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Examination of Optimal Culture Methodologies and Conditions for Tuberculosis Treatment Trials

By

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Master of Science in Public Health

Biostatistics and Bioinformatics

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Examination of Optimal Culture Methodologies and Conditions for Tuberculosis Treatment Trials

By

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Georgia State University

2015

Thesis Committee Chair: Lance A. Waller, Ph.D.

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An abstract of

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Abstract

Examination of Optimal Culture Methodologies and Conditions for Tuberculosis Treatment Trials

By: Keun O. Lee

Introduction: Tuberculosis (TB) is a contagious and airborne disease of the lungs caused by *Mycobacterium tuberculosis* (Mtb) that continues to be a one of top ten causes of death worldwide. In efforts to eliminate TB epidemic, the Tuberculosis Trials Consortium (TBTC) conducts clinical trials, which relies on culture media for assessment of treatment efficacy. The purpose of this study is to examine three different culture systems, Löwenstein-Jensen medium (LJ), selective Middlebrook agar (7H11S), and BACTEC MGIT 960 (MGIT), for the identification of methods and conditions that are optimal for Mtb growth and robust against contamination in detection of Mtb.

Methods: LJ, 7H11S, and MGIT were compared on sensitivity, proportions of Mtb positivity, contamination, and discordance of media results. The two solid culture media, LJ and 7H11S, were additionally stratified by exposure to 8-10% carbon dioxide (CO_2) during incubation. Secondly, independent generalized linear mixed effects models (GLMMs) were used to simultaneously assess the effects of solid medium type, CO_2 exposure, total sputum sample volume, participant HIV status, and time on treatment on culture positivity and Mtb recovery against contamination.

Results: MGIT had the highest sensitivity (98.5%), followed by 72.2% and 77.6% for 7H11S and 7H11S/CO_2, and 67.8% and 69.2% for LJ and LJ/CO_2, respectively. Similarly, Mtb yield was highest for MGIT with 55%, followed by 7H11S/CO_2, 7H11S, LJ/CO_2, and LJ. Contamination varied across different sites. Modeling result showed statistically significant negative effects on solid culture positivity for LJ method, HIV-positive status, and increase in treatment time with adjusted odds ratio (OR) of 0.514 (95% CI: 0.401, 0.658), 0.227 (0.082, 0.629), and 0.379 (0.351, 0.409), respectively. CO_2 exposure showed a positive effect on solid culture positivity with OR=1.367 (1.073, 1.818). In addition, treatment time and sputum sample volume >3mL showed significantly negative effects on Mtb recovery for both solid and liquid media ($p<0.05$).

Discussion: Results indicated that the higher odds of Mtb-positive cultures are associated with 7H11S medium compared to LJ, CO_2 incubation, HIV-negative individuals, and earlier weeks of TB treatment. There were reduced odds of Mtb-positive cultures due to contamination associated with sputum volume > 3mL and longer time on treatment.

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1. INTRODUCTION

Tuberculosis (TB), a contagious and airborne disease of the lungs caused by *Mycobacterium tuberculosis* (Mtb), continues to be a global public health problem and one of top ten causes of death worldwide ("Tuberculosis Fact Sheet", 2017). Despite the average worldwide decline in incidence rate of 1.5% per year since 2000, the number of multidrug-resistant TB (MDR-TB) and latent TB cases and TB's high morbidity among HIV (human immunodeficiency virus)-positive populations call for improvements and continued research in TB treatment. Thus, the identification of efficient and reliable microbiologic methods for detecting Mtb in TB clinical trials is highly essential for treatment development and control of the disease epidemic.

While several molecular methods for detection of mycobacteria in clinical specimens have been developed including polymerase chain reaction (PCR), culture largely remains the reference standard in diagnosis of TB (Huang et. al., 2001). PCR may detect both viable and non-viable mycobacteria, but culture only recovers living mycobacteria, providing a better assessment of active infection than PCR-based methods (Pai et. al., 2000). Solid culture systems such as Löwenstein-Jensen (LJ) slant or Middlebrook 7H11 agar plate have been the conventional methods in detection of Mtb; however, these require extensive time to determine growth and often exceed the recommended 21-day turnaround period Mtb isolation and identification (Lee et al., 2003). Use of mycobacteria growth indicator tube (MGIT) liquid culture media system has been implemented to shorten the delay in TB culture confirmation with studies indicating higher sensitivity and shorter time-to-detection of about 14 days for MGIT compared to 24 days for solid cultures (Hanna et al., 1999). Yet the effectiveness of

culture systems may be undermined by bacterial and fungal contamination, which reduces the proportion of interpretable culture results (Murray et al., 1999; Kassaza et al., 2014). Hence, the identification of culturing techniques and conditions that are optimal for Mtb growth and robust against contamination in Mtb detection is pivotal in clinical research towards the elimination of TB epidemic.

1.1 *Problem Statement*

Active TB treatment trials conducted by the Centers for Disease Control and Prevention's Tuberculosis Trials Consortium (TBTC) require reliable culture methods to determine presence or absence of Mtb. Endpoints for clinical trials in assessing TB treatment efficacy are based primarily on bacteriological status at different time points using solid and liquid media. While LJ solid culture medum have been used widely across TBTC clinical sites, variability between batches exist in its egg-base, which is often locally made and not standardized. The alternative selective Middlebrook agar medium (7H11S) contains antibiotics to avoid contamination, but it has not been tested against the performance of LJ across multiple sites. Although the stimulatory effect of CO_2 on mycobacteria growth for solid media has been demonstrated, there has not been a definitive evidence of improvements in Mtb yield in a clinical trials setting (Gruft & Loder, 1971). Furthermore, MGIT is a liquid media known to be highly sensitive to not only the growth of mycobacteria but also to other bacterial contaminants with reports of various contaminated culture proportions from 5.5% to 15% (Chihota et al., 2010). Therefore, to date, the use of MGIT alone lacks the necessary evidence to replace the role of solid media in studying treatment outcomes in clinical trials.

1.2 *Purpose Statement*

The purpose of this paper is to compare three common culture systems, Löwenstein-Jensen medium, selective Middlebrook agar, and BACTEC MGIT 960, and related conditions on their performance in detection of Mtb in TB treatment trials settings. First, each system will be analyzed against one another in sensitivity based on constructed reference standard, proportions of Mtb positivity (Mtb yield), and contamination (contamination rate). The two solid culture media, LJ and 7H11S, will be additionally stratified by exposure status to 8-10% of carbon dioxide (CO_2) during the incubation period to explore the effect of CO_2 on culture performance. Discordance between results of any two culture conditions and temporal trends of Mtb yield and contamination rate across and by site will be explored. Secondly, independent generalized linear mixed effects models (GLMMs) will be used to simultaneously assess the effects of solid culture medium type, CO_2 exposure, total sputum sample volume, HIV status, and time on treatment on culture positivity and Mtb recovery against contamination. Potential correlations from repeated measurements and the multi-site study design will be accounted for in this longitudinal analysis of the mycobacteriology data.

1.3 *Significance Statement*

Determination of optimal culturing method and condition for detecting Mtb during a clinical trial relying on bacteriologic evidence for treatment efficacy assessment is crucial for obtaining successful study results toward the control of TB. The findings

from this paper will help in standardizing the culture media used across all TBTC mycobacteriology labs in future clinical trials.

2. BACKGROUND

2.1 *TB Overview*

Tuberculosis (TB) is a preventable and curable airborne disease that primarily attacks the lungs of the Mtb-infected individuals. Although the disease occurs in all parts of the world, the highest incidence of TB cases is found in Asia, followed by Africa accounting for 61% and 26% of new cases in 2015, respectively ("Tuberculosis Fact Sheet", 2017). Not everyone infected with TB bacteria develops active TB disease; there is about 10% lifetime risk of active TB disease for a TB-infected individual ("Tuberculosis Fact Sheet", 2017). However, late diagnosis and lack of proper treatments for those with active TB disease could lead to fatality. The risk is even greater for persons with compromised immune systems from HIV, diabetes, and malnutrition ("Tuberculosis Fact Sheet", 2017).

Common symptoms of pulmonary TB disease includes cough with sputum lasting three weeks or longer, chest pains, fever, weakness, and weight loss ("Tuberculosis: Basic TB Facts", 2016). TB has long been diagnosed with sputum smear microscopy, but with the increase in single drug and multi-drug resistant TB cases, the use of more rapid and comprehensive diagnostic tests, such as Xpert MTB/RIF®, has been expanded in recent years ("Tuberculosis Fact Sheet", 2017). Once diagnosed, the drug-susceptible TB disease is treated with a standard 6-month course of 4 antimicrobial drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) under the supervision of healthcare workers

("Tuberculosis: Basic TB Facts", 2016). This long duration of treatment reduces patient adherence and is suspected to contribute to an increase in development of TB drug resistance ("Tuberculosis Fact Sheet", 2017). Thus, many current TB clinical trials aim to find shorter yet effective treatment regimens for TB patients and use bacteriologic biomarkers to assess treatment efficacy.

2.2 *TB Diagnostic Tests*

Löwenstein-Jensen (LJ) Medium

LJ medium is a glycerated egg-based solid medium widely used in the diagnosis of active TB disease. It takes between 3 to 6 week for the isolation of Mycobacteria and another 1 to 2 weeks for Mtb speciation (Naveen & Peerapur, 2012). Although commercially available, LJ medium is also commonly and inexpensively made in laboratories using a local egg supply. Hence, there are concerns over variability in consistency of quality affecting the performance characteristics of LJ medium in detecting Mtb. Unlike 7H11S medium, which contains a set of antibiotics to control for contamination, standard LJ medium does not include antibiotics and uses malachite green instead to inhibit microbial growth.

Middlebrook 7H11 Selective Agar (7H11S)

Middlebrook 7H11 Selective Agar is a solid and selective growth medium used in cultivation of Mycobacteria, which contains antimicrobial agents polymyxin B, carbenicillin, amphotericin B, and trimethoprim lactate to inhibit bacterial and fungal contaminants without inhibiting Mtb growth (National Cancer Institute Term Browser,

2016). While LJ medium has been the solid medium of choice for clinical trials, a related prospective cohort study in TB patients used latent class analysis to compare five different types of solid media to a gold standard concluded that 7H11S was a more reliable solid culture medium than the LJ medium in relation to its ability to recover Mtb in reference to the gold standard with lower contaminated rate (Joloba et al., 2014). Although limited by cost and feasibility, the use of CO_2 incubation for 7H11S and LJ media are both possible and recommended.

Mycobacteria Growth Indicator Tube (MGIT)

The MGIT^{TM} 960 is fully-automated, ready-to-use liquid medium produced by Becton Dickinson Microbiology Systems that uses oxygen-quenching fluorescent sensor technology (BBL^{TM} MGIT^{TM}) and algorithms to detect Mtb positivity (Huang et al., 2001). The approach continuously monitors and records bacterial growth every 60 minutes, making the system easy-to-use for a rapid and sensitive detection of growth including Mycobacteria (Chien et al., 2000). Although liquid media systems boast faster growth of Mtb than in solid media, they do not allow for examination of colony morphology or detection of mixed cultures possible in solid media (Joloba et al., 2014). Similar to antibiotic supplements in 7H11S medium, MGIT contains PANTA, an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin added to the 7H9 broth in MGIT to also control for contaminant growth (Hanna et al., 1999).

3. DATA DESCRIPTION & METHODOLOGY

3.1 *Setting and Specimen Collection*

Between November 2014 and July 2016, 330 participants from 5 TBTC clinical trial sites in New York, Uganda, Vietnam, South Africa, and Kenya were screened and enrolled in TBTC Study 36, a prospective observational study of individual treated for pulmonary TB. All participants were at least 15 years old and had suspected pulmonary TB based on positive smear microscopy for acid-fast bacilli (AFB) or tested positive for Mtb using Xpert MTB/RIF testing with a cycle threshold value ≤22. In addition, participants had no evidence of rifamycin resistance. Initial demographic and behavioral data, including HIV infection status, were collected for each participant at enrollment. Following the enrollment, participants began anti-TB medications using directly observed therapy (DOT) by trained personnel according to the local standard of care; no particular type and regimen of anti-TB drugs were specified to the sites for administration. A total of 1885 sputum specimens were collected from the study participants for smear microscopy, molecular testing and cultures at weeks 0, 2, 4, 6, 8, 13 and 17 following enrollment. Each sputum specimen was prepared and cultured under 5 culture media conditions (LJ, LJ with 5-10% of CO2, 7H11S, 7H11S with 5-10% of CO2, and MGIT) according to the *TBTC Study 36 - Platform for Assessment of TB Treatment Outcomes: Minimum Requirements for TBTC Sites and Laboratories* manual (December, 2014). Culture results were then categorized into three possible outcomes: Mtb-positive, Mtb-negative (no growth or no growth of non-tuberculosis Mycobacteria/ NTM), or Contaminated. Culture results with both Mtb growth and contamination was categorized as Mtb-positive (Figure. 1) for sensitivity and Mtb yield analysis and modeling but as Contaminated in the contamination analysis. For a culture with both NTM growth (Mtb-

negative) and contamination, the result was categorized as Mtb-negative for sensitivity and Mtb yield analysis and modeling but as Contaminated in the contamination analysis. Contaminated culture outcomes (contaminated with no detectable Mtb or NTM growth) were excluded from the sensitivity and Mtb yield analyses but were naturally included in the contamination rate calculation.

3.2 *Data Analysis*

3.2.1 Sensitivity, Mtb Yield, Contamination, and Discordance Analysis

Pairwise discordance between each of the 5 culturing conditions (LJ, LJ with CO2, 7H11S, 7H11S with CO2, and liquid MGIT medium) was determined based on the frequency with which the two media results from the same sputum sample directly contradicted one another (i.e. processed sputum was Mtb-positive under one growth condition and Mtb-negative under another different growth condition). Sensitivity and proportions of Mtb-positive cultures and contaminations were calculated based on the frequencies of culture media results averaged over 5 sites and then for each site individually. For the sensitivity analysis, a reference gold standard was constructed based on the culture results from the same specimen. A sputum sample was considered to be Mtb-positive if at least one culture result was Mtb-positive and considered Mtb-negative if all results were Mtb-negative. Lastly, the contamination rates for cultures under five culturing conditions included frequencies of both contamination and co-contamination. Differences of proportions across sites were tested for independence using a Pearson chi-square (X^2) test. The trends in Mtb yield and

contamination proportions across time were graphed from baseline to week 17 for each of the 5 sites. SAS® statistical software, version 9.4 (SAS, Cary, NC) was used for all procedures in these analyses with significance level set at $\alpha=0.05$.

3.2.2 Nested Logistic Regression Mixed Effects Models

To examine the longitudinal patterns in our mycobacteriology data, we considered four separate 3-level logistic random intercept models with multiple random effects for solid media (LJ and 7H11S) and liquid medium (MGIT). The first two models explored the culture positivity of solid and liquid media against Mtb-negative results by modeling two types of culture outcomes, Mtb-positive and Mtb-negative. The next two models explored the recovery of Mtb in solid and liquid media against contamination by modeling two types of culture outcomes, Mtb-positive and Contaminated. Culture outcomes data in all four models were nested with three levels: sample, participant, and site. In addition, two random effects were included to account for potential within-participant and within-site correlations in the culture results and were assumed to be independent and additive. All predictor variables of interest (solid culture type, time on treatment, exposure to CO_2, sputum sample volume and participant HIV status) were coded using the reference-cell coding scheme.

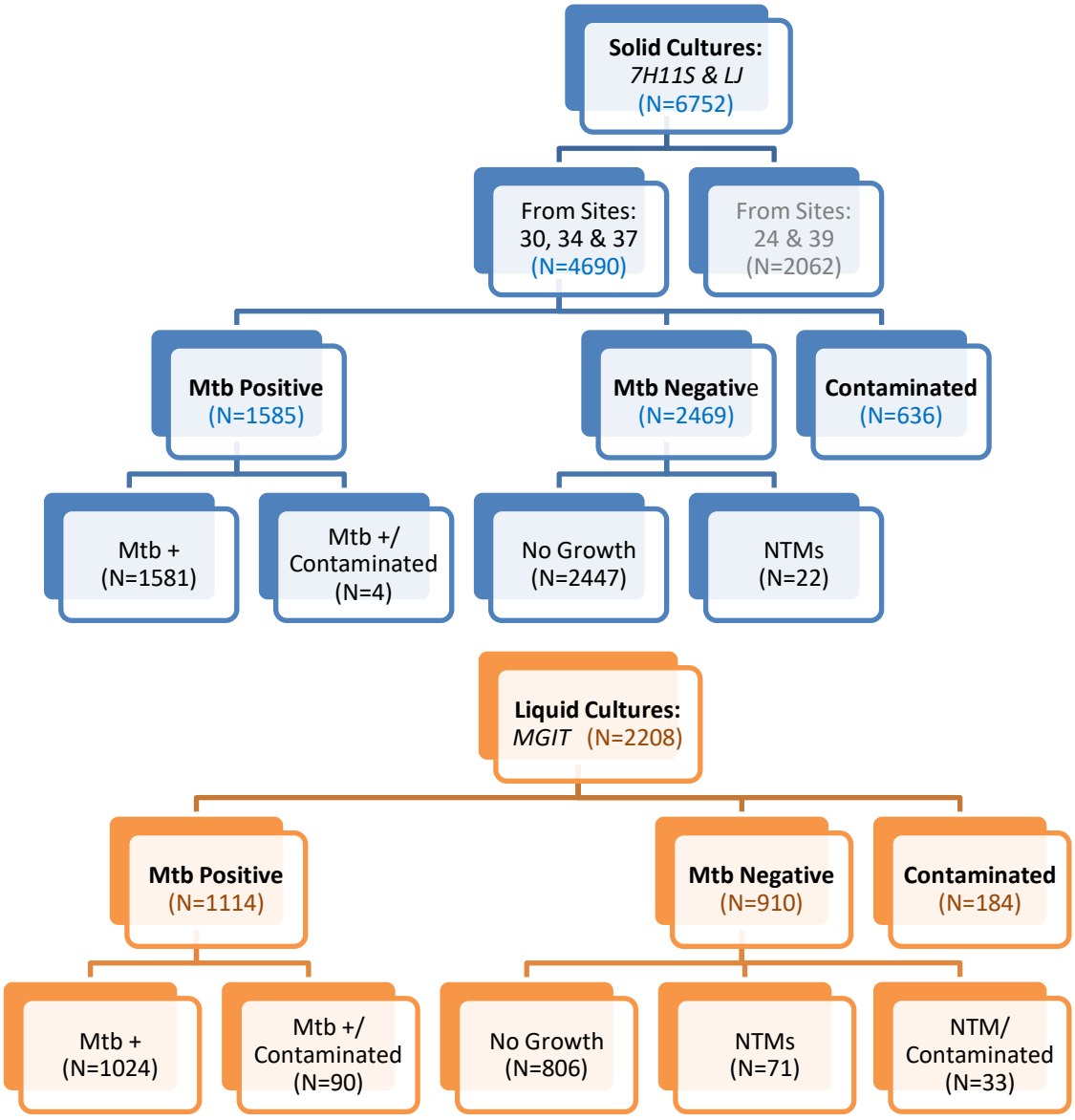
For the solid culture model, observations from the New York and Kenya sites were excluded due to the lack of culture data without CO_2 and observed deviations found in the 7H11S agar formulation from the study guidelines, respectively. No site data were excluded for the MGIT model. The numbers of

culture media results corresponding to different categories of culture outcomes and site exclusion criteria are summarized in Figure 1.

Final models were determined using manual forward selection by adding each predictor variable one at a time and checking for significance of terms. Any covariates corresponding to non-significant beta coefficients at $\alpha=0.05$ were removed from the model while the significant ones remained. The potential for two-way interactions between covariates were also assessed for inclusion in the final models by forming and testing pair-wise products of significant predictors in each of the four models. Fitness assessment of different models in the final model selection was based on information criteria; Akaike's information criterion (AIC) values were compared each time a predictor was added or removed from the current model. For solid models, however, important covariates indicating the solid culture type and CO_2 exposure were always included.

The reference values for the culture positivity solid model was sputum samples from HIV-negative participant collected at the baseline (week 0) visit and cultured on 7H11S medium without CO_2, while the reference values for the liquid model was sputum samples from HIV-negative participant collected at the baseline visit and inoculated on MGIT. Furthermore, the reference values for the Mtb recovery solid model were baseline specimens with volume of 3ml and inoculated on 7H11S medium without CO_2, while the reference values for the liquid model were baseline specimens inoculated on MGIT with sputum sample volume equal to 3ml. All GLMM modeling was done using RStudio with *mgcv* and *lme4* libraries (RStudio Version 0.99.903 – © 2009-2016 RStudio, Inc.).

Figure 1: Categorization of culture media outcomes



Site 24 and 39 are grayed out to indicate exclusion of data from modeling part of the analyses

NTM: growth of non-tuberculosis Mycobacteria; Contaminated: growth other than Mtb or NTMs

LJ: Lowenstein-Jensen culture medium; 7H11S: selective Middlebrook agar medium; MGIT: BACTEC MGIT 960

4. RESULTS

4.1 *Sensitivity, Mtb Yield, Contamination, and Discordance Analysis Results*

There were 8960 culture results from 5 media conditions (7H11S, 7H11S with CO_2, LJ, LJ with CO_2 and BACTEC MGIT 320/960) of sputum specimens collected in the first 17 weeks of TB treatment. For the sensitivity, Mtb-positive yield, and contamination rate analyses and pairwise discordance calculation, Site 39 (Vietnam)'s 7H11S (incubated with and without CO_2) data were excluded due to deviation in the formulation of their 7H11S media from study laboratory guidelines.

Overall, there were 3020 Mtb-positive (38.0%), 3950 Mtb-negative (49.7%) and 978 contaminated (12.3%) cultures. The proportion of Mtb-positive results was highest in MGIT (50.5%), followed by 7H11S (31.8% without CO_2 and 37.7% with CO_2) and LJ (32.0% without CO_2 and 31.4% with CO_2). In contrast, the proportion of Mtb-negative results was lowest in MGIT (41.2%), followed by 7H11S (50.9% without CO_2 and 48.0% with CO_2) and LJ (56.2% without CO_2 and 54.7% with CO_2). The frequencies and percentages of culture media results, overall and by site, are shown in Table 1.a and 1.b, respectively. MGIT always had the highest proportion of Mtb-positive results, followed by 7H11S with and without CO_2 and then LJ with and without CO_2 except for Site 37 (Vietnam), where the proportion of Mtb-positive cultures results were higher for LJ media than 7H11S (both with and without CO_2).

Table 1.a: Frequency and percentages of types of culture media results from all sites

Medium	Positive (%)	Negative (%)	Contaminated (%)
7H11S	262 (31.8)	419 (50.9)	142 (17.3)
7H11S *with CO_2*	537 (37.7)	684 (48.0)	203 (14.3)

LJ	543 (32.0)	955 (56.2)	200 (11.8)
LJ *with CO_2*	564 (31.4)	982 (54.7)	249 (13.9)
MGIT	1114 (50.5)	910 (41.2)	184 (8.3)

7H11S and 7H11S with CO_2 culture data from Site 39 were excluded from the frequency and percentage calculation

NTM: growth of non-tuberculosis Mycobacteria; Contaminated: growth other than Mtb or NTMs

LJ: Lowenstein-Jensen culture medium; 7H11S: selective Middlebrook agar medium; MGIT: BACTEC MGIT 960

Table 1.b: Frequency and percentages of types of culture media results by each site

Site 24 *(New York, US)*	**Medium**	**Positive (%)**	**Negative (%)**	**Contaminated (%)**
	7H11S *with CO_2*	6 (37.5)	9 (56.3)	1 (6.3)
	LJ *with CO_2*	5 (31.3)	10 (62.5)	1 (6.3)
	MGIT	8 (50.0)	8 (50.0)	0 (0.0)
Site 30 *(Uganda)*	**Medium**	**Positive (%)**	**Negative (%)**	**Contaminated (%)**
	7H11S	141 (36.2)	225 (57.7)	24 (6.2)
	7H11S *with CO_2*	392 (42.1)	501 (53.8)	39 (4.2)
	LJ	187 (33.2)	326 (57.9)	50 (8.9)
	LJ *with CO_2*	258 (33.8)	454 (59.4)	52 (6.8)
	MGIT	510 (54.6)	312 (33.4)	113 (12.1)
Site 34 *(South Africa)*	**Medium**	**Positive (%)**	**Negative (%)**	**Contaminated (%)**
	7H11S	54 (32.1)	92 (54.8)	22 (13.1)
	7H11S *with CO_2*	79 (37.6)	113 (53.8)	18 (8.6)
	LJ	40 (24.0)	94 (56.3)	33 (15.3)
	LJ *with CO_2*	58 (26.9)	125 (57.9)	33 (15.23)
	MGIT	135 (46.9)	143 (49.7)	10 (3.5)
Site 37 *(Vietnam)*	**Medium**	**Positive (%)**	**Negative (%)**	**Contaminated (%)**
	7H11S	67 (25.3)	102 (38.5)	96 (36.2)
	7H11S *with CO_2*	60 (22.6)	61 (22.9)	145 (54.5)
	LJ	163 (35.6)	255 (55.7)	40 (8.7)
	LJ *with CO_2*	86 (29.6)	121 (41.6)	84 (28.9)
	MGIT	220 (48.1)	193 (42.2)	44 (9.6)
Site 39 *(Kenya)*	**Medium**	**Positive (%)**	**Negative (%)**	**Contaminated (%)**
	LJ	153 (30.0)	280 (54.9)	77 (15.1)

LJ *with* CO_2	157 (30.9)	272 (53.5)	79 (15.6)
MGIT	241 (47.1)	254 (49.6)	17 (3.3)

Sensitivity:

The sensitivities of culture media, overall and by site, are shown in Table 2.a and 2.b, respectively. MGIT consistently had the highest sensitivity across all sites with overall sensitivity of 98.5%. This was followed by 72.2% and 77.6% for 7H11S and 7H11S with CO_2, and 67.8% and 69.2% for LJ and LJ with CO2, respectively.

Mtb Yield:

The trend in positive-Mtb culture yield against negative-Mtb cultures was similar to the one found with sensitivity: the highest Mtb yield was found with MGIT across all sites, followed by 7H11S media conditions and LJ media conditions. Overall, 55.0% of evaluable MGIT cultures were positive for Mtb, while the Mtb yield was 38.5% and 44.0% for 7H11S and 7H11S with CO_2, and 36.3% and 36.5% for LJ and LJ with CO2, respectively (Table 2.a and 2.b). Chi-square tests results indicated uniform trends in proportions among sites with insignificant p-values for all media except MGIT, which showed significantly different Mtb yield among sites ($p < 0.0001$).

Contamination:

Contamination of different culture systems and conditions varied greatly by site and are also summarized in Table 2.a and 2.b. Overall, the highest rate of contamination was seen in 7H11S with 17.4% (n=143), followed by 7H11S with CO_2 (n=206) with

14.5%, MIGIT (n=307) and LJ with CO_2 (n=249) with 13.9%, and LJ (n=200) with 11.8%. A Pearson Chi-square test results revealed that the contamination rates for all five media were significantly different among sites ($p < 0.0001$), indicating highly variable contamination rate among sites.

Pairwise Discordance:

Pairwise discordance of culture results among different media are summarized in Table 3. Highest rates of discordance observed were seen between MGIT and the two LJ media with and without CO_2 with 11.3% and 11.6% discordance, respectively. 7H11S media showed intermediate discordance in culture results with MGIT at 4.4% and 7.2% for 7H11S and 7H11S with CO_2, respectively. Culture results of 7H11S media conditions, with and without CO_2, were most agreeable with the lowest discordance at 0.96%. The two LJ media were also relatively concordant with discordance at 2.4%. These findings indicated high variability of culture results for the same specimen between solid (LJ and 7H11S) and liquid media.

Table 2.a: Sensitivity, Mtb yield, and contamination rate of culture media

Medium	**Sensitivity %**	**Mtb Yield %**	**Contamination** % (n)
7H11S	72.2	38.5	17.4 (143)
7H11S *with CO_2*	77.6	44.0	14.5 (206)
LJ	67.8	36.3	11.8 (200)
LJ *with CO_2*	69.2	36.5	13.9 (249)
MGIT	98.5	55.0	13.9 (307)

Sputum specimens were collected over the course of first 17 weeks of TB treatment from 330 study participants.

Contamination rate calculation includes cases of both contaminated cultures and co-contamination (Mtb-positive/Contaminated or Mtb-negative/Contaminated cultures).

LJ: Lowenstein-Jensen culture medium; 7H11S: selective Middlebrook agar medium; MGIT: BACTEC MGIT 960.

Table 2.b: Sensitivity, Mtb yield, and contamination rate of culture media for each site

Site 24 *(New York, US)*	**Medium**	**Sensitivity** %	**Mtb Yield** %	**Contamination** % (n)
	7H11S *with CO_2*	75.0	40.0	6.3 (1)
	LJ *with CO_2*	62.5	33.3	6.3 (1)
	MGIT	100.0	50.0	0.0 (0)
Site 30 *(Uganda)*	**Medium**	**Sensitivity** %	**Mtb Yield** %	**Contamination** % (n)
	7H11S	71.6	38.5	6.2 (24)
	7H11S *with CO_2*	76.3	43.9	4.2 (39)
	LJ	62.1	36.5	8.9 (50)
	LJ *with CO_2*	66.3	36.2	6.8 (52)
	MGIT	98.8	62.0	23.2 (217)
Site 34 *(South Africa)*	**Medium**	**Sensitivity** %	**Mtb Yield** %	**Contamination** % (n)
	7H11S	75.0	37.0	13.7 (23)
	7H11S *with CO_2*	79.0	41.2	9.5 (20)
	LJ	61.5	29.9	19.8 (33)
	LJ *with CO_2*	63.0	31.7	15.3 (33)
	MGIT	100.0	48.6	4.2 (12)
Site 37 *(Vietnam)*	**Medium**	**Sensitivity** %	**Mtb Yield** %	**Contamination** % (n)
	7H11S	71.3	39.6	36.2 (96)
	7H11S *with CO_2*	85.7	49.6	54.9 (146)
	LJ	74.4	30.0	8.7 (40)
	LJ *with CO_2*	78.2	41.6	28.9 (84)
	MGIT	97.4	53.3	10.3 (47)
Site 39 *(Kenya)*	**Medium**	**Sensitivity** %	**Mtb Yield** %	**Contamination** % (n)
	LJ	70.8	35.3	15.1 (77)
	LJ *with CO_2*	73.0	36.6	15.6 (79)
	MGIT	98.0	48.7	6.1 (31)

Table 3: Pairwise Discordance among culture media

	7H11S with CO_2	LJ	LJ with CO_2	MGIT
7H11S	0.96	2.9	2.4	4.4
7H11S with CO_2		3.8	4.8	7.2
LJ			2.4	11.6
LJ with CO_2				11.3

Discordance is the percentage of specimens where result of a medium was Mtb-positive and the other was Mtb-negative out of cases where both media were not contaminated and evaluable.

LJ: Lowenstein-Jensen culture medium; 7H11S: selective Middlebrook agar medium; MGIT: BACTEC MGIT 960.

4.2 *Modeling Results & Interpretations*

Based on the high percent of discordance found between liquid and solid culture results and significant difference in proportions of Mtb yield for MGIT culture system and contamination rate of all 5 culture conditions across sites, a solid and liquid mixed effects logistic regression model for Mtb-positive versus Mtb-negative outcomes (culture positivity) and Mtb-positive versus contaminated outcomes (Mtb recovery against contamination) were constructed with site and participants random effects. Line plots of contamination and Mtb yield rate over the first 17 weeks of treatment time in Figure 2.a to 2.d. displayed noticeable trends of decrease in Mtb yield and varying trends of increase in contamination rate over time, overall and among sites. These findings supported the used of GLMM for a longitudinal analysis of repeated participant mycobacteriology data.

Of the 330 participants enrolled for the study, 3 participants' solid culture data from Site 24 (New York; all solid cultures incubated with CO2) and 78 participants' solid culture data from Site 39 (Kenya; different formulation used in making 7H11S media), were excluded for the GLMM modeling of both the culture positivity (against Mtb-negative cultures) and Mtb recovery (against contaminated cultures) to reduce model

convergence issues. Final models after model selection process based on significant predictors and AIC values were the following:

$$logit\ P(y_{ijk}=1) = 4.59 - 0.67LJ + 0.33CO_2 - 0.97Time - 1.48HIV + \mu_k + \mu_{jk} \quad \textbf{(Eq.1.a)}$$

$$logit\ P(y_{ijk}=1) = 5.04 - 0.73Time - 1.09HIV + \mu_k + \mu_{jk} \quad \textbf{(Eq.1.b)}$$

$$logit\ P(y_{ijk}=1) = 3.07 - 0.44Time - 0.02LJ - 0.02CO_2 - 0.06Volume + \mu_k + \mu_{jk} \quad \textbf{(Eq.2.a)}$$

$$logit\ P(y_{ijk}=1) = 5.54 - 0.50Time - 0.07Volume + \mu_k + \mu_{jk} \quad \textbf{(Eq.2.b)}$$

$$\mu_{jk} \sim N(0, \sigma_2^2), \qquad \mu_k \sim N(0, \sigma_3^2)$$

Equations 1.a. and 1.b were final culture positivity models for solid cultures (7H11S & LJ) and MGIT, while equations 2.a. and 2.b. were Mtb recovery models for solid cultures (7H11S & LJ) and MGIT, respectively. The results of four independent mixed effects logistic models are summarized in Table 4.a to 4.d.

Based on the solid culture positivity model (Eq.1.a and Table 4.a), the probability of Mtb-positive culture against culture negativity for a typical (population average) sample collected from HIV-negative participant at week 0 and cultured on 7H11S without CO_2 was 0.990 (95% Wald CI: 0.983, 0.994). After controlling for potential within-participant and within-site correlations, LJ method, HIV-positive status, and treatment time showed statistically significant negative effects on culture positivity with OR of 0.514 (95% CI: 0.401, 0.658), 0.227 (0.082, 0.629), and 0.379 (0.351, 0.409), respectively. In contrast, exposure to CO_2 showed a significant positive effect on culture positivity with OR of 1.367 (1.073, 1.818). The MGIT model for culture positivity (Eq. 1.b and Table 4.b) resulted in probability of Mtb-positive culture for a typical sample collected from HIV-negative participant at week 0 equal to 0.994 (95% CI: 0.986, 0.997).

Similarly, HIV-positive status and treatment time showed statistically significant negative effects on MGIT culture positivity with OR of 0.483 (0.443, 0.526) and 0.336 (0.082, 0.629), respectively.

The two models for Mtb recovery (Eq. 2.a & 2.b) revealed a different set of significant predictors in their models. Based on the solid culture positivity model (Eq.2.a and Table 4.c), the probability of Mtb-positive culture against contamination for a typical (population average) sample with 3mL total sputum volume at week 0 and cultured on 7H11S without CO_2 was 0.956 (95% Wald CI: 0.880, 0.985). After controlling for potential within-participant and within-site correlations, treatment time and sputum sample volume above 3mL showed statistically significant negative effects on Mtb recovery with OR of 0.641 (95% CI: 0.609, 0.675) and 0.945 (0.906, 0.987), respectively. The covariates for LJ medium and exposure to CO_2 also showed a negative effect on Mtb recovery but were not significant with p-values >0.05 They were, however, deemed important for comparing two solid media types and their two conditions and were still included in the final models. The model for Mtb recovery (Eq. 2.b and Table 4.d) resulted in probability of Mtb-positive culture for a typical sample with volume of 3mL collected at baseline equal to 0.996 (95% CI: 0.991, 0.998). Again, treatment time and total sputum volume showed statistically significant negative effects on MGIT Mtb recovery with adjusted OR of 0.928 (0.877, 0.982) and 0.336 (0.082, 0.629), respectively.

Table 4.a: Multivariate model effects on culture positivity for solid media (adjusted odds ratio)

Fixed Effects	OR Estimate	95% CI	P-value
Intercept	98.907	(58.420, 167.448)	< 2e-16
LJ Media	0.514	(0.401, 0.658)	1.28e-07

Exposure to CO_2	1.367	(1.073, 1.818)	0.013
Treatment Time (weeks)	0.379	(0.351, 0.409)	< 2e-16
HIV-Positive Status	0.227	(0.082, 0.629)	0.004
Random Effects	**Variance**	**Standard Deviation**	
Participant (Intercept)	4.930	2.220	
Site (Intercept)	0.00	0.00	

Reference values (comparison group for the OR estimates): Sputum samples collected at baseline (week 0) from HIV-negative participants cultured on 7H11S without CO_2

Table 4.b: Multivariate model effects on culture positivity for liquid medium (adjusted OR)

Fixed Effects	**OR Estimate**	**95% CI**	**P-value**
Intercept	154.002	(72.306, 328.001)	< 2e-16
Treatment Time (weeks)	0.483	(0.443, 0.526)	< 2e-16
HIV-Positive Status	0.336	(0.156, 0.722)	0.005
Random Effects	**Variance**	**Standard Deviation**	
Participant (Intercept)	3.174	1.782	
Site (Intercept)	0.139	0.372	

Reference values (comparison group for the OR estimates): Sputum samples collected at baseline (week 0) from HIV-negative participants cultured on MGIT.

Table 4.c: Multivariate model effects on Mtb recovery for solid media (adjusted OR)

Fixed Effects	**OR Estimate**	**95% CI**	**P-value**
Intercept	21.561	(7.309, 63.600)	2.64e-08
LJ Media	0.985	(0.401, 1.291)	0.913
Exposure to CO_2	0.980	(0.743, 1.293)	0.886
Treatment Time (weeks)	0.641	(0.609, 0.675)	< 2e-16
Sputum Sample Volume (mL)	0.945	(0.906, 0.987)	0.010
Random Effects	**Variance**	**Standard Deviation**	
Participant (Intercept)	1.819	1.349	
Site (Intercept)	0.766	0.875	

Reference values (comparison group for the OR estimates): Sputum samples collected at baseline (week 0) with volume of 3mL cultured on 7H11S without CO_2.

Table 4.d: Multivariate model effects on Mtb recovery for liquid medium (adjusted OR)

Fixed Effects	OR Estimate	95% CI	P-value
Intercept	254.763	(108.054, 600.664)	< 2e-16
Treatment Time (weeks)	0.604	(0.555, 0.656)	< 2e-16
Sputum Sample Volume (mL)	0.928	(0.877, 0.982)	0.009
Random Effects	**Variance**	**Standard Deviation**	
Participant (Intercept)	2.379	1.542	
Site (Intercept)	0.00	0.00	

Reference values (comparison group for the OR estimates): Sputum samples collected at baseline (week 0) with volume of 3mL cultured on MGIT.

5. DISCUSSION

Clinical trials research for TB treatment aims to find shorter yet effective treatment regimens to increase the treatment adherence and success. Moreover, TB clinical trials rely primarily on mycobacteriology evidence for assessing treatment efficacy against active TB disease. Sensitivity of culture systems becomes especially important during the latter part of the TB treatment in clinical trials, where bacillary burden is significantly reduced in sputum samples, which could lead to an increase in false negative results. Hence, it is of a great interest to identify the best available culturing methods and conditions for Mtb growth to provide detection that is sensitive and against contamination.

In this observational multi-site study, five sites enrolled and followed participants with active pulmonary TB and collected sputum samples for up to 7 different time points during their TB treatment (week 0, 2, 4, 6, 8, 13, and 17 after enrollment). Initial analyses of sensitivity, Mtb yield, and contamination rates were performed on all site data except

7H11S culture results from Site 39 (Kenya) to compare the performance of different culturing conditions.

Results revealed that MGIT had both the highest Mtb-positive yield and sensitivity followed by 7H11S with and without CO_2, and lastly LJ with and without CO_2. The sensitivity for MGIT was 29.3 to 30.7 percentage points higher than the two LJ media (with and without CO_2) and 20.9 to 26.3 percentage points higher than the two 7H11S media (Table 2.a). Similarly, Mtb yield was about 18.6 percentage points higher in MGIT than LJ media and 11 to 16.5 percentage points higher than 7H11S media. The effect of exposure of solid media to 8-10% CO_2 during incubation resulted in higher Mtb yield and sensitivity for both 7H11S and LJ. For 7H11S, sensitivity increased by 5.4 percentage points with CO_2, while sensitivity for LJ increased by 1.4 percentage points with CO_2. For Mtb yield, there was a 5.5 percentage point increase in 7H11S and a 0.2 percentage point increase in LJ proportions of Mtb yield with CO_2. In contrast, contamination rates for sites were highly variable with no single culture medium or a condition having the highest or lowest contamination rate across all sites. There were also no observable trends with exposure to CO_2 on contamination rate in solid cultures.

Prior to moving on to GLMM modeling approach for longitudinal analysis of the repeated culture data, the possibility of multiple correlations, within site and within participant, was first explored through visualization of temporal trends in Mtb yield and contamination rate over time by each site and for all sites in Table 2.a-d. While there was a clear negative trend in Mtb yield for all media types over time in every site, there was not a consistent trend observed for contamination rates over time across all sites. In general, pairwise discordance analysis illustrated the highest discordance between liquid

and solid media and not among solid media types, LJ and 7H11S, or among the same medium with different CO_2 conditions. This supported the stratification of data into liquid and solid media results and constructing separate models for Mtb-positive versus Mtb-negative culture results and Mtb-positive versus contaminated culture outcomes. Potential factors investigated in mixed effects logistic modeling for association with culture positivity and Mtb recovery against contamination were the type of solid culture, exposure to CO_2 for solid cultures media, HIV status of participant with active TB disease, total volume of sputum collected, and the time on treatment in weeks.

Multivariate model effects of significant predictors on culture positivity for solid media (7H11S and LJ) showed that odds of culture positivity decreased by 48.6% when using LJ medium compared to 7H11S medium. There was also a significant decrease in odds of culture positivity associated with increase in time on treatment and HIV-positive infection status by 62.1% and 77.3%, respectively. However, exposure to CO_2 during incubation for solid cultures had a positive effect on the odds of culture positivity by a factor of 1.4 over cultures incubated without CO_2. Culture positivity model for MGIT (Eq.1.b) showed two significant effectors, treatment time and HIV-positive status. These predictors lowered the odds of culture positivity by 51.7% for every additional week on TB treatment and by 66.4% for participants who are HIV-positive. These results indicated that higher odds of Mtb-positive cultures are associated with using 7H11S medium compared to LJ, with CO_2 during incubation compared to no additional CO_2, HIV-negative individuals with active TB compared to HIV-positive individuals, and at earlier stages of TB treatment compared to later.

The multivariate model effects of significant predictors on Mtb recovery against contamination for solid media showed that odds of Mtb recovery were reduced by 5.5% per unit increase in total sputum volume (mL) collected from participants. We also observed significant decreases in the odds of recovery associated with increase per week on treatment by 35.9%. The Mtb recovery model for MGIT (Eq.1.d) showed two significant effectors, treatment time and sputum sample volume. Both predictors lowered the odds of Mtb recovery by 39.6% for every additional week on TB treatment and by 7.2% per unit increase in total sputum volume. These results indicated that there are reduced odds of Mtb-positive cultures due to contamination associated with the total sputum volume greater than 3mL and longer time on TB treatment. Unlike culture positivity model results for solid and MGIT culture systems, the type of solid culture methods (LJ vs. 7H11S), participant HIV status, and use of CO_2 during incubation for solid culture had no significant impact on recovery of Mtb against contamination.

There have been various studies comparing the performance MGIT systems with solid media in detecting Mtb. Similar to findings in this study, several other studies have indicated that the MGIT system provide better Mtb yield than solid media; in addition, contamination rates of different media types were reported to be highly variable across different sites and across different studies (Hanna et al., 1999; Chien et al., 2000; Muyoyeta et al., 2009). Variations in contamination rates could be attributed to the dissimilar conditions of specimen quality, type of contaminants present in the samples, transport time and conditions, inoculating and pipetting procedures, contaminants in laboratory cabinets or tubes in different sites and their laboratories (Chien et al., 2000; Muyoyeta et al., 2009). In a study aiming to determine the most reliable solid culture

medium, the 7H11S medium was observed to conform better to reference standards than the LJ medium (Heilig et al., 2014). This was also true in our study with higher sensitivity found in 7H11S compared to LJ (4.4% higher) and 7H11S with CO_2 compared to LJ with CO_2 (18.4% higher).

Modeling results revealed that higher odds of Mtb-positive cultures are associated with using 7H11S medium compared to LJ medium, with CO_2 during incubation, HIV-negative individuals with active pulmonary TB, and in the earlier weeks of TB treatment. Moreover, a second set of binomial logistic regression models showed that there are reduced odds of Mtb-positive culture due to contamination associated with the total sputum volume greater than 3mL and longer time on TB treatment. These results agreed with earlier studies indicating stimulatory effects of CO_2 in growth of mycobacteria in solid cultures (Gruft and Loder, 1971). The association between Mtb positivity with HIV co-infection status demonstrated by this study may be due to the scarce sputum production of HIV-positive individuals (Lora et al., 2015). Since obtaining adequate sputum samples from participants with scarce sputum production is difficult, sputum induction techniques are often implemented on these participants for sample obtainment for pulmonary TB diagnosis (Lora et al., 2015). Additional attention is needed in collecting quality sputum samples from HIV-positive participants. The negative effect shown with sputum volume greater than 3mL on Mtb recovery against contamination may be related to decontamination of specimen during sputum processing. Sputum sample high in volume may be improperly or under-decontaminated, leading to higher contamination that compete with growth of Mtb (Mgit Manual, 2006). Limit on the

volume of sputum samples or changes in specimen processing according to the amount of sputum collected may be helpful reducing contamination rate.

5.1 *Implications*

This study provides longitudinal analysis of multi-site culture data using mixed effects modeling for liquid and solid media. Initial analyses showed that MGIT is the most sensitive culture system with the best Mtb yield compared to two solid media. In addition, 7H11S outperforms LJ in both sensitivity and Mtb yield. Benefits of 8-10% of CO_2 during incubation for solid cultures were also shown with increase in sensitivities and Mtb yields but not necessarily in contamination rates. Modeling results also confirmed a better performance by 7H11S over LJ and the stimulatory effects of CO_2 on Mtb positivity. Results demonstrated association between HIV infection status and culture positivity due to scarce sputum production and reduced quality specimens. Moreover, large sputum volumes showed association to contamination suggesting potential under-decontamination issues during sputum processing.

5.2 *Strengths and Limitations*

Between-participant heterogeneity could exist in repeated data due to correlation between measurements within a participant. Similarly, between-site heterogeneity could also exist for data collected from multiple sites of different countries and regions. For example, population makeup of person afflicted with TB in a certain region may be different from region to region, and the laboratory techniques and procedures used to produce and culture media may also be different from site to site. Failing to account for

these potential heterogeneities may give biased estimation, underestimation, or both (Egger et al, 2013; Chu et al, 2009). Hence, one of the strength of this analysis was the incorporation of random effects in modeling to account for heterogeneities and independent correlations in the study data. The longitudinal exploration of the data allowed for observation of temporal trends in culture positivity and Mtb-recovery against contamination. Mixed effects logistic regression modeling took steps beyond the conventional sensitivity, Mtb-yield, and contamination analyses to compare the performance of different culturing methods and conditions in detection of Mtb. Finally, efforts to reduce bias in estimates and to preserve interpretability were made by constructing two types of independent models, one directly comparing Mtb-positive and negative outcomes and another directly comparing Mtb-positive and contaminated outcomes. Since there were total of three possible culture outcomes (Figure 1), a single binomial logistic model approach would have resulted in either exclusion or re-grouping of the contaminated outcomes with Mtb-positive or negative results. In either case, there would be bias introduced to overestimate or underestimate the proportion of Mtb-positive cultures (Heilig et al., 2014). Therefore, independent binomial modeling approach was used to preserve the contaminated category and to reduce bias in either direction. Although the use of multinomial logistic regression to model all three outcomes at once was considered for more precise estimates, independent binomial model approach was ultimately selected for its better interpretability of results.

In addition to the strengths of this study, there were also several limitations that could have affected the quality of collected culture data and accuracy of analyses results. First issue is the potential variability in the quality of solid media due to the wide use of

non-commercial media. While commercially-made culture media are standardized in its production, in-house made media could be subjected to variability due to difference in sources of ingredients, procedures, and handling techniques. The number of sites using in-house made media for 7H11S and LJ were 4 out of 5 and 2 out of 5, respectively. Similar to the major formulation issue for 7H11S media in Site 39 that led to exclusion of their data in the analyses, their earlier LJ media until April, 2015 were supplemented with antibiotics, which could have reduced overall and site contamination rate for LJ media. There were also other site-specific issues that could have influenced the data and analysis results. For example, Site 37(Vietnam) reported malfunctioning of their CO_2 incubator, which was later replaced but could have led to spurious results in solid cultures.

5.3 *Recommendations*

Based on the results, we conclude that use of 7H11S solid medium with CO2 is the better solid method in detecting Mtb than compared to LJ medium. Furthermore, combinational use of 7H11S with CO2 with highly sensitive MGIT liquid medium will both effectively increase Mtb detection and allow obtainment of pure isolates in TB Phase II clinical trials. Careful consideration for quality of sputum sample from HIV-positive participants and the total volume collected is also recommended.

For the future direction of the study, imputations for missing data (i.e. Site 24, New York, only tested with CO_2 for solid cultures) or a separate summary for the sites where their data was completely excluded from the analysis (i.e. Site 37, Kenya, used disparate formulation for all 7H11S media) could help with loss of information. In addition, other modeling approaches using different methods than GLMM could result in

more insightful and accurate results. For example, instead of using GLMM, Generalized Estimation Equation (GEE) method, which has no underlining distribution assumptions for random effect variances, or Bayesian analysis procedures that allows incorporation of prior knowledge on parameters distribution could be implemented (Chu et. al., 2009). Moreover, additional information criteria including Bayesian information criterion (BIC) and deviance information criterion (DIC) could be used in addition to AIC to find the final models (Chu et. al., 2009). Finally, additional predictors, such as degree of TB disease and country or geographical region of residence for study participants, could also be tested in the model for their potential effects on the culture positivity and Mtb recovery against contamination.

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APPENDIX

Figure 2.a: Overall Mtb yield by culture media type for the first 17 weeks of TB treatment

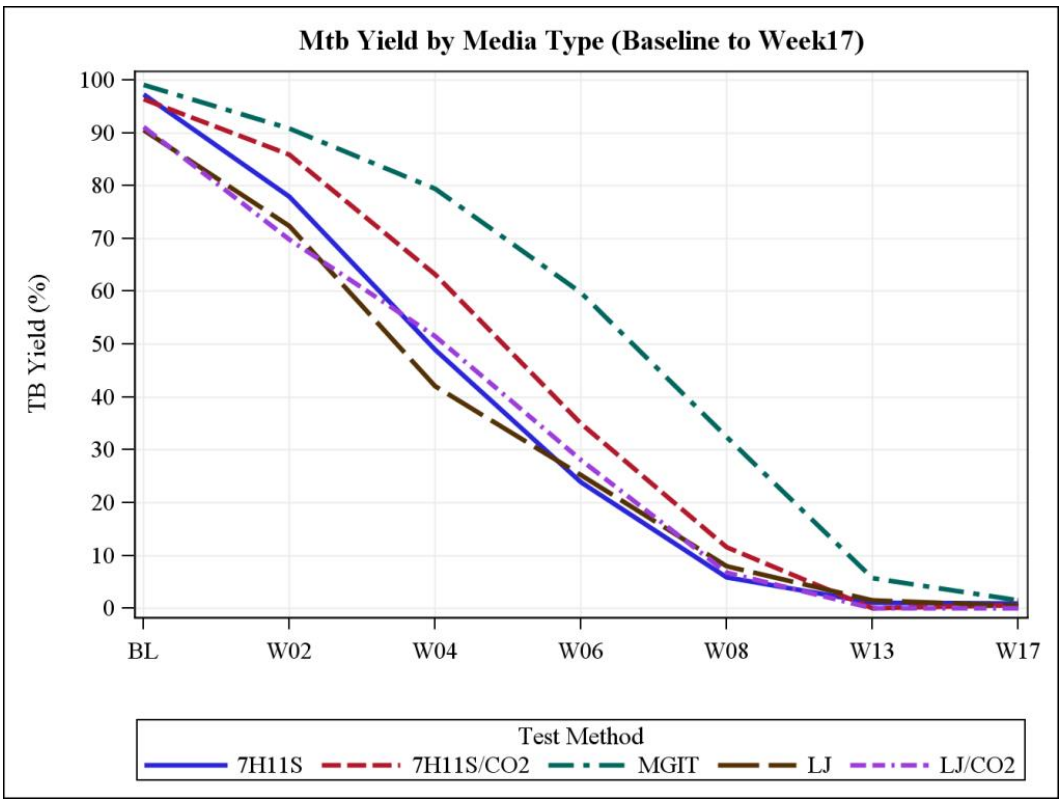
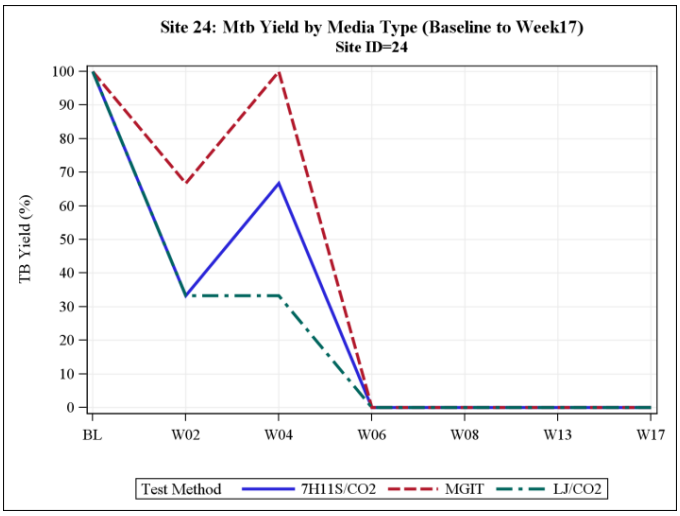
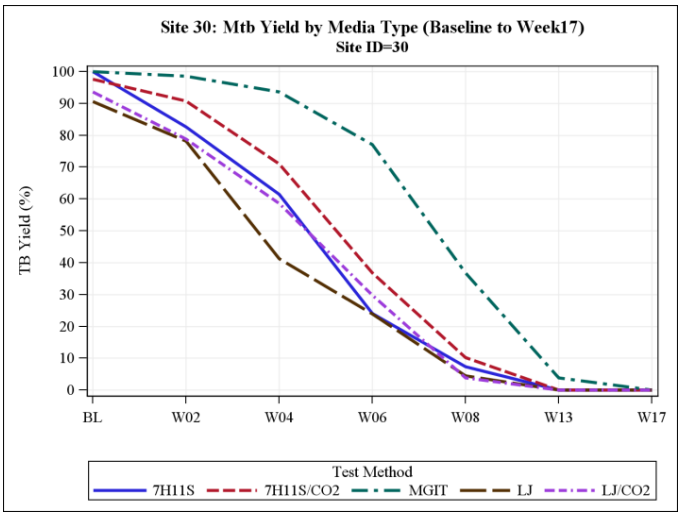
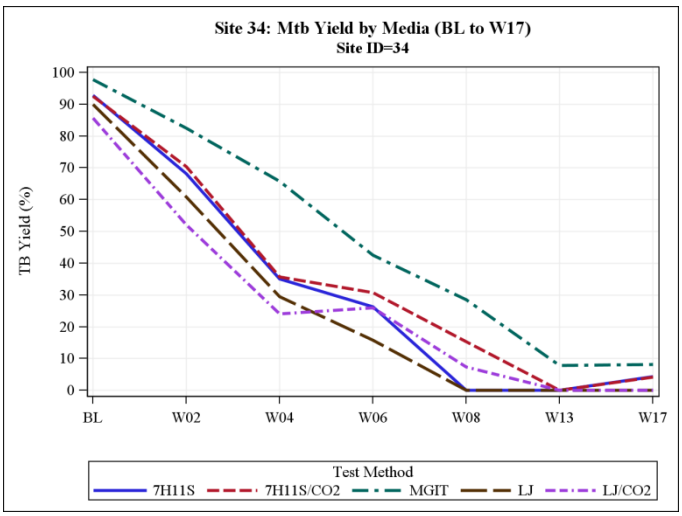
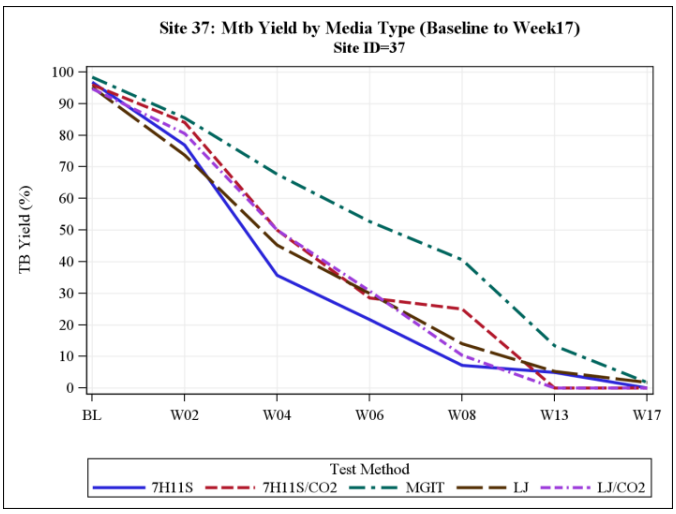


Figure 2.b: Mtb yield by culture media type for the first 17 weeks of TB treatment by site








Site 37: Mtb Yield by Media Type (Baseline to Week17)
Site ID=37
TB Yield (%)
100
90
80
70
60
50
40
30
20
10
0
BL
W02
W04
W06
W08
W13
W17
Test Method
7H11S
7H11S/CO2
MGIT
LJ
LJ/CO2

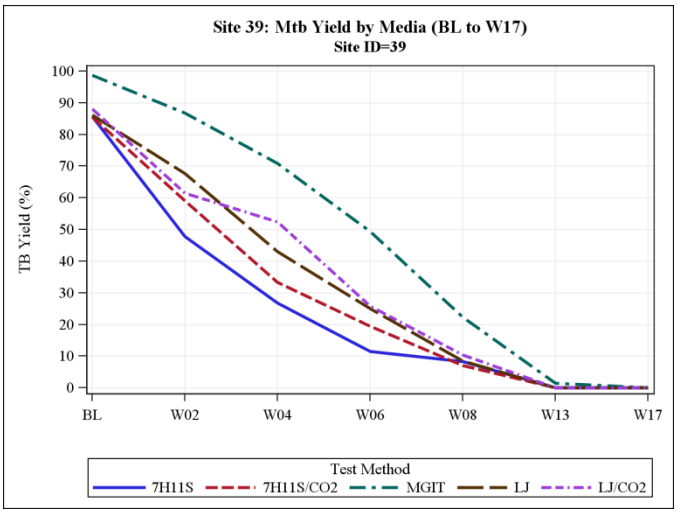

Site 39: Mtb Yield by Media (BL to W17)
Site ID=39
TB Yield (%)
100
90
80
70
60
50
40
30
20
10
0
BL
W02
W04
W06
W08
W13
W17
Test Method
7H11S
7H11S/CO2
MGIT
LJ
LJ/CO2

Figure 2.c: Overall contamination rate by culture media type for the first 17 weeks of TB treatment

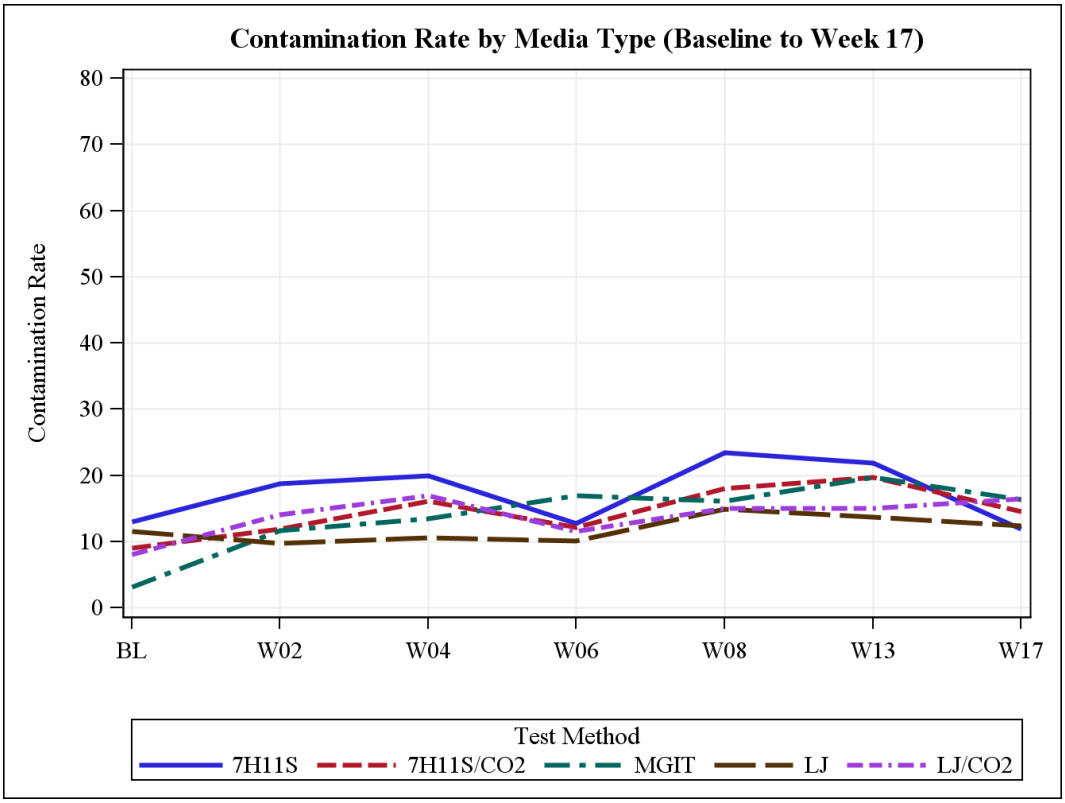
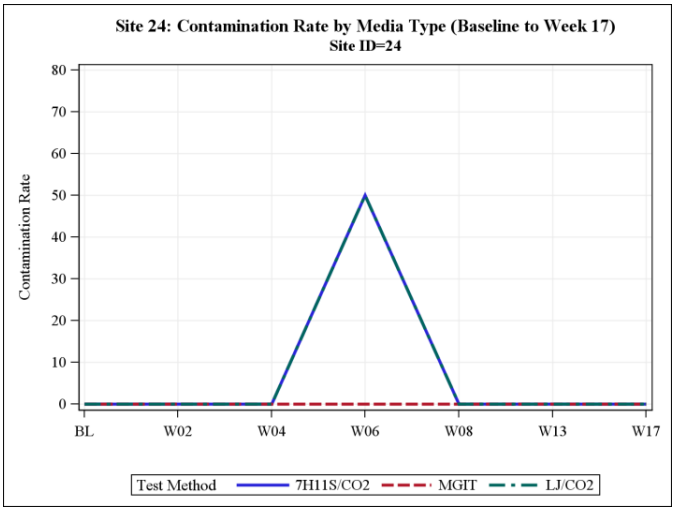
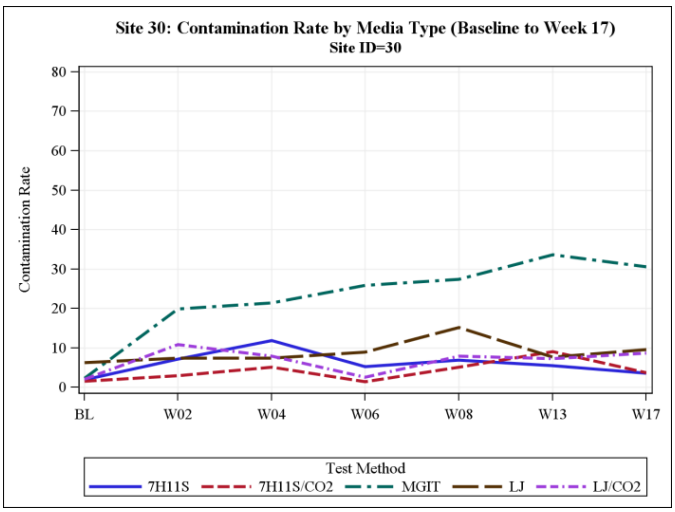
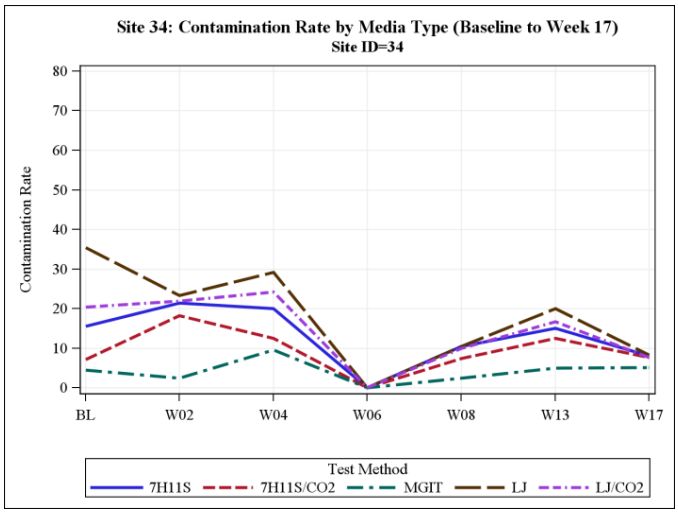
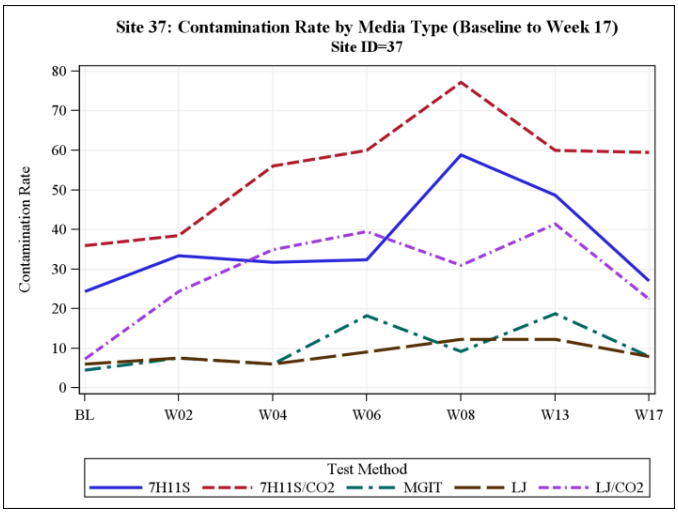


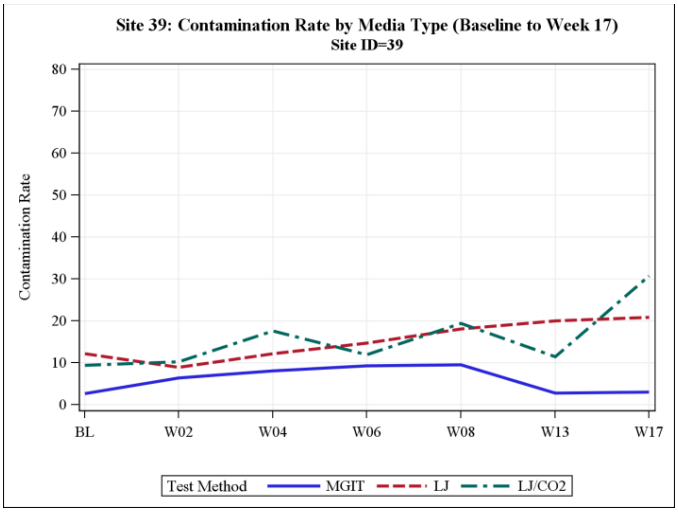
Figure 2.d: Contamination rate by culture media type for the first 17 weeks of TB treatment by site








Site 37: Contamination Rate by Media Type (Baseline to Week 17)
Site ID=37
Contamination Rate
80
70
60
50
40
30
20
10
0
BL
W02
W04
W06
W08
W13
W17
Test Method
7H11S
7H11S/CO2
MGIT
LJ
LJ/CO2


Site 39: Contamination Rate by Media Type (Baseline to Week 17)
Site ID=39
Contamination Rate
80
70
60
50
40
30
20
10
0
BL
W02
W04
W06
W08
W13
W17
Test Method
MGIT
LJ
LJ/CO2