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Original Article

Evaluation of culture of Mycobacterium tuberculosis on blood agar in resource limited setting in Addis Ababa, Ethiopia

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Abstract

Background: *Tuberculosis is one of the major public health problems in Ethiopia. A number of rapid techniques for the isolation and identification of Mycobacterium tuberculosis has been approved by World Health Organization. However, the available laboratories, which have financial constraints, require culture media or techniques that are rapid and inexpensive .*

Objective*: To evaluate the efficacy of 7% sheep blood agar medium for primary isolation of tuberculois from pulmonary tuberculosis suspected patients.*

Methods: *A comparative cross-sectional study was conducted among 212 tuberculosis suspected individuals from five selected health facilities in Addis Ababa, Ethiopia from November 2013 to March 2014. Sputum specimens were collected and examined using AFB smear microscopy, cultured on 7% sheep blood agar and Lowenstein Jensen medium. Molecular characterization using RD9 deletion was done for AFB confirmed isolates*

Results: *The sensitivity, specificity, positive and negative predictive value of 7% sheep blood agar compared with the golden standard of Lowenstein Jensen medium was 96.4%, 98.1%, 94.7% and 98.7%, respectively. The performance of 7% sheep blood agar and sediment smear microscopy was comparable to Lowenstein Jensen. No significant difference in the rate of contamination (p>0.05) where the rate of contamination was 4.7% (20/424) on 7% sheep blood agar tubes and 5.2% (22/424) on Lowenstein Jensen tubes.*

Conclusions: *Mycobacterial growth time was less on sheep blood agar as compared to Lowenstein Jensen, and 7% sheep blood agar medium may be a good alternative of Lowenstein Jensen medium for rapid detection of Mycobacterium tuberculosis from sputum in resource limited settings.*

Keywords: *Sensitivity, Specificity, Mycobacterium tuberculosis, sheep blood agar, Lowenstein Jensen medium*
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Introduction

Nearly one third of the world's population is infected with *Mycobacterium tuberculosis* (MTB) and still accounts for the highest mortality from any infectious diseases worldwide, even surpassing HIV/AIDs, causing 1·5 million deaths in 2018 (1). It is the oldest infectious agent known to humankind and remains a major public health issue. Increased incidence of multidrug-resistant (MDR) isolates has resulted in treatment failures and, these isolates were able to spread in the community (2). The slow growth of mycobacterial cultures is a major problem in order to easily diagnose MTB, which later explains the slow process of evaluating the susceptibility of MTB to antibiotics (3). Thus, the scarcity of accurate and rapid diagnostic tests for *M. tuberculosis* is an important impediment to global TB control (4), It has been defined by an

increased urinary albumin excretion rate (UAER) approximately in the range of 30–300 micrograms per minute (4). In addition, 20% to 30% of subjects with T1D present with microalbuminuria after an average of 5-10 years of diabetes (2).
Current recommendations from the American Diabetes Association concerning screening for microalbuminuria in patients with T1D suggest annual testing after 10 years of age and after an average of 5 years with diabetes (5). Furthermore, these patients are at higher risk of cardiovascular diseases (CVDs) compared with diabetic patients without microalbuminuria which represents a significant burden to health care systems (6).

susceptibility of MTB to the anti-tuberculosis drug (8).

however small improvements in the coverage of testing, detection, and treatment of MDR and rifampicin-resistant tuberculosis were noted with an increase of bacteriologically confirmed tuberculosis rising 10% from 2017. Nevertheless, people with drug-resistant tuberculosis accounted for more than half a million new cases in 2018, and only 1/3 of these were enrolled for treatment (1, 5, 6). Diagnostic algorithms in current use, most in developing countries, are based on tests that have been in clinical use for many years. Moreover, the two greatest challenges to TB control, the TB/HIV epidemic and the growing problem of multidrug-resistant MTB, cannot adequately be addressed solely by sputum smear microscopy. Thus, lack of accurate and rapid diagnostic tests for TB is an important impediment to global TB control (7). Specific media, such as egg-based media, agar-based media, and liquid media are recommended for culturing Mycobacterium species. Such requirements pose logistic and economic problems, especially in resource-limited settings where bacteriological culture facilities are few, and the prevalence of mycobacterial infections, remarkable tuberculosis is high (8). The study conducted in 2020 in Ethiopia indicated high TB burden in countries like Ethiopia; there are also opportunities to identify children with presumptive TB in the framework of integrated community case management. Even though most health facilities prioritize the identification and management of acute childhood diseases; e. g malnutrition, diarrhea, fever, and pneumonia, TB is likely affecting several children evaluated in these clinics accordingly, Ethiopia was the first African country to develop a national childhood TB road map following the development of the global childhood TB road map in 2015 (9).

Currently few papers have reported that primary isolation of MTB on Sheep Blood Agar (SBA) from extra pulmonary site (lymph node) clinical sample after two weeks or more incubation and to determine of This study aimed to evaluate the diagnostic efficacy of 7% SBA media for the primary isolation of MTB from pulmonary sputum for pulmonary tuberculosis (PTB) suspected individuals under routine diagnosis conditions in resource-limited settings in comparison with conventional Lowenstein Jensen.

Methods

Study Design and Study Population

A comparative cross-sectional health facility-based study was conducted from November 2013 to March 2014 among PTB suspected patients in Addis Ababa, Ethiopia. Patients who were clinically suspected for PTB according to the Ethiopia TB and Leprosy National Guideline was included in the study. The protocol was approved by Armauer Hansen Research Institute/All Africa Leprosy, Tuberculosis, Rehabilitation and Research.Training Center (AHRI/ALERT), Ethics Committee before conducting the study. All participants were signed informed consent forms prior to participating in the study.

Sample Collection and Processing

PTB suspected individuals who consented to participate were properly instructed on how to produce productive sputum and submitted an adequate morning-spot-morning sputum specimen to respective health facilities. A minimum of 3-5ml of sputum was collected from each patient in a leak-proof, wide-mouthed plastic container with the cap and stored at the refrigerator before being transported to the AHRI TB laboratory in accordance with laws and guidelines for transport of biohazard material and laboratory specimens.

Laboratory Procedures Smear Examination

Direct and sediment smears were prepared by transferring two drops from unprocessed and processed sputum of the centrifuged specimens on different glass slides. Zihel Neelsen staining was done for direct (before processing), sediments and from culture suspension of all culture-positive specimens to confirm Acid Fast Bacilli (10). The International Union against Tuberculosis and Lung Disease guideline proposed scale of five groups was used for reporting the average number of Acid -Fast Bacilli observed in sputum specimens (11).

Sample Preparation On 7% Sheep Blood Agar and Lowenstein Jensen Medium

Having equal volumes of clinical sputum specimens and 4% NaOH solution was mixed in 50 ml Falcon tube, vortexes until it became homogeneous followed by decontamination, and incubated for 15 minutes at room temperature. At the end of incubation, the 50 ml mark of Falcon tubes were filled with 6.8 pH phosphate buffer saline (PBS) (Merck, Darmstadt, Germany) and then mixed by gentle inversion to neutralize NaOH and stop decontamination. Then they were centrifuged for 15 minutes at 3000 g. Finally, the supernatants were decanted immediately into a splash proof vessel containing a disinfectant, and the sediments were neutralized with Bromo-cresol purple indicator by adding one drop at a time while swirling until the indicator turned yellow from Blue. Immediately 4-5 drops of sediment (80- 100 ul) of this inoculum were dispensed onto culture medium on the LJ slants, as well as 7% SBA slants in duplicate using a Pasteur pipette to transfer the pellet to the media and then incubated at 37^0_C. McCartney bottles of 7% SBA was sealed with Para film in order to prevent desiccation of 7% SBA media (12, 13).

Blood Agar Media Preparation for Primary Isolation of Mycobacterium tuberculosis

Briefly, 7% SBA was prepared as instructed by manufacturers with the basic modifications (8, 13). The blood agar base contains Lab-Lemco' powder, Peptone, Sodium Chloride and Agar which were from Oxoid LTD Oxoid LTD Basingstoke, and Hampshire, England). Crystal violet, Polymyxin-B (Sigma Aldrich, Denmark), Nystatin (Sigma Aldrich, Romania), Nalidixic acid (Sigma Aldrich, Italy),

(and Trimethoprim Lactate Salt powder form drugs were included in the media of 7% SBA. 60 ml of blood was collected from Jugular vein of sheep and defibrinated with sterile glass beads in sterile Erlenmeyer flask and separated from glass beads by pipet boy. Five hundred ml (500 ml) of distilled water with 20 g of blood agar base and 5 ml of crystal violet solution (0.01%) were mixed well and boiled to dissolve the medium completely. It was sterilized by autoclaving at 121°C for 15 minutes and then waits to cool to 45-50 °C in a water bath.

Nystatin solution (3.150 ml), (18 mg Nystatin dissolved in 9 ml Methanol) and antibiotic solution (1.750 ml), (44.4 mg) Polymyxin-B, (5 mg) Trimethoprim and (20 mg) Nalidixic acid in (10 ml) of sterile distilled water were added to the sterilized blood agar base media in order to avoid growth of contaminants when inoculated processed sample. Antibiotics Solution were added in to the base and mixed well. Then 35 ml sheep blood was aseptically added in to the base and mixed well.
Finally, all contents were mixed well by vortexing for a minute, and approximately 7-8 ml was quickly dispensed to each of 14 ml, flat bottom McCartney bottle tubes. The components of PNNT and their final concentrations in 7% SBA were as follows: Polymyxin B, 50 U/ml (88.8 ug/ml); Nystatin, 5 mg/ml; Nalidixic acid, 40 ug/ml; Trimethoprim, 10 ug/ml.
We used a McCarty tube (tightly capped), incubating the culture tubes with distilled water in beakers surrounding the incubated tubes and sealed using para film in order to prevent drying the media.

Detection of Growth, Identification and Reading of Cultures

WHO quantitation scale was used to interpret the colonies growth result on media where a growth index of 10 or more colonies was considered positive (14). All positive growth was in time confirmed by performing smears and staining for AFB. Time taken to growth of colonies on 7% SBA and LJ was monitored daily up to 8 weeks. Isolates were confirmed for AFB by ZN technique and then species identification by PCR based RD9 deletion typing (Huard *et al.*, 2003) and *Mycobacterium* genus typing analysis (15).

Quality Control

Quality control was done by recording the temperature of incubator, fridge up to run positive (H37Rv) and negative control (plain media) in parallel with processed specimen on both media. All of the slopes of medium 7% SBA and LJ prepared in the day were incubated for 48 hours at 37°C to check sterility of the media, and 2 slopes were randomly selected from each batch of medium and incubated at 37°c for 14 days. Prior to interpreting the test we assessed both control media. If there was contamination or growth on the slants of incubated tubes during sterility check and if negative control tubes had growth, the results were invalid and re-prepare the media and re-run the tests. *M. bovis* and *H37Rv* MTB were used as positive control and Qiagen H_2O as negative control in molecular typing of RD9 deletion typing. *M. avium,* H37Rv MTB*, M. bovis were* used as positive control and Qiagen water as negative control in *Mycobacterium* genus typing, respectively.

Statistical Analysis

Data were analyzed using the SPSS version 21.0 for windows (Statistical Package for the Social Sciences Inc, Chicago, IL, USA). Wilcoxon signed-rank test was used to detect Statistically significant differences in time for uneven distribution for culture positivity. Contingency tables were used to compare the diagnostic test versus conventional, with which sensitivity, specificity, PPV and NPV were obtained with their 95% CI for 7% SBA media. P values less than 0.05 were considered statistically significant. Sensitivity and specificity with a fixed false positive rate of 5%, prevalence of smear positive PTB 14.02% from suspected cases in Eastern part of Ethiopia, (16) sensitivity of the SBA in population and 95% confidence interval were used to calculate the sample size

Ethical consideration

The patient's written consent was obtained before the conduct of the study.

Result

Demographic and Clinical Characteristic of Study Subjects

Out of 222 study participants, 10 participants were excluded from analysis due to insufficient volume and poor quality of sputum. Among 212 participants suspected of PTB, 46 (21.7%) were AFB smear positive from the primary sample (i.e., sediment smear), 59 (27.8%) were culture positive on SBA 56 (26.4%) were a culture positive on LJ and 153 (72.2%) were smear and culture negative. The prevalence of TB among suspected individuals was 59/212 (27.8%). 96 (45.3%) were female showing an overall female to male ratio of 1:1.2. The mean age of the participants was 35.1±SD years (ranging from 16 to 80). Of the 212 participants, 39 (18.4%) were HIV positive (Table 1).

Diagnostic Efficacy of Sheep Blood Agar for Primary Isolation of Mycobacterium Species

Diagnostic efficacy of 7% SBA media was done for the primary isolation of mycobacteria considering LJ media a gold standard method. The specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of 7% SBA media were 96.43, 98.08, 94.74 and 98.71%, respectively (Table 2). Seven percent SBA had similar outcomes in diagnostic performance of results with a smear from sediment microscopy but which had a better yield in the examination of sputum in microscopy than direct smear microscopy.

Table 1: Demographic and Clinical Characteristic of participants in Addis Ababa, Ethiopia.

	Variables	Frequency	%
Sex	Female	96	45.3%
	Male	116	54.7%
HIV	Positive	39	18.4%
	Negative	173	81.6%
Sputum appear-ance	Bloody	7	3.3%
	Mucoid	26	12.3%
	Mucopurulent	44	20.8%
	Purulent	87	41.0%
	Salivary	48	22.6%
Sputum grade from direct smear	Negative	166	78.3%
	Scanty	5	2.4%
	1+	9	4.2%
	2+	17	8.0%
	3+	15	7.1%
Direct smear result	Positive	59	27.8%
	Negative	166	78.3%

Table 2: Diagnostic efficacy of SBA over the Gold standard method (1) for 212 participants and (2) for 39 HIV study participants from 5 public's Health institutes Addis Ababa, Ethiopia.

	Variables	Frequency	%
Sex	Female	96	45.3%
	Male	116	54.7%
HIV	Positive	39	18.4%
	Negative	173	81.6%
Sputum ap-pearance	Bloody	7	3.3%
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	Purulent	87	41.0%
	Salivary	48	22.6%
Sputum grade from direct smear	Negative	166	78.3%
	Scanty	5	2.4%
	1+	9	4.2%
	2+	17	8.0%
	3+	15	7.1%
Direct smear result	Positive	59	27.8%
	Negative	166	78.3%

Time to Detection of Mycobacterial Isolates

There was significant difference in the detection day of macroscopic colonies in 7% SBA and LJ. The median days to detect macroscopic colonies on 7% SBA and LJ were 14 days (ranging from 9 to 28) and 20 days (ranging from 12 to 39) days, respectively (P<0.0001). From direct smear grades 1+ on 7% SBA, mean detection days were 14.8 ± 3.1 days but on LJ the mean detection days were 23.4±6.6 days. The smear positive grade of 3+AFB was detected in 13.8 days on 7% SBA, but on LJ were 15.3 days. The rate of contamination was a little lower in SBA 20/424 (4.7%) than LJ 22/424 (5.2%).

Molecular Characteristics of Mycobacterium spp.

Isolates out of the 59 positive cultures in both 7% SBA and LJ, 58 (98.3%) were MTB by RD9 deletion typing. While among 13 smear negative culture positive cases of *Mycobacterium* spp, 7% SBA supports growth of MTB in 10 smear negative culture positive cases and *Mycobacterium* spp in one case whereas LJ media supports growth of MTB in nine smear negative culture positive cases and *Mycobacterium* spp in one case. Out of 59 isolates from both media, 57 isolates were grown on 7% SBA; whereas 56 isolates were grown on LJ. Out of three discordant results, which were growing only on 7% SBA, one isolate was member of *Mycobacterium* spp. Fifty-five isolates from LJ were MTB and one isolate was *Mycobacterium* spp. Two isolates were grown only on LJ

Discussion

In most of clinical microbiology laboratories, standard blood agar is commonly used for isolating bacteria from clinical samples because it is cheap, simple to prepare, and many species can be grown (13). Recent studies reported that SBA could be used for isolation of MTB within 1-2 weeks, and the mean number of colonies was significantly larger on blood agar than on the egg-based medium (13, 15). In our study, the specificity, positive predictive value, and negative predictive value of culture on blood agar were observed to be 98.08, 94.74 and 98.71%, respectively. These values were in agreement with those obtained in a study by Palange i.e., 99.59%, 93.75%, and 98.02%, respectively from India (5).Drancourt and his colleagues illustrated that blood agar has at least equivalent to an egg-based medium for the isolation of MTB from respiratory and lymph node specimens (7). In the present study we evaluated the diagnostic efficacy of 7% SBA in ison with LJ media for primary isolates of MTB from sputum specimens under routine diagnosis. Our finding on primary isolation of MTB on 7% SBA has similar sensitivity with other studies done in Turkey reported 97.5% (17), 94.2% (17), 98.9% (8), 94.2% (13) and 89.3% (5), but disagree with 27.3% sensitivity with other study done in India (18) which may be due to small sample size (only20 smear positives) and difference in drug ingredients concentration. Studies conducted before six decades showed a similar finding in the performance in comparison to LJ media (94.2%), but they used human blood instead of sheep blood (18) reported that sensitivity of direct and concentrated smear microscopy was not show significantly different (15). In this study we have found that sedimentation prior to ZN staining significantly increased sensitivity for TB over direct smear microscopy which is comparable with the results of three previous studies that found higher sensitivity in HIV endemic populations after sputum concentration (19-21). In a study conducted in France they reported that MTB isolates easily grown on blood agar with in average of 1-2 weeks to the diagnosis of TB (11). Blood agar slants are a good substitute of LJ medium for rapid detection of MTB from sputum in resource-limited settings by saving up to one-third of the time, this agrees with that of the previous study (16). So, it is the time to evaluate the new media for their capability to stand alone or with the help of smear microscopy at least as an acceptable culture method. The present study has shown that 7% SBA took short detection time in comparison to LJ. The time to detection (TTD) for macroscopic colonies in 7% SBA was significantly different (P<0.0001) where the median days to detect macro -colonies on 7% SBA and LJ were 14 (range 9 to 28) days and 20 (range 12 to 39) days, respectively. Various studies on SBA TTD from smear positive and negative pulmonary sample revealed in the range of 1-3 weeks (13) Center for Disease Control and Prevention (CDC) recommends that the reports of isolation and identification of MTB complex species should be available within 10 to 14 days or 21 days of specimen collection (10). In our study we showed that growth of *M. tuberculosis* in 7% SBA saves 1/4 time in comparison with LJ and other study showed that the detection time of MTB saves 1/3 time in comparison with LJ and 1/2 time save (13, 16). *Mycobacterium tuberculosis* needs to be enriched and selective media to minimize contamination A contamination rate of 2 -5% is acceptable in laboratories, as a general rule when receiving fresh specimens. Laboratory which experiences no contamination probably uses a method that kills too many of the tubercle bacilli (16) The addition of PNNT to the SBA media performance was excellent and managed to reduce the contamination rate to 4.7% in this study, which is within acceptable range for culture from non-sterile site. This study suggests that isolation of MTB on blood agar will not be an anecdotal unscientific and that contamination of culture will not be a frequent problem if we follow decontamination procedure strictly and use antibiotics solutions to inhibit contaminants (10). Based on CLSI and WHO recommendation, our 7% SBA finding on contamination rate revealed difference from 1.5% in 5% SBA; 1.6 % in 7% SBA versus to 7.8% in LJ medium.

This finding has similarity with that of Shidiki and Pokhrel in which 2% contamination rate on both BA and LJ media (11) and s 1.6% on blood agar, 1.5% on blood agar (8) and 7.8% on LJ medium (16).
Even though our protocol and Mathur *et al*. was the same on media preparation but both of us were used different decontamination procedures. The difference may be due to decontamination because they could not get growth from 4 grade 1+ smear sputum specimens after decontamination using NALC-NaOH method. But our media allowed growth even from scanty smear grade specimens by using 4% NaOH. Other reasons for low contamination rate maybe they used high concentration antibiotics and increase the decontamination time to inhibit contaminants. In previous studies (22) LJ contamination varied from 7.8 to 21.1%. Conclusion Based on this finding 7% SBA may be good alternative of LJ in recourse-limited setting. TB Laboratory without culture facility, sediment microscopy may have equal diagnostic efficacy as 7% SBA, and sediment microscopy should be done at the same time to urgent treatment of the patient. The main advantages of blood agar medium are simplicity of preparation and ability to grow MTB from inoculum easily and recognizable in a short time. The ingredients are easily obtainable, and fresh media can be prepared within a few hours. All these factors should encourage a wider use of cultural methods for the bacteriologic diagnosis of tuberculosis.

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