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Comparison of *Mycobacterium tuberculosis* complex Yield and Contamination Rates using Lowenstein-Jensen with and without Antibiotics in Western Kenya

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Abstract

Background: While molecular methods have been recently endorsed for diagnosis of tuberculosis (TB), mycobacterial culture remains the gold standard. Lowenstein-Jensen (LJ) is often used for the cultivation of Mycobacterium tuberculosis complex (MTBC); however contamination often renders a subset of cultures useless. We compared the MTBC yield and contamination rate of processed sputum inoculated on LJ with antibiotics (LJ PACT) to LJ without antibiotics (LJ).

Methodology: Sputum samples were obtained from people living with HIV enrolled in a TB screening study in western Kenya, processed using NALC/NaOH-Na citrate, then inoculated on LJ PACT and LJ media. Cultures were evaluated weekly with growth identified as acid-fast bacilli by Ziehl-Neelsen bright-field microscopy. MTBC and nontuberculous mycobacteria (NTM) were identified by immunochromatographic and line probe assays.

Results: A total of 700 sputum samples were cultured on both LJ PACT and LJ between March and June 2012. Of those cultured on LJ PACT, 29 (4.1%) grew MTBC, 613 (87.6%) were negative, 12 (1.7%) grew NTM, and 46 (6.6%) were contaminated; on LJ, 28 (4%) grew MTBC, 553 (79%) were negative, 9 (1.3%) grew NTM, and 110 (15.7%) were contaminated. The difference in contamination on LJ PACT and LJ was statistically significant ($p<0.0001$), while the difference in MTBC growth was not ($p=0.566$).

Keywords

pulmonary tuberculosis; diagnosis; culture techniques; Nontuberculous mycobacteria; cross-sectional studies; Kenya; Lowenstein-Jensen

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Introduction

Identification of the etiologic agent *Mycobacterium tuberculosis* complex (MTBC) is required for definitive diagnosis of tuberculosis (TB) [1], a disease responsible for an estimated 9.6 million TB cases and 1.5 million deaths in 2014 [2]. In resource-limited, high TB-burden settings, the primary tool for case detection remains microscopic examination of sputum for acid-fast bacilli (AFB) [3,4]. The limitations of sputum smear microscopy are well known [5,6], and include difficulties in maintaining equipment under field conditions, dependence upon trained, motivated technical staff for reliable results [3], and most importantly the lack of sensitivity, especially among people living with human immunodeficiency virus (PLHIV), who have the highest occurrence of AFB smear-negative TB [7–11].

To address the limitations of microscopy and to facilitate recovery of mycobacteria for drug susceptibility testing, mycobacterial culture facilities have been established in resource-limited settings. Due to the availability of supplies and reagents, ease in preparation, low cost and ability to support ample growth of small numbers of bacilli while inhibiting growth of contaminants, egg-based media such as Lowenstein-Jensen (LJ) were among the first culture media to be introduced [12]. More recently, the World Health Organization (WHO) has endorsed the use of liquid culture for microbiologic confirmation of TB in high-burden settings based on findings of several large demonstration studies [13]. While these studies report an improved sensitivity and a reduction in time to detection of MTBC compared to LJ, increased contamination, as well as the costs and feasibility constraints continue to make liquid culture prohibitive for routine use in many resource-limited countries [14–16].

Kenya is considered a high TB-burden country, with a TB incidence of 233/100,000 [17], and has very limited access to mycobacterial culture. In the few facilities where culture is routinely performed, maintaining acceptable contamination rates remains a challenge and could potentially diminish the utility of culture in this setting. Antibiotic-containing solid media could theoretically reduce contamination rates; however studies describing the potential impact on the yield of MTBC [18,19], specifically on LJ solid media [20] are limited.

As part of a larger intensified TB case finding study (referred to as the parent study) in western Kenya, PLHIV submitted sputum samples as part of a screening and diagnostic evaluation. In the present sub-study, we evaluated the performance of commercially prepared LJ containing the antibiotics polymixin B, amphotericin B, carbenicillin and trimethoprim ([LJ PACT], BD, Sparksville, Maryland) to commercially prepared LJ without antibiotics ([LJ], BD Sparksville, Maryland) for recovery of MTBC and contamination.

Material and Methods

Parent study

We conducted a cross sectional study on sputum samples submitted from PLHIV as part of a TB intensified case finding study ("parent study") [21, 22] The parent study recruited from 15 randomly-selected public HIV care and treatment facilities that had at least 200

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enrolled patients in the Siaya, Bondo and Kisumu East Districts from the former Nyanza Province. Enrolment occurred in a phased manner between May 2011 and June 2012, with each clinical site enrolling participants for 10 weeks. All PLHIV newly enrolled into HIV care and treatment during the study period were screened for eligibility, and if eligible, were invited to participate. Patients were eligible for the parent study if they were documented to be HIV-infected, 7 years or older, and provided informed consent or assent depending on age; exclusion criteria were receipt of any HIV-related care in the preceding two years and diagnosis of TB disease at any time in the previous one year. Three sputum samples were collected from all participants enrolled in the parent study regardless of signs or symptoms. One of two sputum collection strategies was used; collection of the first spot at the time of enrolment, followed by collection of the morning sample at the patient's home, and then collection of the second spot sample when the morning sample was returned to the facility. In some cases we collected both spot samples at least two hours apart at the time of enrolment, with the morning sputum collected the following day from the patients' home and returned to the facility.

Laboratory procedures

Sputum samples collected as part of the parent study were characterized at the point of reception in the laboratory based on appearance (consistency and color). Samples were considered as either salivary (viscous, light and near clear), mucoid (thicker and less viscous, yellow to green in color), or tenacious (sticky, less viscous and clear in color). They were digested and decontaminated using N-acetyl-L-cysteine/ 4% sodium hydroxide-sodium citrate (NALC/NaOH Na-citrate, [final concentration of NaOH 1%]), neutralized with pH 6.8 phosphate buffer solution, concentrated through centrifugation @ 3,000 x g for 15 minutes, decanted, and resuspended in 2.0mL of fresh phosphate buffer solution. Thereafter, 1 LJ PACT slant and 1 LJ slant without antibiotics were inoculated with a maximum of 2 drops each (approximately 50µL per drop) from the same sample. Slants were incubated at 35°—37°C, and evaluated weekly for growth for a total of 8 weeks, and colony morphology observations such as texture and pigmentation were documented. Microscopic evaluations were performed on all colonies observed on both media types for confirmation of AFB; growth consistent with mycobacteria was identified using an immunochromatographic assay (MGIT TBc ID, BD Sparksville Maryland, MD) or the Genotype Mycobacterium CM lineprobe assay (Hain LifeScience, Nehren, Germany). Quality control procedures included inoculation of LJ PACT and LJ with a processed artificial sputum (AS) spiked with an American Type Culture Collection (ATCC) - H37rv control strain for positive control and non-spiked AS for negative control, every 2 months as per established protocol.

Classification of culture results

The study aimed to compare the performance of the two culture media with respect to contamination and MTBC yield. The final outcome was categorized as: MTBC positive, negative, contaminated, or nontuberculous mycobacteria (NTM) positive. Cultures were considered MTBC positive if growth obtained from the cultures was identified as such using the immunochromatographic assay, negative if no growth was observed after the standard 8 weeks of incubation, contaminated if only non-acid-fast organisms grew, and NTM if growth

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was negative for MTBC by the immunochromatographic assay and identified as NTM using the line probe assay.

Statistical analysis

Data were collected on source documents and entered into a password-protected web-based electronic system which used Microsoft Access to store data. Analysis was conducted with STATA 10. Simple descriptive statistics including cell frequencies and their corresponding percentages for categorical data, and summary statistics [(median and interquartile range (IQR)] for continuous data were calculated. We used generalized estimating equations to account for the multiple samples obtained from the same individuals to compare the difference in MTBC positives, negatives, NTM and contaminated obtained from the two media [23].

Ethical Review

The study was approved by the institutional review boards of the U.S. Centers for Disease Control and Prevention and Kenya Medical Research Institute (KEMRI), Kenya.

Results

A total of 388 (49.2%) patients were included in the sub-study, including 126 (32.5%) males and 262 (67.5 %) females, with an overall median age of 30 (IQR, 25–40) years (Table 1). A total of 700 sputum samples were collected, processed and inoculated on both LJ PACT and LJ. Of the LJ PACT slants, 29 (4.1%) were positive for MTBC and 46 (6.6%) were contaminated; of the LJ slants, 28 (4%) were positive for MTBC and 110 (15.7%) were contaminated (Table 2). Twenty-seven specimens were MTBC positive on both LJ PACT and LJ; two specimens that were positive on LJ PACT were either negative or contaminated on LJ, while one specimen that was positive on LJ was contaminated on LJ PACT (Table 2). The difference in contamination on LJ PACT and LJ was statistically significant ($p<0.0001$); however the difference in MTBC yield in the two media was not ($p=0.566$). We further analysed culture yield of MTBC and contamination on LJ PACT and LJ based on sample quality (Table 2). On LJ PACT, 4.1% (21/512) of mucoid samples were positive for MTBC while 3.9% (20/512) were positive on LJ, however tenacious samples had positivity of 22.2% (2/9) on both media though were few in number. On LJ PACT, 31 (6.1%) of 512 mucoid samples and 15 (8.4%) of 179 salivary samples were contaminated; while on LJ, 82 (16.0%) of 512 mucoid samples and 26 (14.5%) of 179 salivary samples were contaminated.

We also determined the percentage of MTBC recovered and the contamination rate based on collection (morning, spot 1 or spot 2). Morning samples were more commonly positive for MTBC (13/167 [7.8%] on LJ PACT, 11/167 [6.6%] on LJ), followed by spot 1 samples (16/355 [4.5%] on LJ PACT, LJ 17/355 [4.8%]) (Table 3). All spot 2 samples (178) were negative for MTBC on both media. On LJ PACT, 14/167 (8.4%) morning samples, 26/355 (7.3%) of spot 1 samples and 6/178 (3.4%) spot 2 samples were contaminated; on LJ, 29/167 (17.4%) morning samples, 55/355 (15.5%) spot 1 samples and 26/178(14.6%) spot 2 samples were contaminated (Table 3).

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Discussion

In the present study, we compared the performance characteristics of LJ PACT to LJ for MTBC culture. To the best of our knowledge, studies evaluating the utility of antibiotic-containing media, specifically LJ, are limited. The most notable, but not unexpected, finding was the significant reduction in culture contamination on LJ PACT compared to LJ (p-value <0.0001) while maintaining similar isolation rates of MTBC; this trend was also observed in a study by Kassaza*et al*, where contamination rates of 32.1% versus 5.0% were reported for LJ and LJ containing antibiotics, respectively [20].

The LJ PACT used in our evaluation contained the antibiotics polymixin B, amphotericin B, carbenicillin and trimethoprim. The *in vivo* and *in vitro* bactericidal activity of the antibiotics in LJ PACT against microorganisms which may be present in respiratory secretions has been previously described [24–28]. This combination of antibiotics is also found in mycobacterial liquid culture media, including MB Redox™[29] and as part of the complete BACTEC MGIT™ media[30].

The yield of MTBC was higher on slants, both LJ PACT and LJ, prepared from morning samples as compared to spot samples. Our findings also suggest samples which were documented as mucoid were more likely to be positive for MTBC when compared to salivary or tenacious samples, although this comparison is limited by the low number of tenacious samples collected. Previous authors have suggested the higher diagnostic yield observed from morning samples may reflect an accumulation of sputum in the lungs overnight resulting in a higher concentration of AFB, compared with samples collected on the spot when bacilli may be shed more sporadically [31]. We also observed more contamination in the morning sample compared with spot samples, this may be due in part to a lack of supervision during collection. While the modest yield of MTBC from the first spot sample suggests that supervision during collection may have resulted in submission of higher quality samples for culture, this was not the case for the second spot sample. Interestingly, all spot 2 samples, regardless of collection as the second or third sample in a series of three, were negative on both media. These findings are consistent with previous reports suggesting the collection of two sputum, including at least one morning sample, for mycobacterial culture may be sufficient for diagnosing TB [31, 32].

We believe these findings are relevant for high TB-burden settings seeking to strengthen diagnostic capacity through implementation of culture, and for laboratories supporting clinical trials. First, while molecular techniques such as the GeneXpert MTB/RIF assay have been recently endorsed by WHO and implemented in many resource-limited, high TB-burden settings [33], mycobacterial culture is the most sensitive test and allows for assessment of viable mycobacteria; as such it remains the gold standard for microbiologic confirmation of TB disease [34]. Mycobacterial culture can be performed on liquid and/or solid media. While liquid culture techniques have been shown to have a higher yield and shorter time to detection of MTBC than solid media such as LJ, liquid culture methods are technically demanding and remain cost-prohibitive for routine use in many high-burden countries [35,36]. One challenge frequently encountered with routine use of liquid culture methods is the increase in overgrowth of bacterial contaminants compared to LJ[35]. The

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higher contamination rates observed with liquid culture potentially increase laboratory costs [16] a major challenge to employing this diagnostic method. An increase in the detection of NTM has also been observed in liquid culture compared to LJ [37]. The use of solid media such as LJ allows for direct observation of colonies, which permits early differentiation of isolates based on morphology [12], and may aid in the detection of MTBC and NTM in patients with mixed mycobacterial populations [37]. All of this demonstrates that solid culture still has a place in TB testing. However, the value of any media type is limited when contamination rates are high, hence the relevance of our findings.

Our findings also have important implications for laboratories supporting clinical research; specifically TB treatment trials where endpoints are established mainly on bacteriological status at baseline and several time points during treatment and follow-up [19]. While liquid culture has been used in some present-day clinical trials, solid media will continue to provide an important link connecting culture results observed on solid media during therapy to clinical outcomes such as treatment failure and relapse [19]. Most clinical trials on TB treatment use colony counts on LJ media as the outcome of interest, and these trials need to have a positive or negative result on as many patients as possible. Contaminated results are not useful and often result in a patient's data not contributing to the analysis, thereby decreasing statistical power and wasting resources.

We acknowledge the following limitations which may have influenced the outcome of our evaluation. While the total number of samples tested in our evaluation was large, the proportion of cultures positive for MTBC was low. Additional studies including a higher yield of MTBC are required to determine whether antibiotic-containing solid media may (negatively) affect MTBC yield. Our comparison included commercially prepared LJ with and without antibiotics. Given the differences in LJ media formulations and sterilization techniques used in the preparation of LJ, performance characteristics for locally prepared media [18], specifically LJ containing antibiotics, may differ from what we have reported here. Patient samples used for our evaluation were collected prior to initiation of anti-TB therapy, and at a single time point. While our data suggests LJ PACT may be useful in clinical trials, this was not assessed in the present study. Future comparisons of solid media for evaluating treatment outcomes should include LJ PACT, as well as selective agar medium. In addition, our study included processed sputum only, and did not include other sample types submitted for mycobacterial culture that may be more prone to contamination. Further studies describing the performance of LJ PACT should include stool samples for example, as previous studies have suggested stool cultures may be useful in the diagnosis of pulmonary MTBC in PLHIV [11, 38].

In conclusion, the use of LJ PACT was associated with a marked reduction in contamination (6.6% versus 15.7%), while having no effect on the proportion of cultures growing MTBC. LJ PACT may be preferable to LJ when contamination is a concern.

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Table 1:

Demographics and clinical characteristics of participants included in study, March through June 2012 (N=388)

Characteristics	Frequency	Percent
Sex		
Male	126	32.5
Female	262	67.5
Median age in years(IQR)	30 (25–40)	
Age-groups(years)		
9–18	14	3.61
19–24	78	20.1
25–34	155	39.95
35–44	68	17.53
45 +	73	18.81
Median Initial CD4 counts (IQR)	349(196–560)	
initial CD4 Categories (cells/mm^3)		
<=250	126	34.05
> 250	244	65.95

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Table 2

Performance of Lowenstein-Jensen with and without polymyxin, amphotericin, carbenicillin and trimethoprim, by sample quality (N=700)

		LJ (without antibiotics)				
		MTBC	Negatives	NTM	Contaminated	Total
LJ PACT	MTBC	27	1	0	1	29
	Negatives	0	523	3	87	613
	NTM	0	2	6	4	12
	Contaminated	1	27	0	4	18
	Total	28	553	9	110	700

Specimen type (LJ PACT)	MTBC n (%)	Negative n (%)	NTM n (%)	Contaminated n (%)	Total
Mucoid	21 (4.1)	453 (88.6)	7 (1.4)	31 (6.1)	512
Salivary	6 (3.4)	154 (87.0)	4 (2.3)	15 (8.4)	179
Tenacious	2 (22.2)	6 (66.7)	1 (11.1)	0 (0)	9
Total	29 (4.1)	613 (87.6)	12 (1.7)	46 (6.6)	700

Specimen type (LJ)	MTBC n (%)	Negative n (%)	NTM n (%)	Contaminated n (%)	Total
Mucoid	20 (3.9)	406 (79.3)	4 (0.8)	82 (16.0)	512
Salivary	6 (3.4)	143 (79.9)	4 (2.2)	26 (14.5)	179
Tenacious	2 (22.2)	4 (44.4)	1 (11.1)	2 (22.2)	9
Total	28 (4.0)	553 (79.0)	9 (1.3)	110 (15.7)	700

* p-Values are as follows MTBC positives = 0.566, Negatives = < 0.0001, NTM = 0.323, Contaminated = < 0.0001

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Table 3:

Yield of Mycobacterium tuberculosis and contamination rates, by sample type and media (N=700)

Sample type & Media	MTBC	%	Contaminated	%
Morning				
LJ PACT	13/167	7.8	14/167	8.4
LJ	11/167	6.6	29/167	17.4
Spot 1				
LJ PACT	16/355	4.5	26/355	7.3
LJ	17/355	4.8	55/355	15.5
Spot 2				
LJ PACT	0/178	0.0	6/178	3.4
LJ	0/178	0.0	26/178	14.6