CULTURING, IDENTIFICATION AND DRUG RESISTANCE OF MYCOBACTERIUM TUBERCULOSIS IN SPUTUM SPECIMEN

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Abstract: The development of low-cost, fast, and reliable methods for detecting Mycobacterium tuberculosis (MTB) infection and drug susceptibility is critical for tuberculosis control. The new microscopic examination of liquid drug susceptibility assay (MODS) examines early MTB colonies in a liquid medium, which permits for more convenient diagnosis and testing of drug susceptibility. The sensitivity of MODS (91%) was superior as compared to the sensitivity of different culture methods (92%). The MGIT and MODS were used to monitor tuberculosis-positive sputum samples for isoniazid and rifampin susceptibility. Concordance between MODS and MGIT was found in 89 percent of cases. MODS are the fastest method used for diagnostic and susceptibility testing (median, 10.0 or 9 days). MODS is a fast, low-cost, responsive, and particular method for detecting and testing MTB susceptibility; it is specifically well-suited utilize in growing countries with high infection rates and a growing number of multidrug-resistant cases.

Introduction
Tuberculosis (TB) is a major contributor to the worldwide burden of disease and has gain considerable attention in recent years, particularly in low and middle-income countries (Pio et al, 1999). The genus Mycobacterium belongs to the family of Mycobacteriaceae. The most familiar of the species are Mycobacterium tuberculosis, the causative agents of tuberculosis (TB). Tuberculosis (TB) is an infectious airborne disease. The name Mycobacterium, meaning Fungus like bacterium is obtained from the mold-like appearance of Mycobacterium tuberculosis, when expand in liquid media. They are non-motile, aerobic, non-copulating and non-sporing. It seems to like to grow in a well-ventilated area of the upper layers of the lungs. Tuberculosis generally impact the lungs in humans known as Pulmonary tuberculosis but it can also harm intestine, meninges, bones, and joints, lymph glands, skin and other tissues of the body mutually called extra pulmonary tuberculosis. It occurs more in people with a weakened immune system and adults. When this disease affects animals like cow; this is known as bovine tuberculosis (Gleissberg et al, 2001).

Mycobacterium tuberculosis has a double duration of 18 hours and clinical practice can take about 6 to 8 weeks to develop. Resistance to dehydration and can survive the expected cough. The cell wall contains a complex lipid chain such as mycolic acid, simple chain fatty acids to form acid-fast components, wax D and phosphatides (Levinson W, 2010). In some microorganisms the presence of fats and waxy substance typically interfere with the staining. These microorganisms cannot be stained by Grams stains unless some special treatments square measure done. It is estimated that from two thousand to two thousand ten at least 9 cases were reported over the year. One and half million people die due to this disease in every year. While, the 95% deaths have been found in moderate income countries. TB is mostly found in children in the poorest countries where more than a hundred thousand children die every year. In United States about ten to twelve million people are infected by the Mycobacterium
tuberculosis. In contrast to the decline of infectious disease within the African nations has dramatically accumulated. TB is next main reason of death among young people from infectious disease in these countries having nearly half percent of HIV infected population. Isolation, cultivation, identification and drug resistant mycobacteria in sputum specimen are very important to upgrade the identification as part of the worldwide TB control efforts. Over 60 other species of mycobacteria have been recognized, some of which are low-grade pathogens in man (Ali et al, 2010). The development of antimicrobial resistance is one of the many challenges. Tuberculosis has the potential to improve genetic mutations that make it more resistant to many effective antibiotics (WHO, 2010).

After the introduction of effective antibodies within the nineteen fifties, TB incidence declined steady. The rate of nearly 2000 deaths annually, have continued to decrease. In current demonstrated that one third of the world’s population is sick with this disease. The occurrence of tuberculosis in United States has declined over the years and now it is almost decease. Much 1907 incontestable the presence of Gram-positive granules is cold symptoms pus wherever there is no proof of acid-fast bacilli however that might could manufacture infectious disease once injected into susceptible animals. Much suggested that these granules are nonacid fast quick kind of tubercle bacilli. The mode of direction is on face to face intake of duster bacilli contain in bead nuclei of spit up sputum tubercle bacilli square measure no heritable from persons with active sickness who are excreting viable bacilli by means of coughing, sneezing or talking.

Mycobacteria square measure quickly killed by ultraviolet light even through glass, and by heat at 60 C for 15 to 20 min. Culture may be killed by exposure to daylight for two hours but bacillus in sputum may remain alive for 20 to 30 hours. Sputum smear microscopy is used as an important or pressing priority to spot or diagnose infectious disease within the patients in specific analysis space. The most usual specimen for diagnosis of pulmonary tuberculosis is sputum which consists of pus and mucus secretions coughed up the sputum into a very clean wide-mouthed container. If the sputum is scanty, a 24 hours sample also tested. Sputum samples on three days raised colony which was found in usually broth and micro plate sources with very little visibility. We compared sensitivity, specificity, pollution levels, effect time, and cost of these two tests (Kidenya et al, 2013). Mycobacterium species smegmatis mc2155 (Carriere et al, 1997; Jacobs, Jr, et al, 1993; Riska & Jacobs, Jr, 1998) and mc24502 used to distribute existing phages grown in Middlebrooks 7H9 media containing 0.2% glycerol, 0.5% serum albumin (BSA), fraction V, 0.2% dextrose and 0.09% NaCl (selected SADC) with 0.05% Tween-80 and 150 g / ml hygromycin (for mc24502). (Svetoslav Bardarov et al, 2002).

**Laboratory methods**

**Sample collection**

Patients with tuberculosis suspected of having high-risk or tuberculosis-resistant. MTB samples among victims in HIV- contaminated hospitals, samples each patient collected only one study, 20 of which came from abdominal washing of 12 patients those were unable to give sufficient sputum specimen (Moore et al, 2006). The most usual specimen for identification of pulmonary tuberculosis is liquid body substance that consists of pus and mucus secretions coughed up from the respiratory organ. Patient instructed to cough up the sputum into a clean wide mouthed container. If the sputum is scanty, a 24 hours sample also tested. Sputum samples on three days raised probability of detection (Kunkel et al, 2016).

**Growth detecting using (LJ) medium**

It is a particular media known for the cultivation and isolation of *Mycobacterium tuberculosis* commonly called LJ medium (Elbir et al, 2008). It adds Congo red and malachite green to inhibit the growth of unwanted/contaminated bacteria. The contaminated specimen (100 ml) was placed in two test tubes of solid LJ media which was being made by following usual procedure (Central TB Division, 2009). For

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preparation of LJ media, 1st a table salt solution consisting of 2.3 g of monopotassium phosphate (KH2PO4), 0.23 g of MgSO4, 0.6 g of magnesium 2-hydroxypropane -1,2,3-tricarboxylate, 3.5 g asparagine (amino acid), 11 ml glycerol, 20 ml glycerol (grade reagent), was first developed. - Malachite green, 3.0% solution (originate with 3.0 g malachite green dye and 100 ml of ddH2O). The components were mixed in a series of ddH2O, then lowered to 25°C and deep-freeze. Then 600 ml of table salt solution is mingled with 1000 ml of hatched eggs. Whole eggplant was spread on aseptic vessels, sealed in 6-7 ml volumes then placed in incubator in sealed container for 55 minutes at 85uC. LJ center is later refrigerated for up to 4 weeks because replicating time of M. tuberculosis was slow (15–20 hours) as compared with to bacteria (Kidenya et al, 2013). When *M. tuberculosis* is allowed to grow on LJ medium they appear as brown, granular colonies. Each classification was tested for morphology and pigmentation (Cattamanchi et al, 2009). The church of the emergence of the colonies was noted. In the event of contamination, sputum specimen was again contaminated and re-designed for patient guidance and excluded from the study (Davis et al, 2011). In addition, whether no growth was showing for eight weeks even contamination was there, the cultures were thrown away or lab forms were properly filled. Additionally, to the cultural characteristics, usual Ziehl-Neelsen slides are create with feature colonies that appear earlier week 8 to ensure the appearance example acid-fast of bacilli (Kiraz et al, 2007).

**DST using BACTEC MGIT**

DST was accomplished by using BACTEC Mycobacteria Growth Indicator (MGIT) Tube 960 SIRE Kits, Sparks, USA, Becton Dickinson, the ASTM Set Carrier and the BACTEC MGIT machine with fundamental isolates acquired from Lowenstein Jensen (LJ) media. The manufacturer’s DST laboratory protocol is available there. The BACTEC-MGIT system delivered an automatic result after strict adherence to the procedure. As a quality check, each test batch included the reference strain H37Rv MTB (ATCC 27294). The final vital concentrations for Isoniazid and rifampin were 0.1 and 0.4 g/ml for Isoniazid and 1 g/ml for rifampin (Ejigu et al, 2008).

**Microscopic observation of drug susceptibility (MODS)**

The Microscopic observation of drug susceptibility (MODS) assay test was performed in both indirect & direct methods. The tests were performed on 12 well cultured plates. In which each test contained a 2 mL Middlebrook 7H9 broth, 200 microliter (µL) suspension, and 34 microliter (µL) of significant antimicrobial drugs. The suspension of McFarland, approximately 104 CFU / mL of isolates was used. The test tube (control) was set on the 1st row of the plate. In every plate, two antibiotics combined with a combination of isoniazid and streptomycin (I&S) and ethambutol and rifampicin (E&R) were administered randomly (WHO, 2013). A direct method test was performed with 140 microliter (µL) of a contaminated sputum sample which is prepared from the pump-extracted mixture as indicated. Indirect tests were performed with appropriate MTB colonizeation equipment. Bad control contained only MODS media. Good control consisted of MODS and MTB media with a combination of 1: 100 and 1:10 PZA. All the plates were placed at 37°C and tested twice a week after daily contamination for up to 42 days. Detectable serpentine clusters also known as pellicle formation shown good culture. Growth seen in both drug free and drug containing sources were resilient, while drug free trials and no growth in drug induced media outlets shown that testing may be separated from experimental drugs (park et al, 2002). Broth from Mycobacteria Growth Indicator (MGIT) tubes was poured into MODS sources to determine whether the cord formed in the acidic area could be detected easily. Intermediate broth for DST persistence of streptomycin, isoniazid, rifampicin, and ethambutol (SIRE) was prepared in a laboratory (Owusu and Newman et al, 2020).

**Results**

**Culture Detection of *M. tuberculosis* by LJ Media, and MODS Assay**

During the study period, 321 suspected cases of pulmonary tuberculosis which were enrolled, and all were included in the research. 51.1 percent of the people tested positive for HIV, while 60.4 percent tested positive for ZN. According to medical criteria, 62.3 percent of the cases were pulmonary tuberculosis. Liquid broth detected 89% pulmonary tuberculosis cases, 95 percent confidence interval, 84.7 percent to 93.3 percent while LJ detected 77.0 percent, or 95 percent of confidence interval, is 71.2 percent to the 82.8 percent and their pvalue = 0.0007. The liquid assay correctly identified 95.9% of the 121 patients without tuberculosis as negative, while the LJ solid assay correctly identified 93.4% as negative. As a result, sensitivity of liquid assays was 89 percent and its specificity which was 95.9 percent.

**TAT for MODS and LJ medium**

In each sample, turnaround time which was calculated from date of the following inoculation for example sample processing and availability date

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of positive-results concurrent identification or susceptibility results. The median TAT for MODS was nine days, by a range for example five to twenty nine days. Whereas LJ’s was 21 days and an IQR which was used for almost fourteen  – twenty eight days. The period to positive culture results was evaluated about 138 samples which were positive including both micro of broth culture or L. Hence the amount of time it required to diagnose tuberculosis using micro broth culture was substantially faster than using LJ culture (P-value less than 0.0001).

**Comparison of MODS with BACTEC-MGIT**

The results which obtained by the MODS or BACTEC-MGIT that were in agreement usually for almost 55 isolates 94.5 percent, 23 susceptible, 32 resistant have 0.894 and 58 isolates at the low concentrations that tested for usually INH susceptibility (0.1 g/ml). Among the discordant samples, one strain which tested vulnerable with MODS while resistant with such as MGIT, or two strains that tested resistant mostly with MODS yet susceptible with the MGIT. For 57 strains tested for RMP susceptibility, there was full agreement between MODS and MGIT results (98.3%); 38 susceptible, 19 resistant. With MODS, the one discordant isolate was susceptible, but with MGIT, it was resistant. For fifty seven strains 98.3 percent, the analyses including its two methods which detecting the MDR-TB were very similar; 19 were MDR or 38 were not MDR is 0.961.

**Discussion**

Drug-resistant tuberculosis (DRTB) is a major obstacle to global TB control development. HIV including AIDS care or treatment programmes which are also involve in jeopardy as a result previous emergence of the XDR-TB between the patient of HIV-infected in South Africa, as well as more mortality that comes with it (Gandhi et al, 2006). The key diagnostic method involve in DOTS strategy, sputum smear microscopy, is the inadequate which resolve this kind of emerging problem in the TB regulation. Some work has been done in this field recently, or new methods that detecting the TB drug resistance. Traditional solid-media of culture that takes three –four weeks to produce outcomes or have sensitivity from seventy six to eighty four percent; while twenty three and four is much better than the sputum smear of microscopy, but it still misses a significant percentage of following TB cases. Liquid media is the modern recommended culture or DST method of the choice, as it quicker and more sensitive than solid culture (Moore et al., 2006). Therefore total of 25 studies testing the MODS were found in our study. MODS had 92 percent pooled sensitivity and a 96 percent specify city, while MGIT had an 87 percent pooled sensitivity and a 98 percent specify city. Even in stratified studies, however, there was significant variability in these figures. Both tests had low reagent and material costs. In the same studies 38, the average or normal time from the receipt to analysis MODS took 9.2 days to complete inside the lab or 11.5 days usually for the MGIT, which was quicker than with traditional solid or liquid cultures. Hence proportion of infected specimens for the both assays that was minimal, and they were comparable to traditional cultures.

**Conclusion**

The study tell that liquid assay example micro of the broth culture including MODS with *Mycobacterium tuberculosis* reported in the literature are early, feasible, or inexpensive tool for evaluating pulmonary tuberculosis and outperforming traditional LJ of slant culture.

**Conflict of Interest**

The authors declared absence of conflict of interest.

**References**

Pio A., Luelmo, F., Kumaresan, J. and Spinaci, S. (1997). National tuberculosis programme review: experience over the period 1990-95. *Bulletin of the World Health Organization*, 75(6), 569.

Ali, W., Naseem, A., Hani, M., Shahzad, M. and Ali, F.M. (2010). Status of health professional awareness about resistant tuberculosis. *Pak J Chest Med*, 16: 4-8.

Bardarov, Jr., S., Dou, H., Eisenach, K., Banaee, N., Ya, S. U., Chan, J., and Riska, P. F. (2003). Detection and drug-susceptibility testing of M. tuberculosis from sputum samples using luciferase reporter phage: comparison with the Mycobacteria Growth Indicator Tube (MGIT) system. *Diagnostic microbiology and infectious disease*, 45(1), 53-61.

Carriere, C., Riska P. F., Zimhony O., Kriakov J., Bardarov S., Burns J., Chan J., & Jacobs W. R., Jr. (1997). Conditionally replicating luciferase reporter phages: improved sensitivity for rapid detection and assessment of drug susceptibility of Mycobacterium tuberculosis. *J Clin Microbiol* 35, 3232–3239.

Cattamanchi, A., Davis, J.L., Worodria, W., den Boon, S., Yoo, S., et al. (2009) Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. *Int J Tuberc Lung Dis*. 13: 1130–1136.

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Caviedes L, Lee T S, Gilman R H, et al. Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. *J Clin Microbiol* 2000; 38: 1203–1208.

Central, TB Division (2009) Revised National TB Control Programme Training Manual for Mycobacterium tuberculosis Culture & Drug susceptibility testing. New Delhi: Directorate General of Health Services, Ministry of Health and Family Welfare.

Davis, J.L., Huang, L., Worodria, W., Masur, H., Cattamanchi, A., et al. (2011) Nucleic acid amplification tests for diagnosis of smear-negative TB in a high HIV-prevalence setting: A prospective cohort study. *PLoS One* 6: e16321.

Ejigu, G. S., Woldeamanuel, Y., Shah, N. S., Gebeyehu, M., Selassie, A., & Lemma, E. (2008). Microscopic-observation drug susceptibility assay provides rapid and reliable identification of MDR-TB. *The International Journal of Tuberculosis and Lung Disease*, 12(3), 332-337.

Elbir, H., Abdel-Muhsin, A. M., & Babiker, A. (2008). A one-step DNA PCR-based method for the detection of Mycobacterium tuberculosis complex grown on Lowenstein-Jensen media. *The American journal of tropical medicine and hygiene*, 78(2), 316-317.

Gandhi, N.R., Moll, A., Sturm, A.W., et al. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368: 1575–1580.

Gleissberg, V.G., Maximova, Z.D., Golubchikova, V.T., Wares, D.F. and Banatvala, N. (1999). Developing nursing practice as part of the collaborative TB control programme, Tomsk, Siberia. *The International Journal of Tuberculosis and Lung Disease*, 3(10), 878-885.

Jacobs, W.R., Jr., Barletta, R.G., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, T., Sarkis, G. J., Hatfull, G. F., and Bloom B. R. (1993). Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 260, 819–822.

Kidenya B-R, Kabangila R, Peck R-N, Mshana S-E, Webster L-E, Koenig S-P and Fitzgerald D-W (2013). Early and efficient detection of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. *PLoS one*, 8(2), e57527.

Kiraz N, Et L, Akgun Y, Kasifoglu N, Kiremitci A (2007) Rapid detection of *Mycobacterium tuberculosis* from sputum specimens using the FASTPlaque TB test. *Int J Tuberc Lung Dis* 11: 904–908.

Kunkel, A., Zur Wiesch, P. A., Nathavitharan, R. R., Marx, F. M., Jenkins, H. E., & Cohen, T. (2016). Smear positivity in paediatric and adult tuberculosis: systematic review and meta-analysis. *BMC infectious diseases*, 16(1), 1-9.

Levinson W, (2010) Review of medical microbiology and immunology. The McGraw-Hill Companies.

Moore D A J, Evans C A W, Gilman R H, et al. Microscopic observation drug susceptibility assay for the diagnosis of TB. *N Engl J Med* 2006; 355: 1539–1550.

Moore, D. A. J., Evans, C. A. W., Gilman, R. H., Caviedes, L., Coronel, J., Vivar, A., … Friedland, J. S. (2006). Microscopic-observation Drug-Susceptibility Assay for the Diagnosis of TB. *New England Journal of Medicine*, 355(15), 1539–1550.

Owusu, E., & Newman, M. J. (2020). Microscopic Observation Drug Susceptibility (MODS) Assay: A Convenient Method for Determining Antibigram of Clinical Isolates of *Mycobacterium tuberculosis* in Ghana. *Medical Sciences*, 8(1), 5.

Park, W.G., Bishai, W.R., Chaissen, R.E., Dorman, S.E. (2002). Performance of the microscopic observation drug susceptibility assay in drug susceptibility testing for *Mycobacterium tuberculosis*. *J. Clin. Microbiol*. 12. 4750–4752.

Riska P. F., & Jacobs W. R., Jr. (1998). The use of luciferase-reporter phage for antibiotic-susceptibility testing of mycobacteria. *Methods Mol Biol* 101, 431–455.

Steingart K-R, Sohn H, Schiller I, Kloda L-A, Boehme C-C, Pai M and Dedukuri N, (2013). Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane database of systematic reviews*, (1).

World Health Organisation. Guidelines for Surveillance of Drug Resistance in Tuberculosis. WHO/TB/2003.320-WHO/CDS/CSR/RMD/2003.3. 2003.
World Health Organization, Global Tuberculosis Program: Global Tuberculosis Control. WHO Reported (1999) 181. Geneva: WHO, 1999.

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