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Diagnostics

Accuracy of the color plate micro-colony detection for the diagnosis of *Mycobacterium tuberculosis* complex in Northwest Ethiopia

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ABSTRACT

Background: Accurate and timely tuberculosis diagnosis is the primary step for initiating effective treatment. The color plate agar-based culture test (TB-CX test) is low cost, simple to use and detects *Mycobacterium tuberculosis* faster. Therefore, the main objective of this study was to compare the diagnostic accuracy and time to detection of positive cultures using color test and Löwenstein Jensen culture.

Methods: A comparative cross-sectional study was conducted at University of Gondar Hospital. A total of 200 sputum samples were collected from TB patients and processed for direct smear microscopy and cultures.

Results: Sixty-five percent were found positive on both methods and 4 (2%) were positive on LJ culture and negative on the color plate. The median time for detection of MTB growth was significantly shorter using color plate test (Median 12 days) than LJ culture (Median 21 days) ($P < 0.0001$). The overall sensitivity and specificity of the color test compared to LJ culture were 97% (95% CI: 93–99) and 100% (95% CI: 94–100), respectively.

Conclusions: The color plate test for micro-colonies allows early and accurate MTB diagnosis in a median time of 12 days. This rapid method could be an option for diagnosis of pulmonary TB in resource limited settings.

1. Introduction

Tuberculosis (TB) is among the top threats to public health worldwide. According to the World Health Organization (WHO) 2017 report, 10.4 million people were diagnosed with new TB cases and an estimated 1.7 million deaths were attributed to TB in 2016 [1]. Worldwide, nearly one third of all TB cases are estimated to be undetected or unreported each year [2]. Several methods for rapid and accurate *Mycobacterium tuberculosis* diagnosis have been developed; however, some of these techniques are costly, require technical expertise, need sophisticated equipment with sustained electrical power source or long turnaround time, making their implementation difficult to sustain in low-income countries [3,4].

TB diagnosis delays leads to prolonged periods of infectivity with increase in transmission in the community and disease progression with increase co-morbidity and mortality. TB transmission in contacts of smear-negative patients has been documented to be as high as 17% [5]. In resource limited countries, the most widely used TB diagnosis test is sputum smear microcopy which has low sensitivity to detect the TB bacilli. Löwenstein-Jensen (LJ) culture requires 4–8 weeks to give results, while BACTEC mycobacterial growth indicator tube (MGIT) 960 culture is more rapid but needs specialized equipment, is prone to contamination, is too costly, and is not always available in resource constrained settings [6,7]. Molecular techniques are also costly, and require skilled personnel and also sophisticated equipment [7]. GeneXpert MTB/RIF assay (Xpert assay, Cepheid, Sunnyvale, CA, USA) is more rapid to perform but has high cost and operational concerns limit its feasibility to be used as first-line diagnostic test in resource-limited countries [8,9].

In recent years, the development and evaluation of rapid and low cost culture methods is considered as a priority in the end TB strategy of TB. Several new methods have been developed to reduce the time to

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diagnosis of TB, such as the liquid media microscopic observation drug susceptibility assay (MODS), the nitrate reductase assay (NRA) or the colorimetric redox-indicator assay [10–13]. Micro-colonies detection is described as an alternative method for definitive diagnosis of TB and provides more rapid results and presumptive species identification [14–17].

One of the new low cost culture method that reduces the time to diagnose TB is the color TB-CX test, a thin-layer agar (TLA) method that allows the initial identification of *M. tuberculosis* complex based on its characteristic cording morphology under the microscope [18]. *M. tuberculosis* colonies show irregular wavy margins and appear like small spirals initially, but as they mature, these small colonies acquire cording with distinct irregular margins and ultimately spread over the entire area when viewed through the microscope [19,20]. The color TB-CX test detection principle relies on the microscopic detection of micro-colonies in solid media. The advantage of TLA plate compared to MODS is the use of a standard microscope instead of an inverted one [17].

Most of the TLA studies were conducted in developed countries or Latin America [7,8,16,17,20–22] and one was from high HIV prevalence settings [23]. The sensitivity of TLA culture is close to LJ method, but faster (10–15 days *vs.* 30–40 days) [7,19,22,24].

The 2, 3 diphenyl-5-(2-thienyl) tetrazolium chloride (STC) is an oxidation-reduction indicator that is stable in an incubator and change color when microorganism grows [25–27]. The addition of STC to the TLA media results in the growth of red TB colonies which makes them visible to the naked eye at the early stages of growth. This makes daily checking of the plates faster, as there is no need to check every plate under the microscope [27]. Several studies indicates that TLA is inexpensive with variable cost due to supplies and reagents cost in different countries, from US$ 0.36 to 5.8 [15,17,24,28].

In Ethiopia, TB is being diagnosed mainly using smear microscopy. The GeneXpert and culture are restricted to limited number of health facilities. A rapid, inexpensive, simple to use and accurate diagnostic options is required for the diagnosis of susceptible and drug resistance TB in Ethiopia especially in rural area to improve laboratory capacity. To the best of our knowledge, no published study is reported in Ethiopia to evaluate the diagnostic accuracy and time to detection of color TB-CX test for pulmonary TB diagnosis. The present study was undertaken to determine the diagnostic accuracy and time to detection of positive cultures using color TB-CX test as compared to conventional LJ.

2. Materials and methods

2.1. Study design and setting

A comparative cross sectional study was conducted at University of Gondar Hospital TB Directly Observed Treatment, Short course (DOTS) clinic or MDR TB treatment center. All patients with microbiologically confirmed or clinically suspected pulmonary TB ≥ 15 years of age from March 2016 to August 2017 who agreed to participate were included in this study. The University of Gondar Hospital is located in Northwest Ethiopia and serves as a referral hospital for the region that offers a wide range of services including diagnosis and treatment of TB and MDR-TB cases. The DOTS clinic is operated under the National Tuberculosis and Leprosy Control Program (NTLCP) of Ethiopia with smear microscopy method for acid fast bacilli (AFB), culture and GeneXpert® for detection of TB and rifampicin resistant TB cases. Chest radiographs and pathological investigations are also used to support the diagnosis. The University of Gondar TB culture laboratory is a recently established TB diagnostic referral laboratory performing smear microscopy, culture using LJ and MGIT 960 system (Becton Dickinson Microbiology System, Sparks, MD, USA) and drug susceptibility testing using line probe assays and MGIT 960 phenotypic method.

2.2. Data collection, transportation and quality control

Data were collected using pre-coded, pre-tested structured questionnaire that assess the socio-demographic, clinical characteristics and laboratory result of study participants. A single morning or spot sputum sample was collected per patient and transported to the University of Gondar hospital TB culture laboratory immediately after collection and then kept at −20 °C. No decontamination procedure was performed prior to storage or during transportation. The reference strain *M. tuberculosis* $H_{37}R_v$ (American Type Culture Collection number 27294) was used as the reference growth control for each batch of color TB-CX plate. Blind rechecking was performed for all ZN smear slides. All color TB-CX plates were read blindly to the results of reference standard method.

2.3. Culture and identification of M. tuberculosis complex

A total of 200 (170 drug susceptible and 30 rifampicin resistant (RR) TB) sputum samples were collected at the University of Gondar hospital TB DOTS clinic and MDR TB treatment center settings. Ziehl Neelsen smear microscopy was performed directly from patient specimens, and AFB results reported as smear negative or smear positive (scanty, 1+, 2+, 3 + according to bacterial load). The single sputum specimen (approximately 5 ml) was collected per patient and was decontaminated and processed using color plate disinfectant solution for Color TB-CX test (0.5–1.5 ml sputum) or conventional sodium hydroxide-*N*-acetyl-L-cysteine method for LJ (2–4 ml sputum).

2.3.1. Color TB-CX test

Color TB-CX test is a thin layer agar (Middle brook 7H11) based plate with four quadrants allowing the detection of drug-susceptible (drug free), isoniazid (INH), rifampicin (RIF), and ciprofloxacin (CFX) resistant *M. tuberculosis*. All color TB-CX tests were prepared at The Ohio State University, USA as described previously [29], and transported to Gondar, Ethiopia and stored at 4 °C for up to 4 months from the date of manufacture; Color TB-CX tests not used within 4 months were discarded. All color TB-CX test batches were quality control checked for the control quadrant (drug free) using the laboratory strain *M. tuberculosis* $H_{37}R_v$ upon arrival to Ethiopia. The color TB-CX test disinfectant solution was prepared by dissolving tri-sodium phosphate (2 g), ammonium sulphate (50 mg), magnesium sulphate (5 mg) and ferric ammonium citrate (2.5 mg) with 10 ml of sterile distilled water by heating and stirring with a magnetic stirrer for 4 h and then filter twice and sterilized. After sterilization, sterile 100 IU/ml of penicillin and 1 ml of red food coloring reagent were added on the disinfectant solution. Briefly, 0.5–1.5 ml of sputum samples were transferred to a 15 ml disposable plastic tube inside a bio-safety cabinet and 1–3 ml color plate disinfectant (1:2 dilutions) was added. This mixture was then gently mixed for 15 s. Then, two drops of the sputum-disinfectant mixture were deposited onto the control quadrant (drug free) of the color TB-CX test, and the test plates was then double-sealed in a Ziploc bag and incubate at 37 °C. Plates were removed from the incubator three times per week for 6 weeks and *M. tuberculosis* colony growth (micro-colonies) was assessed using light microscope under 10X objective without removal from the double-sealed, plastic bag. If any contamination does occur in a part of a color plate, it does not prevent the interpretation whether the plate is positive or negative and considered as partial growth or not. A positive culture was identified by the characteristic colony morphology of *M. tuberculosis* complex growth, considering consistency, colony border and cord formation. Non-tuberculosis mycobacteria (NTM) were recognized by their lack of cording [30].

2.3.2. Lowenstein–Jensen (LJ) culture

A total of 2–4 ml of the remaining sputum sample was placed in a 50 ml falcon tube and decontaminated with equal volume of Sodium

hydroxide-*N*-acetyl–L cysteine solution (NaOH-NALC) (4% NaOH, 2.9% sodium citrate) and incubated at room temperature for 15 min and then phosphate buffer saline (PBS) (pH 6.8) was added to the preparation followed by centrifugation at 3000 × *g* for 15 min [31]. The supernatant was discarded and then a suspension was made by adding 2 ml PBS to the sediment. After vortex mixing, 2–3 drops of the suspension were inoculated into two LJ medium tubes (either pyruvate or glycerol) and incubated at 37 °C and read once per week for 8 weeks All samples showing growth on culture was confirmed by typical colony morphology, cord formation on AFB smear microscopy and Capillia MPT64 TB assay (Alere Medical Pvt Ltd, SD BIOLINE, Gurgaon, Haryna, India) and reported as "culture positive for *M. tuberculosis* complex".

2.4. Sample size determination

Based on standard sample size calculation for diagnostic test evaluation [32], assuming a sensitivity of 0.9 for the color TB-CX test verses LJ culture, an alpha error of 0.05 and a precision of 0.1 and the prevalence of pulmonary TB in Gondar as 18% [33], the sample size was estimated as greater than 192 TB cases and therefore, 200 TB cases were included in the study.

2.5. Statistical analysis

All data were entered and cleaned in EpiData v. 3.1 (EpiData Association, Odense, Denmark) and analyses were performed using SPSS version 20 (Statistical Package for the Social Sciences, Chicago, IL, USA). Descriptive statistics were employed. The Wilcoxon rank test for paired samples was performed to compare time to detection of growth in the color TB-CX test and LJ excluding contaminated specimens in any of the culture media. Time to detection of growth was compared using Mann–Whitney *U* test for unpaired comparisons for color TB-CX test based on smear microscopy status. A P value < 0.05 was considered statistically significant. Sensitivity, specificity and predictive values were calculated for the detection of colonies using the color TB-CX test including 95% confidence intervals (CIs), as compared to the conventional method using MedCalc version 11.5.1.0 (MedCalc software, Mariakerke, Belgium). Kappa coefficient was also calculated.

2.6. Ethical approval

The study was ethically approved by University of Gondar Ethical Review Board (Ref. No: O/V/P/RCS/05/19/2016). Demographic and clinical data and sputum samples were collected from all volunteer participants after obtaining written informed consent. The Color TB CX-test results did not influence the treatment of participants and all infected patients were treated accordingly using routine diagnosis. Confidentiality for all collected data was preserved using secret codes for each participant.

3. Results

3.1. Demographic and clinical characteristics

A total of 200 study participants were enrolled in this study. The median age was 30 years (range 16–85, Interquartile range (IQR) = 22–45) and 60.5% were between 15 and 34 year of age. Majority of study participants were males, 125 (62.5%). Out of 200 participants, 25.5% were previously treated for TB disease, 12.5% were HIV positive, 3.5% had rifampicin resistant (RR)/MDR-TB contact history, 13.5% had family history of TB, 56.5% were urban dwellers, 57% were literate and 117 (58.5%) were ZN smear negative (Table 1).

Table 1
Characteristics of study participants (n = 200).

Characteristics	n (%)
Previous anti-tuberculosis treatment	
Yes	51 (25.5)
No	149 (74.5)
Family history of tuberculosis	
Yes	27 (13.5)
No	173 (86.5)
RR/MDR TB contact history	
Yes	7 (3.5)
No	193 (96.5)
HIV status	
Positive	25 (12.5)
Negative	175 (87.5)
Sex	
Male	125 (62.5)
Female	75 (37.5)
Age group, years	
15–24	67 (33.5)
25–34	54 (27)
35–44	26 (13)
45–54	28 (14)
≥ 55	25 (12.5)
Place of residence	
Urban	113 (56.5)
Rural	87 (43.5)
Educational status	
Literate	114 (57)
Illiterate	86 (43)
ZN smear score	
Negative	117 (58.5)
Scanty (1–9 bacilli/100 fields)	9 (4.5)
1+ (10–99 bacilli/100 fields)	38 (19)
2+ (1–10 bacilli/field)	18 (9)
3+ (> 10 bacilli/field)	18 (9)

RR = Rifampicin mono resistance, MDR TB = Multi-drug resistant tuberculosis, ZN = Ziehl-Neelsen, HIV= Human Immunodeficiency virus, n = number.

3.2. Accuracy and time to micro-colony detection of color TB-CX test

From 200 samples, 130 (65%) were found positive on both methods, 4 (2.0%) were positive on LJ and negative on the color TB-CX test, 1 (0.5%) was positive on the color TB-CX test and contaminated on LJ, 1 (0.5%) had growth on LJ and contaminated on the color TB-CX test, 55 (27.5%) were negative on both tests, 6 (3.0%) were negative on LJ and contaminated on the color TB-CX test, 3 (1.5%) were negative on the color TB-CX test and contaminated on LJ. From 131 color TB-CX test positive, 12 plates were showed partial growth with contaminant and reported as positive for color plate.

The contamination rate was 7 (3.5%) on the color TB-CX test and 4 (2%) on LJ medium. The overall positivity of the sputum samples for all the diagnostic methods used was 136 (68%). We excluded all contaminated samples in both tests, and thus only 189 study participants were used for statistical analysis. All smear-positive samples yielded mycobacterial growth on both the color TB-CX test and LJ. The median time in days from sample processing to detection of *M. tuberculosis* growth was significantly shorter for the color TB-CX test (Median 12 days, IQR 9–16) than LJ (Median 21 days, IQR 14–21) ($P < 0.0001$) (Fig. 1). The minimum time for detection was 6 days for color TB-CX test and 14 days for LJ and the maximum was 26 days for color TB-CX test and 56 days for LJ.

In addition, 70% of positive samples were detected within the first two weeks on color TB-CX plate agar, and 35.4% on LJ. The median time to detect a positive culture showed major differences between color TB-CX plate and LJ. In smear positive samples, the time to detection of positive cultures on color TB-CX plate was a median of 11 days (IQR: 9–13) while 21 days (IQR: 14–21) for LJ ($P < 0.0001$). In smear negative samples, the time to detection of positive cultures on color TB-CX plate was a median of 15 days (IQR: 11–18) while 21 days (IQR: 21–28) for LJ ($P < 0.0001$). For the color TB-CX plate, the median time to detection was significantly shorter ($P < 0.0001$) for

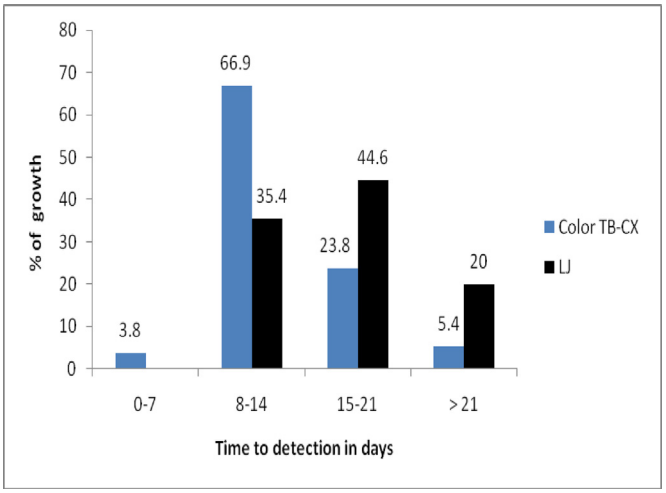

Fig. 1. Cumulative percentage of positive *M. tuberculosis* isolations based on time to detection in Color TB-CX test and LJ medium.

smear-positive samples (Median, 11 days) than for smear-negative ones (Median, 15 days).

M. tuberculosis growth in the form of micro-colonies appearance was first observed on the color TB-CX plate as early as six days, and well-formed micro-colonies were generally observed after 9–15 days through the naked eye without the use of microscope (Fig. 2).

There was no statistical difference in time to detection of bacilli related to the grade of positivity in direct smear examination in both culture methods ($P > 0.05$). Samples inoculated on color TB-CX plate were found positive in median of 12, 9.5, 11, 10.5 and 15 days for smear positive 3+, 2+, 1+, scanty and smear negative, respectively. For LJ culture, positive results were found in median of 21, 14, 21, 17.5 and 21 days for smear positive 3+, 2+, 1+, scanty and smear negative, respectively. All smear positive samples were also positive on color TB-CX test. However, a significant difference was observed among smear negative samples in which LJ cultures were more sensitive to detect bacilli ($p < 0.001$) (Table 2).

The overall sensitivity and specificity of color TB-CX test compared with conventional LJ culture were, respectively 97% (95% CI: 92.5–99.2) and 100% (95% CI: 93.5–100) after excluding contaminated specimens on either culture medium. The positive predictive value and negative predictive value of color TB-CX test were 100% and 93.2% respectively as compared to LJ culture. The kappa coefficient was 0.95 (95% CI: 0.894–0.988; $p < 0.001$).

4. Discussion

Early diagnosis of TB has become a priority in the recent years for both early treatment and decreasing TB transmission worldwide. Previous studies have been carried out in various contexts to evaluate the performance of TLA or color plate [3,19,23,24,28,30,34] in order to introduced a rapid and easy new test for diagnosis of TB and drug resistant strains especially in resource limited settings. The importance of evaluation of color TB-CX test in Ethiopia is because, as a low resource country, Ethiopia is increasing its laboratory capacity for improving culture and drug susceptibility tests. However, currently with increase in the number of TB cases in many regions of Ethiopia, an improved TB diagnostic method is needed. The TB-CX test also offers a cost effective, rapid, easy to use MTB diagnosis. In addition, it also has capacity for drug susceptibility testing for INH, RIF, and Fluoroquinolone or PZA which will improve treatment and decreasing transmission.

In this study, the majority of the isolates (70.8%) were detected within the first two weeks on color TB-CX test, and thus color TB-CX test has a clear advantage over the conventional LJ medium for early detection of mycobacteria. Four smear-negative isolates were recovered only on LJ but not on color TB-CX test. All smear-positive samples yielded mycobacterial growth on both color TB-CX test and LJ. One possible explanation for these missing growths on color TB-CX test might be low number of bacilli and may be deterioration of some media within a batch because of storage temperature effect during transportation of the color plate as it was prepared in USA and transported to Gondar, Ethiopia. Indeed, there was one occasion that a batch of color TB-CX plate passed our positive control in USA, however, upon transport to Gondar, Ethiopia, this batch failed the quality control and was discarded.

The median time to detection of *M. tuberculosis* growth on the color TB-CX test was shorter than LJ, 12 and 21 days respectively; and for color TB-CX test, growth detection was shorter in smear-positive *vs.* smear-negative sputa. Similarly other studies showed that time to detection was remarkably shorter for thin layer agar than for LJ (12 *vs.* 44, 11.5 *vs.* 30.5, 10.1 *vs.* 20, 7 *vs.* 25, 14 *vs.* 23 and 7 *vs.* 17 days, respectively) [15,20,23,30,34,35]. Similar to our findings, other studies also revealed that TLA detection time was shorter for smear positive samples [14,19,35]. Moreover, TLA had nearly identical time to detection compared with MGIT 960 (11 *vs.* 12.6 and 11 *vs.* 9.6 days, respectively) [36]. However, TLA was able to differentiate between *M. tuberculosis* and non-tuberculosis mycobacterium (NTM) at the time of detection, which is a great advantage compared to BACTEC MGIT 960, even though there were not NTM detected in this study. Minimum training is required prior to performing the color TB-CX test to recognize the typical cord formation characteristic of *M. tuberculosis* that differentiates it from bacterial or fungal contaminants.

The contamination rate of color TB-CX test and LJ was comparable in this study. The color TB-CX test contamination rate was low in our study and comparable with other studies [30,35]. Some studies reported higher contamination rate between 12.3% and 26% [15,23,24,28]. The reason for the low contamination rate observed in this study might be explained using penicillin as a sole antibiotic agent during preparation of color TB-CX test disinfectant as well as higher concentration of amphotericin B in the medium in order to inhibit the growth of bacteria and/or fungi. In addition, some studies reported that smear negative sputum and longtime interval from specimen collection to processing favors increased contamination rate due to overgrowth of contaminants in the collected sputum.

In our study, the sensitivity and specificity of color TB-CX test were 97% and 100% respectively. Similar findings were observed in a multicenter study conducted in Latin America [35] and Belgium [28]. In addition, the sensitivity of color TB-CX test in this study was higher than other TLA studies reported in Colombia (73.5%) [16], Indonesia (86%) [30], Colombia (83%) [15], Kenya (74%) [23] and a systematic review estimated 87% overall pooled sensitivity [24]. This could be explained by the fact that our study only used sputum samples, but other studies carried out in various settings, using extra-pulmonary samples in addition to sputum, large numbers of smear negative samples and some used both solid and liquid culture as reference standard that might create the disparities in sensitivity of TLA or color plate in different studies.

Our data also revealed that the color TB-CX test had not only a specificity of 100% but also an excellent positive predictive value of 100%, which shows a high level of confidence that a positive result represents a true result. The agreement between color TB-CX test and LJ was excellent with the Kappa coefficient of 0.95. This showed a higher concordance as compare to the previous reports that showed lower concordance (Kappa = 0.79) for pulmonary samples only [30], and (Kappa = 0.52) for sputum and other respiratory samples [16].

In addition to good sensitivity and short time to TB detection, the color TB-CX test has other advantages. It does not require additional sophisticated laboratory. *M. tuberculosis* colonies grow faster and are easy to visualize by the naked eye as red dots due to the addition of STC indicator in the medium. It also avoids bio-safety risks as the color TB-

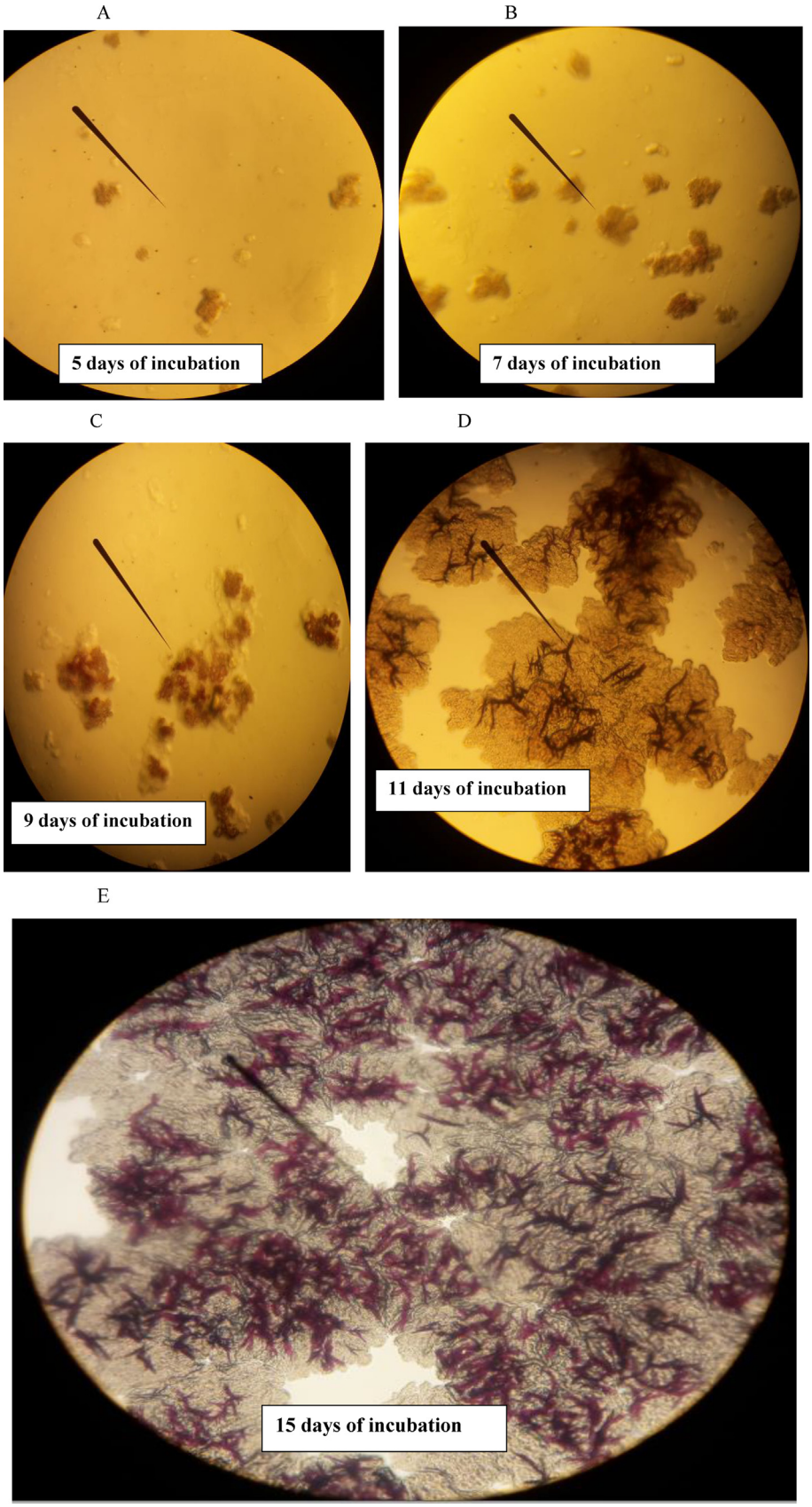

Fig. 2. Micro-colony morphology of mycobacteria observed with characteristic cord formation A) after 5 days of incubation, B) after 7 days of incubation, C) micro-colonies observed at 9 days, D) Micro-colonies observed at 11 days, and E) well formed micro-colonies observed after 15 days. Microphotographs (using mobile camera) observed with 100x magnification (10× objectives) using light microscope.

Table 2
Mycobacterium tuberculosis by color TB-CX test micro-colony and LJ method based on smear result.

Direct ZN smear	n[a]	No of positive (%)		P[b]
		Color TB-CX test	LJ	
Negative	107	48 (44.9)	52 (48.6)	P < 0.001
Scanty	8	8 (100)	8 (100)	
+1	38	38 (100)	38 (100)	
+2	18	18 (100)	18 (100)	
+3	18	18 (100)	18 (100)	

[a] Excluding sputum samples contaminated in either LJ or Color TB-CX test; LJ = Löwenstein-Jensen; ZN = Ziehl-Neelsen.
[b] X^2.

CX plate is covered with Zipper storage (Ziploc) bag and reading and identification of the growth was performed without opening the plates. MGIT 960 culture has a similar time to detection and sensitivity [28] but it is costly and requires sophisticated equipment. Moreover, the MODS test is accurate and rapid yet inexpensive method both for TB diagnosis and drug sensitivity testing, but requires inverted microscopy and presents higher degree of contamination [10,21,24,37]. GeneXpert is also rapid test for TB detection and rifampicin drug resistance, but it is costly to be used in high incidence settings as first diagnostic tool [8,38].

A major disadvantage of color TB-CX test is laborious, requiring time for repeated observation of *M. tuberculosis* growth, which may limit their use to only laboratories that process moderate to low numbers of specimens for culture. In our study, it took around 3–4 min to read a plate microscopically and checked for *M. tuberculosis* micro-colony growth three times per week for 6 weeks. The color TB-CX test is more suitable for a laboratory with low to medium workload and may be a good addition to routine LJ culture.

In summary, the color TB-CX test is a rapid, simple to use and accurate TB diagnostic method and could be an alternative option for diagnosis of pulmonary TB from medium to low workload laboratories in resource limited settings. It could be an excellent addition to routine TB culture at reference, regional and referral hospital laboratories in addition to LJ. Implementation studies are urgently required to evaluate the performance of the color TB-CX test for extra-pulmonary samples in addition to sputum samples and the cost-effectiveness and impact of its introduction into programmatic settings.

Conflict of interest

We declare that we have no competing interests.

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Authors' contribution

AS: Conceived and designed the study, carried out the study, analyzed and interpreted the data, wrote the first draft and final write up of the manuscript; BG: Designed the study, analyzed and interpret the data and, edit and approve the final manuscript, agreed with manuscript results and conclusions; HK: Made the color plates, edit and approve the final manuscript; JMB: Critical review, edit and approve the final manuscript; CE: Critical review, edit and approve the final manuscript; SW: Designed the study, analyzed and interpret the data and edit and approve the final manuscript, agreed with manuscript results and conclusions; JT: Made the color plates, analyze and interpret the data and, edit and approve the final manuscript. BT: Designed the study, analyzed and interpret the data and, edit and approve the final manuscript, agreed with manuscript results and conclusions.

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