Addis Ababa University

College of Health Sciences

School of Allied Health Sciences

Department of Medical Laboratory Sciences

In vitro starvation model for Assessing Phenotypic Drug Tolerance in *Mycobacterium tuberculosis* Lineages of Ethiopia

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This is to certify that the thesis prepared by Wondimu Ashagre Awoke, entitled: *In vitro* starvation model for Assessing Phenotypic Drug Tolerance in *Mycobacterium tuberculosis* Lineages of Ethiopia and submitted in partial fulfilment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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My Father surely your goodness and love will follow me all the days of my life, and I will follow your footsteps, may your soul rest in peace with eternal life in the kingdom of LORD.

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Abbreviations

AAU: Addis Ababa University

AFB: Acid Fast Bacilli

AHRI: Armauer Hansen Research Institute

AIDS: Acquired Immuno deficiency Syndrome

BSA: Bovine Serum Albumin

BSC: Bio Safety Cabinet

DOT: Directly Observed Treatment/Therapy

DST: Drug Susceptibility Test

EMB: Ethambutol

FDA: Fluorescein Diacetate

FM: Foamy Macrophages

HIV: Human Immunodeficiency Virus

ILI: Intra-Cytoplasmic Lipid Inclusions

INH: Isoniazid

LB: Lipid Body

LED: Light Emitting Diode

LJ: Lowenstein Jensen

LPA: Line Probe Assay

MDR: Multi drug Resistant

MIC: Minimum Inhibitory Concentration

MTB: *Mycobacterium tuberculosis*

MTT: Micro Titer Technique

MOTT: Mycobacteria Other Than Tuberculosis

OADC: Oleic-Acid Albumin Dextrose Catalase

OD: Optical Density

PBS: Phosphate Buffer Solution

RIF: Rifampicin

SDW: Sterile Distilled Water

SOP: Standard Operating Procedure

SPSS: Statistical Packages for Social Sciences

STM: Streptomycin

TB: Tuberculosis

TG: Triacylglycerol

WHO: World Health Organization

XDR-TB: Extensively Drug Resistant Tuberculosis

ZN: Ziehl Neelson

Abstract

Background: *Mycobacterium tuberculosis* persist in the human host for decades and reactivation can occur at any point. It becomes dormant and phenotypically drug tolerant when exposed adverse conditions. Understanding of the signals and processes which allow the bacteria to achieve this feat could potentially be used as a baseline to design new types of drugs or modify old drug regimens for improved cure and avert development of drug resistance.

Objective: To assess the level of phenotypic drug tolerance of *Mycobacterium tuberculosis* using *in vitro* nutrition starvation model.

Methods: Three *MTB* lineages and one standard susceptible reference strain (H37Rv) were tested by different test methods at different time point from March to September 2017. All lineages tested to be sensitive to first-line anti-TB drugs. Log phase (highest colony count on week 3-4) culture from Lowenstein Jensen medium sub cultured to Middle-brook7H9 with 10% Oleic Acid Albumin Dextrose Catalase as a normal, Phosphate Buffer Solution (PBS) (PH 7.2) and Sterile Distilled water (SDW) as starvation media were used. Each week culture growth reading was performed, Acid Fast Stain (AFS) by Ziehl Neelson(ZN), Lipid Bodies (LB)by Sudan black stain and viability by Fluorescein DiAcitate (FDA) staining. On week 0, 3 and 6 drug susceptibility test was done by colorimetric MTT assay. Graph pad prism 6 and SPSS V20 used for data analysis.

Results: A total of 576 experiments were performed using4 strains of *Mycobacterium tuberculosis* subcultured on SDW, PBS and 7H9 and. Of these, 324 microscopic tests using ZN(108)acid fastness, FDA(108) viability, and Sudan black stain(108) for lipid bodies, 108 culture growth reading done. After week 6 acid fastness, viability and culture growth decreased. 144 phenotypic DST done using MTT assay. A higher inhibitory drug concentration was required at the 6^{th} week compared to the baseline and C50 (RMP=0.5;INH=0.1; STM=2.0 and for EMB=4.0), yet the proportion of lipid body containing bacilli increased continuously in all lineages.

Conclusion: Our study showed that the mycobacteria lineages behaved similarly in all media systems and reached stationary phase at similar time. The increased drug concentration observed at the 6^{th} week coincided with the decline in viable bacilli in all media systems, thus attributing this phenomena to lipid body accumulation alone was difficult.

Keywords: *Mycobacterium tuberculosis*, LB%, Drug Tolerance, and MTT Assay.

1. Introduction

1.1. Background

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), continues to be a world leading killer and a serious global health problem primarily affecting poor people in many developing countries. Lineages of MTB include Indo-Oceanic (lineage 1), East Asian (lineage 2), East-African Indian (lineage 3), Euro-American (lineage 4), West African 1 (lineage 5), West African 2 (lineage 6), *Aetopicus vetus*(L7) proposed name, based on Single nucleotide polymorphism (SNP)-based population analyses gives phylogenetically information (1,2).

Based on WHO 2017 report Ethiopia is among 30 TB High Burden Countries; 10.4 million people fell ill with TB and 1.67 million died from the disease. Over 95% of TB deaths occur in low and middle-income countries. TB is a leading killer of HIV-positive people: 35% of HIV deaths were due to TB. Globally an estimated 490 000 people developed multidrug resistant TB (MDR-TB). WHO estimates about one-third of the world's population has latent TB, which means people have been infected by TB bacteria but are not (yet) ill with the disease and cannot transmit the disease (3).

The pathogen enters to the host by inhalation of an infectious aerosol and replicates in the alveolar macrophages, but in a great majority of cases, the host's immune defense causes bacteriostasis that leads the pathogen to go into a state of non-replicate, drug-resistant dormant bacteria. When the host's immune system is weakened, this dormant pathogen replicates and leads to active tuberculosis (3,4).

Most cases of active tuberculosis arise from the small fraction of people who have had the dormant organism for years. Since the pathogen under dormancy is resistant to antimicrobial drugs, the ability of the organism to survive long periods in such a state creates great difficulty in the control of tuberculosis. Molecular mechanisms that allow the pathogen to go into dormancy, survive in the host for decades under such conditions, and resume replication upon weakening of the immune system of the host are poorly understood (5,6).

The ability of the pathogen to go into the phenotypically drug-resistant non-replicating dormant state in such latent infection is a major obstacle to curing the disease since currently available drugs cannot kill latent *MTB*. The emergence and spread of multidrug-resistant

(MDR) or extensively drug resistant (XDR) TB complicates this problem especially with the spread of AIDS worldwide (7).

Tubercle bacillus has amazing ability to persist in the human host and can cause latent infection. The current TB chemotherapy while effective in killing growing bacilli is largely ineffective in killing persistent or dormant bacilli, leading to prolonged therapy. There is considerable recent interest to study mechanisms of persistence and dormancy in mycobacteria(8).

Various Mycobacterial factors have recently been identified that may be involved in persistence or dormancy and recovery of dormant organisms. The phenotypic resistance to anti tuberculosis drugs in persistent and dormant bacilli presents a major challenge for effective control of the disease. The host immune system is critical in controlling latent TB infection from reactivation. The complex interaction between the bacteria, drugs, host and the environment underscore the need for a combined approach that incorporates chemotherapy, immunotherapeutic agents, and improved socioeconomic, nutritional and even conducive psychological factors for more effective control of active and latent TB (9).

The development of antibiotic resistance in non-replicating dormant bacteria which is described as ‘phenotypic drug-resistance or drug-tolerance is due to changes in the physiological state of the bacteria and not conferred by any inheritable genetic resistance mechanism. Several studies have explored possible stress conditions that *MTB* would face in the host where the pathogen goes into the latent state. Convincing evidence obtained in recent years has shown that the glyoxylate cycle plays a critical role in the persistence of the pathogen in the host. However, the origin of the fatty acids and the nature of the storage form of fatty acids are not clear. They have shown that the pathogen stores energy as triacylglycerol (TG) as it goes into dormancy-like state *in vitro* and uses this stored energy to survive during starvation and product as the dominant contributor to storage of TG that occur when *MTB* is exposed to different single stress factors(10).

In the current study assessed the importance of phenotypic drug tolerance for the control of tuberculosis using three lineages and one growth control H37Rv. Lipid accumulation and Phenotypic drug tolerance increased in MTB under nutrition starvation. This approach provides a new perspective to the understanding of latent infection and suggested some novel targets for the control of tuberculosis and provided a new straight forward *in vitro* model that could help to test the activity of drugs against dormant bacilli from a novel perspective.

1.2. Statement of the problem

Despite the decades of effort in TB control and the Directly Observed Treatment (DOT) program in Ethiopia, TB continues to be one of the public health threats. The advances in microbial genetics,biochemical differentiationand host immunity provide an opportunity for renewed investigation of this persistent threat to human health. Resistance usually is said to develop due to inadequate TB management, including improper use of medications, improper treatment regimens and failure to complete the treatment course. (11)

There is an increasing report of drug resistance, yet no methods to my knowledge to evaluate the efficacy of existing drug/regimens as they are used in human and there is no clear understanding of the continued TB transmission. However, the contribution of phenotypic drug tolerance and the mechanism how MTB bacilli adapts and takes advantage of the granuloma environment in host cells is little explored. (12).

Several animal models of latent TB have been developed. However, it is unlikely that any of them truly represent the human latent TB. In vitro nutrient starvation models of latent MTB suitable for screening chemical libraries to discover drugs that can kill latent MTB are not available (13).

Yet, the evidences showing that the bacilli accumulate neutral triacylglycerol (lipid bodies) claimed to contribute for the adaptation and phenotypic drug tolerance merit closer look. Such kind of study information is not available in Ethiopia and even limited in African context. Hence it is time to explore if there could be difference between different mycobacterial lineages in their ability to accumulate lipid bodies and their drugs responses. (14).

1.3. Significance of the study

The data generated from this study enabled us better understand about the accumulation of lipid bodies is associated with drug tolerance and there was a difference between different lineages in their ability to accumulate lipid bodies. In other word, the results have direct implication on how to manage, need for rigorous assessment to our first line TB treatment in light of the lipid bodies containing bacilli load in patient sample. Laboratory models showed that *Mycobacterium tuberculosis* exist in a non-growing, drug-resistant state that persist *in vitro* and this work definitely will serve as a baseline study.

2. Literature Review

Mycobacterium tuberculosis enters the host by inhalation of an infectious aerosol and replicates in the alveolar macrophages until the host's immune defense causes bacteriostatic, which leads the pathogen to go into non-replicative drug-resistant dormancy. The source of energy used by dormant and reactivating *M. tuberculosis* within the host remains unclear. The dormant pathogen can survive for decades till the host's immune system is weakened and active tuberculosis develops. Even though fatty acids are thought to be the major energy source required for the persistence phase, the source of fatty acids used is not known (6).

Bacterial strains and culture conditions, *MTB* H37Rv was grown in Middle brook 7H9 (supplemented with 0.05% Tween 80, 10% oleic acid-albumin-dextrose-catalase enrichment, and 0.2% glycerol), in Dubos-Tween-albumin medium (prepared from Dubos broth base and Dubos medium albumin). They postulate that the pathogen uses triacylglycerol (TG) as a storage form of fatty acids. And their result shows that Induction of TG synthesis in *M. tuberculosis* during gradual depletion of O2. Autoradiogram showing oleic acid incorporation into TG. Dichromate-sulfuric acid charring of lipids showing TG accumulation in *M. tuberculosis* cells going into the non-replicative state without exogenous oleic acid and after 6 h of incubation with 0.64 M oleic acid–0.5% bovine serum albumin (BSA) (7,8).

During the dormant phase *MTB* persists in lung granulomas by residing in foamy macrophages (FM) that contain abundant lipid bodies (LB) in their cytoplasm, allowing bacilli to accumulate lipids as Intra-cytoplasmic Lipid Inclusions (ILI). Quantitative analysis of detailed electron microscope showed that the presence of ILI resulted in a reversible block of division without causing a change in the mycobacterial replication rate. Fluctuation between ILI either partially or fully extending throughout the mycobacterial cytoplasm was suggestive of bacterial cell cycle events. Foamy macrophages constitute a well-defined cellular system in which to study changed metabolic states of intracellular mycobacteria that may relate to persistence and reactivation of tuberculosis (9).

A novel *in vitro* multiple-stress dormancy model for *MTB* by applying combined stresses of low oxygen (5%), high CO2 (10%), low nutrient (10% Dubos medium) and acidic pH (5.0), are conditions that *MTB* is thought to encounter in the host. Under this condition, *MTB* stopped replicating, lost acid-fastness, accumulated triacylglycerol (TG) and wax ester (WE), and concomitantly acquired phenotypic antibiotic-resistance. The new *in vitro* multiple stress dormancy model efficiently generates *MTB* cells meeting all criteria of dormancy, and this

method is adaptable to high-throughput screening for drugs that can kill dormant *MTB*. A critical link between storage-lipid accumulation and development of phenotypic drug-resistance in *MTB* was established. Storage lipid biosynthetic genes may be appropriate targets for novel drugs that can kill latent *MTB* (10).

In the case of the study on *Mycobacterium tuberculosis*, the primary objective of examining the comparability of *in vivo* and *in vitro* grown bacilli was accomplished by an examination of various biochemical characteristics. The differences observed are striking and indicate that tubercle bacilli in their natural infectious state are dissimilar to those grown inartificial culture medium (11).

Reactivation, where the bacilli resume replication and cause further clinical disease, can occur at any point. Thus, there is a large reservoir of potential disease and further transmission. An understanding of the signals and processes which allow the bacteria to achieve this feat will help us to design new laboratory approach and types of drugs or modify old drug regimens to eradicate all bacilli from infected individuals. Infection by *Mycobacterium tuberculosis* is able to persist in the human host for decades in an apparently dormant state where it is presumed to reside in a hypoxic environment. This can be mimicked by the Wayne culture model in which progressive oxygen depletion causes the bacteria to shift into a non-replicating state (12).

The pathogen able to go into the phenotypically drug-tolerant non-replicating dormant state in such latent infection is a major impediment to curing the disease since currently available drugs cannot kill latent MTB. The emergence and spread of multidrug-resistant (MDR) or extensively drug resistant (XDR) TB complicates this. Development of drugs that can kill phenotypically drug-resistant *MTB* in patients with latent infection(13).

TB latent infection is an extensively urgent need of research. The development of antibiotic resistance in non-replicating dormant bacteria which is described as ‘phenotypic drug-resistance or ‘drug-tolerance is due to changes in the physiological state of the bacteria and not conferred by any inheritable genetic resistance mechanism. Typically, the phenotypic drug-resistance of dormant *MTB* is exemplified by resistance to the sterilizing antibiotic (Rifampicin) and is regarded as one of the hallmarks of latent TB. Several animal models of latent TB have been developed. However, it is unlikely that any of them truly represent the human latent TB. *In vitro* models of latent *MTB* suitable for screening chemical libraries to discover drugs that can kill latent *MTB* are not available (14).

Metabolic processes that are critical for the pathogen to go into dormancy, survive under this non-replicating drug-resistant state, and get reactivated when the immune system of the host is weakened remain poorly understood. It has been recognized for more than half a century that the pathogen inside the host utilizes fatty acids as the major energy source and that glyoxylate cycle plays a critical role in the use of fatty acids as the main carbon source. Convincing evidence obtained in recent years has shown that the glyoxylate cycle plays a critical role in the persistence of the pathogen in the host. However, the origin of the fatty acids and the nature of the storage form of fatty acids are not clear. They have shown that the pathogen stores energy as triacylglycerol (TG) as it goes into dormancy-like state *in vitro* and uses this stored energy to survive during starvation (15).

Bacteria were subjected to single-amino-acid starvation (Middlebrook 7H9 liquid plus 10% oleic acid-albumin-dextrose-catalase [OADC; Becton Dickinson] and 0.05% [wt/vol] Tween 80 without amino acid supplementation) and complete starvation (no nutrients, i.e., sterile distilled water). Lysis was measured by monitoring the optical density at 600nm (OD600), and viability was measured by determining the numbers of CFU per ml on Middlebrook 7H10 agar containing 10% (vol/ vol) OADC supplemented with 40ug of amino acid per ml as required. Both the wild type and tryptophan auxotroph grew normally in supplemented medium (Middlebrook 7H9 liquid containing 10% OADC, 0.05% [wt/vol] Tween 80, and 40ug of amino acid per ml). There was no difference in their ability to survive complete starvation (15,16).

The cultures in water showed an immediate drop in OD 600 to below the limit of detection and an accompanying log reduction in CFU. There was no further death over 7 weeks. The fact that these bacteria have a remarkable ability to survive complete starvation for long periods of time suggests that there must be a coordinated response to the lack of nutrients which changes their physiology from actively growing cells to persistent (non-growing) cells. In the non-supplemented medium, the wild-type strain grew normally; however, the tryptophan auxotroph showed an unusual phenotype. Although the OD600 remained stable, indicating that the cells did not lyse, there was a 2-log reduction in cell viability over the course of the 7-week experiment. Whatever response the bacteria made to complete starvation was not induced solely in response to tryptophan depletion (16).

The survival experiment over a longer time scale to determine if this pattern of survival would be maintained. Over 15 weeks, the OD 600 of the tryptophan auxotroph remained

constant in non-supplemented medium, while the OD 600 in water rapidly declined in the first week. Again, this did not mirror the viability counts. The wild type showed a small (<1 log) reduction in viability after extended stationary-phase growth (17 weeks) in 7H9 medium, while the tryptophan auxotroph lost viability over this time scale with a 4-log reduction at 13 weeks and a complete loss of viability (approximately 8-log) by 17 weeks. In contrast, there was no difference in the two strains ability to survive in water, with both strains losing less than 0.5-log viability over 17 weeks. They also determined whether the tryptophan auxotroph could be recovered from the non-supplemented medium by sub culturing into supplemented liquid medium, but no growth occurred after repeated subculture, thus indicating that the cells were nonviable and non-culturable(17,18).

Following Hartman, who first suggested the use of Sudan black B, in place of red Sudan, as a bacterial fat stain (Hartman, 1940), Burdon, Stokes, and Kim-brought (1942) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing, from suspensions of the organisms in alcoholic Sudan black solution(19).

The *Mycobacterium tuberculosis* has DosRregulon. It's a genetic program induced by conditions that inhibit aerobic respiration and prevent bacillus replication. DosRregulon assists in metabolic homeostasis and enables rapid recovery from non-respiring dormancy. The DosRregulon encodes novel metabolic mechanisms essential for M. tuberculosis to survive in the absence of respiration and to successfully transition rapidly between respiring and non-respiring conditions without loss of viability (20).

Four clinical isolates and standard reference strain *Mycobacterium tuberculosis* (H37Rv) were used for the study. FDA staining method used to check viability. Log phase cultures were used for the study after subculturing from original LJ slopes. Middlebrook 7H9 broth supplemented with 10% bovine serum albumin and 0.5% Tween 80 was prepared. The metabolically active cells ranged from 60 to 90% on day zero in FDA-EB stain method. Proportion of metabolically active cells on day 7 reduced to 14 to 30% in controls and 7.5 to 40% in cells treated with CPC (21).

Use of fluorescein diacetate staining to detect viable *Mycobacterium tuberculosis. Tuberculosis* has long been a common problem prevailing in developing countries. Present study focused on the rapid identification of live *Mycobacterium tuberculosis* among treatment failure cases. Assumed as multidrug resistant tuberculosis, were studied through fluorescein diacetate staining under light emitting diode (LED) fluorescence microscope (22).

For *Mycobacterium tuberculosis* drug susceptibility test, the proportional method and BACTEC 460 are widely accepted. Later, the colorimetric method using MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used as an alternative method for rapid and indirect determining cellular growth. The MTT method of *Mycobacterium tuberculosis* was developed by using bacterial suspension prepared from colonies on solid media (23).

The MTT tube assay in 1 ml Middlebrook 7H9 broth was completed within 4 days for Rifampicin (RIF) and within 7 days for Isoniazid (INH).When MTT assay results with 279 *MTB* clinical isolates were compared with those of the conventional proportion method on Löwenstein-Jensen medium, high specificity and sensitivity values of 100% and 94.1%, respectively, for RMP susceptibility testing, and 99.5% and 89.2%, respectively, for INH susceptibility testing were obtained. The accuracy of the MTT method for RMP and INH was >0.97 concordance with the proportion method. The MTT method is simple, inexpensive and rapid. The high level of agreement with the conventional proportion method suggests a potential to rapidly detect drug-resistant *Myacobacterium tuberculosis* in developing countries, as only basic microbiological equipment is need(24).

Drug susceptibility testing, drug treatment was performed in both actively growing *M. tuberculosis* H37Rv cells and dormant SS18b cells at 37°C with shaking. Mid-logarithmic-phase *M. tuberculosis* cultures were diluted to an OD of 0.05 and split into 10-ml samples, and drugs were added at the concentrations indicated in the procedure, with an untreated sample serving as a control. Serial dilutions of the cultures were plated on 7H10 medium supplemented with glycerol, OADC, and STR (in the case of SS18b) at day 7 after addition of drugs. The STR-dependent phenotype was checked by plating the same dilutions on 7H10 medium without STR. CFU were counted after 4 to 5 weeks of incubation at 37°C (25).

3. Objective

3.1. General objectives

To assess the level of phenotypic drug tolerance of *Mycobacterium tuberculosis* using *in vitro* nutrition starvation model.

3.2. Specific objectives

- To evaluate *in vitro* starvation model for *Mycobacterium tuberculosis*.
- To test lipid body (LB) accumulation pattern among different MTB lineages circulating in Ethiopia.
- To assess the effect of LB accumulation on efficacy of anti-TB drugs.

4. Hypothesis:

- H0: There is no difference in phenotypic drug tolerance with lipid body accumulation in MTB lineage isolates.
- HA: There is difference in phenotypic drug tolerance with lipid body accumulation in MTB lineage isolates.

5. Materials and method

5.1. Study area and setting

This study conducted in Addis Ababa, Ethiopia, at AHRI TB Laboratory on previously isolated of *Mycobacterium tuberculosis* from different projects in Ethiopia. AHRI was founded in 1970 through the initiative of the Norwegian and Swedish Save the Children organizations seconded by the Ministry of Health of Ethiopia. The Institute established as a bio-medical research institute, hold different activities cover basic (immunology and molecular biology), epidemiological and translational research. It has a network of national and international collaborators in peer reviewed grant projects, clinical trial partnerships, student's capacity building.It's Located next to the All Africa Leprosy Rehabilitation and Training Hospital (ALERT).

5.2. Study design and period

Experimental study design was employed from March to September 2017 at AHRI on stored clinical isolates. Purposive convenient sampling method was used for strain selection from AHRI Cryo-bank representing L3, L4, and L7 lineages so far identified in Ethiopia.

5.3. Inclusion and exclusion criteria

5.3.1 Inclusion criteria: *Mycobacterium tuberculosis* Lineages, Lineages which is capable to grow in culture media and found at AHRI TB laboratory.

5.3.2 Exclusion criteria: all non-mycobacteria tuberculosis or Mycobacteria Other Than Tuberculosis (MOTT) not included.

5.4. Sample size determination and Sampling

5.4.1. Sample size determination

A total of four strains, one standard growth control (H37Rv) and three common lineage isolates selected, form previous isolates of other different research projects.

5.4.2. Sampling procedures

Purposive convenient sampling technique was employed to include study isolates which meet the inclusion criteria. From the list of isolates in the cryo-box one unique lineage/strain was taken by checking representativeness. We picked the next lineage isolate if the selected

isolate different lineage with the previous one which was found and picked out from cryo-box, thawed in biosafety cabinet and inoculated on LJ slant.

5.5. Laboratory Methods

5.5.1 Culture-nutritional starvation model

Lineages (strains) from -80^{0}c freezer; and inoculate all lineages on LJ media. After 3-4weeks of incubation later inoculate on enriched and starved media. Then from the new sub-cultured every week up to week eight the two procedures viability (FDA), lipid body (Sudan black+ ZN) and every three weeks at three test time point drug susceptibility test by MTT method was done.

Starvation experiment: Lineages response to stress: Sub cultured on LJ media by diluting the growth with 3ml 7H9 medium, 3ml PBS and 3ml SDW. 7H9 Middlebrooks supplemented with 0.2% (vol/vol) glycerol, 10% (vol/vol) OADC enrichment, and 0.025% (vol/vol) Tween 80 (E), PBS (P) and SDW (S) prepared and used. For eight weeks, three sets of bottles were prepared, One for nutrient enrich medium, and two for starvation, took pure colony from fresh LJ medium compare the turbidity of the test suspension with the McFarland Standard in PBS, take 20µl of suspension to 3.8ml PBS incubate standing at 37^{o}C in sealed bottles (6).

5.5.2 ZN/AFB Staining

ZN was done for the consecutive eight weeks by taking each isolatestrains from LJ slant. The smears for ZN, were done from bacterial suspension sediment, after dilution 1:20 from specific media that were SDW, PBS and 7H9.The standard operating procedure was followed (Annex II).

5.5.3 Sudan Black staining for LB

Totally 108 Sudan black stains were done. LB staining was done at eight-time points in eight weeks, dual staining of *MTB* with the combination (add Sudan black reagent to dried smears and wait for 15 min followed by the ZN procedure) used to reveal acid-fast staining property and neutral lipid accumulation in the same cell. We applied this dual staining procedure to examine acid-fastness of *MTB* cells and lipid body accumulation within the *MTB* cells under nutritional stress weekly. We checked the proportion of lipid bodies from the total of acid fast bacilli%LB+AFB =100(Total LB - Positive AFB / Total AFB)(9).

5.5.4 Fluorescein Di-Acetate staining for Viability check

Totally108 FDA Stain was done to check viability of *MTB.*Weekly for every samples at eight time points to evaluate the proportion of dead cells among viable bacilli and the result shows that decreasing of viability. This helped us to know the persistence of *MTB* bacilli in all starved medium. (20).

5.5.5 MTT Assay

MTT assay was done using 96 wells ELISA plate to all nutrients enriched and starved tubes of all lineages. This *in vitro* dormancy model used for drug screening and viability of mycobacterium cells. The MTT assay based on 3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide done on 3 weeks. Contamination checked by growing subcultures on nutrient agar medium. The assay was done using one bacterial control tube with no drug and another tube with bacteria and drug. We use the MTT assay to quantify the phenotypic drug resistance bacilli of 0-day, 3-weeks and 6-weeks incubation at 37^{o}c in nutritional-stress medium. Thus, MTT assay method can be used with the nutritional-stress model to screen chemical changes to detect compounds that show lethal activity against dormant bacilli. This method is adaptable to high throughput screening. Took pure colony from fresh LJ medium compare the turbidity of the test suspension with the McFarland Standard in SDW, took 20µl of suspension to 3.8ml SDW incubated standing at 37^{o}C in sealed bottles to see their drug tolerance activity of *MTB* lineages (21).

Totally four sample isolates of *Mycobacterium tuberculosis* from AHRI TB laboratory were used and inoculated in duplicate LJ tubes and checked for growth every week. After a month (at log phase) we sub cultured all in to a new LJ slope. All experiments were started from old culture for each lineage. We used enrichment (7H9+OADC) and two starvation there was (SDW & PBS) model.

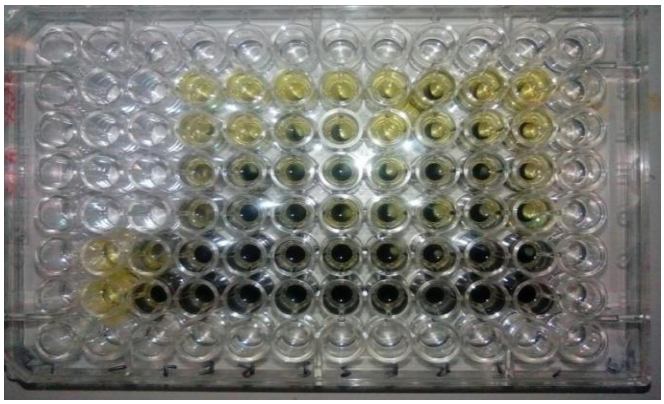

Figure 1: Representative photo of MTT rapid test, with the lowest concentration of drugs prevented growth of *MTB* and no change of color from yellow to purple in the above wells.

5.5.6 Work Flow

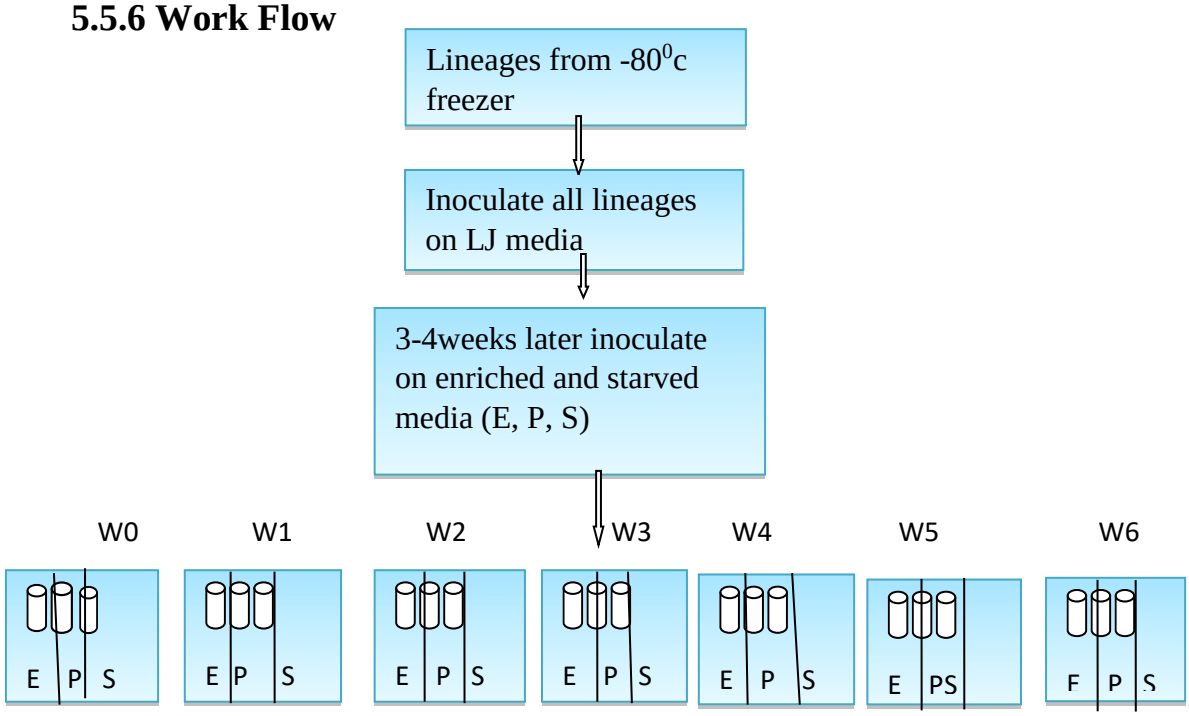

Figure .2: Activity flow diagram, NB: LJ=Lowenstein Jensen, E= enrichment media, P= PBS, S= Sterile distilled water, W=Week.

5.6. Data Quality Assurance

We kept it as pre-analytical phase isolate selection based on our purpose and identification of the isolates by their laboratory ID, date isolated, and name of project documented.

Analytical phase the selected strain labelled with our Lab ID and analyses done parallel with known growth control results were interpreted, and during post-analytical steps we record and presentation of final results. H37Rv was used as a positive growth control, and name of

the lineage, Lineages identifiers are labeled on the cryo tube, contacting with the PI of that project, written information on log books and all are isolated at AHRI TB laboratory.

Post analytical for each isolate, the drug susceptibility, lipid body accumulation pattern and change in drug susceptibility with exposure to the different culture models were documented, also applicable to all lab test procedures which we engaged.

5.7. Data analysis and interpretation

We used SPSS version 20, and graph pad prism version 6 for data entry and analysis. We assessed the trend and association in lipid body accumulation with drug tolerance.

5.8. Ethical consideration

We used lineages of *MTB* isolates, previously isolated and stored in cryobox in deep freezer (-80c) from different studies. Confidentiality of patient's record was protected, and approval for this study obtained from AHRI/ALERT Ethical Review Committee and the Ethical Committee of Addis Ababa University, College of Health Sciences, School of Allied health science, Department of Medical Laboratory Sciences, we worked on stored isolates, so there is no direct risk to patients.

5.9. Dissemination of results

Study results will be presented at Addis Ababa University, College of Health Sciences, school of Allied health science, Department of Medical Laboratory Sciences and Armauer Hansen Research Institute. A copy of the thesis will be made available at the Addis Ababa University, College of Health Sciences, and School of Allied Health Science, Department of Medical Laboratory Sciences library and Armauer Hansen Research Institute. We intend to publish the findings of the study in a peer reviewed local and/or international journals.

5.10. Operational Definition

- ✓ **Phenotypic drug tolerance/resistance:** non-inheritable resistance to a drug conferred by a specific metabolic state (usually dormancy).
- ✓ **Minimum Inhibitory Concentration:** the lowest concentration of drug that prevented growth of microorganisms.
- ✓ **Dormant MTB:** non-replicating bacilli maintaining full viability at a very low metabolic rate, organism show minor susceptibility or Phenotypic drug resistance to antibiotics targeting functions required for growth.

6. Results

In this experimental study four strains of *Mycobacterium tuberculosis (MTB)*L3, L4, L7and standard growth control H37Rv were used. The level of phenotypic drug tolerance of tuberculosis lineages was assessed using *in vitro* nutrition starvation model in each strain at three time point on week 0, 3 and 6.With minimum inhibitory concentration of the drug (MIC) based on the McFarland standard. We also conducted over eight weeks, AFB, LB and Viability staining in duplicate using ZN, Sudan black/ZN dual stain and FDA respectively.

A total of 4 (100%) isolate strains subcultured on SDW, PBS and 7H9.576experiments were performed. Of these, 324 microscopic tests using (108(ZN) acid fastness, 108(FDA) viability, and 108(Sudan black stain) lipid bodies), 108 culture growth reading done. After week 6acid fastness, viability and culture growth decreased, but proportion of lipid body containing bacilli increased. 144 Drug Susceptibility Test(DST) were done using rapid MTT test. Based on Minimum Inhibitory Concentration (MIC) in µg/ml (RMP=0.5; INH=0.1; STM=2.0 and for EMB=4.0). All lineages, were sensitive on week 0 and on week 3 but at the 6th week the inhibitory concentration was higher than MIC.

Lipid bodies accumulated in *MTB* cell and Lipid body positive cells proportion becomes higher over the eight weeks. The percentage of changed in the proportion of lipid body containing bacilli was lower in lineage 7 in all media systems but this difference did not attain statistical significance (Fig .2). Also on re-culturing of the strains on to LJ slant we saw better growth of the strains recovered from the 7H9 medium.

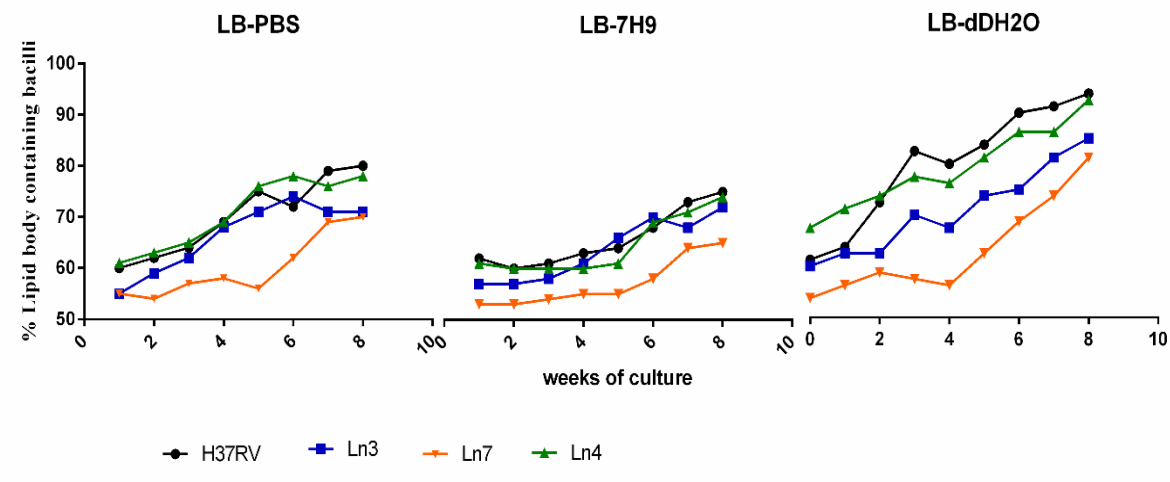

Figure 3: The change in the percentage of Lipid body containing bacilli over eight weeks of culture in the different media systems in the different lineages.

The colony forming units on the LJ were used to estimate the number of bacilli per inoculum in each medium type with the McFarland method.All strains accumulated most LB in SDW medium than PBS and 7H9 and least in 7H9. Generally lineage 7 stored the least lipid bodies than other lineages, lineage 3 medium accumulation but lineage 4 and H37Rv accumulated more lipid bodies with in eight weeks.

The proportion of viable bacilli compared to dead once as determined by the - FDA (Florescence Diacetate) Staining increased until week four and started to decline from week 6 onwards in all media systems (Figure 2). Culture growth was better in 7H9 than the two starved Medias (PBS and SDW).

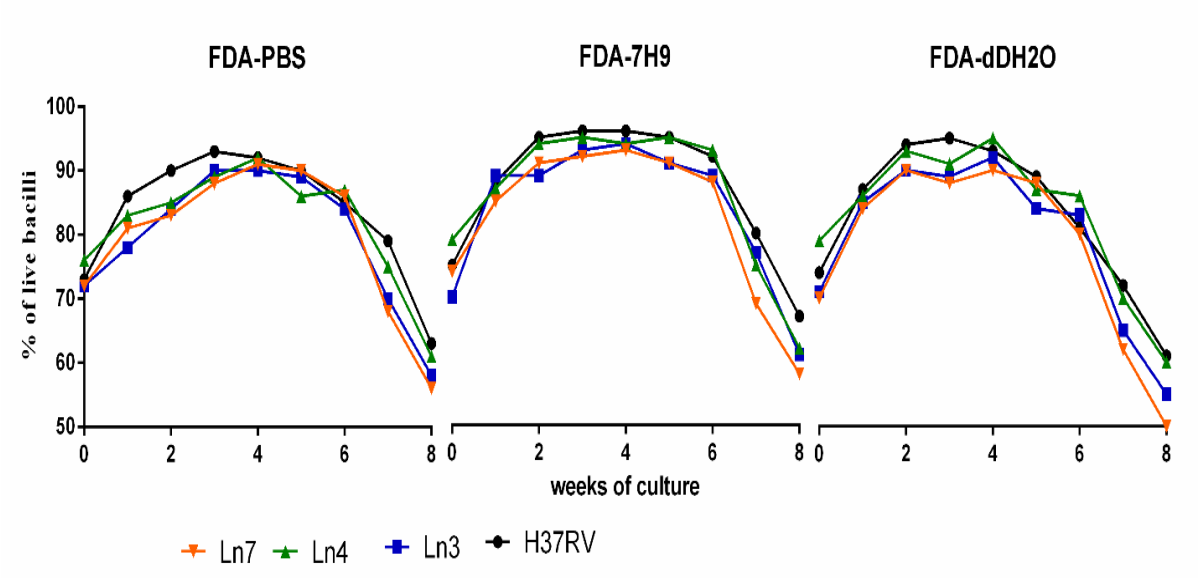

Figure 3: The change in the percentage of live bacilli over eight weeks of culture in the different media systems. Ln7= Lineage 7, Ln4=Lineage 4, Ln3=Lineage 3, H37Rv=Growth Control, FDA=Florecin Diacetae, PBS=Phosphate Buffer Solution, sDH2O=sterile Distilled Water and 7H9=Middle Brook Liquid Media.

The rapid colorimetric drug susceptibility test (MTT Assay)

Standardization of MTT assay for INH, RIF, EMB and STM, from the stock using serial dilution in 96 micro wells plate. Six final concentrations of drugs were used to determine the critical concentration to be used in the direct MTT assay. Four clinical isolates of MTB from the different lineages were checked and confirmed to be sensitive to the test drugs. Isolates were then sub cultured onto LJ and incubated for 3-4 weeks at 37^{o}C. Innocula were prepared by suspension of colonies from 3-4 weeks old LJ cultures in sterilized distilled water to a turbidity equal to that of a 0.5 McFarland standard. A 0.5 ml of inoculum was incubated into

multiple screw capped tubes containing 3ml 7H9 broth, 3ml sterile distilled water and 3ml PBS. Thus for each isolates tested, there were incubated in micro titer 96 well plate at 37c until the day of MTT assay(7 days) 1 positive and one negative control (21).

Table.1: The MTT MIC/C50 standard used, AHRI Sept, 2017.

	I	R	E	S	I	R	E	S	GC(+)	GC(-)	
	1 µg/ml	2 µg/ml	16 µg/ml	4 µg/ml	1 µg/ml	2 µg/ml	16 µg/ml	4 µg/ml	A	B	
	0.5	1	8	2	0.5	1	8	2	GC(+)	GC(-)	
	0.25	0.5	4	1	0.25	0.5	4	1			
	0.125	0.25	2	0.5	0.125	0.25	2	0.5			
	0.0625	0.0125	1	0.25	0.0625	0.0125	1	0.25			
	0.0312	0.0625	0.5	0.125	0.0312	0.0625	0.5	0.125			

MIC/C50 for INH, 0.1 µg/ml or less; RMP, 0.5 µg/ml or less; for EMB, 4.0 µg/ml or less and STM, 2.0 µg/ml or less. The reading of the MTT assay showed that the unit of drug concentration in µg/ml changed at the 6th week for all lineages and drugs tested (Table .1). Analyzing the result for each medium and lineage by lineage (Figure .4) and overlapping the proportion of the live and lipid body containing bacilli showed that the increased drug concentration coincided with the time when the population of live bacilli started to decline.

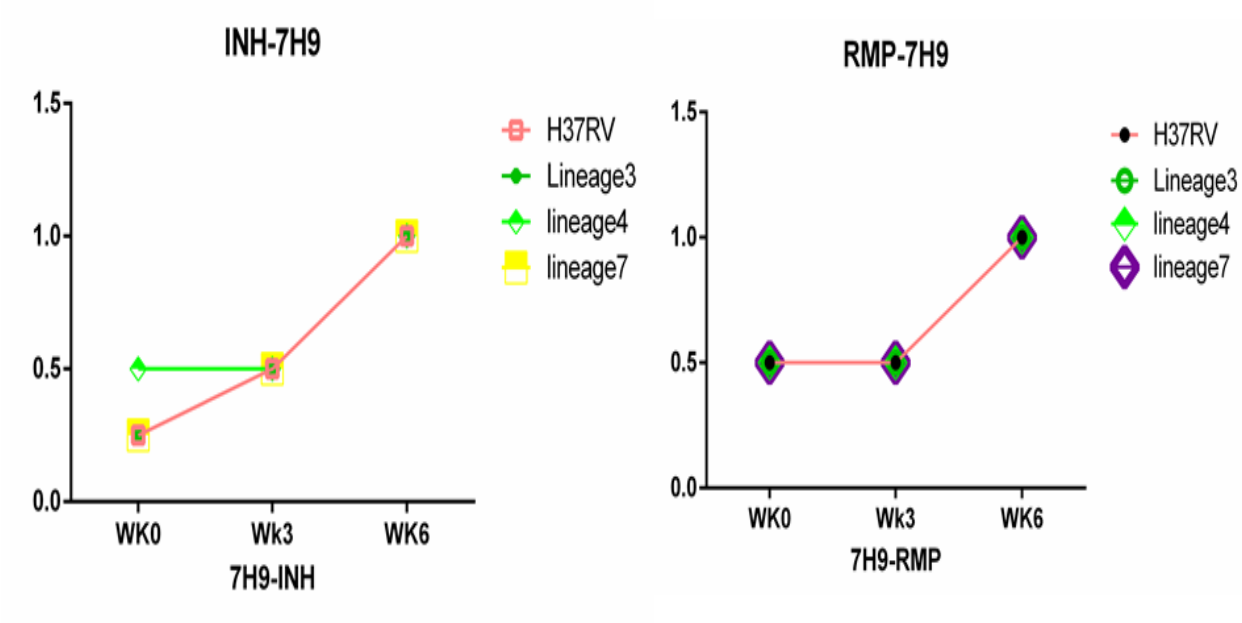

Figure 5: Representative graphs showing the change in the minimum inhibitory concentration at the 6th week for INH and RIF in 7H9 meduim.

MTB Lipid body accumulation and all the 4 drugs concentration which enabled to inhibit the growth of strain(H37Rv)both increased that means the accumulation of lipid bodies contribute bacterial drug tolerance.Viable bacilli cells of H37Rvstrain declined after week 6 which were inoculated in starvation(SDW &PBS) and enrichment (7H9) media models.A higher inhibitory drug concentration was required at the 6th week compared to the baseline and C50 (RIF =0.5;INH=0.1; STM=2.0 and for EMB=4.0), yet the proportion of lipid body containing bacilli increased continuously in all lineages.

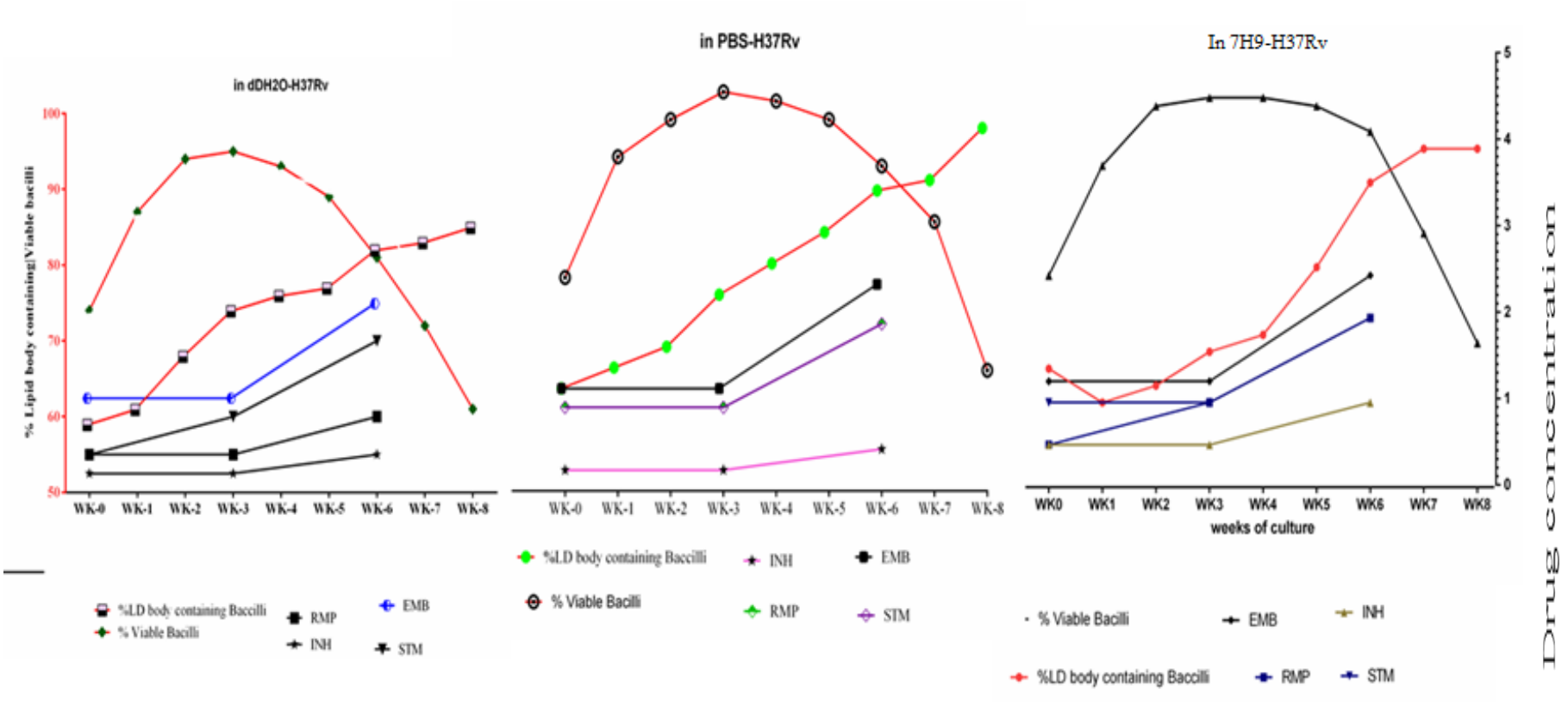

Figure 6: Representative graph showing that the higher drug concentration coincided with decline in the proportion of live bacilli.

7. Discussion

Mycobacterium tuberculosis is a major concern for the global community . It's a world leading killer and a serious global health problem primarily affecting poor people in many developing countries. WHO estimates about one-third of the world's population has latently infected with TB, it's due to the dormant nature of the bacterium (2).

In previousstudies researchers used multiple stress like oxygen depletion, increase carbondioxide, acidic PH and they have shown that the pathogen stores energy as triacylglycerol as it goes into dormancy-like state *in vitro* and uses this stored energy to survive during starvation. In our study four *mycobacterium tuberculosis* strains assessed for their Phenotypic drug tolerance related to lipid bodies accumulation based on nutrient starvationmodel.Thelipid bodies proportion and their drug tolerance continuously increased up to week 8 in all lineages(13).

In this study viability decreased and culture growth decreased gradually after week six, but lipid bodies proportion and their drug tolerance continuously increased up to week 8 in all lineages (10).

Acid-fast staining was performed by conventional Ziehl-Neelsen method to diagnose acid fastness. Due to the lack of mycolic acid biosynthesis under the nutritional starvation, MTB cells become non-replicative and they lose acid-fast staining property probably due to the shutdown of mycolic acid synthesis. Thus exhibit INH-tolerance at a higher frequency and at an earlier time point as compared to the development of Rifampicin tolerance (26).

The exponentially growing and nutrient starved *MTB* have unique capacity to synthesize and store lipid bodies and ability to catabolize stored lipid bodies as an energy source during starvation. Lipid bodies are accumulated in the cell wall of the bacteria and associated with slow growth and antibiotic tolerance. Lipid bodies in the form of lipid droplets provides energy via oxidation process (28).

The outcome when exposing mycobacteria to nutrient starvation, was that non replicating and logarithmically growing *MTB* differ in their lipid body accumulation and drug tolerance, due to the very different characteristics of the strains in nutrition starvation. This highlights the importance of considering the starved form of *MTB* in future vaccine and drug studies. (29)

Accumulation of lipid bodies and loss of acid-fastness in *MTB* cells under nutritional stress. Over laid images of the dual stained *MTB* are shown. Magnified view of three different *MTB* cells, representing three different subsets of *MTB* cells in terms of acid fastness and neutral lipid

staining property, were observed in the *MTB* population under nutritional-stress. Nutritional starvation conditions caused accumulation of lipid bodies' result in detectable tolerance against different concentrations at different time point, an important indicator of true dormancy (6).

Of the 3 strain isolates and 1 growth control, all 4(100%) were sensitive to all the four first line drugs instantly; at week 6, lineages showed that more tolerance than the previous weeks. The lineages got more tolerant to all first line drugs that were tested. The availability of a simple *in vitro* dormancy model would be an asset for TB drug discovery program and would complement those that exist already, such as the Wayne or starvation model (27).

Intracellular lipid bodies' accumulation suppresses *MTB* growth. Here expressed differences between lineages and their accumulation pattern, lending further support to our hypothesis that lipid bodies significantly increase with increasing drug tolerance (28).

In one study they tried to show possible connection between INH resistance phenotype and loss of acid-fastness owing to the absence of mycolic acid. However, development of drug tolerance has been assessed only in few in vitro dormancy models (23, 26).

Significant resistance was noted moderate levels of Rifampicin (5 mg/ml). In the widely used Wayne model of dormancy based on hypoxia, the maximum phenotypic rifampicin-resistance obtained against a very low concentration of rifampicin (0.1 mg/ml) was about 21%after 8 days and gradually decreased to 17% tolerance after 14 days and to 12% after 22 days (26).

In multiple stress model, by 9 days under stress 100% of the cells were resistant to 0.1 mg/ml of rifampicin. Under the multiple-stress condition the rifampicin resistance against a 50 times higher concentration of rifampicin (5 mg/ml) increased gradually to approximately 5% and 12% within 9 and 18 days respectively. In the nutrient starvation model about 60% tolerance against rifampicin (at 1 mg/ml) was reported after 42 days under the stress, but in our case more drug tolerant was observed on week six for all lineages in all medium (23).

We observed from Sudan black staining slides containing lipid inclusion bodies and shows tolerant to first line anti TB drug. There was progressive increase in proportion of lipid accumulated bacilli from the total of AFB, as detected by weekly reading from the slide under nutrition starvation. Loss of acid-fastness seems to be an important trait for non-replicating dormant cells from in vitro cultures. Other study shows that the linkage between lipid accumulation and drug resistance was strongly supported. Moreover, the higher percentage of acid-fast positive cells were noted in tgs1 mutant, with impaired TG accumulation ability under

multiple-stress, suggests links between loss of acid-fastness, lipid accumulation and development of phenotypic antibiotic resistance during the development of true dormancy (30).

We developed a novel *in vitro* nutritional starvation dormancy model for *MTB* by applying nutrient enrichment (7h9+OADC) and two starvation (SDW & PBS) models. Under this condition, *MTB* declined replicating, decreased acid-fastness, accumulated lipid bodies, and increased the *MIC* through time. This indicates phenotypic antibiotic tolerance. Lineages with ability to accumulate lipid bodies lesser, exhibited a lesser degree of antibiotic tolerance (30).

One study stated that phenotypic tolerance occurs when the environmental or physiological status of the bacteria change. For example, environmental factors such as low pH, depletion of certain nutrients, and high magnesium or calcium concentration induce phenotypic tolerance. The inhibition of growth occurs in the stationary phase and is the most common cause of reduced drug susceptibility in all bacteria (31).

Some approved drugs for tuberculosis, such as rifampicin and moxifloxacin, are genuinely active *in vitro* against non-replicating *MTB,* but at far higher concentrations and with far less reduction in bacterial numbers than under replicating conditions. The same is true also in our study, in which a higher inhibitory drug concentration was required at the 6^{th} week compared to the baseline and C50 (RMP=0.5;INH=0.1; STM=2.0 and for EMB=4.0); yet the proportion of lipid body containing bacilli increased continuously in all lineages (32).

8. Limitation and strength of Study

Limitation of the study

- Type of lipid bodies stored in the strains was not known or characterized.

Strength of the study

- It’s an experimental study and we go through different procedures based on nutritional starvation model we assessed lipid bodies, viability and drug tolerance of *Mycobacterium tuberculosis.*

9. Conclusions

- Sudan black staining method to films of the strains cultivated on 7H9, PBS and SDW media revealed that stainable fatty material in the form of cytoplasmic inclusions,
- *In vitro* nutrition starvation dormancy model efficiently generates *MTB* meeting all criteria of dormancy, and this method is adaptable to high-throughput screening for drugs that can kill dormant *MTB.*
- The link between lipid accumulation and development of phenotypic drug tolerance in *MTB* was established. The more lipid bodies' accumulated bacilli may be appropriate for novel drugs that can kill latent *MTB.*

10. Recommendations

- This *in vitro* dormancy model used for drug screening and viability of mycobacterium cells.
- Experimental *in vitro* nutritional starvation modelused for the assesement of phenotypic drug tolerance of tuberculosis is one approach to study further about*MTB,* &how it becomes MDR-TB.
- Further studies are required to understand how *MTB* develop drug resistance.
- MTT Assay must be performed only in level II BSC with proper bio-safety conditions mycobacteriological laboratories.

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12. Annexes

Annex 1: Media preparation procedures

I. Lowenstein-Jensen Glycerol/ pyruvate preparation

Lowenstein-Jensen Glycerol/ pyruvate medium is prepared with fresh egg and used for the isolation and differentiation of Mycobacterium species (spp).

Procedure

1. Dissolve 37.2 g of the LJ base medium in 600mL of purified water(DW) containing 12 mL of glycerol/pyruvate.

2. Heat with frequent agitation to completely dissolve the medium in water bath at 100^0C.

3. Autoclave at 121°C for 15 minutes.

4. Prepare 1000mL of a uniform suspension of fresh eggs under aseptic conditions. Avoid whipping air into suspension during the collection and mixing.

5. Add 2ml amphotericin B.0.075gm dissolved in 3ml distilled water

6. Add 0.036gm polymixin B dissolved in 2ml distilled water

7. Aseptically mix the 1000ml of egg suspension with 600mL of the sterile Lowenstein-Jensen Medium cooled to 50-60°C, avoiding air bubbles.

8. Dispense the finished medium into sterile screw cap test tubes. Place the tubes in a slanted position and heat at 85°C for 45 minutes.

II. Preparation of the Middle brooks 7H9-S broth

7H9-Supplemented (7H9-S) = 7H9 broth + 10% OADC+ 0.5 % glycerol + 0.1 % casitone

For 200 ml of 7H9-S media:

- Weigh 0.94 g of 7H9 powder and dissolve in 180 ml of distilled water mix until complete solubilization.
- Weigh 0.2 g of casitone and add to the previous solution until complete solubilization. Warm the solution if necessary.

- Autoclave the broth in a 250 ml flask.
- After autoclaving and cooling, add 20 ml of OADC (oleic acid dextrose catalase) enrichment and 1 ml of sterile glycerol. Mix well.
- Check in the incubator for sterility (leave one night in the incubator and check the day after if there is no turbidity and if the media is still transparent).
- Store the medium protected from direct light at 4°C.

III. Phosphate Buffered Solution

1. To prepare 1.5 L of phosphate buffer:

- Combine 7.1 g disodium phosphate (Na2HPO4), 6.8 g monopotassium phosphate (KH2PO4), and 1,500 ml distilled water in a 2 or 4 L flask.
- Stir with magnetic stirring bar on magnetic stirrer.
- Check pH, which should be 6.8. Adjust if necessary. Add disodium phosphate to raise pH
- Add monopotassium phosphate to lower pH.
- Using a smaller flask or beaker, dispense buffer into smaller volume containers for storage (ideally, 50 ml are dispensed into 60 ml sterile Nalgene tubes; alternatively, 100 - 200 ml sterile containers can be used).
- Label containers with the buffer name, date prepared, expiry date, batch lot number, and technician initials.
- Cap the tubes and sterilize in the autoclave.

Annex 2: Preparation of antibiotics

I. Preparation of antibiotics stock solutions

- Isonized (INH): Weigh 2 milligrams of isoniazid powder and dissolve in 2,0ml of distilled water, making a stock solution of 1 mg/ml.
- Filtration with a 0,22µm syringe filter; aliquot and store frozen (-20°C) until use.

- Rifampicin (RMP): Weigh 20 milligrams of Rifampicin powder, dissolve in 2,0 ml of absolute methanol, making a stock solution of 10 mg/ml. Sterilize by filtration with a 0,22 µm syringe filter; aliquot and store frozen (-20°C) until use.
- Ethambutol (EMB): Weigh 2 milligrams of ethambutol powder and dissolve in 2,0 ml of distilled water, making a stock solution of 1 mg/ml. Sterilize by
- Filtration with a 0,22µm syringe filter; aliquot and store frozen (-20°C) until use.
- Streptomycin (SM): Weigh 2 milligrams of streptomycin powder and dissolve in 2.0 ml of distilled water, making a stock solution of 1 mg/ml.
- Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

II. Preparation of antibiotic working solutions

Each antibiotic working solution is prepared at 4 times the final concentration to be tested in the plate.

- INH: add 10 µl of stock solution (1 mg/ml) to 2490 µl of 7H9-S broth to make a 4 µg/ml INH solution.
- RMP: make two dilutions! Add 250 µl of the stock solution (10 mg/ml) to 2250 µl of 7H9-S and from this concentration, add 20 µl to 2480 µl of 7H9-S broth to make a 8µg/ml RIFAMPCILINE solution.
- EMB: add 160 µl of the stock solution (1 mg/ml) to 2340 µl of 7H9-S broth to make a 64µg/ml EMB solution.
- SM: add 40 µl of the stock solution (1 mg/ml) to 2460 µl of 7H9-S broth to make a 16µg/ml SM solution.

Annex 3: Procedure for specimen collection and processing

I. Laboratory procedure for selection and culturing of isolates on LJ medium

- Randomly select all MTB Lineages and H37Rv strain from -80^{o}c at AHRI TB Lab.
- Inoculate these isolates on LJ media to get pure colony

- After 21-28days incubation check ZN, LB% and FDA.

II. Preparation of Inoculums from growth on solid medium

It is very important to have fresh growth on a solid medium (21-28 days old). Older cultures may result in unreliable susceptibility test results.

Prepare a bacterial suspension of 1mg/ml as follow:

McFarland 1.0 standard

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with the McFarland Standard. A McFarland Standard is a chemical solution of barium chloride (BaCl2) and sulfuric acid (H2SO4); the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration. McFarland Standards should be stored in standing position at 4°C to 8°C and Protect from light during 12 weeks.

Procedures.

- Prepare one enrichment media (7H9) and two starved media(PBS and DW)
- Take a full loop of bacterial growth with a sterile loop and put it in sterile tubes with five to ten glass beads just covering the bottom of the tubes with 3ml of 7H9-S broth, 3ml of PBS and 3ml of DW (try not to take any medium when removing growth).
- Vortex the tubes for at least 1 minute to break the clumps until a fairly turbid suspension are obtained.
- Transfer the supernatant to new sterile tubes and leave it to sediment for 15 minutes.
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard by adding corresponding medias
- To make 1:20 dilution for testing first-line drugs add 20ul of suspension to 3.8ml media (7H9-S broth PBS and DW).
- Add 3.8ml media(7H9,PBS,DW) to sterile tube and label W0-W6
- Add 20ul of suspension to all tubes corresponding to the medium from W0-W6

- Mix and take 100ul from 1:20 dilution for MTT assay weekly from all media but don't forget diluting of enrichment media after W0.
- Centrifuge left over of 1:20 dilution for all media weekly
- Make two smears from sediment to LB and FDA staining
- Stain the first slide primarily with Sudan black followed by ZN staining to see LB%
- Stain the second slide with FDA staining the see the viability of the bacteria.

III. Procedure for Zehel Nelson staining

1. Cover the smear with 1% carbolfuchsin for 5min.
2. Heat the slide to steaming with the flame from a Bunsen burner.
3. Wash off the stain with distilled water.
4. Flood slides with 3% acid-alcohol for 2min.
5. Wash off the acid-alcohol with distilled water and tilt the slides to drain.
7. Flood the slides with 1% methylene blue and let stand for 1-2 minute.
8. Wash off the methylene blue with distilled water.
9. Tilt the slides to drain.
10. Allow slides to air dry in the slide rack.

NB: Result interpretation was 1-9 bacilli/100 fields = AFB found **(exact number)**, 10-100/100 fields = AFB found **(+)**, 1-10/field = AFB found **(++)**, >10 AFB/field = AFB found **(+++)**.

IV. Sudan Back staining

Standard Procedure for Sudan Back staining to check lipid bodies

Sudan black staining has become an important tool to monitor lipid body accumulation suspected to contribute for phenotypic drug tolerance.

1. Label the slides and make a smear and let it to air dry
2. Place slides on staining rack and flood with Sudan black for 15min
3. Wash off the stain with distilled water

4. Cover the smear with carbolfuchsin for 5min.
5. Heat the slide to steaming with the flame from a Bunsen burner.
6. Wash off the stain with distilled water.
7. Flood slides with 3% acid-alcohol for 2min.
8. Wash off the acid-alcohol with distilled water and tilt the slides to drain.
9. Flood the slides with methylene blue and let stand for 1-2 minute.
10. Wash off the methylene blue with distilled water.
11. Tilt the slides to drain.
12. Allow slides to air dry in the slide rack.

Using a bright field microscope, Sudan black smears are examined with the 100X oil objective. The AFB appear bright red against the background material counterstained blue with central or terminal dot which what we call it LB. count LB containing bacilli per 100 bacilli and report LB%.

V. Fluorescein Diacetate (FDA) staining

Viability check staining procedure using Fluorescein Diacetate (FDA)

- Prepare 2 smears (not too thick) and let them dry (preferably in a BSC). Don't fix the smears with heat. Air dries the smears for at least 1 hour to avoid washing off during staining.
- Place a piece of filter paper on the bottom of the Petridish; humidify with sterile distilled water.
- Put the dried and not fixed smears in a Petridish on a support (i.e. match sticks).
- Cover the smears with the FDA stain, close the Petridish and keep them at 37°C for 30 minutes.
- Rinse carefully with a bit of water avoiding washing out the smears.
- Cover the smears with 0.5% acid alcohol, let act for 3 minutes and rinse carefully again with a bit of water.

- Cover the smears with 0.5% potassium permanganate (or if not available, with 0.3% methylene blue), let act for 1 minute and rinse carefully again with a bit of water.
- Cover the smears and bottom of the Petridish with 5% phenol solution for 10 minutes to kill the bacilli and then rinse carefully with water.
- Rinse smears carefully with water and remove them from the Petridish.
- Let the smears dry in the incubator and examine as soon as possible with a fluorescent microscope (200x magnification if well visible in your system (LED); with classical mercury vapour fluorescence microscopes a higher magnification of 1000x may be needed; fluoresce in/auraminefilterset). Look for green fluorescing rods of typical shape. The fluorescence of one field disappears after a minute or so, change fields fast enough. Next day little fluorescence will be left, but re staining is possible.

VI. Procedure for MTT assay

Preparation of the MTT plate for first-line drugs: INH-RMP-EMB-SM

In a 96-well flat-bottom microtiter plate there is enough space to test two isolates against the four drugs in six two-fold dilutions of each drug.

- ✓ Add 100 µl of 7H9broth, PBS or DW to columns 2-11from rows B to G
- ✓ Add 100 µl of the working solution of INH to well B2 and B6
- ✓ Add 100 µl of the working solution of RMP to well B3 and B7
- ✓ Add 100 µl of the working solution of EMB to well B4 and B8
- ✓ Add 100 µl of the working solution of SM to well B5 and B9
- ✓ With a multi-channel pipette make dilutions from rows B to G (columns 2-5 and 6-9) discarding the last 100 µl after mixing in row G.
- ✓ Add 100 µl of 7H9broth, PBS or DW to wells B10 and C10
- ✓ Add 100 µl of 7H9broth, PBS or DW to wells B11 and C11; these will represent the negative and sterility controls of the test.
- ✓ Add 200 µl of sterile distilled water to all the outer wells left without broth; these will prevent evaporation during incubation of the plate.

Inoculation of the plates

Using tips with filters inoculate the plates B2-B5 through G2-G5 and B10 for positive control with 100 µl of the sample 1 and B6-B9 through G6-G9 and C10 (dilution 1:20) to all wells included GC (+) well but not in the well containing the negative control.

Incubation of the plates

- After inoculation, seal the plates in plastic bags and incubate at 37 °C for 7 days.
- After 7 days incubation at 37^0C, add 10 ul of the MTT solution (5 mg/ml) to each well and the plate was reincubated overnight.
- If a violet precipitate (formazan) appeared in the MTT well, add 50ul of the SDS-DMF solution to these wells.
- Reincubated for 3 h.
- A change in color from yellow to violet indicated the growth of bacteria.
- The minimal inhibitory concentration (MIC) of each drug is interpreted as the lowest concentration of the antibiotic that prevents a change in color of the MTT.
- MIC values are scored for each isolate for comparison with the results obtained with the proportion method.
- Positive and negative controls: the positive control should show positive growth and the negative control should show no growth within the incubation protocol period. If negative control shows a growth, investigate procedures, could be a cross-manipulation and check for all reagents for possible source of contamination

Detection of contamination

The incidence of contamination varies from laboratory to laboratory depending of several factors. The recommendation is that up to 5% contamination rate is acceptable. Liquid media are more susceptible to contamination than solid media. It is extremely important to take care during the manipulation and to work with sterile material.

- Any well with a turbid appearance is suspected of contamination and result of this well is not valid.

- At the moment that you add the MTT if you observe directly a change of color the well could be contaminated and has to be discarded.

Precautions

- All work should be carried out in a proper biological safety cabinet (class II).
- All materials should be sterilized by autoclaving prior to disposal.
- Plate should be kept in the incubator together in a box to avoid that someone moved the plate by accident.
- Plate should be closed in both sides by a small tape for security.

VII. Quality control (QC)

It is important to perform a quality control of drug susceptible to first line drugs. Add H37Rv strain as a QC strain which is susceptible to all anti-tuberculosis drugs. If the susceptible H37Rv shows some resistance, then all the results obtained within during the experiment become invalid and the test should be repeated.

Two diagrams of the plate are shown below as an example.96-well plate for first-line drugs.

A											
B	I 1µg/ml	R 2 µg/ml	E 16µg/ml	S 4µg/ml	I 1 µg/ml	R 2 µg/ml	E 16µg/ml	S 4µg/ml	GC(+) A	GC(-) B	
C	0.5	1	8	2	0.5	1	8	2	GC(+)	GC(-)	
D	0.25	0.5	4	1	0.25	0.5	4	1			
E	0.125	0.25	2	0.5	0.125	0.25	2	0.5			
F	0.0625	0.0125	1	0.25	0.0625	0.0125	1	0.25			
G	0.0312	0.0625	0.5	0.125	0.0312	0.0625	0.5	0.125			
H	2	3	4	5	6	7	8	9	10	11	12

MTT microtiter assay, ELISA 96 wells plate 12x8.

MTT assay reagents we need for this project

For a single sample we need 260µl MTT and 1300µl detergent per sample

Totally we have 150 sample so 260µl* 150sample = 39000µl (39ml) MTT Reagent

And 1300µl*150sample = 195000µl (195ml) detergent

1. Preparation and inoculation of plate

- Serial dilutions of the drug are prepared directly in a sterile 96-well flat bottom microtiter plate using a volume of 100 µl of 7H9-S medium.
- Add 100 µl of the inoculums McFarland 1.0 dilution 1/20 of sample 1 or sample 2 in the corresponding well.
- Incubate at 37°C for 7 days

2. Addition of the indicator after 7 days

- After 7 days of incubation add in each well 10ul MTT solution of 5mg/ml.

- Re-incubation overnight for color development
- If a violet precipitate (formazan) appeared in the MTT well, 50µl of the SDS-DMF solution will added to these wells and the plate was re-incubated for 3 h.

3. Reading the plate after 24-48 hours

- A change in color from yellow to violet indicated the growth of bacteria
- The MIC is defined as the lowest drug concentration that prevented this color change.

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

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Signature: _____

Date of submission: 20/04/2018

This thesis has been submitted with our approval as advisors.

Advisor: **KASSU DESTA (PhD, Candidate)**

Signature: _____

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Place: Addis Ababa, Ethiopia.

Advisor: **ENDALAMAW GADISA (PhD, POST DOC.)**

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Place: Addis Ababa, Ethiopia.