/BacT, MB Redox and ESP II culture systems.^{6,8,10}

The MODS method is similar to TLA. Both are non-commercial, low-cost methods, easy to implement in TB diagnostic laboratories. Both employ microscopic examination of the characteristic *M. tuberculosis* microcolonies for early detection of growth and presumptive identification. The median time (days) to detection of growth obtained for MODS (9 days)²⁰ was equivalent to that observed in this study for TLA (7–11 days), and both studied populations had a similar proportion of

multibacillary specimens (78% vs. 76%). In contrast to MODS, however, TLA does not require an inverted microscope for observation of microcolonies, or the more biohazardous liquid cultures, it can be incubated in a candle jar, and it allows differentiation of *M. tuberculosis* from NTM based on the morphology of the microcolonies. Both methods, however, had the limitation of requiring time for observation of cultures, which may limit their use to laboratories that process low to moderate numbers of specimens for culture.

The sensitivity obtained with TLA was slightly better than with LJ medium, although the difference was not statistically significant. Nevertheless, this result is distinct from those of other studies that found that the sensitivity of TLA was lower.^{22,25} The contrasting results might be explained by the lower rate of TLA contamination observed in the present study, which, while slightly higher than for LJ (5.1% in TLA vs. 3.0% in LJ), was lower than reported in the previous studies. The lower contamination rate observed might be explained by the use of a higher concentration of amphotericin B and smaller-sized TLA plates.

Because the population disease prevalence varied in the different participant laboratories, we chose to analyze the results by calculating LRs, which are useful in comparing diagnostic tests and, unlike predictive values, are independent of the prevalence of the disease. With AFB-positive specimens the positive LRs (+LR) for LJ and TLA were not significantly different, but with AFB-negative specimens the +LR value for LJ was slightly higher. The explanation for this difference is not clear, but the analysis may be affected by the higher rate of contamination observed in TLA. No significant differences were observed when comparing negative LRs for the two culture methods.

Delays in TB diagnosis have been associated with prolonged periods of infectivity and disease progression, with consequent increases in community transmission and patient morbidity and mortality.^{29,30} Moreover, TB transmission in contacts of smear-negative patients has been documented to be as high as 17%.³¹ Thus, culture is particularly important in the diagnosis of paucibacillary pulmonary and extra-pulmonary disease, where smear tests are often negative. In this study, LJ detected 35 (3.2%) and TLA culture 47 (4.4%) patients who were false-negative on direct smear. Although the addition of positives by TLA is small, the results are influenced by the high number of sputum specimens included (754/1118 specimens) and the higher contamination rate found on these specimens.

As the number of NTM was limited in this study, it is not possible to evaluate the TLA performance for differentiation, by microscopy, of *M. tuberculosis* microcolonies from NTM. However, the experience gained by the different laboratories in participating in the study showed that microcolony characteristics on TLA can be used for preliminary identification of *M. tuberculosis*, allowing earlier reporting.

Although the impact on TB management services was not specifically evaluated in this study, all participant laboratories agreed that by using TLA they were able to provide more rapid responses to physician requests concerning AFB growth and whether the growth was compatible with *M. tuberculosis*, without a major increase in costs or laboratory workload. We previously reported an approximate cost for TLA plates of less than US\$3.00. The examination of the plates under the microscope takes around 1–2 min for an experienced technician.²²

The data presented here demonstrate that TLA is an easily implemented technique that does not require special laboratory equipment, and that it has at least the same sensitivity in detecting *M. tuberculosis* as conventional culture methods, but is much more rapid. It should be considered as an economic alternative or a complementary method for laboratories wishing to reduce the time to detect TB in cultures.

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R É S U M É

CONTEXTE : Laboratoires du diagnostic de tuberculose (TB) en Amérique Latine.

OBJECTIFS : Evaluation des cultures sur agar en fine couche (thin-layer agar, TLA) par comparaison avec le Loewenstein-Jensen (LJ) pour le diagnostic de la TB.

SCHÉMA : Etude prospective de phase II dans six laboratoires. Les échantillons ont comporté des expectorations et des échantillons extrapulmonaires provenant de patients avec un diagnostic clinique de TB. Les échantillons respiratoires ont été décontaminés par la méthode NaOH/NALC, et tous les échantillons ont été centrifugés. On a exécuté selon les procédés recommandés la coloration de Ziehl-Neelsen (BAAR), la culture sur LJ et TLA et l'identification. La sensibilité et les ratios de probabilité (LR), la durée de détection de la croissance et le taux de contamination ont été calculés pour les deux milieux.

RÉSULTATS : On a étudié 1118 échantillons cliniques. Les cultures ont détecté *Mycobacterium tuberculosis*

dans tous les échantillons à bacilloscopie positive, alors que dans les échantillons à bacilloscopie négative, le LJ a détecté 3,2% de *M. tuberculosis* et le TLA 4,4%. Les valeurs de sensibilité ont donc été respectivement de 92,6% (IC95% 87,9–95,9) et de 84,7% (IC95% 78,8–89,0) pour TLA et LJ. Les ratios de probabilité positive et négative ont été similaires. La contamination est survenue dans 5,1% des échantillons avec TLA et dans 3,0% avec LJ. La durée médiane de détection d'une culture positive a été de 11 jours (IC95% 9,3–15,0) pour TLA et de 30,5 jours (IC95% 26,9–39,0) pour LJ ($P < 0,0001$).

CONCLUSION : La TLA est une technique de culture facile à mettre en œuvre, elle n'exige pas d'équipement particulier et a la même sensibilité que les méthodes conventionnelles tout en étant plus rapide. Elle devrait constituer une alternative pour les laboratoires désirant raccourcir la durée de détection de *M. tuberculosis* dans les cultures.

RESUMEN

MARCO DE REFERENCIA: El diagnóstico de la tuberculosis (TB) en América latina.

OBJETIVOS: Evaluación del cultivo en agar de capa delgada (TLA) para el diagnóstico de TB, comparado con el cultivo en medio Lowenstein-Jensen (LJ).

MÉTODOS: Fue este un ensayo prospectivo de fase II realizado en seis laboratorios. Las muestras de las vías respiratorias y extrapulmonares provinieron de pacientes con diagnóstico clínico de TB. Las muestras de las vías respiratorias se descontaminaron mediante el método de NaOH/NALC, todas las muestras se centrifugaron y se prepararon frotis para tinción de Ziehl-Neelsen y cultivos en LJ y TLA. La identificación bacteriológica se realizó siguiendo los procedimientos recomendados. Para ambos medios de cultivo se calculó la sensibilidad, los cocientes de verosimilitud, el tiempo de detección del crecimiento y la tasa de contaminación.

RESULTADOS: Se estudió un total de 1118 muestras clínicas. Los cultivos detectaron *Mycobacterium tuber-*

culosis en todas las muestras con frotis positivos para bacilos ácido-resistentes; en contraste, solo el 3,2% de las muestras con frotis negativos dieron cultivos positivos en el medio LJ y el 4,4% en TLA. La sensibilidad del cultivo en TLA fue del 92,6% (IC95% 87,9-95,9) y en LJ fue del 84,7% (IC95% 78,8-89,0). Los cocientes de verosimilitud positivos y negativos fueron equivalentes. La tasa de contaminación para el TLA fue del 5,1% y del 3,0% para el LJ. La mediana del tiempo necesario para la detección de un cultivo positivo fue 11,5 días en el TLA (IC95% 9,3-15,0) y 30,5 días en el LJ (IC95% 26,9-39,0; $P < 0,0001$).

CONCLUSIÓN: El cultivo en TLA es una técnica de ejecución sencilla, no requiere equipo específico, presenta una sensibilidad equivalente a la de los métodos convencionales y es también más rápida. Este medio debería constituir un método alternativo para los laboratorios interesados en disminuir el tiempo de detección de la TB en los cultivos.