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Development of *Mycobacterium tuberculosis* ribosomal RNA as a drug target

BY

DAVID K. KIULUKU

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Signature; …… Date; ……

David K. Kiuluku

This thesis has been submitted for examination with our approval as university supervisors:

Prof. Francis Mulaa

Department of Biochemistry

University of Nairobi

Signature; ……. Date; ……

Dr. Betty Mbatia

Department of Biochemistry and Biotechnology

United State International University (USIU)

Signature; ……. Date; ……

DEDICATION

To my family and my wife, Julliet, I affectionately dedicate this work to you. Your support has been a never ending spring of hope, your belief in me is a constant source of strength.

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Interdependence has a higher value than independence; this is what the pursuit for my masters has taught me in depth. The research community is like a bee hive, each member great and small working together each playing his/her role for the benefit of all concerned.

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LIST OF ABBREVIATIONS

ACP	Acyl Carrier Protein
AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine triphosphate
ART	Anti- Retroviral Treatment
BCG	Bacillus Calmette-Guérin
Boc	t-Butoxycarbonyl
CDC	Centre for Disease Control
Cs	Cycloserine
CTAB	N-cetyl-N-, N, N-trimethylammoniumbromide
DEPC	diethyl pyrocarbonate
DMPK	Drug Metabolism Pharmacokinetics
DNA	Deoioxyribonucleic Acid
DOTs	Direct observed Treatment-Shortcourse
EDTA	Ethylenediamin tetraacetic acid
EMB	Ethambutol
Fc	Fragment Crystallization
FQ	Fluoroquinoline
GATDD	Global Alliance for Tuberculosis Drug Development
GMP	guanosine monophosphate
HDCR	Huisgen dipolar cycloaddition reaction
HIV	Human ImmunodeficiencyVirus
INF	Interferon

IRIS	Immune reconstitution inflammatory syndrome
Kat G	Catalase per oxidase
MDR	Multidrug resistant
MIC	Minimal Inhibitory Concentration
MTb	Mycotuberculosis
NaCl	Sodium chloride
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NOI	Reactive Oxygen Intermediate
OI	Opportunistic Infection
PAS	Para-aminosalicylic acid
PCR	Polymerase Chain Reaction-Restriction Fragment Length olymorphism
pH	Potential of Hydrogen
PZA	Pyrazinamide
Rif	Rifampicin
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediate
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SAR	structure-activity relationship
SD	System Diagnostics

SDS	Soduim Dodecyl Sulphate
SM	Streptomycine
TB	Tuberculosis
TBTA	tris(benzyltriazolylmethyl)amine
TE	Tris-EDTA
TLR	Toll like Receptor
TRC	Tuberculosis Research Centre
WHF	World Health Forum
WHO	World Health Organization
XDR	Extreme Drug resistant

ABSTRACT

Ribonucleic Acid (RNA) is an important drug or biochemical probe target. (i) For every protein, a drug target is coded for by an RNA that could equally be targeted; (ii) many pathogenic organisms including viruses have RNA genomes that could be potentially targeted. (iii) it is now well established that many new generation antibiotics are targeted at ribosomes which are effective drug targets to treat bacterial infections. Despite the critical role that RNA plays in biology and thus as a potential therapeutic and chemical biology target, only a small number of RNA drug targets have been developed and exploited. The objective of this study was thus to develop rational methods to exploit *Mycobacterium tuberculosis* RNA as a drug target. To accomplish this objective, we developed a method to isolate and label *Mycobacteria tuberculosis* ribosomal Ribonucleic Acid (rRNA) transcripts, as well as a study design for identifying all small molecules, or drugs, that bind the RNA with high affinity and specificity developed. This study has enabled the development of an rRNA probe that can be used for screening potential small molecules that target Tuberculosis (TB) rRNA. The long term goal is to develop a RNA motif-ligand database that can be mined against RNA sequences to rationally design small molecules that specifically target RNAs. This study showed that it is possible to target antibiotics to certain RNA structures. This was evident from the colour development that showed that there are sites on RNA that participate in specific interactions with antibiotics.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. BACKGROUND

RNA is an interesting target for development of therapeutics or chemical probes of biological function. This is however limited by the understanding of how to target RNA with small molecules and natural products. RNA forms complex tertiary structures that impart diverse functions (Batey *et al.,* 1999). For example, RNA catalyses reaction regulates gene expression and encodes proteins and plays other essential biochemical roles. This makes RNA a very interesting and important target for screening and development of drugs. It is however a very underutilized target, mainly because of the limited information available on RNA-ligand interactions that could facilitate rational design.

One advantage of using TB RNA as a drug target is that the secondary structure information, which includes the motifs that comprise an RNA, can easily be obtained from sequence information by free energy minimization or phylogenetic comparison. RNA tertiary structures are composites of the secondary structural motifs and the weak bond that form between them. More importantly, RNA motifs are known to have similar properties both as isolated systems and as part of larger RNAs. A good example, are the aminoglycoside antibiotics that are known to affect the structure of the bacterial rRNA A-site similarly when they bind the entire ribosome or oligonucleotides they mimic the bacterial rRNA A-site (Aminova *et al.,* 2008). Studies on the binding of aminoglycoside and strepmine dimers to RNA loops and hairpins have facilitated the development of the new generation of compounds to combact multidrug resistance (Vaiana *et al.,* 2006). Studies show that the identification of RNA motifs that bind small molecules can be useful for targeting the whole RNAs from which they are derived (Guan & Disney 2012).

However, since RNAs can adopt diverse structures, internal and hairpin loops, an understanding of how to develop and targets RNA with small molecules and other ligands like natural products has been elusive. Current methods used to study and identify RNA ligand interactions include systematic evolutions of ligands by experimental enrichment ("SELEX") (Disney, 2005), structure-activity relationship (",SAR") chemical microarrays (Barrett *et al.,* 2006) and homology modeling(Mwangi *et al.,* 2013; Gitonga *et al*, 2013). Current antibiotics, has led to the need of drugs that target new areas of the pathogenic bacteria. One of the viable targets is the ribosomal RNA (Shasmal and Sengupta, 2012). This follows the role played by the ribosomal RNA in the coding for the ribosomal protein and also the role the RNA plays in the initiation of the mutations against antibiotics, e.g. in the case of mycobacteria resistance to streptomycin (Meier *et al.,* 1994).

Emergence of TB strains that are resistant to and the viomycine, has led to shift of interest from protein target to understanding and coming up with drugs that target the RNAs (Akbergenov *et al.,* 2011*)*. The introduction of TB chemotherapy in the 1950s, along with the widespread use of Bacillus Calmette-Guérin (BCG) vaccine, had a great impact in reduction of TB incidence. However, despite these advances, TB still remains a leading infectious disease worldwide, especially in the third world countries (Peabody *et al.,* 2006).

M. tuberculosis is a particularly successful pathogen that latently infects about two billion people, accounting for about one third of world population (WHO, 2012). Each year, there are about eight million new TB cases and two million deaths worldwide. TB has been on the increase largely owing to HIV infection, immigration, increased trade, and globalization (WHO, 2003). The increasing emergence of drug-resistant TB, especially multidrug-resistant TB (MDR-

TB, resistant to at least two frontline drugs such as isoniazid and rifampin), is particularly alarming. MDR-TB has already caused several fatal outbreaks (CDC, 1993) and poses a significant threat to the treatment and control of the disease in some parts of the world, where the incidence of MDR-TB can be as high as 14 % (Ryan, 1993). The standard TB therapy is ineffective in controlling MDR-TB in high MDR-TB incidence areas (Kimerling et al., 1999 ;De Cock & Chaisson 1999). Fifty million people have already been infected with drug-resistant TB (Ryan, 1993). There is much concern that the TB situation may become even worse with the spread of HIV worldwide, a virus that weakens the host immune system and allows latent TB to reactivate and makes the person more susceptible to re-infection with either drug-susceptible or drug-resistant strains (Zhang 2005). The lethal combination of drug-resistant TB and HIV infection is a growing problem that presents serious challenges for effective TB control. In view of this situation, the World Health Organization (WHO) in 1993 declared TB a global emergency (WHF, 1993).

No new TB drugs have been developed in about 30 years. There is therefore an urgent need to develop new TB drugs (Zumla *et al.,* 2013). Although TB can be cured with the current therapy, the six months needed to treat the disease is too long, and the treatment often has significant toxicity. These factors make patient compliance to therapy very difficult, and this noncompliance frequently selects for drug-resistant TB bacteria (Zhang 2005). The current TB problem clearly demonstrates the need for a re-evaluation of our knowledge of the current TB drugs and chemotherapy and the need for new and better drugs that are not only active against drug-resistant TB but also, more importantly, shorten the dosage period (Lienhardt *et al.,* 2010). Direct observed treatment shortcourse (DOTS) are currently the best TB therapy; they have a

cure rate of up to 95 % and are recommended by the WHO for treating every TB patient (Ryan, 1993). However, DOTS alone may not work in areas where there is high incidence of MDR-TB, where its cure rate is as low as 50 % (Kimerling et al., 1999; De Cock & Chaisson 1999). In such situations, WHO recommends the use of DOTS-Plus, which is DOTS plus second-line TB drugs for the treatment of MDR-TB and TB (Ryan, 1999). However, treatment of MDR-TB with DOTS-Plus takes up to 24 months and is not only costly but also has significant toxicity (Zhang 2005).

In this study we proposed RNA as a possible drug target. RNA is an important target for developing therapeutics and chemical genetics probes because of the important roles it plays in many biological processes. RNA is the central figure in protein synthesis in which the controlled action of ribosomal, transfer, and messenger RNAs creates all cellular protein. RNA also catalyzes chemical reactions in biological systems. In all of these cases, the interplay of RNA secondary and tertiary structure elements is responsible for the RNA's biological activity (Doudna 2000). Though most drug discovery efforts are directed towards proteins, RNA is a validated and important drug target that remains largely unexploited. In the vast majority of cases, small molecule RNA inhibitors were identified. Although these screening efforts have been valuable for finding small molecules that modulate the activity of RNA, they fail to identify the motifs that small molecules prefer and therefore do not provide enough information to enable the rational design of potent and specific compounds. The screening efforts have to be able to show RNA–small molecule binding partners to aid the rational design of compounds that target RNA. In the current study a selection-based approach that requires only a single round of selection to identify the RNA secondary structure elements was developed. The aim of this study

was to screen for *Mycobacteria tuberculosis* RNA motifs that interact with kanamycin A and streptomycin antibiotics. The reason for using kanamycin A is because they are potent inhibitors of protein synthesis and RNA splicing in vitro and in vivo. As the first class of compounds known to bind specifically to subdomain of larger RNA sequences, they are useful for understanding the design principles required to produce new classes of therapeutics to bind RNAs. These aminoglycosides make hydrogen binding, electrostatic and hydrophobic contacts that contribute to their high binding affinity for the RNA target (Richard, *et al*., 1999). Additionally, this ligand has been immobilized onto resin and used for selection experiments. Streptomycin can be used to expand the knowledge on ligand–RNA interactions and also for comparison

1.2. LITERATURE REVIEW

1.2.1. Morphological Characterization of Mycobacterium tuberculosis

Mycobacterium tuberculosis is an acid fast bacteria, which can form acid-stable complexes when certain structures of arylmethane dyes are added (Uhia *et al*., 2011). All species of mycobacteria have ropelike peptidoglycan that are arranged in such a way to give them properties of an acid fast bacteria.

1.2.2. Genome structure

Mycobacterium tuberculosis is composed of about 4,200,000 nucleotides long circular chromosomes with a G+C content is about 65 %. (Ouellet *et al*., 2011). The genome of *M. tuberculosis* was studied generally using the strain *M. tuberculosis H37Rv* genome which contains about 4000 genes. Genes coding for lipid metabolism constitute 8 % of the genome (Mohn *et al*., 2008).*Mycobacteria tuberculosis* complex studies have shown that different species have 95-100% DNA relatedness based on DNA homology studies. The 16S rRNA gene is exactly the same for all the species. Different school of thought suggest that they should be grouped as a single species while others argue that they should be grouped as varieties or subspecies of *M. tuberculosis* (Brzostek *et al*., 2009).

1.2.3 Challenges to Mycobacterium tuberculosis to current tuberculosis drugs

The currently prevailing scenarios of drug-resistant tuberculosis (TB) are particularly alarming, and pose a significant threat to the control of the disease globally (Zignol *et al.,* 2006). In a recent report on the Anti-tuberculosis Drug Resistance Surveillance Global Project published by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (The Union), the proportion of MDR, denoting resistance to at least rifampicin (RIF) and isoniazid (INH), in new cases ranged from 0 % to 22.3 % (WHO, 2008). The highest

proportion of MDR-TB reported was 60 % among previously treated cases. It has been estimated that 489 139 cases of MDR-TB emerged in 2006, and the global proportion of such resistance among all cases amounted to 4.8 %. The proportion of extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional bacillary resistance to fluoroquinolones (FQs) and at least one second-line injectable drug, among MDR-TB cases in different settings, ranged from 0 % to 30 % globally. Around 40 000 cases of XDR-TB are estimated to emerge globally each year. Understanding the mechanisms of mycobacterial resistance to the anti-tuberculosis drugs not only enables the development of more rapid molecular diagnostic tests and furnishes implications for designing new anti-tuberculosis drugs, it also helps to implement measures to prevent the development of such resistance (Zhang, 2005). Rapid diagnosis of MDR-TB has been reviewed recently (Ling *et al.,* 2008; Chang, *et al.,* 2009).The current TB drugs can be divided into two categories: bacteriostatic and bactericidal drugs. The bacteriostatic drugs include ethambutol (EMB) and para-aminosalicyclic (PAS), whereas the bactericidal drugs include isonicotininyl hydrazide (INH), rifampicin (RIF), streptomycin (SM), and fluoroquinolones (FQ). However, the distinction between static and cidal drugs is only relative, because some static drugs can be cidal under certain conditions e.g with a higher drug concentrations/smaller inoculum, or change in bacterial physiological status). For example, pyrazinamide (PZA) can show cidal activity against small numbers of non-growing bacilli at acid pH but primarily shows static activity for growing bacilli with active metabolism (Changsen et al., 2003). Cidal drugs exhibit higher activity over static drugs in reducing the number of bacilli in the lesions. The current TB drugs can also be categorized as either first-line drugs or second-line drugs. The first-line drugs include INH, RIF, PZA, EMB, and SM; the second-line

drugs include kanamycin, amikacin, capreomycin, cycloserine (CS), PAS,ETH/PTH, thiacetazone, and FQ (Ahmad & Mokaddas 2009).

1.2.4 TB-HIV co-infection challenges

Human Immunodeficiency Virus (HIV) and Mycobacterium tuberculosis (TB) co-infection cases have been prevalent from when Acquired Immune Deficiency Syndrome (AIDS) was first described in Zambia , Haiti , and New York City (Masur *et al.,* 1982). The co-infection (HIV and TB) have been increasing despite vast amount of progress in both anti-HIV and anti-TB therapy with an estimate ranging as high as 30% both in the United States and particularly in developing countries (Awoyemi *et al.,* 2002). HIV sero positivity has been found to be a significant risk factor beyond simple co-incidence in the reactivation of latent tuberculosis (Selwyn *et al.,* 1989). The reactivation of latent TB has been elucidated by CD^{4+} T cells been activated by TB infection which in turn recruit gamma (γ) and produce pro-inflammatory cytokines such as IFN which activate macrophages that are mandatory for the formation of granulomas to walls of infection sites (Orme *et al*., 1993).

Subsequent studies have found that anti-tuberculosis T cells are rapidly depleted following acute HIV infection (Geldmacher *et al.,* 2010). On the hand , pulmonary tuberculosis infection has been found to accelerate HIV viral replication within lung segments (Nakata, K., *et al*., 1997). Immunological failure and poor CD^{4+} T cell count recovery observed in patients diagnosed with incident tuberculosis during anti-retroviral therapy (ART) may be atrributed to this phenomena (Hermans *et al.,* 2010). 80% incidence reduction in TB is achieved with the initiation of ART which is critical in preventing treatment failure in the absence of anti-TB therapy (Miranda *et al.,* 2007). There are concerns with regards to co-intiation of anti-TB and anti-retroviral therapy.

Among the concerns is that of immune reconstitution inflammatory syndrome (IRIS) (CDC, 2002). IRIS is thought to be the product of ART-induced rapid recovery of antigen-experienced CD^{4+} T cells, which initiate a massive, Th1-predominant inflammatory response to a disseminated yet previously clinically silent opportunistic infection (OI) (Sharma *et a.,* 2005).

1.2.5 Validation of small molecules-RNA interaction

Dervan and co-workers developed an elegant paradigm of targeting DNA with small molecules that exhibit exquisite sequence specificity (Nakatani *et al.,* 2006). A "modular node" has been developed drawing inspiration from DNA-binding small molecule of natural products, wherein the unique pairing of pyrrole, imidazole, and hydroxypyrrole rings are used to selectively recognize all combinations of Watson-Crick base pairs. Sequence-specific polyamides inhibit or induce transcription of specific genes in vitro and in cell culture by targeting 6-16 DNA base pair segments (with binding affinities typically in the low- to subnanomolar range) (Yu *et al.,* 2005). The ability of drugs to bind on RNA has been demonstrated by various classes of antibiotic which bind to the defined regions of the prokaryotic ribosome. Aminoglycoside such as macrolide,tetracycline, and oxazolidinone antibiotics exert their antibacterial effects by binding to ribosomal RNA(rRNA). Ribosomes unlike other forms of RNA have a catalytic component and have abundant form of cellular RNA.

The ribosome, unlike other forms of RNA, represents a catalytically competent and abundant form of cellular RNA. The "holy grail" in small molecule-RNA binding would be the selective targeting of a single cellular RNA (such as an mRNA transcript, a regulatory RNA, etc.), resulting in functional perturbation of a specific cellular process. RNA ligand binding sites typically consist of a single type of secondary structure whether small molecules or protein

which give greater weight to secondary structure (Lang *et al.,* 2009). Secondary RNA structure thus becomes the key determinant in defining the ability of a drug to bind to a particular RNA ligand.

1.2.6 RNA targeting

Given the increased understanding of potential roles of RNAs in the maintenance of normal health and in contribution to disease, directly targeting RNAs is an increasingly compelling therapeutic strategy. Since its inception, the modern pharmaceutical industry has focused almost exclusively on proteins as drug targets, either by design or happen stance (Yu *et al.,* 2013). Rationally designing or identifying traditional small-molecule drugs that selectively interact with nucleic acids, carbohydrates, or lipids in a safe and efficacious manner has been challenging. There are a small number of cytotoxic chemotherapeutic agents that primarily interact with DNA, and aminoglycoside and macrolide antibiotics that bind to bacterial ribosomal RNAs. However, these examples are the exception rather than the rule, and additional therapeutic platforms are needed to expand the list of druggable macromolecules. Antisense oligonucleotides represent a promising drug platform that has the potential to target, in a selective manner, all RNAs in a cell, thus dramatically expanding the druggable universe and in the process producing medicines that will have a major impact on patients' lives (Silver 2011).

Drug discovery efforts have historically focused on the search for compounds that modulate the protein products of genes. The vast majority of drugs available today either act at the protein level, or the drugs themselves are proteins. These compounds are usually agonists or antagonists of receptors, or they inhibit or stimulate enzymes or protein–protein interactions. However, the interest in specifically targeting RNA is increasing, both for target validation and/or therapeutic purposes, not least with the introduction of RNAi a few years ago. In addition, there are many

ongoing efforts aimed at targeting mRNA with small molecules, antisense oligonucleotides, ribozymes or aptamers. When it comes to nucleotide RNA (ncRNA) as a potential new drug target category, with no protein being produced there is obviously no alternative but to attempt to find ways to affect the RNA transcript itself. Current drug therapy targets only a few hundred endogenous targets, mainly proteins, such as receptors and enzymes (Bernstein *et al.,* 1952).Genomics and transcriptomics efforts have identified many RNA novel candidate drug targets that need to be validated.

1.2.7 Cu-Catalyzed Azide- Alkyne Cycloaddition (CuAAC)

Bioorthogonal click chemistry is a two-step reaction and needs a pair of functional groups. First there is incorporation of biorthogonal functional moiety (chemical reporter) of a compound into a substrate. Second the reporter is covalently linked to an exogenous probe through a click reaction, which allows for detection and isolation of the target. It is important that the covalent reaction between the two compounds proceed rapidily and selectively in a physiological environment with biocompatible pH (6–8), and temperature (37 °C), which have less by products and be nontoxic. The chemical reporter should be inert *in vivo* and small enough to modify the target substrate without any functional and spatial interference. This has led to development of a few bioorthologonal click reagents for biological use (Prescher & Bertozzi 2005). Demko & Sharpless 2002 coined the widely well known example of click chemistry the copper-catalyzed azide-alkyne cycloaddition (CuAAC) which was described as a better version of Huisgen's [2 + 3] cycloaddition with little solvent dependence and better adherence of click chemistry principles (Kolb *et al.,* 2001). Dramatic acceleration of the cycloaddition reaction was significantly changed by the presence of copper which changed the reaction mechanism and improved the

yields. It can be performed below room tempetature and has been widely used to make covalent connections between substrates tagged with functional groups such as azides.

Figure 2:A reaction of an azide R and an alkyne R acting as a handle to immobilize a ligand catalyzed by cu^{+} adopted from work of Kolb *et al.*, 2001.

1.2.8 The antibiotic platform

The biggest challenge to immobilization is the strong dependency on the linker length and it concentration (Song *et al.,* 2009). Wong and co-workers explored the use of Cu(I)-catalyzed azide-alkyne cyclooaddition to attach small molecules on micro-titer plates (Fazio *et al.,* 2002). First, there is an alkynylated hydrocarbon chain is adsorbed on the slide by hydrophobic interactions; then azido compounds are combined through CuAAC. The azide provides a way to immobilize ligands on plates either by azide-alkyne click chemistry or Staudinger ligation, and enables to analyze ligand-rRNA interactions, and to indentify novel RNA-biding substrates, to promote more in-depth RNomics research.

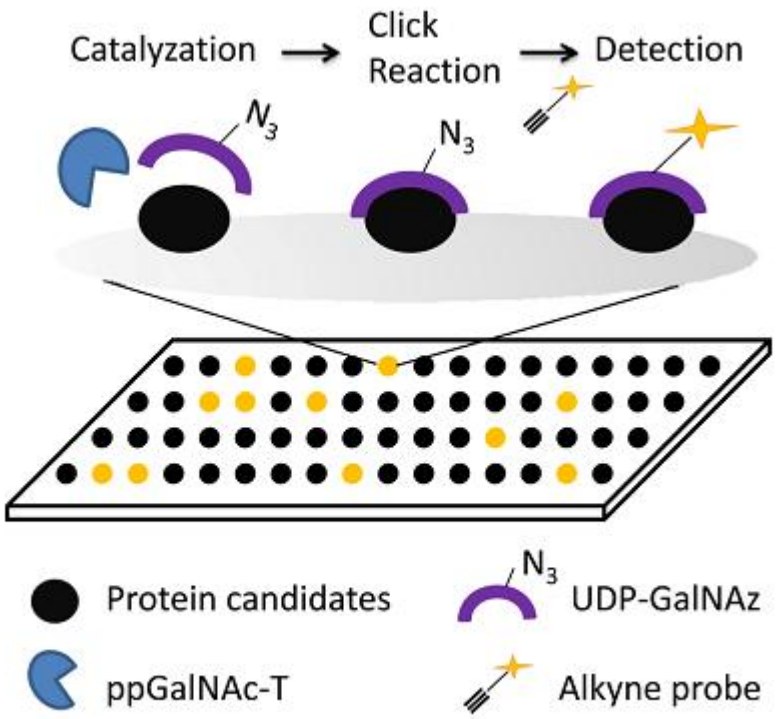


Figure 3: ppGalNAc-T substrates profiling by click chemistry

Glycan enzymes catalyzed the connection of azide labeled donors to substrates. Then protein complex reacted with alkyne labeled fluorescence probe by click reaction. Substrate proteins can be detected ultimately by fluorescence scanning.

1.3 Statement of research problem

Development of anti-*tuberclosis* drugs that target rRNA and are not susceptible to the current resistance mechanisms and less susceptible to the development of resistance is novel and has not been attempted previously. Though most drug discovery efforts are directed towards proteins, RNA is a validated and important drug target that remains largely unexploited therefore it is novel for development of anti-tubercular drugs

1.4 Justification

Whole cell screens are useful for TB drug development; however we must recognize the potential problem of developing drugs active against growing tubercle bacilli: However this is only limited to the rapidly replicating cells. To circumvent this problem therefore we need a target based approach where the drug of interest is directed to specific molecules. Target-based antibacterial drug discovery approach has enormous advantages. These include predicting the phenotype, selectivity and sensitivity which makes the approach superior to whole cell screening. This will represent a paradigm shift from previous approaches, which focused on just finding another drug. With recent development in genetic tools and TB genomics, high throughput screening, new technology of combinatorial chemistry provide an exciting opportunity to discover new magic bullets that kill persisters and shorten the current TB treatment.

1.5 General objective

Development of *M. tuberculosis* mRNA screening assay platform, as a possible drug target by immobilization of the ligands to a functionalized azide strips.

1.5.1. Specific objectives

- Isolation of pure Mtb colonies from sputum of TB infected patient.
- Development of a mRNA screening assay platform.
- Screening of RNA target against selected antibiotics.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

The reagents, chemicals and culture media used in this study were of analytical grade obtained from: Sigma (st Louis, MO, USA), Difco laboratories (MI, USA), Fisher scientific (Leicestershire, UK), Applied Biosystems (New Jersey, USA) and OXOID (Hampshire, UK). The kits were obtained from Roche (Mannheim, Germany) and GE Healthcare (Buckinghamshire, UK).

2.1.2. Oligonucleotides

The oligonucleotides used for PCR were obtained from IDT (Leuven, Belgium). They include: 16s F27 forward primer, 20-mer; 5'…*AGA GTT TGA TC(AC) TGG CTC AG*…3', 16s R 1492 reverse primer, 22-mer; 5'…*TAC GG(CT) TAC CTT GTT ACG ACT T*…3'. The oligonucleotides used for insert confirmation were obtained from IDT (leuven, Belgium). They include: SP_6, 18-mers; 5'…*ATT TAG GTG ACA CTA TAG*…3', T7, 23- mers; 5'…*TAA TAC GAC TCA CTA CTA TAGGG*…3'.

2.2 Isolation of pure *Mycobacteria tuberculosis* colonies from sputum as a specimen

2.2.1 Collection of specimen (sputum) from tuberculosis patients

The sputum was collected as described in the WHO report (2007). Morning specimens of 3-5 ml of sputum containing purulent material were expectorated into universal bottles by the TB patients. These bottles were well labeled with the information of the Health Centre and the patients details. The bottles were closed securely and sent to the Central Tuberculosis Reference Laboratory for analysis.

2.2.2. Processing of specimen by NaOH-NaCl method

The samples were processed using a modified protocol of Petroff (1915) routinely applied to sputum prior to culture. Each sputum sample was transferred to 50 ml centrifuge tube with a screw cap and was treated with equal volume of 4 % NaOH and 2.9 % NaCl. The sample was left to stand for 20 minutes at room temperature with periodic vortexing. This was followed by addition of equal volume of neutralization buffer (140 g/L potassium phosphate monobasic and 8 ml 1 % phenol red) at a pH of 6.8. The mixture was then centrifuged at 3000 Xg for 30 minutes. The supernatant was poured off and the pellet resuspended in 2 ml phosphate buffer (pH 6.8). The resuspended pellet was used for Zieln-Neelsen (ZN) staining, Lowenstein- Jensen (LJ) solid culture and for inoculation of Mycobacteria Growth Indicator Tube (MGIT).

2.2.3. Zieln-Neelsen (ZN) staining

Pre-cleaned glass slides were used. A portion of resuspended pellet above was transferred using a microbial loop was used to apply the resuspended pellet to the slide by slowly spreading the liquid to make a thin film; the size of the smear was 1 cm by 1.5 cm in size. The smear was allowed to dry completely; care was taken not to create an aerosol by working under the safety cabinet. The smear was fixed at 80 ˚ C for 15 minutes. The slides were then placed in a suitable staining device. Carbolfuchsin stain was used to saturate the slide; the underside of the slide was heated carefully by passing a flame. Tap water was used gently to wash the slide until no colour appeared in the effluent. Methyl blue was flooded to the slide and two minutes allowed for complete counter staining and finally washed with tap water. The smear was air dried, immersed in oil and examined under a microscope at x 1000 magnification.

2.3 Growth media

2.3.1 Mycobateria Growth Indicator Tube (MGIT)

The MGIT was obtained commercially containing 7.0 ml of modified Middlebrook 7H9 broth base. The media composition was modified by adding nalixidic antibiotic 0.05µml. There is a fluorescent sensor embedded in silicone on the bottom of the tube. The tube is flushed with 10 % CO_2. The processed cells (section2.2.2) were inoculated into the MGIT tubes. The growth was detected by bactec 960 machine used as an indication of *M. tuberculosis*.

2.3.2. Lowenstein-Jensen solid culture

The medium was prepared by dissolving 37.3 g of formula in 600 ml of distilled water as its composition appears in appendix 1. In addition, 12 ml glycerol was added. The mixture was mixed well and heated with frequent agitation for complete dissolution. The medium was dispensed into appropriate containers and sterilized in autoclave at 121 ^{0}C for 15 minutes. It was cooled to 46 ^{0}C. Meanwhile, one litre of whole eggs was prepared aseptically. It was swirled to mix without introducing air bubbles. The egg fluid was added slowly to the base to obtain a homogeneous mixture without bubbles. It was distributed into screw-capped tubes (universal bottles). The tubes were placed in a slanted position and inspissated at 90 ^{0}C for 45 minutes to coagulate the medium. The prepared media was stored at 4 ^{0}C. The processed cells (section 2.2.2) were inoculated onto the medium and incubated in controlled room at 37 ^{o}C up to 8 weeks.

2.4 Development of a mRNA screening assay platform

2.4.1 Genomic DNA extraction

Total bacterial genomic DNA of of the isolates was extracted using the procedure described by Kate Wilson (1987). In each case, 5 ml of an overnight culture was subjected to centrifugation (Eppendorf centriguge) at 10,000 rpm for 5 minutes and the pellet resuspended in 547µl of TE

pH8. Into this, 233µl of cell lysis buffer containing, lysozyme, sodium dodecyl sulphate (SDS), cetyltrimethyl ammonium bromide (CTAB) and proteinase K was added and the mixture incubated for 1 hour at 37 ºC. DNA was then extracted sequentially with Phenol/Chloroform/Isoamyl alcohol (25:24:1) and Chloroform/Isoamyl alcohol (24:1). The DNA was precipitated with 0.6 volumes isopropanol alcohol and the pellet resuspended in 50 µl of TE buffer (pH 8) containing RNase to get rid of RNA. DNA was quantified using a nanodrop 1000 (Thermo Scientific, USA) and its quality checked on a 1 % agarose gel before storage at -20ºC.

2.4.2. DNA analysis by TAE/agarose/Ethidium Bromide gel electrophoresis

The quality of genomic DNA was analysed on 1% (w/v) agarose (Sigma,USA) gel in 1× TAE buffer. A 1 % TAE /agarose/EtBr gel was prepared by heating up 0.35 g agarose in 35 ml of 1× TAE buffer to boiling. The hot agarose solution was cooled down to about 60ºC before ethidium bromide was added (final concentration: 0.5 µg/ml). The agarose solution was then poured into the gel casting chamber and appropriate combs placed in position. After the gel had polymerised, it was transfered into the electrophoresis chamber with the slots facing the cathode and covered with 1× TAE buffer (running buffer). A 6 × orange DNA loading dye (Fermentas, USA) was premixed with the DNA samples in the ratio 1:6 µl of sample (final concentration:1×) prior to loading of the samples into the sample wells in the gel. The samples were electrophoresed at 5 V/cm (80V) for genomic DNA and 10 V/cm (120V) for PCR products for 45 min-1 hr. The current was supplied by an electophoresis power supply (Consort EV265 Hoefer Inc). A 1kb DNA ladder (Fermentas, USA) and a 250 bp DNA ladder (Roche, Germany) were run alongside DNA samples. The DNA bands were visualised under a UV transilluminator (Herolab E.A.S.Y 442K, Germany).

2.4.3 PCR amplification of 16S rDNA

For a 50 µl PCR polymerase chain reaction (PCR), the PCR reagents were mixed in a thin-walled PCR reaction tube (Simport, Canada) (Table 1). The primers used were 16S F27 (forward) and 16S R 1492 (reverse) are listed in section 2.1.2. The reaction mixture ,reagent, volume of concentation are given in Table 1.

Table 1: PCR reagents added to the 16S rDNA PCR reaction mixture

Reagent	Volume	Final concentration
Water PCR Grade	36.5µl	
10× PCR buffer	5µl	1×
$MgCl_2$ (25mM)	4µl	2 Mm
dNTPs (10 mM)	1µl	200 µM
Forward primer (50 µM)	1µl	1µM
Reverse primer (50 µM)	1µl	1µM
DNA template	1µl	
Taq DNA polymerase (5U/µl)	0.5µl	1.25 U
Final volume	50µl	

The reaction mixtures were overlaid with a layer of mineral oil to prevent evaporation during the PCR cycling reaction. The reaction was run in a TProfessional thermocycler (Biometra, Germany) according to the cycling conditions (Table 2).

Table 2: PCR thermocycling parameters

	Temperature	Time (min)	Cycling steps	
Step 1	94ºC	5 min	Initial denaturation	
Step 2	94ºC	1 min	Denaturation	35 times
Step 3	55ºC	1 min	Annealing	
Step 4	72ºC	2 min	Extension	
Step 5	72ºC	7 min	Final extension	
Step 6	4ºC	Pause		

The quality of the PCR products was checked on a 1% agarose gel as described in procedure (2.4.2) above. Purification of the product was done as described below.

2.4.4 Gel purification of PCR products

PCR products from the gel were purified using Illustra GFX PCR DNA and Gel band purification kit (GE Healthcare, UK).. The products were electrophoresed on a 1.5 % agarose gel as described in procedure (2.4.2). Using a sterile scalpel, under UV light, agarose bands containing the sample of interest were excised from the gel and weighed. To the gel slices of about 0.5 g, 500µl of capture buffer type 2 was added in micro-centrifuge tubes and incubated at

60 ºC until the agarose slices dissolved. The solution (sample mix) was then mixed by inversion and briefly centrifuged to collect the solution at the bottom of the tube. An aliquot of 600µl of the sample mix was transferred into an assembled micro-spin column and collection tube. This was incubated at room temperature for one minute and centrifuged at 16,000× g for 30 seconds to bind DNA. The flow through was discarded and 500 µl of wash buffer type 1 added to the GFX micro-spin column. The assembled column and collection tube were again centrifuged at 16,000× g for 30 seconds. To an assembled GFX micro-spin column and sample collection tube, 20µl of elution buffer type 6 (sequencing buffer) was added, incubated for one minute at room temperature and centrifuged

2.5. Transformation of JM109 *E. coli* cells

2.5.1 Preparation of competent cells

Cells were prepared according to the method described by Christine *et al.,* (1997). LB media was prepared in two flasks (100 ml and 10 ml each) and pH adjusted to 7.2 and sterilized at 121 ^{0}C for 15 minutes. The 10 ml tube was inoculated with *E. coli* colony and incubated over night on a thermoshaker at 37 ^{0}C as pre-culture. Five ml of the pre-culture was inoculated in the 100ml tube and incubated on a thermoshaker for 3 hours at 37 ^{0}C. The culture was chilled on ice for 15 minutes and centrifuged at 4000 rpm in the Jouan tabletop centrifuge for 10 minutes at 4 ^{0}C. The supernatant was discarded and the pellet re-suspended in 30ml of 0.1M $CaCl_2$ and kept on ice for 30 minutes. The re-suspended cells were centrifuged again and the supernatant discarded. The cell pellet was finally re-constituted in 6 ml of cold 0.1M $CaCl_2$ and 15 % glycerol. Aliquots 0.5ml of the cells were pipetted into an epperdoff tubes and stored at -80 ^{o}C.

2.5.2 Assessment of competent cells

A 5 µl aliquot of puC18 plasmid was mixed with 100 µl of thawed competent cells in 1.5 ml eppendorff tubes gently. The cells were placed on ice for 30 minutes. The mixture was heat shocked for 90 seconds and immediately placed on ice for 10 minutes before addition of 600 ml of SOC solution was added and cells placed in water bath at 37 ^{0}C for 90 minutes. Meanwhile 8 µl of IPTG and 200 µl of X-gal were evenly spread on LB /ampicillin plate and allowed to dry. After the incubation period 200 µl of transformed cells were spread on the LB/ ampicillin/IPTG/X-gal plate and allowed to dry. The plates were then sealed and incubated at 37 ^{0}C for 16 hours for colony development. Competence was confirmed by development of blue colonies.

2.5.3 Transformation of competent cells with recombinant plasmid containing 16s rRNA insert

A ligation mixture was prepared as shown in Table 3 below:

Table 3: Ligation reaction mix

Item	Quantity
16s DNA insert	4µl
Double distilled water	3.5µl
T4 buffer 10*	1µl
T4 ligase	0.5µ
PGEM-T vector	1µl
Total volume	10µl

5µl of ligation mixture was put in sterilized 1.5 ml eppendorff tube, 100 µl of competent cells was then added and mixed gently.The mixture was placed on ice for 30 minutes. The cells were transformed by heat shocking at 42 ^{0}C for 90 seconds and tube was rapidly placed on ice and chilled for two minutes. 600 µl SOC medium was added to the cells and incubated at 37 ^{0}C for 90 minutes. This was done to allow recovery of the transformed cells and express the antibiotic resistant marker. Meanwhile 8 µl of IPTG and 200 µl of X-gal were evenly spread on LB /ampicillin plate and allowed to dry. After the incubation period 200 µl of transformed cells were spread on the LB/ ampicillin/IPTG/X-gal plate and allowed to dry. The plates were then sealed and incubated at 37 ^{0}C for 16 hours for colony development. The success of transformation was confirmed by development of blue and white colonies after 16hrs. Both the blue and white colonies were each inoculated in 100 ml LB pre-treated with 100 µl ampicillin. The two cultures were incubated at 37 ^{0}Cfor 16 hours and growth established by development of turbidity. The cultures were used for plasmid extraction

2.6 Plasmid DNA extraction

For each of the blue and white bacterial culture colonies, 1ml was aliquoted into a 1.5 ml sterilized eppendoff tube and centrifuged at 14000 rpm for 5 minutes. The culture from the blue colony acted as a control. The supernatants were discarded and the pellets re-suspended in 100 µl of solution 1(2.5ml of 2M Glucose, 5µl of 0.5M Tris-HCl, pH 8.0). 200µl of solution 2(20µl of 0.2M NaoH, 10µL of 1% SDS) was added to the two tubes and mixed by repeated inversion. To the 150µl of solution 3M sodium acetate (NaOAc) was added and inverted repeatedly to facilitate precipitation. The two samples were centrifuged at 5000rpmfor 5 minutes and the supernatant carefully removed and placed in two new eppendorf tubes. Phenol/chloroform 0.5ml was added and vortexed for 5 seconds followed by centrifugation at 5000 rpm for 3 minutes. The

aqueous upper layer was removed and put in new epperdoff tubes; the two steps were repeated severally to eliminate precipitates at the interface of the two phases. Chloroform was added to the final aqueous phase and spanned briefly, the aqueous solutions were transferred to new eppendoff tubes and 2.5vol of cold isopropanol added and placed in -20 freezer for 2 hours to precipitate plasmid DNA. The tubes were centrifuged at 4000 rpm for 10 minutes and the supernatant discarded by pipetting taking care not to disturb the pellet. 400 µl of 70 % ethanol was added to wash the cells that were spanned down for 3 minutes and the ethanol removed by pipetting. The tubes were allowed to air dry for five minutes and the pellet re-suspended in 30 µl of deionised distilled water. The negative and positive plasmid quality was determined by electrophoresis on a 1% agarose gel.

2.7 PCR amplification of T7 and Sp6 promoter

Amplification of T7 and Sp6 promoter was carried out as described in section 2.4.1.3 with replacement of the primers with T7 and Sp6. The reaction mixtures were overlaid with a layer of mineral oil to prevent evaporation during the PCR cycling reaction. The reaction was run in a TProfessional thermocycler (Biometra, Germany) according to the cycling conditions in Table 2. The quality of the PCR products was checked on a 1% agarose gel as described in procedure section 2.4.2 above and rplasmid taken for sequencing.

2.8 Phylogenetic analysis of the sequences

The 16S rDNA gene sequences obtained from sequencing were analyzed with the standard nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) search at National Center for Biotechnology Information (NCBI) obtained from; http://www.ncbi.nlm.nih.gov/BLAST. The deduced sequence was matched with known 16S rDNA gene sequences at NCBI using the BLAST algorithm. The isolate sequences, including the ones retrieved from the database were

then aligned in MUSCLE (Edgar 2004) and phylogenetic trees constructed based on the nucleotide sequences with the Bayesian phylogenetic method in MrBayes software obtained at http://mrbayes.net. The trees were then visualised using Fig Tree software obtained at http://tree.bio.ed.ac.uk/.

2.9 Linearization of recombinant DNA and negative control plasmid DNA

The plasmid was nicked with Not1 which cleaves downstream of the T_7 promoter

Table 4: Not1 restriction of plasmid

Reagent	Volume
Recombinant Plasmid	8µl
Sure/Buffer H 10*	5µl
Not1	1µl
Double distilled water	6µl
Total	20µl

The sample was incubated in a water bath at 37 ^{0}C for 2 hours after which 5µl of proteinase K was added and incubation period extended for 30 minutes. The sample was extracted twice with 400µl of phenol-chloroform and the pellet precipitated with 2.5 volumes of 95 % ethanol. The pellet was re-suspended in 25 µl of double distilled water. Linearization of the plasmid was confirmed by running 5µl of plasmid on a 1% agarose gel electrophoresis.

2.10 *Invitro* transcription of RNA

RNA run-off was done following the protocol of Milligan *et al.,* 1989. The reagents composition are shown in Table 5.

Table 5: *Invitro* transcription reagents

Reagents	Volume
Transcription Buffer 5*	5µl
NTPs 10*	4µl
100mM DDT	2.5µL
DEPC-Treated Water	6.5µl
Linearized plasmid	1µl
T_7 RNA Polymerase	1µl
Dig-UTP	5µL
Total	25µl

The sample was incubated at 37 ^{0}C for 4 hours.

2.11 Glyoxal denaturation of RNA samples

The secondary structure of RNA produced was denatured before running on a gel. The denaturing reagents are shown in Table 8 below.

Table 6: Denaturant reagents

Reagent	Volume
RNA	7µL
100mM sodium phosphate	4.5µL
DMSO	22.5µL
Deionized glyoxal	6.6µl
DEPC treated water	4.0µl
Total	44.6µl

The sample was mixed thoroughly with the reagents and boiled for 1 hour at 50 ^{0}C and thereafter chilled for 3 minutes.

2.12 Glyoxal/DMSO electrophoresis of RNA

A 1 % agarose gel was prepared by dissolving 1.0 g of agarose in 100 ml of 10 mM sodium phosphate, pH 7.0. The agarose was boiled and left to cool until no steam was formed. EtBr of 2 µl was then added and the gel cast to a thickness accommodating 60 µl. The comb was removed and the gel placed in the electrophoresis chamber. The gel was covered with 10 mM sodium phosphate buffer to a depth of 1 mm. Glyoxal loading buffer of 12 µl was mixed thoroughly with 45 µl of each sample and 60µl of the mixture loaded per lane and electrophoresed at 80V and the remixing of the buffer done at interval of 30 minutes to redistribute the electrolyte in the buffer. Electrophoresis was done for 3 hours until the bromophenol blue travelled to at least 80 % of length of the gel.

2.13 Generation of lead Compounds

2.13.1 Preparation of azide functionalized Paper strip

This protocol was done according to Aminova *et al*., 2008. Paper strips strips (1″ × 3″) were cleaned in piranha solution (70:30 H_2SO_4 : 30 % (v/v) aqueous H_2O_2) overnight at room temperature . Strips were functionalized with amines by submersion in a 3 % (v/v) solution of 3-aminopropyltriethoxysilane in 95 % ethanol for 1 hour at room temperature using. The solution was prepared prior to use by stirring for 10 min at room temperature. Arrays were then washed with 95 % ethanol and cured by heating at 110 °C for 1 h. After cleaning (sonicated for 1 h in water), the amine-functionalized arrays were coated with ~2 mL of a 2% (w/v) agarose solution, and the agarose was allowed to dry to a thin film at room temperature. The agarose was then oxidized by submersion in 20 mM Sodium iodide ($NaIO_{4)}$ for 3 hours at room temperature followed by extensive washing in water (3*30 min with frequent water changes). Slides were then submerged in a 20 % (v/v) aqueous ethylene glycol solution for 1 hour, and then washed with water (6 × 100 mL for 15 min each) Alkyne-functionalized arrays were afforded by submerging the slides in an aqueous solution of 10 mM 3-azidopropargylamine and 10 mM Sodium hydrogen carbonate ($NaHCO_{3)}$ overnight. The following morning arrays were quenched for 5 min by incubation of 20 mL Sodium cyanoborohydride ($NaCNBH_{3)}$ solution (200 mg in 70 mL ethanol and 130 mL phosphate buffered saline) and dried to a clear film on the bench top.

2.13. 2 Spotting the library onto the azide functionalized surface

Dilutions of each ligand were spotted on the array at concentrations of 50 µM in 1X spotting solution (10 mM Tris•HCl, pH 8.5, 1 mM Cu_2SO_4, 100 µM ascorbic acid, 100 µM Tris (benzyltriazolylmethyl) amine (TBTA) and 10 % (v/v) glycerol). Approximately 500 µL of each antibiotic solution was delivered to the surface of an alkyne-functionalized slide using a pipette

treated with Depc and completely sterilized). The arrays were then incubated at room temperature for ~3 h. The arrays were washed in buffer (8 mM Na_2HPO, pH 7.0, 1 mM EDTA, and 180 mM NaCl; 3 × 15 min each) to remove uncoupled antibiotic and coupling reagents including Cu^2, rinsed with nanopore water, and left to dry at room temperature.

2.13.3 Hybridization and detection of the labelled RNA

Hybridization solution was prepared by pipetting 7µl of a labeled RNA (from procedure Table 5) in to a micro-centrifuge tube along with 50 µl of water. The micro-centrifuge tube was then placed in a boiling water bath for 5 minutes to denature the probe. The denatured probe was quickly chilled in ice and immediately added to an appropriate amount (3.5 ml) of pre-warmed hybridization buffer (DIG Easy Hyb, Roche, Germany).

After pre-hybridization, the pre-hybridization solution was poured off, and the hybridization solution added into the hybridization bag. The antibiotic strip was incubated with the hybridization solution at 50 ºC in an air bath for 16 hours (overnight) with gentle agitation. The following day the strip was washed twice for 5 minutes in ample (50 ml) low stringency buffer (2× SSC, 0.1% SDS) at room temperature, and twice for 15 minutes in pre-heated high stringency buffer (0.5× SSC, 0.1% SDS) at 65ºC under constant agitation.

Chromogenic detection method was used to localize the probe-target hybrids on the strip using the DIG wash and Block buffer set kit (Roche, Germany). After the stringency washes the membrane was transferred to a plastic container containing 50 ml of washing buffer and incubated for 2 minutes at room temperature. The wash buffer was discarded, and the membrane incubated in 20 mL of blocking solution for 30 minutes under constant agitation. The blocking solution was discarded, and the membrane incubated in 10 ml of antibody solution for 30

minutes with gentle shaking. The antibody solution was prepared by diluting Anti-Digoxigenin-AP 1:5000 (150 mU/mL) in blocking solution. After this incubation the antibody solution was discarded and the membrane rinsed twice (2 × 15min) in 50 ml portions of washing buffer, then equilibrated in 20 mL of detection buffer.

The strip was finally flooded with 10 mL of colour substrate solution and incubated in the dark for 16 hours at room temperature without shaking. The colour substrate solution was prepared by adding 200µl of NBT/BCIP stock solution to 10 mL of detection buffer. When the colour reaction had produced bands of required intensity the reaction was stopped by rinsing the membrane in 50 mL of TE.

CHAPTER 3: RESULTS

3.1 Collection of sputum from TB patients

Sputum was expectorated into a universal bottle and capped tightly for processing.

3. 2 Identification of MTB using MGIT

The mycobacteria Growth indicator tube (MGIT) was used for detection of mycobacterium growth. Growth was confirmed visually by observing presence of a non-homogeneous light turbidity in the medium as shown in figure 3.2

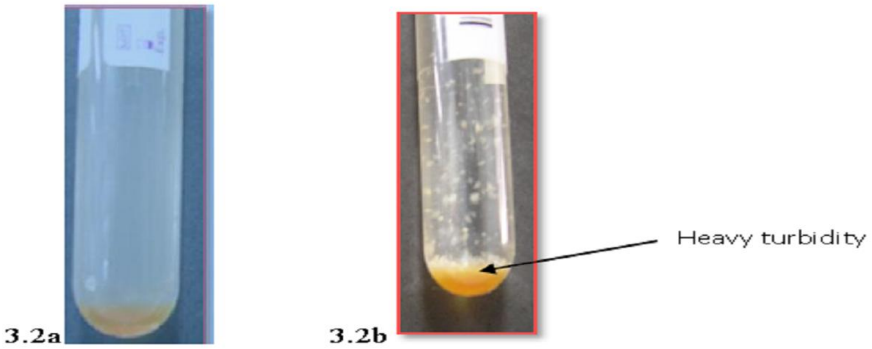


Figure 3.2: 3.2a indicate pure growth of *M. tuberculosis* grown in MGIT tube, 3.2b; *M. tuberculosis* growth with contanamination

3.3 Morphological characterization of Mycobacterium

The morphological feature of the isolates was determined by acid fast test (AFT). Mycobacterial cell walls contain a waxy substance composed of mycolic acids. These are ß-hydroxy carboxylic acids with chain lengths of up to 90 carbon atoms. Basic fuchsin binds to negatively charged groups in bacteria. Acid- fast bacteria will appear red in colour under the microscope while non acid fast bacteria will appear blue in colour (figure 3.3).

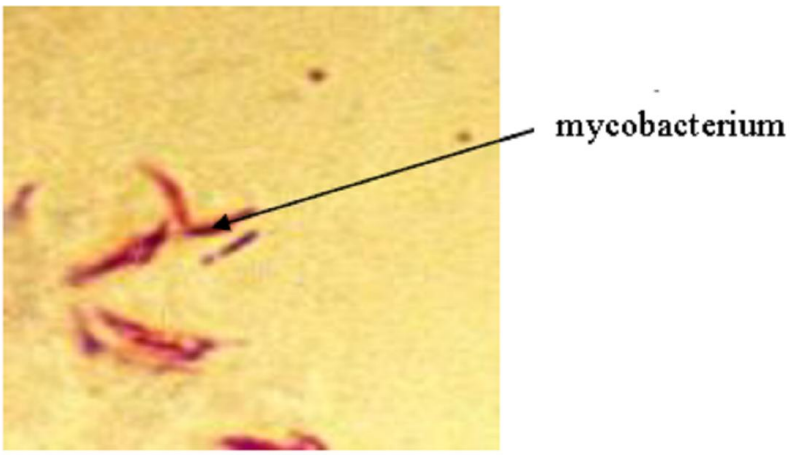


Figure 3.3: Zn stained photograph of the *M. tuberculosis* isolate. The arrow indicates acid fast bacilli.

3.4 Isolation of Mycobacterium tuberculosis

The mycobacterium(mtb) was cultured in Lowenstein and Jensen media and their growth appeared white and buffy (figure3.4).

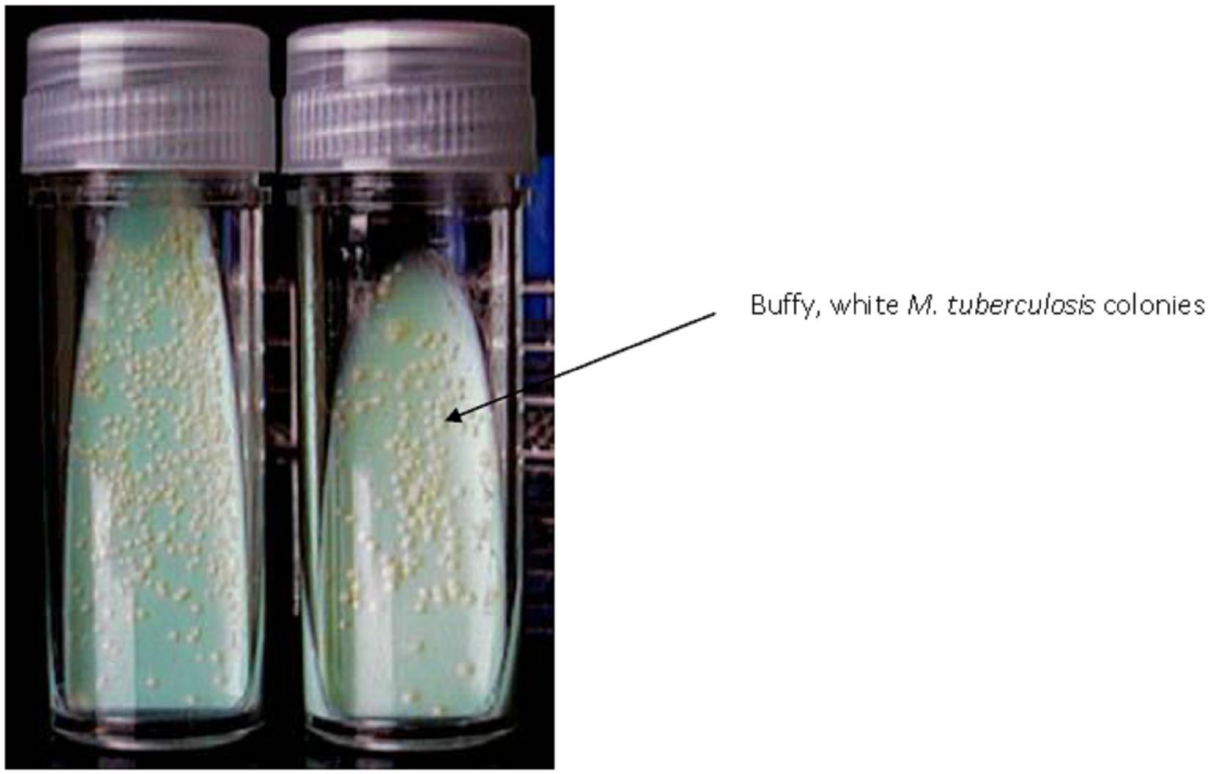


Figure 3.4: Mycobacterium colony in Lowenstein and Jensen media

3.5. Analysis of genomic DNA

The quality of genomic DNA extracted from the Mycobacterium bacterial isolate was analysed on a 1 % agarose gel figure 3.5 and its concentration estimated using a nanodrop 1000 (Thermo Scientific, USA)

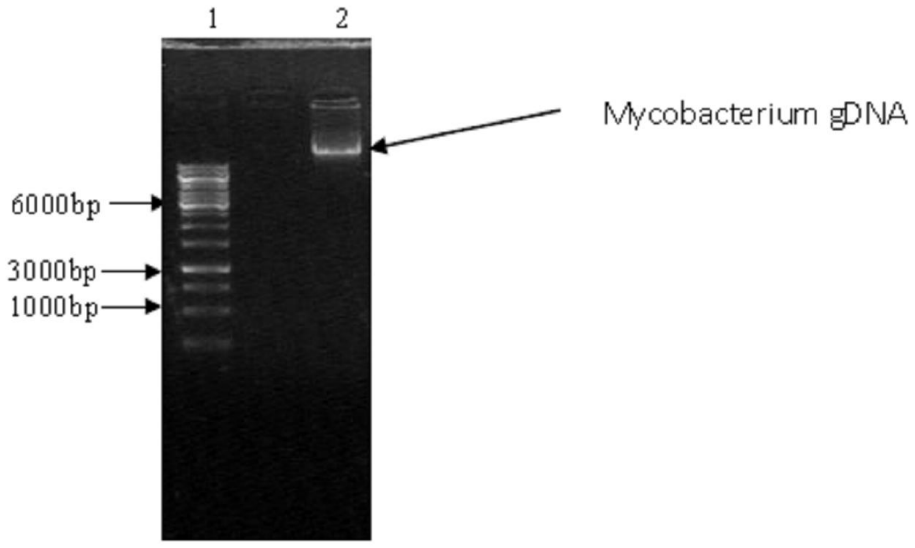


Figure 3.5: Agarose gel analysis of genomic DNA extracted from *M. tuberculosis* indicating high molecular weight DNA. Lane 1 is: 1kb DNA molecular weight marker (Fermentas, USA), and Lanes 2 is genomic DNA isolate from *M. tuberculosis*

3.6 Amplification and analysis of 16S rDNA

The 16S rDNA PCR amplicons from the mycobacterium bacterial strain was gel purified and analysed on a 1 % agarose gel before sequencing. The 16S rDNA PCR product was approximately 1500bp long. The cleaned 16s (3.6b) was gel purified using cleaning kit from GE healthcare using manufacturers instruction.

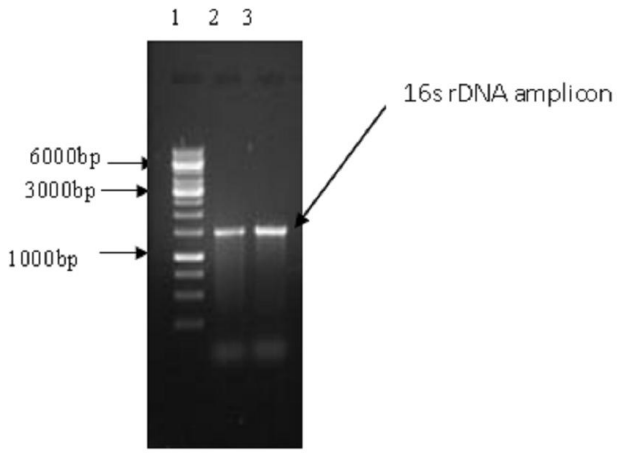


Fig. 3.6 a

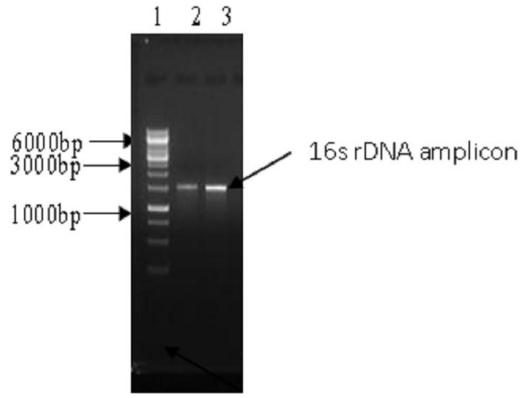


Fig. 3.6b

Figure 3.6: Agarose gel analysis of 16s rDNA PCR amplicons. Figure 3.6a; Lane 1 is: 1 kb DNA molecular weight marker (Fermentas, USA) and Lane 2&3 are: 16S amplicon from Mtb. Figure 3.6b: Agarose gel analysis of cleaned 16s rDNA PCR amplicons. Lane 1 is: 1 kb DNA molecular weight marker (Fermentas, USA) and Lane 2&3 are: cleaned 16S amplicons from *M. tuberculosis.*

3.7 Analysis of JM109 bacterial cells competence

Competent cells from JM109 series that had been prepared were transformed with pUC 18/19 and plated on LB/Amp/IPTG/agar plate. Blue colonies developed after 16 hours (figure 3.7).

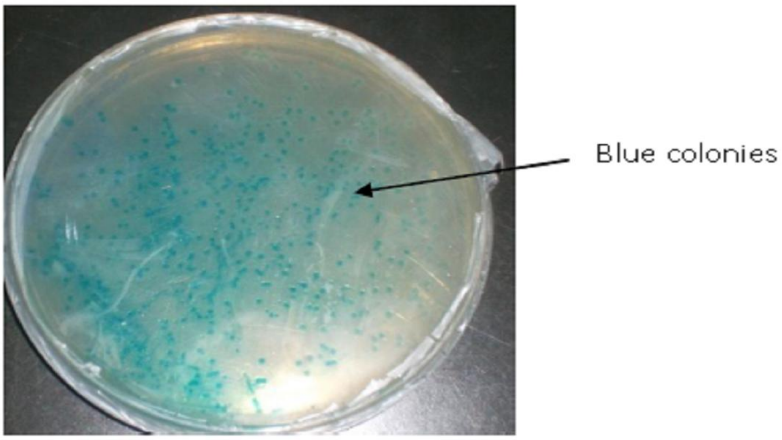


Figure 3.7: Blue colonies formed on LB/ampicillin/IPTG/X-gal agar plate

3.8 Screening for recombinant colonies

Competent cells whose competence had been tested were transformed with a ligation mixture of PGEM-T,16S rDNA and plated on LB/Amp/IPTG/agar plate .White and blue colonies developed after 16 hours (figure 3.8b) .

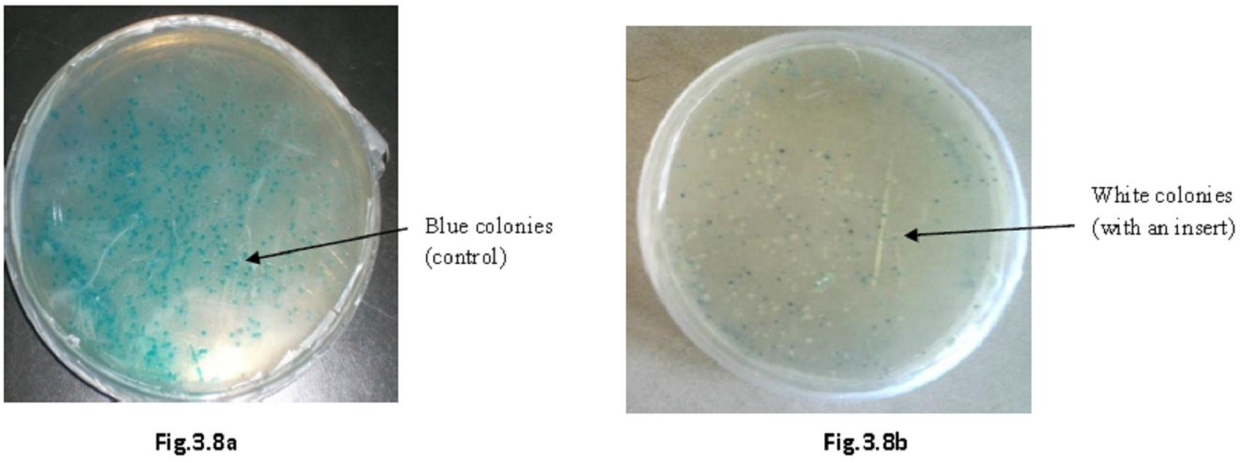


Figure 3.8: Fig 3.8a; blue colonies (without an insert), Fig.3.8b; White and blue colonies (with an insert) on LB ampicillin/X-gal /IPTG agar plate

3.9 Analysis of white and blue plasmid DNA

Plasmid DNA from both the white and blue colonies were respectively extracted and analysed on a 1% agarose gel. The recombinant DNA was approximately 4500bp and non-recombinant DNA was 3000 long (figure 3.9a). The plasmids were amplified and the amplicons analyzed on a 1% agarose gel before controlled digesting. The PCR product was approximately as shown in figure 3.9b.

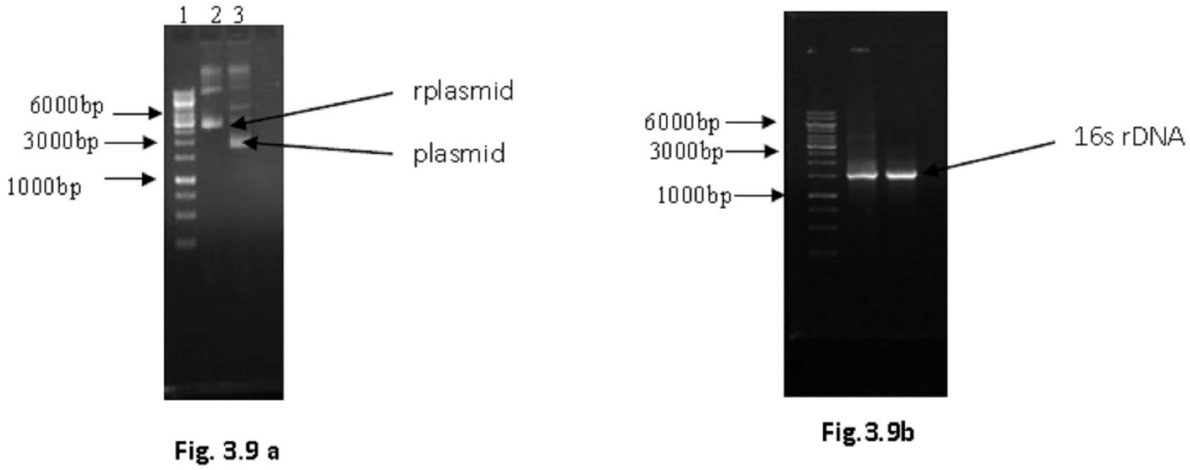


Figure 3.9: Fig. 3.9a; Agarose gel analysis of plasmid DNA. Lane 1 is: 1 kb DNA molecular weight marker (Fermentas, USA) and Lane :2 is a recombinant plasmid and lane 3: is a negative control plasmid DNA. Fig.3.9b; Lane 1 is: 1 kb DNA molecular weight marker (Fermentas, USA) and Lane 2&3 are: T7 and SP6 amplicon from *M. tuberculosis*.

3.10 Analysis of controlled digested recombinant product

The plasmid DNA was digested with Not 1 restriction enzyme and the quality of digested DNA was analysed on a 1% agarose (figure 3.10)

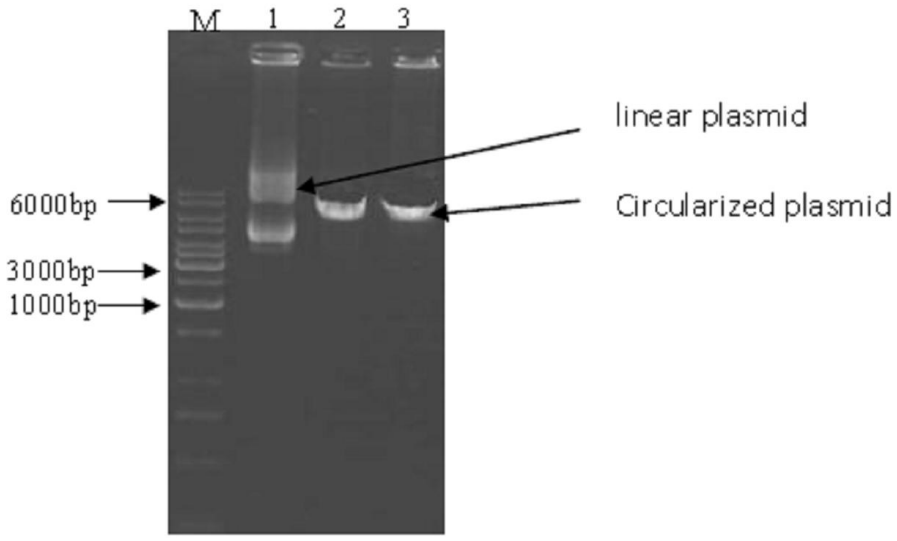


Figure 3.10: Agarose gel analysis of a nicked recombinant plasmid.Lane M: 1 kb DNA molecular weight marker (Fermentas, USA) ,lane 1: nicked rplasmid DNA with Not1 restriction enzyme and lane 2 and 3 an intact rplasmid DNA

3.11 PCR synthesis and analysis of Digoxigenin (DIG) labelled RNA probes

DIG labelled probes were synthesised by PCR (Table 7). The labelling of the probes was achieved by incorporation of DIG-11-UTP in the PCR reaction. Successful incorporation of DIG-11-UTP in the PCR products was analysed on a 1% agarose gel (figure 3.11).

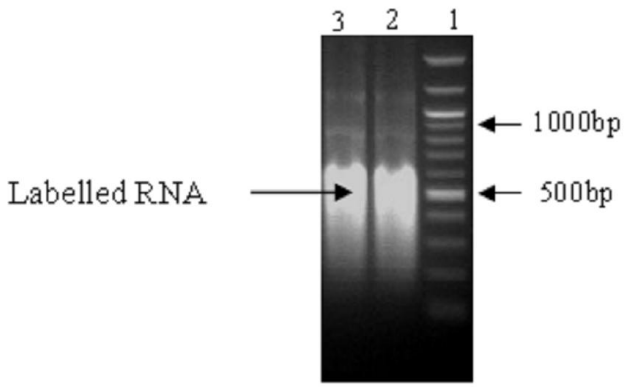


Figure 3.11: Agarose gel analysis of Invitro transcribed RNA. Lane 1 is: 1 kb DNA molecular weight marker (Fermentas, USA) and Lane 2&3 are: 16s Invitro transcribed RNA from *M. tuberculosis*.

3.12 Phylogenetic tree analysis of 16S sequences from Mtb

To confirm the relatedness of our isolate, a phylogenetic tree was drawn using muscle and this ascertained we were truly working with ribosomal fragment from *M. tuberculosis*.

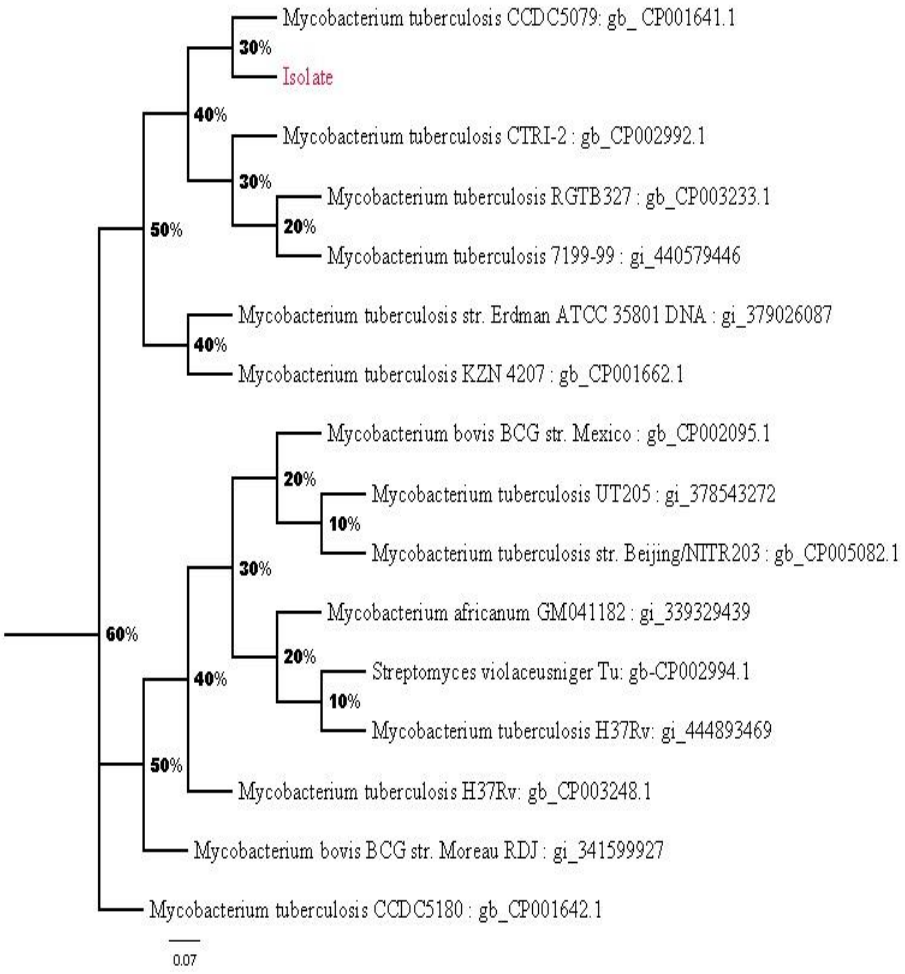


Figure 4: Phylogenetic tree showing that the isolate was a *Mycobacterium turberculosis* ccdc 5079 from homology studies of 16S rRNA gene

The tree was constructed using Mr. Bayes, a program for the Bayesian inference of phylogeny that is based on the Markov Chain Monte Carlo (MCMC) method. Numbers at the nodes show percentage of posterior probabilities indicating topological robustness of the Phylogenetic tree.

3.13 Screening for RNA –antibiotic interaction

Hybridization was done using the labelled RNA to screen for interaction of RNA and selected antibiotic. The probe-target hybrids were detected by polyclonal anti-DIG Fab fragments

conjugated with alkaline phosphatase (AP) (figure 20). Nitro blue tetrazolium chloride (NBT) and BCIP (5-Bromo-4-Chloro-3-indoyl phosphate toluidine salt) were used as Chromogenic substrates. In this reaction BCIP is the AP-substrate, which reacts further after dephosphorylation to give a dark-blue indigo-dye as an oxidation product while NBT serves as the oxidant and also gives a dark-blue dye. This intensifies the colour and thus makes the detection more sensitive. Figure 19 shows the reaction scheme for the dye-generating redox reaction.

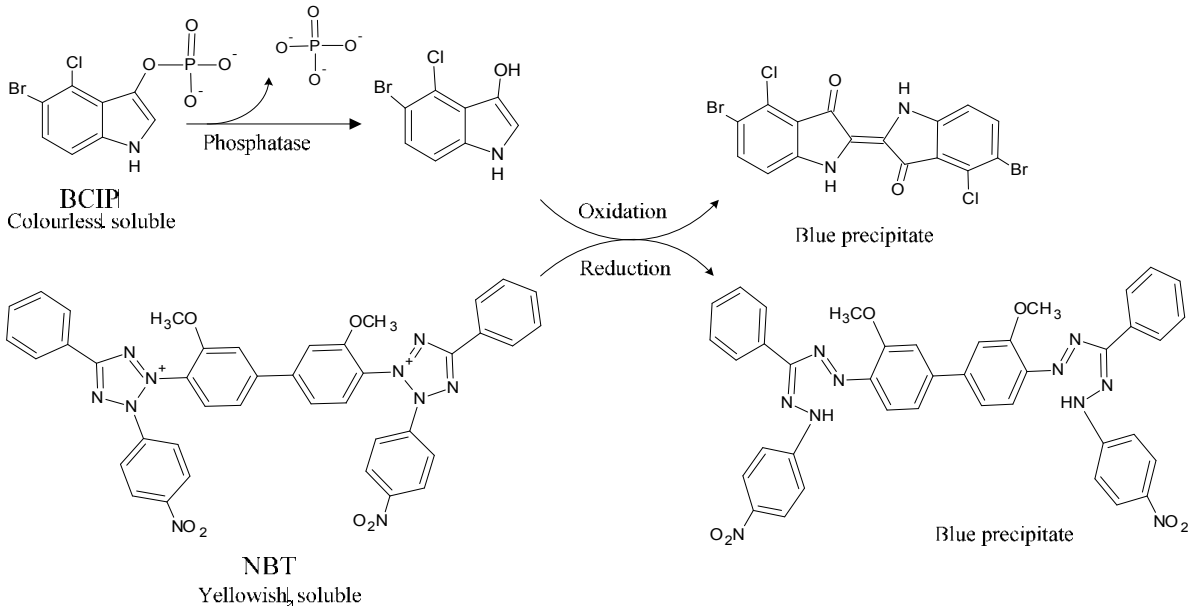


Figure 3.13: Fig 3.13a; BCIP/NBT reaction scheme for the dye-generating redox reaction.

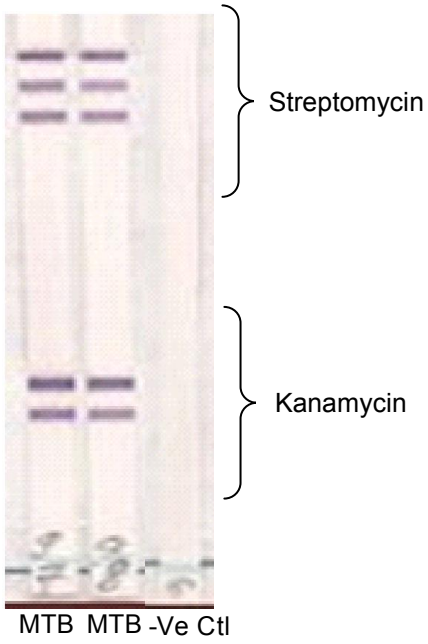


Figure 3.13b; Hybridization of target- probe interaction (labelled MTB rRNA) on antibiotic (Kenamycin and streptomycin) platform with a negative control (–ve Ctl; water)

CHAPTER 4: DISCUSSION

Tuberculosis is one of the devastating bacterial diseases with increasing rate of morbidity and mortality despite the presence of effective chemotherapy and Bacillus-Calmatte-Guerin (BCG) vaccine. The success of Mycobacterium tuberculosis lies in its ability to spread by aerosol droplets, evade the host immune system and to persist in pulmonary granulomas (Vohra et al., 2006).

Various families of antibiotics have been produced that target essential mechanisms of the bacteria. These targets together with the specific antibiotics include: protein synthesis example; streptomycin, DNA replication; Flouroquinolines(FQ) , cell wall synthesis; isoniazid, enzymes involved in transcription; rifampicin. However subsequent research has elucidated many forms of the *Bacillus tubercle* that are; persistent, static and resistant which make the treatment a long course of approximately 8 months. This study was therefore motivated by the fact that despite the RNA being the central and largest molecule in gene expression no drug has been produced to target the RNA of infectious bacteria. RNA plays a key role in a variety of biological systems that cement its status as a therapeutic target. Therefore, developing compounds that modulate RNA activity is important. In this study we were able to show that it is possible for RNA to bind to immobilized kanamycin A and streptomycin, these agreed with other studies that had postulated that off-target RNA could be involved in binding with antibiotics that target A-site of ribosome. It is important to note that RNAs are being discovered in genomic sequences. Most of these represent untapped potential as target for therapeutics or chemical generation probes. Our study sought to develop a general method to identify ligands that bind to highly structured RNA scaffolds. Critical to this study were selection of ligands and the use of azide based antibiotic platform based screening to score ligand library for binding. Antibiotic platform allow for facile

screening for binding of target biomolecules including proteins and RNAs (Aminova *et al.,* 2008) .

From this study i was able to identify Mtb through smears and isolation through cultures, the bacilli were rod shaped, 4 μm in length and 0.5μM in width figure 5. As much as 50-60 % of AFB (acid fast Bacilli) culture-positive clinical specimens may fail to reveal AFB on smear made from the specimen (Salman *et al*., 2006). As a consequence, culture techniques play a key role in the diagnosis of mycobacterial disease. We used liquid and solid medium for growth of Mycobacterium that was according to CDC report, 1993. In liquid medium we used MGIT which consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. Addition of the MGIT PANTA is necessary to suppress contamination. The tube also contains an oxygen-quenched fluorochrome, tris 4,7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen was utilized and was replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome was no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The intensity of fluorescence was directly proportional to the extent of oxygen depletion and the growth was read by Bactec 960. This was flagged after four days which meant the machine system is sensitive and by the time there was positive reading there were 10^5-10^6 CFU. Manually it was observed by presence of non-homogeneous light turbidity appearance in the medium when the growth is pure while when there was contamination it had heavy turbidity as shown in Figures3.2a and 3.2b. Lowenstein-Jensen media was used for isolation, it is selective for *M. tuberculosis* and it is an egg based, L-Asparagine and Potato Flour are sources of nitrogen and vitamins. Monopotassium Phosphate and Magnesium Sulfate enhance organism growth and act as buffers.

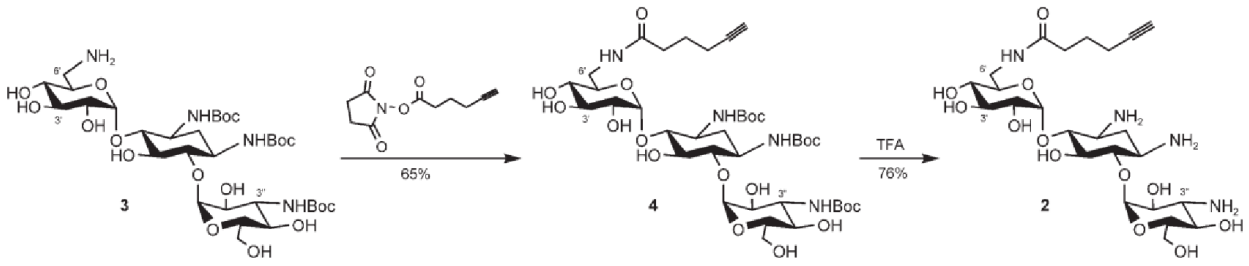
Glycerol and the Egg Suspension provide fatty acids and protein required for the metabolism of Mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Sodium Citrate and Malachite Green are selective agents to prevent growth of most contaminants and allow early growth of Mycobacteria. The colonies appeared after the fourth week and they appeared white, creamy and buffy Figure 3.4. Characterization of bacterial isolates relied on morphological characteristics. The morphological features of the isolate were determined by acid fast test (AFT) Figure 3.3 and molecular tools which are confirmatory and effective tools for *M. tuberculosis* identification figure 3.12. *M. tuberculosis* have unusual cell walls that are waxy and nearly impermeable due to the presence of mycolic acid, and large amounts of fatty acids, waxes, and complex lipids. These organisms are highly resistant to disinfectants, desiccation and are difficult to stain with water-based stains such as the Gram stain. The *M. tuberculosis* bacilli appeared red while the nonacid fast appeared blue Figure 3.3.

Recombinant cells were able to grow in the presence of ampicillin, that property is acquired from amp^r in the rplasmid. Selection was made possible by development of blue and white colonies. The presence of insert was confirmed by PCR amplifying the isolated plasmids using T7 and SP 6 primers that recognized regions flanking the multiple cloning sites, allowing the size of the insert to be determined prior to submission for sequencing and invitro transcription. The size of the PCR product was consistent with insert from the white colonies screened as shown in Figure 3.9a and 3.9b.

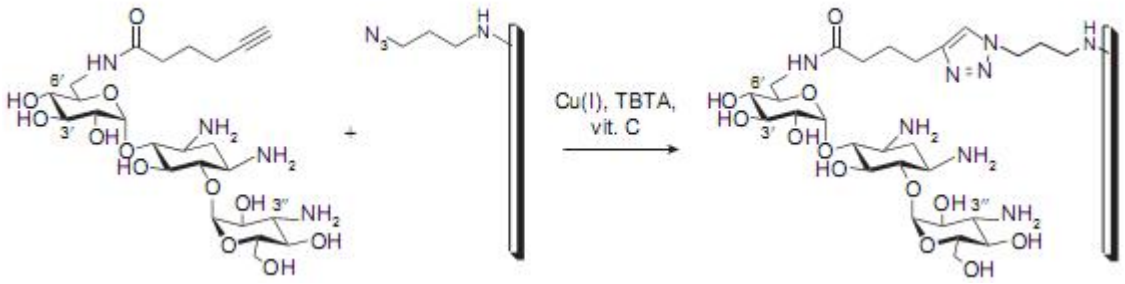
The 16S rDNA sequence obtained was used to search similar sequences at NCBI using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). The sequence confirmed the

organism as Mtb as shown by figure 18 and therefore the signals obtained during ligand-rRNA interaction were truly from mtb's rRNA.

The recombinant plasmid with a control (Figure 13), was nicked with Not 1, a restriction enyzme, upstream the T7 promoter site in pGEM T. The nicked plasmid was run parallel with uncut plasmid Figure 16 and result showed that the supercoil plasmid travelled further compared to nicked plasmid this is because it was more retarded along the matrix of the agorose gel as opposed to the supercoiled plasmid. T7 RNA polymerase was used for invitro transcription and subsequent labelling of the RNA molecules using 11 DIG – UTP for chromogenic detection system. The RNA were approximately 0.5 bp, Figure 3.11.Kanamycin A was immobized at 6'-NH_2 position which is the most nucleophillic.This position is usually acetylated(Disney,M.D and Childs-disney,L.,2007) this allowed a defined immobibilization through reaction of the alkyne handle with azide-displaying glass slide using Huisgen dipolar cycloaddition as shown by scheme 1



Scheme 1 from Disney ,M.D :Synthesis of 6' -N-5-hexynoate kanamycin A (2) used for immobilizationon to azide displaying glass slides by click chemistry.Boc= tert-butoxycarbonyl;TFA= trifluoroaceticacid (Aminova *et al*., 2008).



Scheme 2 from Aminova *et al*., 2008: Schematic of the immobilization of hexanoate kanamycin onto azide-functionalized agarose slides via a Huisgen dipolar cycloaddition reaction.

Scheme 1 shows immobilization of kenamycin A which was reacted with succinimidyl-5-hexynoate to protect the active groups and was deprotected by trifluoroacetic acid this was the same scheme used to immobilize streptomycin to the slide. Formation of triazole between the activated azide and ligand through alkyne handle is shown by scheme 2.

The immobilized ligands was screened for interaction of ligands and RNA motifs .This surface is robust enough for ligand screening because it offers optimal surface. (Afanassiev *et al*., 2000). Agarose provides a three dimensional surface for high ligand loading and a versatile surface to accomondate a variety of immobilized chemistries (scheme 2).The ability to complete selections at different ligand loading on the same surface demonstrates that this platform can be used to complete multiple selections in parallel in a single array as shown by Figure 20. This has also been reported by Aminova et al., 2008. In this case labelled RNA was incubated with the platform, where the DIG labelled RNA interacted with immobilised antibiotics. A conjugated ant-DIG antibody was then added and colour development visualised by incubation of the slides with BCIP/NBT substrate. Both sets of the experiment demonstrated that colour formation in both streptomycin A and kanamycin was not due to simple charge–charge interactions but through a specific interaction between the functional groups displayed by the ligands (scheme 1

and 2). The interaction can be attributed to the presence of grooves on RNA, which are either deep or shallow made of negatively charged phosphate backbone that facilitate the interaction .This binding has effect on RNA biological activity by; preventing the binding of the relevant macromolecule (protein or RNA), distorting the RNA active confirmation and forcing an alternative confirmation of the RNA, inhibiting RNA catalysis, and competitive binding for a cofactor binding size.

CHAPTER 5: CONCLUSION

This study has shown that there are a handful of bioactive compounds that can bind RNA motifs as demonstrated by binding of RNA to streptomycina and kanamycin. This information will facilitate construction of RNA motifs database that can specifically be recognized by small molecules such as antibiotics. This will lead to development of new antimicrobial compounds with less susceptibility to the development of resistance, and therefore extended clinical profiles. These would provide valuable new weapons in the arsenal of compounds used to treat infection caused by persistent Mtb and will reduce toxicity and shorten treatment time. The advantages of targeting RNA over targeting proteins include:

- More sites are accessible at the RNA level, whereas the active site of a protein is often the only target.
- Proteins that share a common substrate like ATP or ligands are difficult to inhibit specifically.
- It is possible to develop multivalent drugs to target RNA or drugs that target a RNA sequence that is essential for encoding an important sequence of a protein.

By expanding the results disclosed herein and probing diverse chemical space for binding RNA via microarray, more scaffolds are likely to be identified. Future developments in using microarray-based screening of other small molecules (which have triazole would be possible to immobilize) would benefit from using chemoinformatics approaches to compute chemical diversity to rationally design libraries with broad chemical landscapes. By using statistical analysis of binders, general ligand features for binding RNA can be elucidated quickly. Further studies are therefore required to determine the internal RNA loop-drugs affinity capacities which

will necessitate production new family of antibiotics. This will not only shorten the length of treatment but also lower the cost for cure.

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Appendix:

Media composition for L and J

Formula/liter

L-Asparagine…….3.6g

Monopotassium phosphate……2.5g

Magnesium sulfate…….0.24g

Sodium citrate…….0.6g

Malachite Green……..0.4g

Potato flour…….30g

Supplement:

Glycerol,12ml

Egg suspension 1000ml

added up to 10 ml. The mixture was autoclaved