

**OPTIMIZATION OF rTDMH AS A REAGENT TOWARD IMPROVING THE
SENSITIVITY OF THE RT-PCR BASED DIAGNOSIS FOR *MYCOBACTERIUM*
*TUBERCULOSIS***

by

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Submitted to the Graduate Faculty of
Graduate School of Public Health in partial fulfillment
of the requirements for the degree of
Master of Public Health

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH
GRADUATE SCHOOL OF PUBLIC HEALTH

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Alexandra Bhatti, M.P.H.

University of Pittsburgh, 2011

Current diagnostic tools being used for tuberculosis lack the speed and sensitivity necessary to successfully combat the current tuberculosis epidemic. Real-time Polymerase Chain Reaction, RT-PCR, can provide the rapid and specific diagnosis that is currently in demand in the global community. Its disadvantage is that due to the waxy and robust nature of the *M. tuberculosis* membrane, not enough genomic DNA is present to provide for amplification in a RT-PCR. It was previously found in our laboratory that hydrolysis of one of abundant glycolipid of mycobacterial envelope, Trehalose, 6,6'-dimycolate, by a recombinant TDM-specific hydrolase caused rapid lysis of cell (Yong et.al. manuscript submitted). In this study, we tested if rapid lysis by TDM-specific hydrolase (rTDMH) can be exploited in conjunction with the RT-PCR to develop a sensitive diagnosis of tuberculosis.

Results demonstrated that by incubation of both attenuated *M. tuberculosis*, and virulent *M. tuberculosis* with rTDMH for lysis and subsequent usage of this lysate in a RT-PCR assay, yields sensitive amplification of mycobacterial DNA. rTDMH-mediated lysis could facilitate amplification of even 10 bacilli, the rTDMH treated cells show amplification while lack of treatment failed to detect these bacilli. These results were consistent in *in-vitro* liquid culture and

in complex sputum samples spiked with the mycobacteria, showing that incubation with rTDMH can improve the sensitivity of the RT-PCR.

Statement of Public Health relevance: Using rTDMH with RT-PCR as an improved diagnostic tool for tuberculosis due to the rapid, accurate and sensitive nature of the assay could provide the global community with a much better method of diagnosing a disease that has plagued the world for thousands of years. Tuberculosis infects 9 million people and kills 3 million people every year and presently one-third of the world's population is infected with it. A better diagnostic tool could result in reducing the spread of disease; reducing the mortality associated with disease, especially in HIV infected individuals; and on a broader scale, could reduce the economic burden associated with the disease.

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ACKNOWLEDGMENTS

I would first like to thank Dr. Anil Ojha for guiding me and helping me develop my research skills, and preparing me for my future endeavors in research. It was through his expertise, feedback and guidance that I was able to complete this study. I would like to also thank Dr. William Pasculle from UPMC, for without the samples ascertained from the hospital, I would not have been able to accomplish my study to the same depth. In addition, I would like to express a great deal of gratitude to Dr. Alice Tarun, for allowing me to use her Real-Time PCR machine, as well as those that work in her lab. Usage of that machine was the fundamental portion of my study. I would also like to recognize Dr. Rodger Beatty, Dr. Jeremy Martinson, and Dr. Martha Terry, for their guidance, suggestions, and support during this thesis development process. Their help and contributions were invaluable. Lastly, I would like to thank my colleagues in Dr. Anil Ojha's lab: Kathleen Kulka, Yong Yang PhD and Mohammed Islam PhD, who have been there offering their encouragement, expertise and guidance every step of the way. Particularly, I would like to thank Yong Yang, who had experience with the subject matter of my study and was able to offer much technical support along this journey.

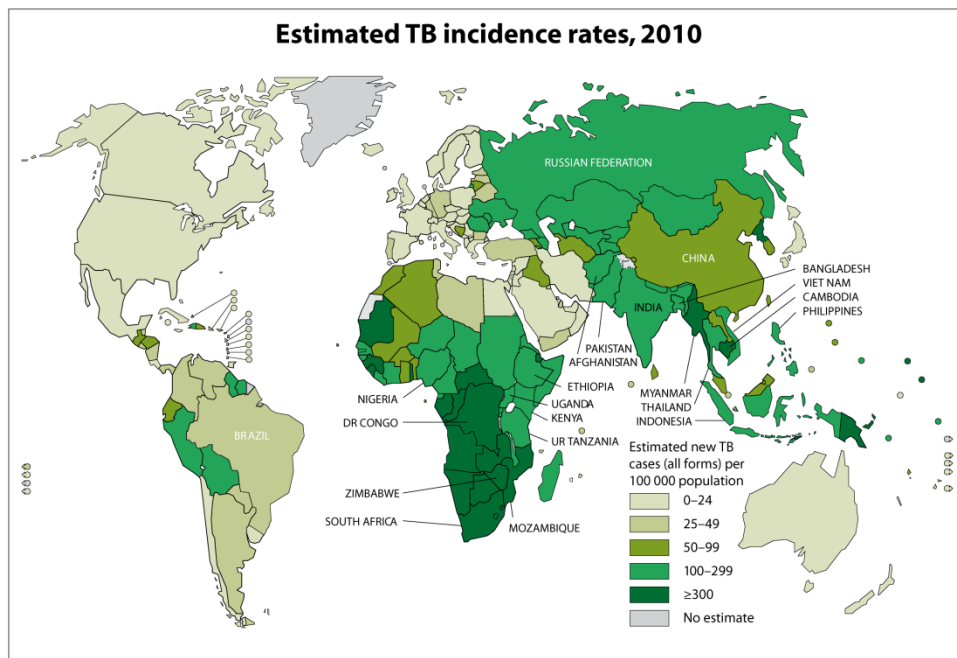
1.0 INTRODUCTION

1.1 THE TUBERCULOSIS EPIDEMIC

Mention the disease tuberculosis to any person and they most assuredly have heard of it. Tuberculosis is not a novel disease that has yet to capture the attention of the general public. In fact, it has had a presence in the global community for thousands of years. Skeletal remains dating from as early as 4000BC show the existence of tuberculosis, and tubercular decay has been found in the spines of mummies from 3000-2400BC. As tuberculosis presents a variety of symptoms, it was not until the 1820s that it was identified as a unified disease, and in 1839 it was formally labeled tuberculosis by J.L. Schonlein[1]. That being said, there is still a sense of apathy that exists about it, and perhaps this is due to its silent nature and that its presence is not pronounced as much as others. As research on tuberculosis has progressed, the way in which it is viewed has in turn changed to looking at it as a spectrum, one end being asymptomatic, noninfectious disease to the other end being, infectious and symptomatic. This characteristic of tuberculosis is what makes it challenging to address. Nevertheless, the impact of this disease is notable, and more attention must be allotted to it.

Presently, one-third of the world's population is infected with tuberculosis and thus it is one of the most wide-spread diseases[2]. The World Health Organization projects that approximately one billion people will be infected with tuberculosis between the years of 2000

and 2020. Annually, over nine million people around the world become infected, and there are approximately two to three million deaths that are directly attributable to tuberculosis[3]. Moreover, tuberculosis, AIDS, and malaria are responsible for over half the deaths due to infectious diseases each year. In some cases these deaths arise from co-infections, where the individual is infected with both tuberculosis and HIV. When observing the global impact of disease, there appears to be a socio-economic trend associated with it. The highest incidences are seen in countries with the lowest gross national products, this being predominantly in regions of Africa, Eastern Europe, South East Asia, Asia, and parts of South America. A particular burden is seen in sub-Saharan Africa, with ≥ 300 new cases per 100,000[2]. Figure 1 demonstrates the global burden of disease and includes all forms of tuberculosis, including multi-drug resistant (MDR-TB) and extremely drug resistant tuberculosis (XDR-TB).



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

Source: *Global Tuberculosis Control 2011*. WHO, 2011.



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Figure 1: Global Incidence of TB in 2010 (World Health Organization)

The prevalence of MDR-TB and XDR-TB continues to increase in the global community. MDR-TB is a form of tuberculosis that has adapted a tolerance to at least two of the best antibiotics used as treatment, isoniazid and rifampicin. XDR-TB is, in turn resistant to the previously mentioned first-line drugs as well as any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin)[4]. Of the new cases of tuberculosis 3.3% are MDR-TB. Figure 2 depicts the global burden of MDR-TB and XDR-TB[5].

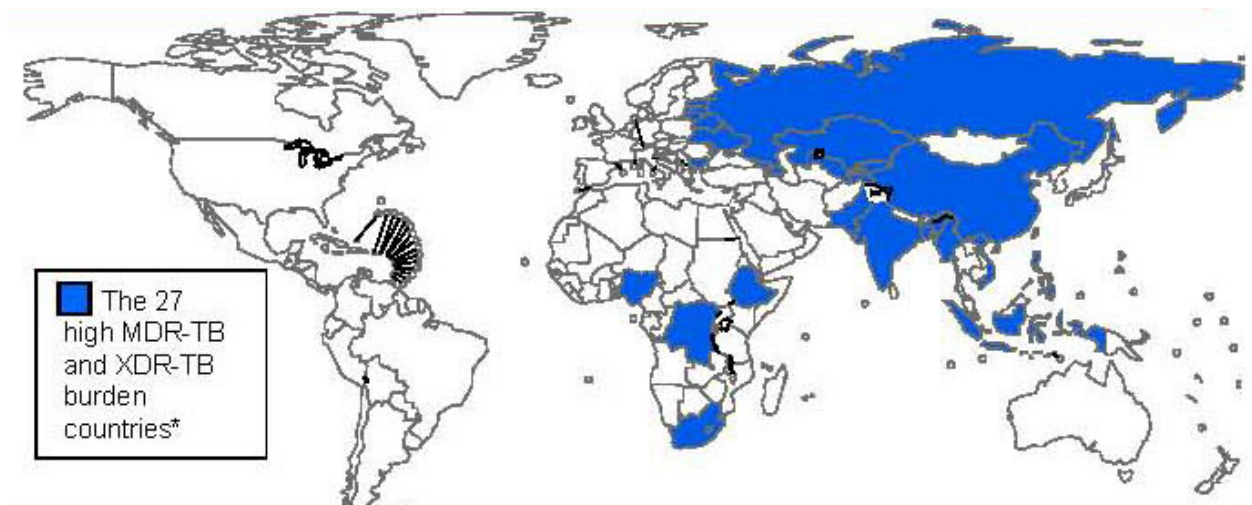


Figure 2: Global Burden of MDR-TB and XDR-TB (World Health Organization)

When comparing Figures 1 and 2, there is an interesting difference to note. In Figure 1, the highest burden of disease is in Africa, whereas when evaluating the global burden of MDR-TB and XDR-TB, the highest is seen in Eastern Europe, Asia and South East Asia. This may be because accessibility of antibiotics may be higher in Eastern Europe, Asia versus Africa, thus

increasing the likelihood that drug resistance may appear, or because surveillance may not be as good in parts of Africa and thus the true numbers are underrepresented.

Tuberculosis has a significant presence in the HIV-infected community as well. It is the leading cause of death amongst persons with HIV infection[6]. In fact, almost one in three deaths amongst people with HIV are due to tuberculosis. People who have both HIV and latent tuberculosis are 20 to 30 times more likely to develop active tuberculosis[7]. In 2009, there were 9.4 million new cases of tuberculosis, of which 1.2 million (13%) were among people living with HIV[6]. The highest burden of HIV in new tuberculosis cases is in sub-Saharan Africa (see Figure 3).

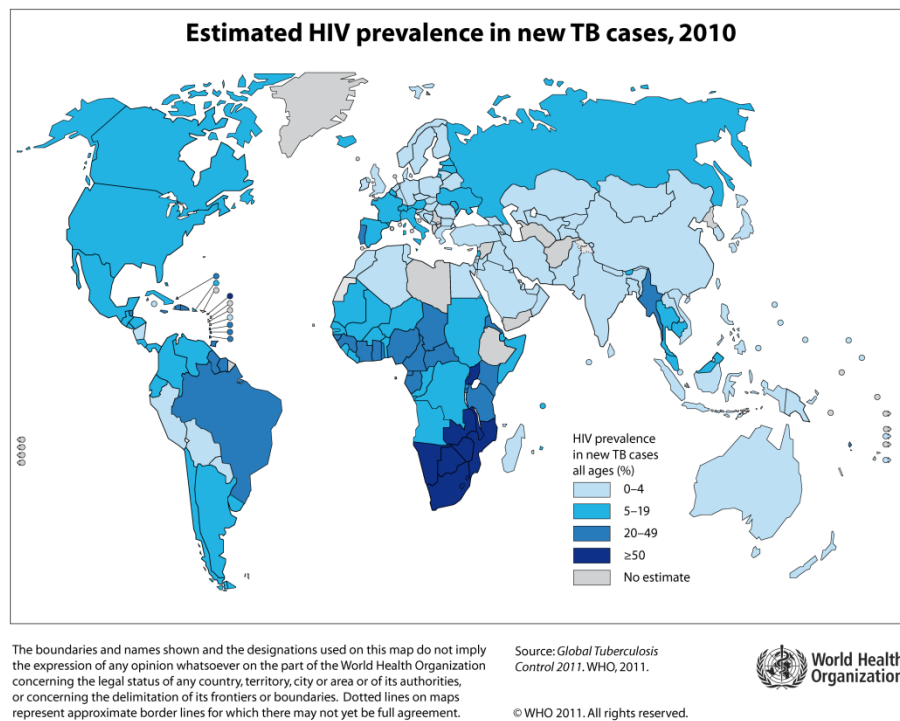


Figure 3: Estimated HIV prevalence in new TB cases in 2010 (World Health Organization)

2.0 BACKGROUND: THE TUBERCULOSIS DISEASE

2.1 PATHOGENESIS

M. tuberculosis, is the causative pathogen for disease tuberculosis. *M. tuberculosis* is a bacterium that typically attacks the lungs; however, it potentially can attack any part of the body such as the kidney, spine, and brain. If left untreated, tuberculosis can be fatal. Tuberculosis is a droplet infection spread from person to person through the air[8]. When an infected individual coughs, sneezes, and sings, droplets containing the bacteria may be introduced into the air and surrounding individuals may be exposed to said droplets and unknowingly inhale the air containing them and become infected. Tuberculosis infections begin as the mycobacterium begins to replicate in the small air sacs of the lungs, and from there, some may enter the blood stream and spread throughout the body [9].

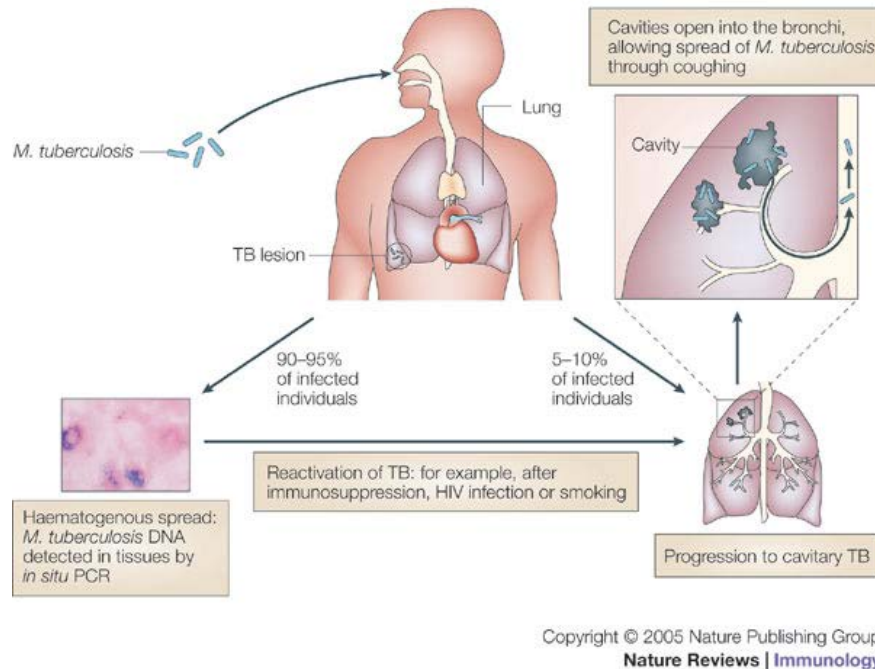


Figure 4: Phases of infection of *M. tuberculosis* (Rook, Dheda, & Zumla)

Infection of *M. tuberculosis* produces a spectrum pathology. Historically, it was thought that there were two forms of tuberculosis, latent and active; now it is becoming more apparent that these are the two ends of the spectrum. The latent form is non-infectious and can reside in the host's body dormant for years, if not his lifetime. It is asymptomatic and cannot be spread from person to person. However, an asymptomatic infection is always at risk for it becoming active and various stressors on the body may cause this such as immune suppression with HIV. The infected individual will test positive with the skin or blood test; however sputum smears and chest x-rays will be negative [10].

The active disease causing form is when it is reproducing in the host. In this scenario, the host is presenting symptoms of disease and is infectious, meaning the host may further infect others, further propagating the disease. This individual will test positive if given the skin test or blood test and may have an abnormal chest x-ray or positive sputum smear or culture. A person may have one of two different subsets of the disease, pulmonary or extrapulmonary. Typically,

tuberculosis infection occurs in the lungs (pulmonary); however, as aforementioned it can be found in other parts of the body (extrapulmonary) [10].

2.2 MYCOBACTERIUM TUBERCULOSIS CELLULAR STRUCTURE

M. tuberculosis belongs to the genus mycobacteria, which are actinomycetes. A separate classification for mycobacterial species is primarily because of their acid-fast staining that originates from their unique envelope structure. The mycobacterial cell envelope is divided in distinct layers of plasma membrane, a core cell wall complex and a membrane-like outer layer made of extractable lipids. In fact, over 60% of the cell envelope is comprised of lipid, which provides intrinsic tolerance to many antibiotics, and resists against killing by acidic and alkaline compounds, osmotic lysis via complement deposition, resistance to lethal oxidations and survival inside macrophages [11]. See Figure 5 for a schematic diagram of the cell envelope.

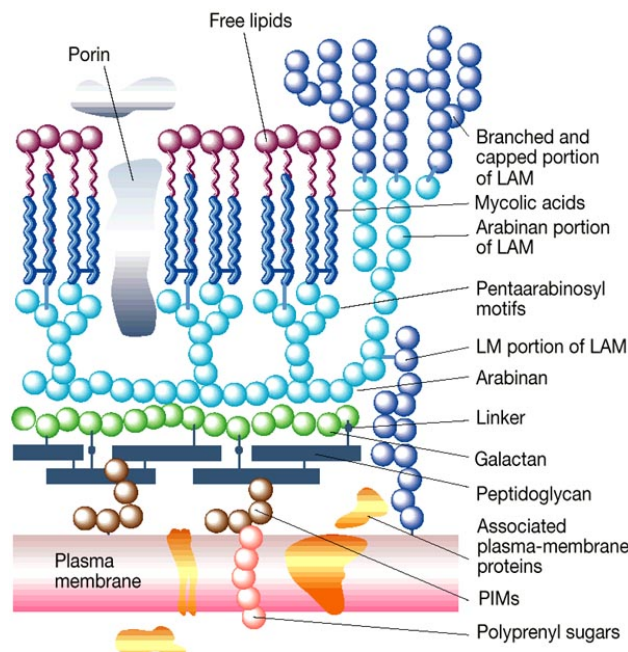


Figure 5: Schematic diagram of the cell envelope of *M. tuberculosis* (Park & Bendelac)

2.2.1 Trehalose 6,6'-dimycolate (TDM)

One of the most abundant extractable lipid of the envelope is Trehalose 6,6'-dimycolate (TDM), which is responsible for the cording morphology of the virulent *M. tuberculosis* [12].

2.2.2 TDM hydrolase

A hydrolase of TDM, rTDMH, was discovered in *Mycobacterium smegmatis* called MsmeG_1529. *M. smeg.* is commonly used in laboratory work to research other mycobacteria such as *M. tuberculosis* because of its similarity; however, it has attractive qualities, such as being non-pathogenic, growing faster and able to be used at a BSL-1 level. This species shares more than 2000 homologs with *M. tuberculosis* and has the same unique cell membrane structure. This discovery prompted the search for the similar hydrolase in *M. tuberculosis*. Such a hydrolase was found, subsequently isolated and *M. tuberculosis* culture was then treated with rTDMH and results showed that rTDMH was able to successfully, disrupt the cellular membrane and thus lyse *M. tuberculosis* [12]. This was done in Dr. Anil Ojha's lab following a process by which 10^6 *M. tuberculosis* bacilli were incubated with various concentrations of recombinant rTDMH for 2 days in phosphate buffer saline with Tween- 80, PBST. It was found that the viability of the population was reduced by 100-fold or more with 0.8 μ M or higher concentrations of the enzyme. It was also done in various other media environments including a detergent free Sauton's media agar plate. This was done, in particular, to exclude the impact that tween-80 may

have on the mycobacterial envelope [12]. The results were overwhelmingly supportive of rTDMH's ability to reduce mycobacterial viability. The results can be seen in Figures 6 and 7 below where TDMH was spotted on a lawn of *M. tuberculosis* and buffer on another as a control.

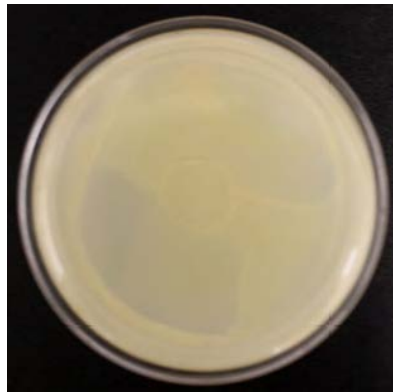


Figure 6: Buffer treated bacilli on Sauton's agar plate

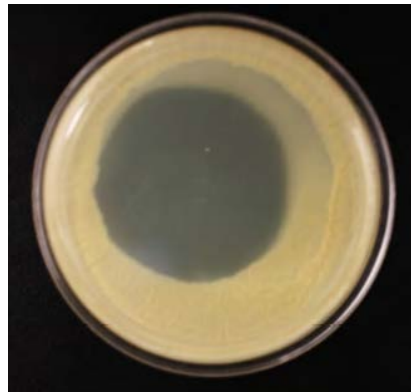


Figure 7: TDMH treated bacilli on Sauton's agar plate

2.3 DIAGNOSIS

Diagnosing an individual with tuberculosis follows a series of tests and steps that consist of medical history examination, tuberculin skin test, chest x-ray and bacteriologic examination.

What the majority of the population recognizes and has experienced is the tuberculin skin test. This test can recognize latent tuberculosis as well as active; however further testing must be done to confirm infectious tuberculosis infection. The test is performed by injecting a small amount of fluid called tuberculin into the skin in the lower part of the arm. The test is read in 48 to 72 hours by a trained health care professional who looks for an induration on the arm. An induration is a hard, raised area where the injection was. What determines a positive result is the size of the reaction to the site. It also depends on the individual's risk factors for infectious tuberculosis and progression to tuberculosis disease such as immunosuppression or exposure to other tuberculosis infected individuals. The disadvantage of the skin test is that it may produce false negatives, especially if the individual is co-morbid with sarcoidosis, Hodgkin's, lymphoma, and malnutrition [13].

If said individual tests positive with the skin test, a chest x-ray is performed to rule out pulmonary disease. A posterior-anterior chest radiograph is used to detect chest abnormalities. Lesions may appear anywhere in the lungs and may differ in size, shape, density and cavitation. These abnormalities may suggest tuberculosis infection but cannot definitively diagnose it.

Sputum samples are gathered and examined for tuberculosis via acid fast bacilli (AFB) using microscopy. AFB is rather simple and quick but again it cannot confirm the presence of *M. tuberculosis* as some acid-fast bacilli are not *M. tuberculosis*. Individuals with positive AFB smears are considered infectious, and then a culture is grown to definitively identify if the pathogen is *M. tuberculosis* [14]. The sputum culture is the cornerstone trademark test for definitive diagnosis of tuberculosis. The culture is grown in 7H9 broth or on 7H10 or 7H11 agar plate. Following this, the specimen may be tested for drug susceptibility, which requires an additional four weeks of testing.

There are challenges associated with using sputum cultures as a diagnostic tool. One such challenge is that the mycobacterium is very slow growing and it may take four to 12 weeks for the blood or sputum culture to grow. It is imperative to look at the HIV-infected community as a separate cohort as it is affected by tuberculosis differently. There are several drawbacks of this tool that are more specific to HIV-infected individuals. Tuberculosis is often difficult to diagnose in HIV-infected individuals because of the presence of nonspecific symptoms, atypical or normal chest radiograph findings. First, HIV-infected individuals do not produce much sputum and the bacterial count may be low, thus resulting in false negatives in sputum cultures. Second, because of the amount of time it could possibly take to diagnose someone, or the possibility of a false negative, the individual may be put on antiretroviral therapy (ART) for HIV, which could induce the latent tuberculosis to become active [15]. In addition to this, the potentially infected individual may have active tuberculosis and be exposing others to the disease, particularly other HIV-infected individuals with compromised immune systems when they go to a clinic for example [14].

When evaluating diagnostic tools, they should be examined for the following qualities: sensitivity, specificity, predictive value, speed, reproducibility, cost effectiveness, safety and simplicity. As far as laboratory techniques are concerned, there are various others that may be used for the recognition of tuberculosis, two of which were previously mentioned, AFB and sputum culture. However, in addition to those, there are others that may be potential candidates. Others that exist include those that are used through methods of culturing such as, micro colony detection on solid media; septi-AFB method; radiometric BACTEC 460; MGIT 960 mycobacteria detection system; MB/BacT system; ESP culture system II; microscopic observation of broth cultures [14]. As aforementioned, one consistent disadvantage with these

culture techniques is that the minimum time required is at least two weeks and may take up to 12 weeks. For the purpose of this paper, only those that are used for the detection of *M. tuberculosis* directly from clinical samples will be detailed.

There are other methods that are used as an alternative to smears. There is the phenotypic method, in which there is the FAST plaque technique which is a phage based technique which uses a mycobacteriophage to detect the presence of *M. tuberculosis* directly from sputum samples. There is Capture ELISA which can be used in the detection of tuberculosis in urine and this technique has 93% sensitivity and 95% specificity [14]. Detection of LAM in sputum samples is another that is based on the capture antibody from a murine source (murine monoclonal antibody against LAM). The rabbit anti-serum against *M. tuberculosis* is used as a source of detection against LAM in sputum. Next, there is antigen detection in bodily fluids. Here, free mycobacterial antigen at a specific concentration can be detected in biological fluids such as cerebrospinal fluid. The sensitivity here is from 40-50% and specificity is 80-95% [13].

Recently, nucleic acid amplification was integrated into the diagnostic algorithm of TB. This technique was further improved by molecular-beacon-based real-time polymerase chain reaction, or RT-PCR. RT-PCR is a technique by which specific, target DNA is simultaneously amplified and quantified. PCR entails the use of a pair of primers that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase such that a copy is made of the designated sequence. After making this copy, the same primers can be used again, to make not only another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to exponential amplification. This process is done through a series of heating and cooling to extend and anneal [14].

The components necessary to perform a RT-PCR in this study are the sample DNA, molecular probe, Applied Biosystems Taq Man master mix (contains the polymerase), forward and reverse primers. The primers used are tuberculosis specific 16s rRNA primers. 16s rRNA is used for phylogenetic studies; it is highly conserved between different species of bacteria and archaea and also contains hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. This is why it is used in bacterial identification in RT-PCRs and what makes RT-PCRs able to be so specific. The molecular beacon probe is a short oligonucleotide probe with a loop-and-stem structure. One portion of it is labeled with a fluorophore and the other with a quencher [16,17]. The loop contains a sequence that is complementary to the wild-type sequence of *M. tuberculosis*. When the complementary sequence is recognized it anneals to the amplicon on the beacon and will fluoresce.

In comparison to the other two techniques commonly used in tuberculosis diagnosis, AFB microscopy and sputum culture, the RT-PCR is a more rapid, specific and reliable technique, taking less than a day to attain results. However, it has one particular limitation which is highly relevant in the context of mycobacteria. The sensitivity of the technique is dependent on the efficiency of the DNA extraction from the target organisms. The robust membrane makes it difficult to lyse the mycobacteria by simple detergent such that DNA is exposed and as a result more mycobacteria are required to elicit ample amplification in the PCR. Therefore, an efficient lysis of *M. tuberculosis* is important for sensitive diagnosis of Tuberculosis. Until then, although this technique is promising, it will have this aforementioned drawback.

2.4 PUBLIC HEALTH SIGNIFICANCE

Tuberculosis remains a significant concern in the global community for many reasons, mostly because of the sheer magnitude of its reach. It continues to become a more complex problem with the emergence of its drug resistant varieties, MDR-TB and XDR-TB. With millions currently infected with the disease and millions more being infected each year, it is imperative to address this problem. Due to the nature of the pathogen, and its disproportionate effect socioeconomically speaking, it has been quite difficult to control and eliminate. In resource limited areas, the disease has a higher burden because of poor surveillance (in diagnosis), poor living conditions, treatment availability, and co-infection with other pathogens such as HIV.

One of the ways in which it can be addressed is by developing a better diagnostic tool for it. If a more sensitive and rapid tool was available, cases of tuberculosis could be identified more quickly and subsequently isolated and treated, thus preventing further spread of the disease and hopefully decreasing mortality rates. A better diagnostic tool, particularly in HIV infected individuals, could also significantly reduce deaths related to tuberculosis, which, as aforementioned, is responsible for one out of every three deaths [3].

With that being said, if the envelope of *Mycobacterium tuberculosis* could be disrupted such that bacterial DNA was released, this lysate could be used in conjunction with RT-PCR as a more sensitive and rapid diagnostic tool. The study explored whether through the use of an enzyme, TDMH, the bacterial membrane could be disrupted and then used in a RT-PCR, resulting in greater amplification.

3.0 THESIS AIMS

Tuberculosis affects more than just those who are infected with the causative pathogen, but also has an economic burden and puts others, especially vulnerable populations, at risk for disease. There is a clear demand for a better diagnostic tool to identify *M. tuberculosis*. Existing diagnostic tools, such as sputum culture, are time intensive and not accurate enough to address the needs of the community. RT-PCR is an attractive tool for the diagnosis of tuberculosis because of its sensitivity, specificity, and rapid nature. As a result, finding a way to use it successfully is desired.

It is known in our lab that the TDM-hydrolase can be used to remove the TDM glycolipid that is abundantly present on the membrane such that the envelope is disrupted leading to cellular lysis. The purpose of this study is to explore whether the product of this lysis, the lysate, could be successfully used in a RT-PCR scenario and provide improved amplification. If this indeed worked, it could be used as a diagnostic tool for *M. tuberculosis*.

The three chief aims of this thesis begun with developing the enzyme, rTDMH and testing its activity after which the first was, testing its effectiveness with the attenuated form of *M. tuberculosis*, MC² 7000 and running a RT-PCR. Second, testing if the enzyme is effective in more complex samples such as sputum, using MC² 7000. The final aim was examining the effectiveness of the enzyme in virulent *M. tuberculosis* in sputum samples.

Hypothesis: Can we use the rTMDH-mediated lysate of *M. tuberculosis* with the RT-PCR assay to enhance the assays sensitivity?

AIM #1: Test the utility of rTMDH in RT-PCR based detection of *M. tuberculosis* using liquid culture of attenuated *M. tuberculosis*.

AIM #2: Test the utility of rTDMH in simulated sputum sample spiked with attenuated *M. tuberculosis* in RT-PCR

AIM #3: Test the utility of rTDMH in simulated sputum sample spiked with virulent *M. tuberculosis* in RT-PCR

4.0 MATERIALS AND METHODS

4.1 MYCOBACTERIAL STRAINS USED

The mycobacteria used take a week to grow after the culture is passaged. When cells die, they are lysed, or broken open and cellular components including DNA are released. Because of this, younger cultures are used for the experiments. The amount of bacteria present in culture is determined by using a spectrophotometer. The desired optical density for the experiments was approximately 0.5.

4.1.1 *M. tuberculosis: mc² 7000*

mc² 7000 is the attenuated form of *M. tuberculosis*. and thus is a biosafety level 2 approved agent. The culture is grown at 37 degrees Celsius in a 50ml polystyrene bottle containing 7H9OADC media with 0.05% tween and pantothenic acid (100mg/ml).

4.1.2 Virulent *M. tuberculosis* (Edmonds)

Virulent mycobacteria are non-attenuated and pathogenic and due to its dangerous nature, it must be used in a biosafety level 3 laboratory. The culture is similar to that of its attenuated form

however, with the omission of pantothenic acid. Thus the culture is still grown at 37 degrees Celsius in a 50ml polystyrene bottle consisting of 7H9OADC media with 0.05% Tween.

4.2 SPUTUM

Dr. Anil Ojha PhD, had and has a standing IRB to obtain sputum samples for the studies. The sputum samples were provided by Dr. William Pasculle from UPMC. The samples did not have bacteria in them upon receiving them; they were later spiked with one of the two mycobacteria for the purpose of the experiment. Samples were stored at 4 degrees Celsius and placed on ice when they were removed.

4.3 AIM #1: TEST THE UTILITY OF RTMDH IN RT-PCR BASED DETECTION OF *M. TUBERCULOSIS* USING LIQUID CULTURE OF ATTENUATED *M. TUBERCULOSIS*.

4.3.1 Protocol for the development of rTDMH enzyme

4.3.1.1 Transformation protocol

50ul of competent *E. coli* cells were taken from -80 degrees Celsius freezer and 1ul of plasmid was added and mixed very gently. This was then placed in a 42 degrees Celsius water bath for one minute, after which, it was placed back on ice for two minutes. This temperature shock

enabled the plasmid to enter the *E. coli*, by disrupting the membrane. Following this, 1 ml of LB broth was added and placed on a shaker for 1 hour at 37 degrees Celsius. Next, it was spun down at 5K rpm for five minutes; 800ul were removed and the pellet was resuspended in the remaining liquid. The cells were then plated out on LB+CB agar plates for 12 to 18hrs. Carbenicillin, CB, is a bacteriolytic antibiotic used for selection. Only those colonies that are resistant to the antibiotic will remain, and those colonies are the ones that contain the plasmid. After, the colonies were scraped and placed in 2ml LB broth and placed on 37 degree shaker overnight.

The next day the sample was transferred to a flask containing one liter of LB broth and 1ml CB and placed on 37 degree Celsius shaker for three to four hours or until the optical density, o.d., read 0.6 to 0.8. After, IPTG was added (for .24 grams of IPTG in 1ml) and placed on 30 degree Celsius shaker at 160 rpm for 3 hours. The cells were spun down in four different bottles at 10K rpm for five minutes at 4 degrees Celsius and the supernatant was removed. After this, 10ml lysis buffer + 100ul imidazole were added to the cell pellet and resuspended and placed on ice for 30 minutes then sonicated 10 times. The sonication process entailed 10 seconds of sonication then 20 seconds on ice. After this the samples were spun down at 1200rpm for 30 minutes at 4 degrees Celsius. While the samples were being spun the agarose that would be later used was made. It involved combining 2ml agarose and 10ml binding buffer and this was washed 3 times. The final time the supernatant was removed and just the agarose remained.

The supernatant of the sonicated cells that were spun down for 30 minutes was added to the agarose and resuspended and put on a rotary at 4 degrees Celsius for one hour after which the sample was spun down at 4 degrees Celsius for five minutes at 2000 rpm and washed five times with 5ml of wash buffer. The final time, the pellet was resuspended in 4ml of wash buffer. This

product was then transferred to a column and placed over a glass tube to let it run through and the elution buffer was added in 500ul increments and let run through in separate microcentrifuge tubes seven times. The elution is done by Ni²⁺ chromatography; in which, the protein is His-tagged which binds to the beads and allows the rest to flow through. The protein is then eluted out by using imidazole containing elution buffer to release it from the beads. A SDS PAGE gel was run and three lanes were chosen that had the most heavily concentrated bands and those were used for the dialysis.

4.3.1.2 Dialysis

Dialysis is a method used to concentrate the enzyme in glycerol. A spectra/por dialysis reservoir was filled with a large volume of the appropriate dialysis buffer. The dialysate volume was 100 times the sample volume. (Example: 10ml sample → 1 L of dialysate). The reservoir was a larger 2L beaker. Following this, the dialysis tubing was cut into appropriate lengths; the closure was opened. The tubing was then inserted into the opened closure and re-clamped with approximately 3 to 5 mm extending from the closure. After this, the sample was loaded into the dialysis tubing through the open end and adjusted for length. The tubing was clamped perpendicular to the previous with the second closure. Next, the dialysis sample was placed in the appropriate dialysis buffer and a clean magnetic stir bar was dropped into the dialysis reservoir and placed at 4 degrees Celsius overnight. The next day it was transferred to a fresh centrifuge tube and immediately stored at -20 degrees Celsius.

The concentration of the enzyme was determined by using a spectrophotometer set at 260nm. Standards using BSA were measured by the spectrophotometer and plotted to develop a trendline and subsequent equation to determine the concentration of the measured sample.

4.3.2 Protocol for testing the activity of the rTDMH enzyme

4.3.2.1 Plating rTDMH on 7H11 agar plates in comparison to standard control of storage buffer

50ug of rTDMH were added to 10^7 mc² 7000 bacteria, and stored overnight at 37 degrees Celsius and the following day serial dilutions were made. Corresponding volume of storage buffer was added to another set of 10^8 mc² 7000 cells as a comparison. 10ul of each dilution was plated out on 7H11 agar plate and left for two weeks in a 37 degree Celsius incubator. See Figure 8 for a depiction of the plate layout

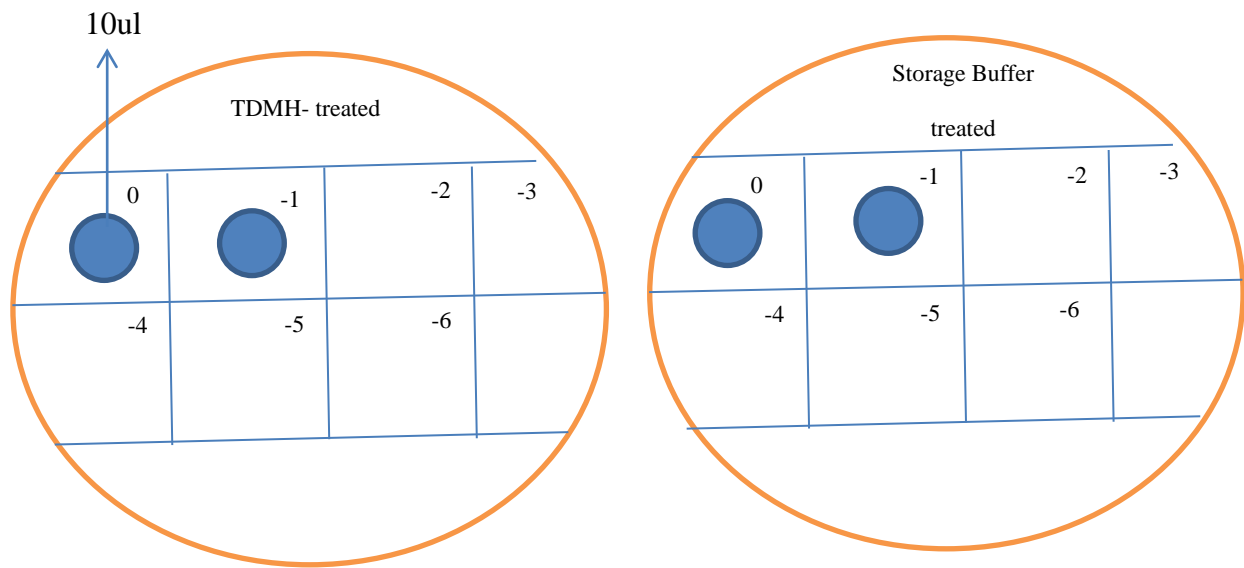


Figure 8: Diagram of Enzyme Testing on 7H11 agar plates by dilution factor

4.3.3 Protocol for genomic DNA preparation and isolation using rTDMH

The stock concentration of rTDMH used was 13ug/ul. 10^9 cells were taken and washed with 10ml PBS and resuspended in a microfuge tube in 1ml of PBS-asparagine (PBS-A). PBS-A was

used as it was determined to be the optimal buffer for rTDMH in comparison to PBS, PBS-tween, and NaCl. 20ul or 260 ug of rTDMH was added to one microfuge tube and placed on a rotator for two hours at 37 degrees Celsius. In a corresponding tube, 20ul of storage buffer was added to 10^9 cells in PBS-A as a standard control.

After two hours, 100ul of 10% sodium dodecyl sulfate (SDS) was added and mixed gently after which 10ul of 50mg/ml proteinase K was added, gently mixed, and placed at 55 degrees Celsius for 30 minutes. Following this, 200ul of 5M NaCl was added and gently mixed. NaCl blocks the binding of DNA to centrimide, which is present in the CTAB solution. At this point, the CTAB was preheated at 65 degrees Celsius and then 160ul of it was added to the sample, gently mixed and placed at 65degrees Celsius for 10 minutes. An equal volume of Chloroform: isoamyl alcohol (24:1 ratio) was added and shaken gently to mix and spun for five minutes at high speed. 900ul of the supernatant was transferred to a fresh microfuge tube and the extraction was repeated this time 800ul of the aqueous layer was transferred to a fresh microfuge tube. To the 800ul, 560ul (0.7 x the vol) of isopropanol was added and mixed via inversion until DNA had precipitated out of solution. Then the sample was incubated at room temperature for five minutes after which it was spun down at max speed for 10 minutes and the supernatant was removed and 1ml of 70% Ethanol was added to wash the DNA pellet and once again was spun at max speed for five minutes. The supernatant was removed and the pellet was allowed to air dry and then covered with 50ul of TE buffer and an electrophoresis gel was run to verify presence of DNA.

4.3.4 Incubating attenuated form of *M. tuberculosis*, mc²-7000 with rTDMH and subsequently run RT-PCR with sample Incubation Protocol

4.3.4.1 Incubation Protocol

First 10^9 cells were attained. 1ml of growing culture was taken and the optical density (o.d.) was measured. An o.d. of 4 means 10^9 cells. To achieve 10^9 bacteria, 4 was divided by the measured, o.d.

- Example: o.d. = x \rightarrow 4/x = #ml needed to reach 10^9 cells

The cells were subsequently spun down at 5K rpm for 10 minutes and the supernatant was discarded. The cells were resuspended in 10ml of PBS (to wash cells), and spun again at 5K rpm for 10 minutes. Once again the supernatant was discarded, and cells were resuspended in 1ml PBS-Asparagine (4g/L) and transferred to 1.5ml microfuge tube. The desired dilutions were then made. Of the dilutions, 10ul of each dilution was placed into a microfuge tube. 1ul of rTDMH (or 11 -17 ug) was added to each of the samples. For the non-protein control, 1ul (or corresponding volume) of storage buffer was added to matching sample set. The samples were then incubated at 37 degrees for 30 minutes. Following incubation the samples were removed and placed on a 75 degree hot plate for 20 minutes to inactivate the enzyme or any other cellular components or nucleases that could inhibit the reaction. Following this, the RT-PCR was set up

4.3.4.2 RT-PCR protocol

The protocol was completed on ice and in a dimly lit room as the molecular probe is light sensitive. The RT-PCR reaction had a total volume of 10ul. After samples were loaded to the

wells and covered, the plate was wrapped in foil to protect it from light. The components of the RT-PCR are as follows:

- 1ul of sample.
- 5ul Applied Biosystems Taqman master mix
- 4ul Millipore water
- 0.2 16S ul Molecular beacon probe (commercially available from Sigma-Aldrich)
- 0.1ul 16SRNA primer (Forward and Reverse primer mix)

RT-PCR Conditions for Applied Biosystems master mix (Using Applied Biosystems RT-PCR machine in Dr. Alice Tarun's, PhD, lab)

- 95 degrees 30 sec, 58 degrees 1min, 72 degrees 30 sec * 40 cycles

RT-PCR Samples

The dilutions used were the following: bacteria per 10ul RT-PCR reaction: 10^4 , 10^3 , 10^2 , 10, and 1. Data from the RT-PCR were plotted in an excel graph to compare the results between the storage buffer standard and the rTDMH treated bacteria. “NC” is negative control, “Protein” was the TDMH treated, “Buffer” was the storage buffer standard treated, and “PC” was the positive control, which consisted of purified genomic DNA.

4.3.4.3 Confirmatory Gel Electrophoresis

The electrophoresis gel separates segments of DNA by size via a current. Because DNA is negatively charged, the DNA will “run” through the gel from the anode to the cathode. Larger

pieces run slower than smaller and through this the pieces are separated by size. A standard ladder was run in one lane so that the size of the bands present in the sample could be determined. The gel that was run was a 1% Agarose gel and the components of which were as follows:

- 0.5 grams Standard Agarose
- 50 mL TBE buffer

These components are heated in a microwave for one minute, after which 0.4ul ethidium bromide was added, mixed thoroughly and added to the gel mold. After solidified it was placed in the apparatus in TBE buffer and 5ul of sample were added per well, with 3ul of a 100 base pair ladder added as a standard comparison to identify the size of the DNA.

4.4 AIM #2: TEST THE UTILITY OF RTDMH IN SIMULATED SPUTUM SAMPLE SPIKED WITH ATTENUATED *M. TUBERCULOSIS* IN RT-PCR

4.4.1 Spiking Sputum Samples with mc² 7000

First 10^9 cells were obtained. 1ml of growing culture was taken and the optical density (o.d.) was measured. An o.d. of 4 meant 10^9 cells. To achieve 10^9 bacteria, 4 was divided by the measured, o.d. The o.d. of the bacteria used was 0.482

- Example: $\text{o.d.} = x \rightarrow 4/x = \text{\#ml needed to reach } 10^9 \text{ cells}$

The cells were subsequently spun down at 5K rpm for 10 minutes, and the supernatant was discarded. The cells were resuspended in 10ml of PBS (to wash cells), and spun again at 5K rpm for 10 minutes. Once again, the supernatant was discarded and cells were resuspended in 1ml

PBS-Asparagine (4g/L) and transferred to a 1.5ml microfuge tube. The desired dilutions were then made. The sputum samples were spiked with the desired dilution value in 450ul of sputum. For example, if the dilution made was 10^8 then 50ul was added to 450ul of sputum to achieve a value of 10^7 bacteria per ml of sputum. The dilutions made were 10^6 , 10^5 , 10^4 , 10^3 per ml of sputum.

4.4.2 Protocol for Clarification of Sputum

First, a decontamination fluid was made by mixing equal volumes of sterile 1M NaOH solution and 0.1M sodium citrate, and then 5mg N-acetyl-L-cysteine was added per ml. One volume of decontamination fluid was added per volume of sputum sample and placed on shaker at 25 degrees Celsius for 20 to 25 minutes, after which the samples were spun down at 12K x g for five minutes, washed with 20mM Tris HCl and centrifuged again and the pellet was resuspended in PBS-Asparagine with 0.8 x the original sputum volume.

4.4.3 rTDMH Incubation and RT-PCR

The samples were treated with 11ug (2ul) rTDMH and a corresponding standard was treated with 2ul of storage buffer and placed at 37 degrees Celsius for 30 minutes. Following this, the samples were placed on a heat block at 75 degrees Celsius for 20 minutes to inactivate the enzyme and other lysate products that could inhibit the RT-PCR. After heat inactivation the RT-PCR was set up according to the RT-PCR protocol detailed in 4.4.2 and a subsequent electrophoresis gel was run to confirm results seen in RT-PCR data.

4.5 AIM #3: TEST THE UTILITY OF RTDMH IN SIMULATED SPUTUM SAMPLE SPIKED WITH VIRULENT *M. TUBERCULOSIS* IN RT-PCR

Because of the nature of the pathogen, the following was done in Bioscience Tower 3 on the University of Pittsburgh campus, in a Biosafety level 3, BSL-3 laboratory facility.

4.5.1 Spiking Samples with virulent *M. tuberculosis*

First, 10^9 cells were attained. 1ml of growing culture was taken and the optical density (o.d.) was measured. An o.d. of 4 meant 10^9 cells. To achieve 10^9 bacteria, four was divided by the measured, o.d. The o.d. of the bacteria used was 0.61.

- Example: $\text{o.d.} = x \rightarrow 4/x = \text{\#ml needed to reach } 10^9 \text{ cells}$

The cells were subsequently spun down at 5K rpm for 10 minutes and the supernatant was discarded. The cells were resuspended in 10ml of PBS (to wash cells) and spun again at 5K rpm for 10minutes. Once again the supernatant was discarded, and cells were resuspended in 1ml PBS-Asparagine (4g/L) and transferred to 1.5ml microfuge tube. The desired dilutions were then made. The sputum samples were spiked with the desired dilution value in 450ul of sputum. For example, if the dilution made was 10^8 , then 50ul was added to 450ul of sputum to achieve a value of 10^7 bacteria per ml of sputum. The dilutions made were 10^6 , 10^5 , 10^4 , 10^3 per ml of sputum.

4.5.2 Sputum Clarification

First, a decontamination fluid was made by mixing equal volumes of sterile 1M NaOH solution and 0.1M sodium citrate, and then 5mg N-acetyl-L-cysteine was added per ml. One volume of decontamination fluid was added per volume of sputum sample and placed on a shaker at 25 degrees Celsius for 20 to 25 minutes, after which the samples were spun down at 12K x g for 5 minutes, washed with 20mM Tris HCl and centrifuged again and the pellet was resuspended in PBS-Asparagine with 0.8 X the original sputum volume.

4.5.3 rTDMH treatment

The samples were treated with 11ug (2ul) rTDMH and a corresponding standard was treated with 2ul of storage buffer and placed at 37 degrees Celsius for 30 minutes. Following this, the samples were placed on a heat block at 75 degrees Celsius for 20 minutes to inactivate the enzyme and other lysate products that could inhibit the RT-PCR. After heat inactivation, the RT-PCR was set up according to RT-PCR protocol detailed in 4.4.2, and a subsequent electrophoresis gel was run to confirm results seen in RT-PCR data.

5.0 RESULTS

5.1 AIM #1 TEST THE UTILITY OF RTMDH IN RT-PCR BASED DETECTION OF *M. TUBERCULOSIS* USING LIQUID CULTURE OF ATTENUATED *M.* *TUBERCULOSIS*

5.1.1 Test the activity of rTDMH that was developed

10^7 mc² 7000 bacteria were treated with 50 ug of protein overnight at 37 degrees Celsius. After which, six serial dilutions were made and 10ul of each sample was placed on the 7H11 agar plate for 17 days. Looking at Figure 12, the storage buffer treated bacteria, at -4 there are approximately 18 colonies. If 18 colonies were present from 10ul of a 1mL dilution that meant that there were 18×10^6 bacteria per ml. This was done by multiplying 18 by 100, as 10 ul was taken from the original dilution and from there, because it was the -4 dilution, it was multiplied by 10^4 , thus giving, 18×10^6 . The same was done with the rTDMH treated plate, which can be seen in Figure 13. There appears to be roughly, 9 colonies at the -3 dilution. This means there are roughly 9×10^5 bacteria per ml. This is roughly a 2 log difference at 10^7 bacteria which, shows that the protein developed is decidedly active (See Figures 9 and 10).

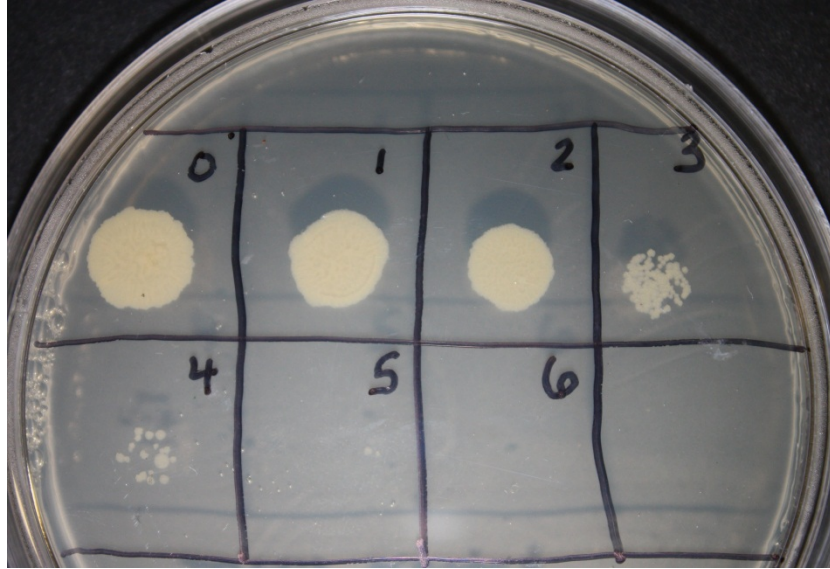


Figure 9: Storage Buffer treated MC² 7000 for 17 days to test protein activity

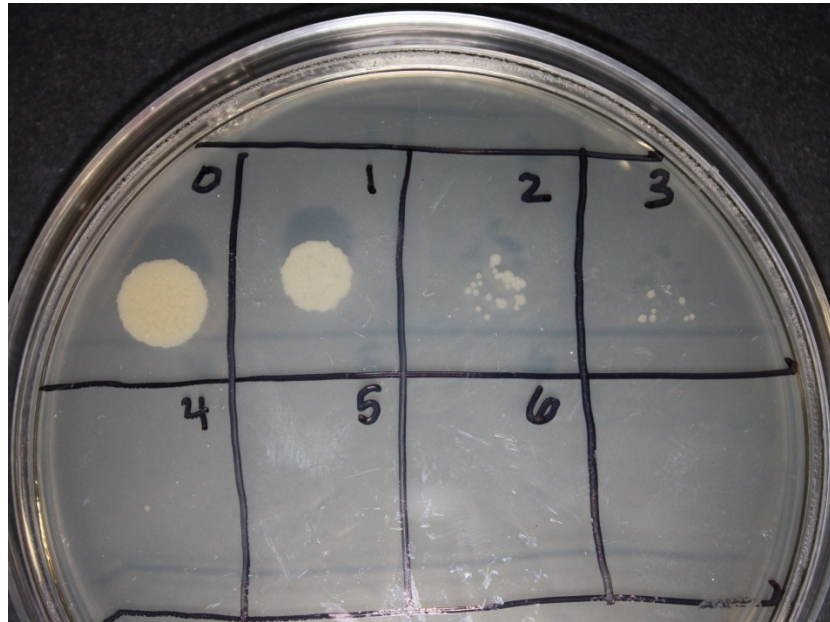


Figure 10: rTDMH treated MC² 7000 for 17 days to test protein activity

5.1.2 Genomic DNA preparation and isolation using rTDMH

An electrophoresis gel was run to compare the results between the standard laboratory CTAB DNA preparation and isolation procedure and the protocol developed using rTDMH (see Figure 11).

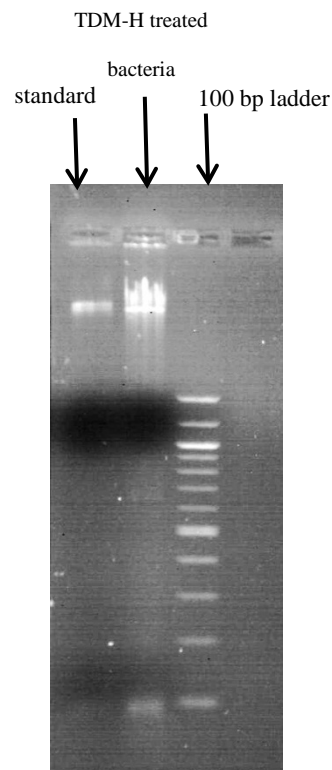


Figure 11: Genomic Isolation using rTDMH Electrophoresis Gel

Resulting data shows that, first rTDMH can be used to effectively isolate genomic DNA from mc² 7000. Second, that it produces a greater amount of DNA than the commonly used technique. Third, it is a significantly faster technique. The current technique takes over a day to complete and involves more procedures and in a laboratory setting this is invaluable.

5.1.3 Test the utility of rTDMH in RT-PCR using liquid culture of attenuated *M. tuberculosis*

The purpose of this experiment was to see if the rTDMH mediated lysate could be successfully used in a RT-PCR reaction. In addition to this, a comparative amplification between rTDMH treated and storage buffer treated was desired. It was expected that the rTDMH lysis would result in earlier and greater amplification of the sample versus the standard control. The dilutions used were the following, bacteria per 10ul RT-PCR reaction: 10^4 , 10^3 , 10^2 , 10, and 1. Data from the RT-PCR were plotted in an Excel© graph to compare the results between the Storage buffer standard and the TDM-H treated bacteria. (See Figures 12, 13, 14, 15, and 16).

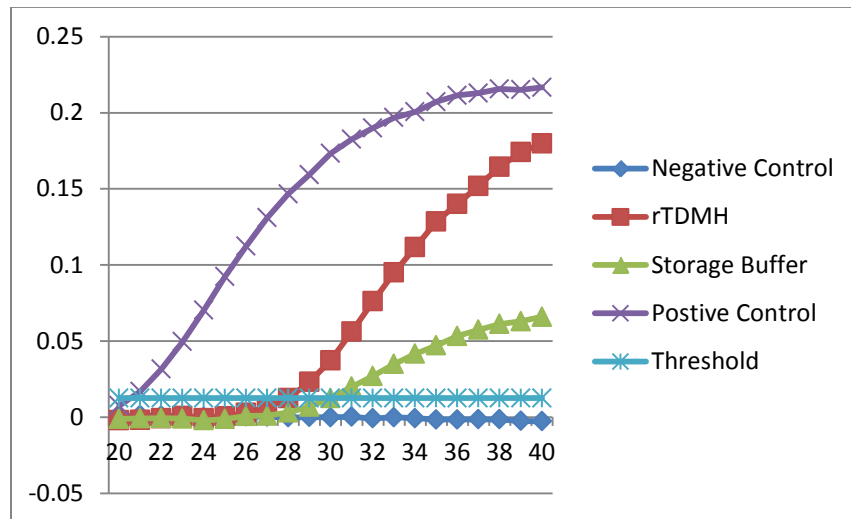


Figure 12: 10^4 bacteria per RT-PCR reaction

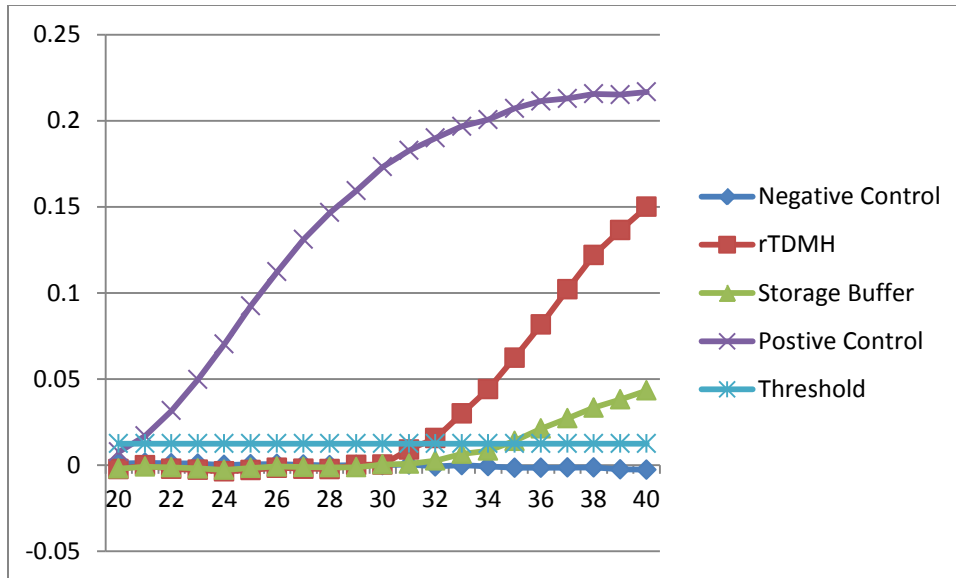


Figure 13: 10^3 bacteria per RT-PCR reaction

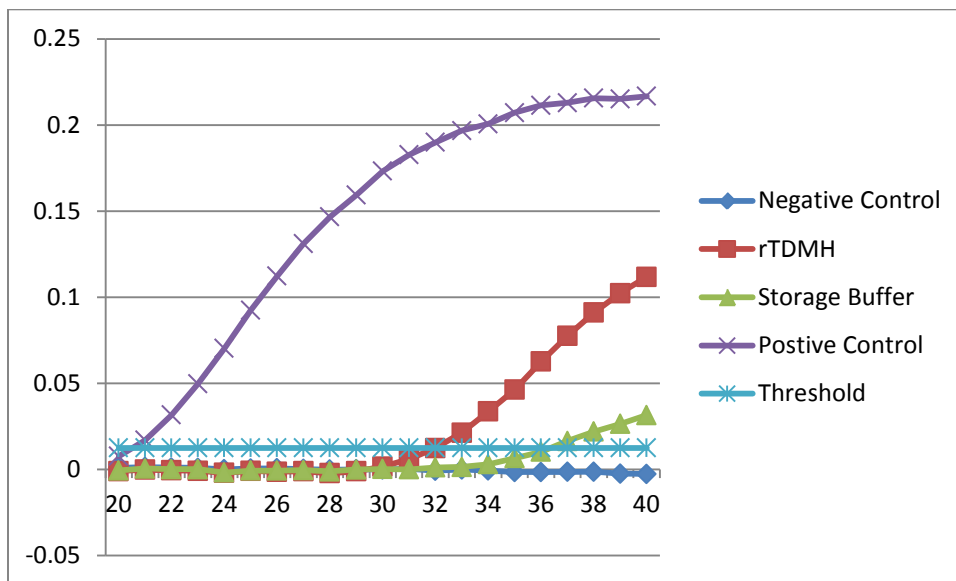


Figure 14: 10^2 bacteria per RT-PCR reaction

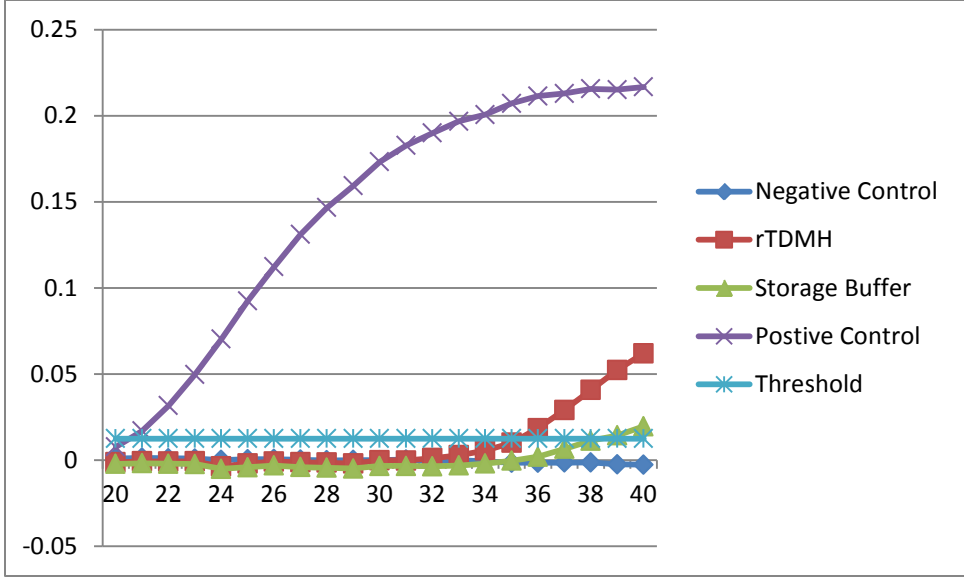


Figure 15: 10 bacteria per RT-PCR reaction

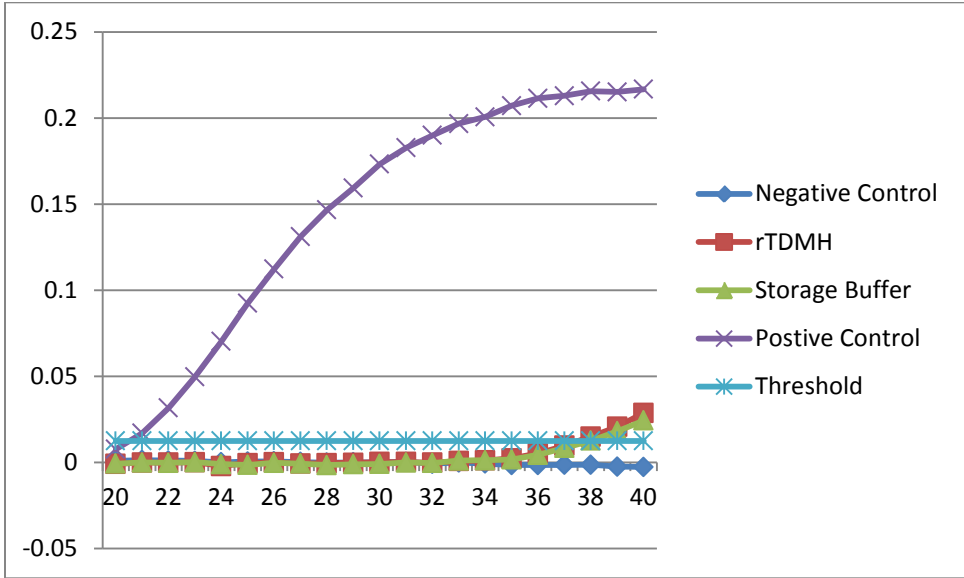


Figure 16: 1 bacteria per RT-PCR reaction

Tables 1 and 2 below compare the Ct values between those samples treated with storage buffer versus those treated with rTDMH. Ct value is the cycle in which the sample passes the threshold. The threshold is where the sample is amplifying exponentially. This value is what is used to compare the efficacy of the enzyme. If the samples have a one cycle difference that means that there is a two-fold difference between the amounts of DNA. If there is a 3.3 cycle difference that means that there is a 10-fold difference between the concentrations of DNA. Figure 17, nicely graphs the Ct value difference between the different samples. In every sample, the rTDMH treated sample has a lower Ct value, meaning it is reaching the threshold sooner. Take into considering 10^3 bacteria per a RT-PCR reaction as an example. Here it is seen that the Ct value of the storage buffer is 36.3, whereas the rTDMH treated sample is 31.98. This is a 4.32 cycle difference.

Table 1: Ct Values for Storage Buffer Treated Samples

Storage Buffer	C _T	C _T SD
1	37.95259	3.502306
10 ²	Undetermined	3.502306
10 ³	36.30987	3.502306
10 ⁴	34.6563	3.502306
10 ⁵	29.96415	3.502306

Table 2: Ct Values for rTDMH Treated Samples

rTDMH	C _T	C _T SD
1	37.60346	3.502306
10 ²	35.25035	3.502306
10 ³	31.98085	3.502306
10 ⁴	31.56207	3.502306
10 ⁵	27.93839	3.502306

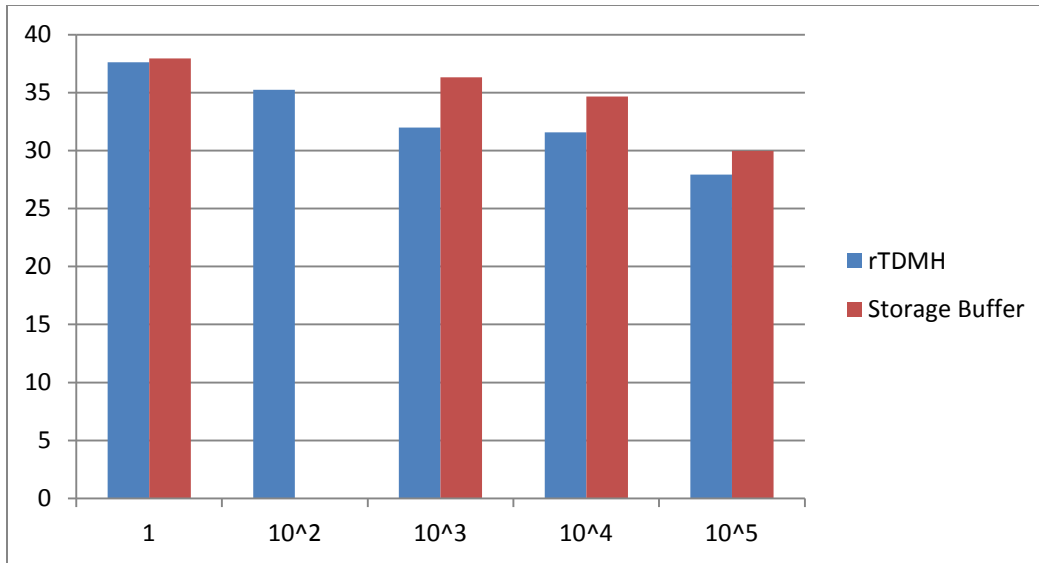


Figure 17: mc²7000 in liquid culture

The bacteria treated with rTDMH show a significantly higher level of amplification than those untreated, and the difference is most pronounced in reaction with 10 bacteria. As seen in figure 14, with 10 bacteria amplification is seen at the 35 cycle in rTDMH treated bacteria, whereas the storage buffer control is no longer eliciting any amplification. There is as well a trend that occurs from the highest dilution to the lowest, where at 10⁴ bacteria per reaction; amplification from rTDMH treated starts at the 28th cycle. The amplification subsequently begins later and later, such that at 1 bacterium, there is little to none seen. In every sample, the rTDMH treated bacteria has a higher and earlier amplification.

An electrophoresis gel was then run with a 100 base pair ladder to confirm the RT-PCR results. From left to right, the lanes are as follows: 100BP ladder, negative control (NC), 1 storage buffer (SB), 10 SB, 10² SB, 10³ SB, 10⁴ SB, 1 TDMH, 10 TDMH, 10² TDMH, 10³ TDMH, 10⁴ TDMH, and positive control (PC) (see Figure 17).

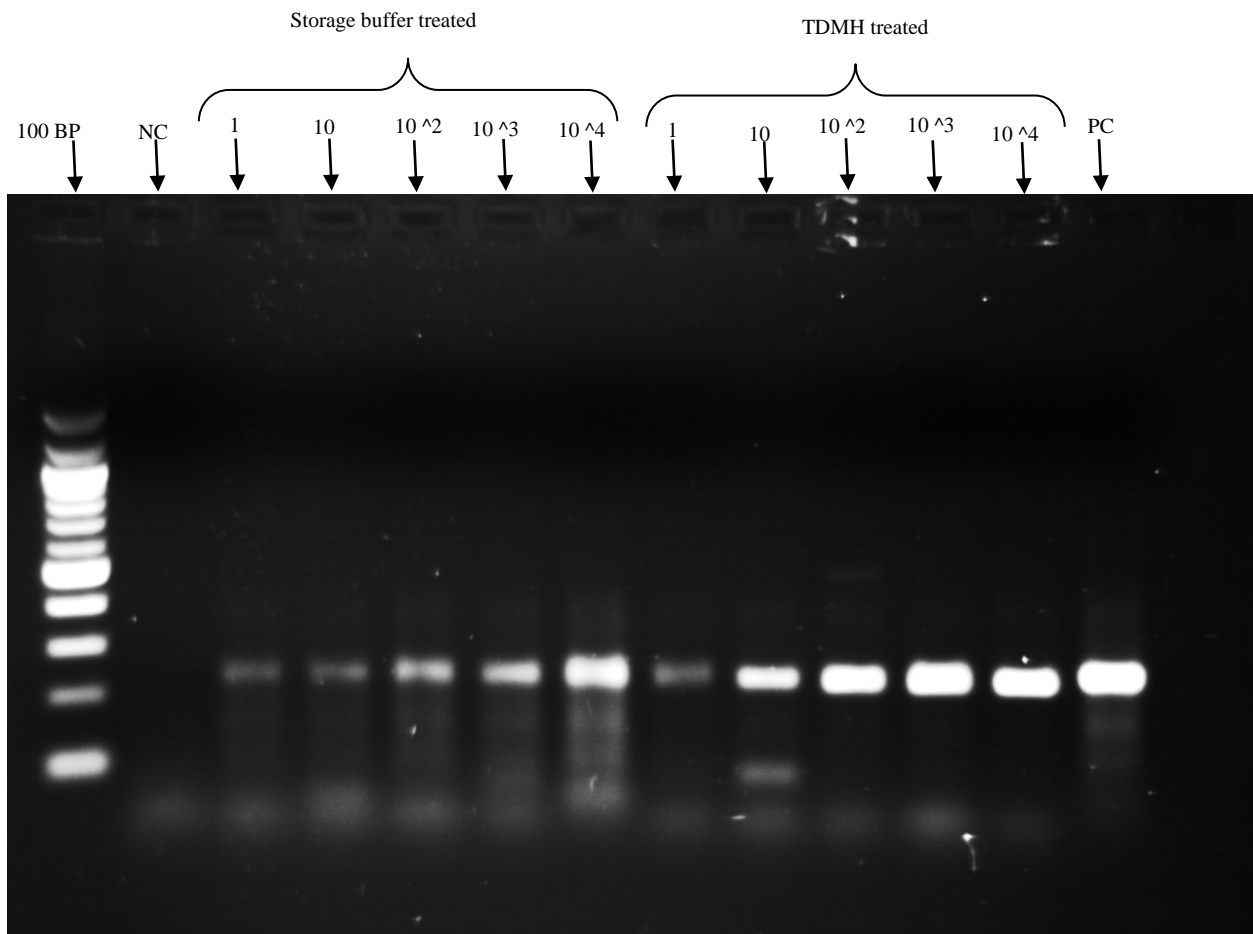


Figure 18: Electrophoresis gel of rTDMH and Storage buffer treated *mc*² 7000

Gel electrophoresis of the DNA in Figure 16 is fully consistent with the plots shown in Figures 11-15, in a way their lower Ct values show an increased amplification in bacteria that are treated with rTDMH first before the RT-PCR. Moreover, samples treated with enzyme storage buffer had less amplification, which faded away as the bacterial number decreased below 10^3 . Overall, these findings show that the attenuated *mc*² 7000 can be successfully treated with rTDMH and subsequently used in a RT-PCR reaction and not only elicit an amplification but a greater amplification than the cells that are not treated with rTDMH. Our next goal was to test whether rTDMH could be used in clinically relevant *M.tb* samples in which bacteria are present

in chemically complex environments. We chose to test this in sputum as this is the most common clinical sample used for diagnosis of Tuberculosis.

5.2 AIM #2: TEST THE UTILITY OF RTDMH IN SIMULATED SPUTUM SAMPLE SPIKED WITH ATTENUATED *M. TUBERCULOSIS* IN RT-PCR

The purpose of this experiment was to test if the activity of rTDMH was preserved in more complex chemical environments. Sputum in particular, is what is most commonly used for tuberculosis identification; with that said, if the activity was indeed persevered then it would be a promising potential option for tuberculosis diagnosis. Spiking a sputum sample is the closest alternative to an actual clinical sample. In this RT-PCR, the negative control had rTDMH in it as well to ensure that it was not the rTDMH that was showing background noise in the gel or amplification data. The dilutions of bacteria used were 10^4 , 10^3 , 10^2 , 10^1 bacteria per RT-PCR reaction (see Figures 18, 19, and 20).

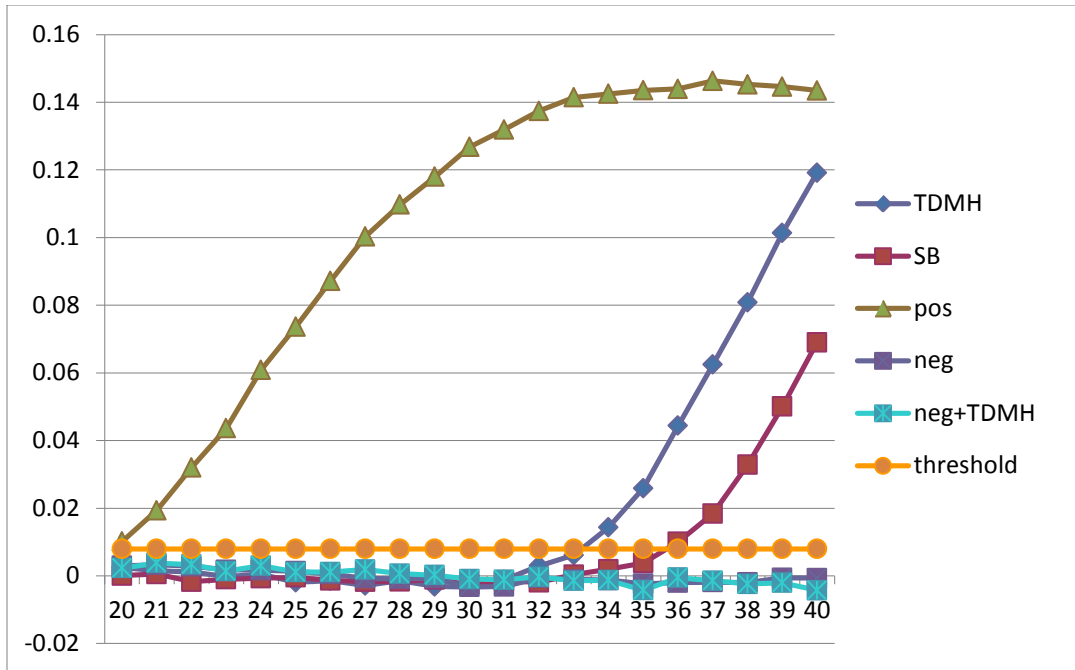


Figure 19: 10⁴ bacteria per RT-PCR reaction

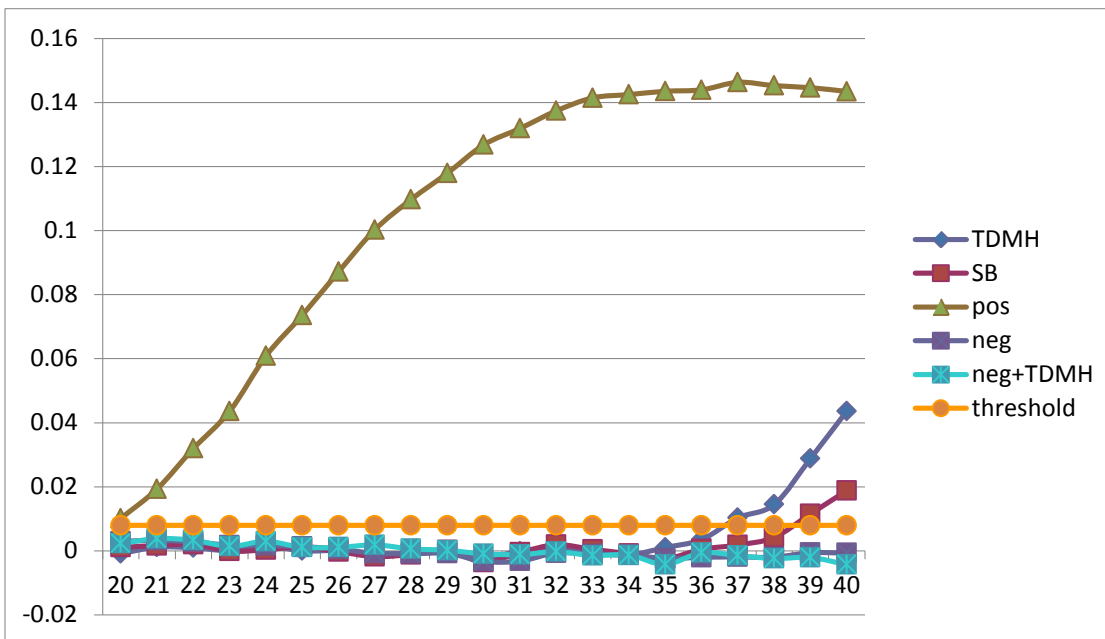


Figure 20: 10² bacteria per RT-PCR reaction

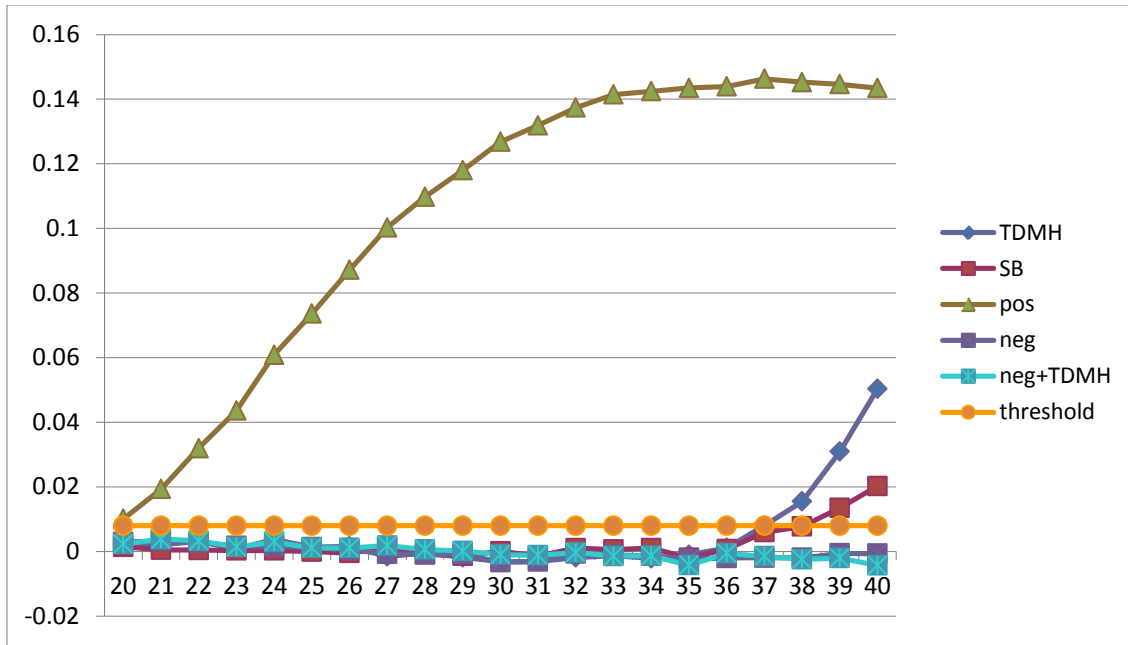


Figure 21: 10 Bacteria per RT-PCR reaction

Tables 3 and 4 depict the samples Ct values along with the average Ct value for each sample. Samples that are labeled: “undetermined”, mean that the sample never reach the threshold. With that said, in reality the average Ct value is in fact lower. In addition Figure 22 compares the average Ct values for the samples. In the case of 10^3 , although it appears as though the storage buffer treated sample has a lower average Ct value, this is not the case as it does not take into consideration the undetermined Ct value of the other sample and an accurate average was not attained. The actual average Ct value would be lower. Again, it is seen that the rTDMH treated samples produce a lower Ct value than those treated with the storage buffer. Take into consideration the lowest concentration of bacteria per a RT-PCR reaction. There is roughly 2.01 cycle difference between the two samples. Recall that for every cycle difference there is a two-fold difference in the concentration of DNA.

Table 3: Storage Buffer Sputum Samples with mc²7000

Storage Buffer	C _T	C _T 2	C _T Mean	C _T SD
10 ⁴	35.7551	36.18729782	35.97119891	1.77002
10 ³	35.36415	Undetermined	35.36415	1.77002
10 ²	38.51189	Undetermined	38.51189	1.77002
10	38.03784	37.69691086	37.86737543	1.77002

Table 4: rTDMH Treated Sputum Samples with mc²7000

rTDMH	C _T	C _T 2	C _T Mean	C _T SD
10 ⁴	33.22733	33.17180252	33.19956626	1.77002
10 ³	35.51291	36.52143097	36.017170485	1.77002
10 ²	33.0528	36.89831924	34.97555962	1.77002
10	36.03351	37.12096405	36.577237025	1.77002

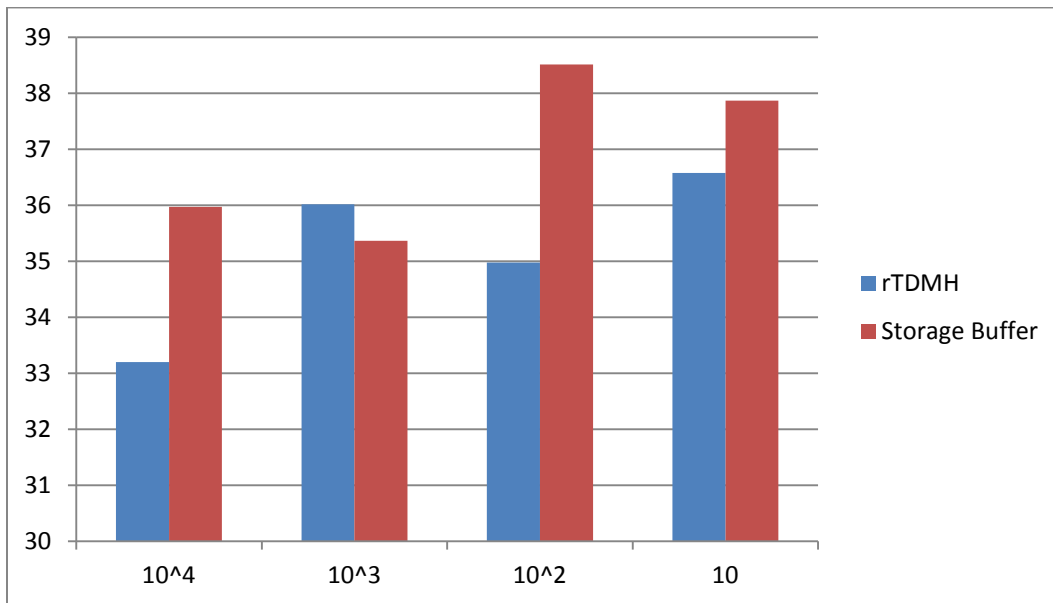


Figure 22: mc²7000 spike sputum samples in liquid culture

Results show that although the amplification is not as early as in the *in-vitro* culture, there is still an early amplification seen in samples treated with rTDMH versus those untreated. An electrophoresis gel was then done to compare to the results seen in amplification plots. Results in Figure 21 show the presence of the desired bands in samples treated with rTDMH, and faint to

no bands in samples treated with just storage buffer. To confirm that it is not the rTDMH itself that is creating this band, the negative control in the gel has rTDMH in it. Note the NC, negative control, does not have a band.

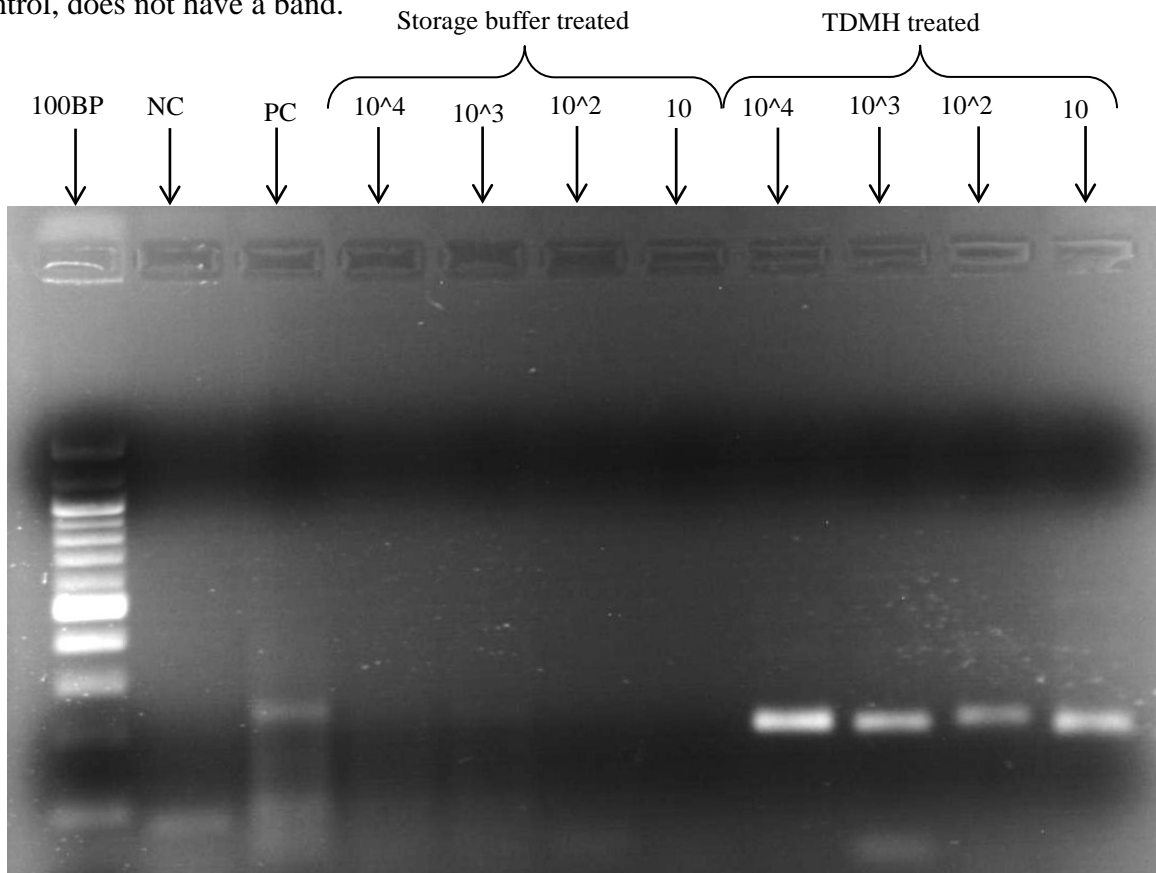


Figure 23: Electrophoresis Gel of Sputum Samples Spiked with MC2 7000

Results from AIM 3 led to the question, would similar results be seen in virulent *M. tuberculosis* as well?

5.3 AIM # 3: TEST THE UTILITY OF RTDMH IN SIMULATED SPUTUM SAMPLE SPIKED WITH VIRULENT *M. TUBERCULOSIS* IN RT-PCR.

This experiment was done because although mc²7000 is very similar to the virulent form, it still is not as sturdy as the virulent form. To accurately test the utility of the enzyme in a RT-PCR for an actual diagnostic tool, it must be tested in the most natural, typical setting, this being virulent *M. tuberculosis* in sputum. The dilutions for the RT-PCR reaction were 10⁴, 10³, and 10² per RT-PCR reaction. The resulting amplification plots can be seen in Figures 22, 23, and 24.

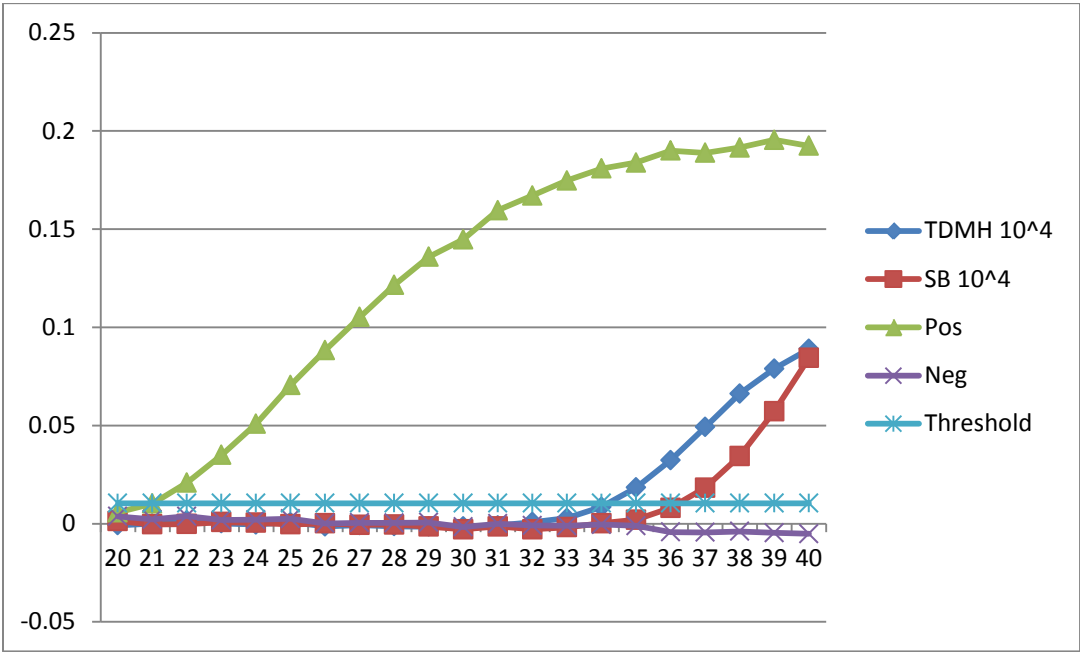


Figure 24: 10⁴ virulent bacteria per RT-PCR reaction

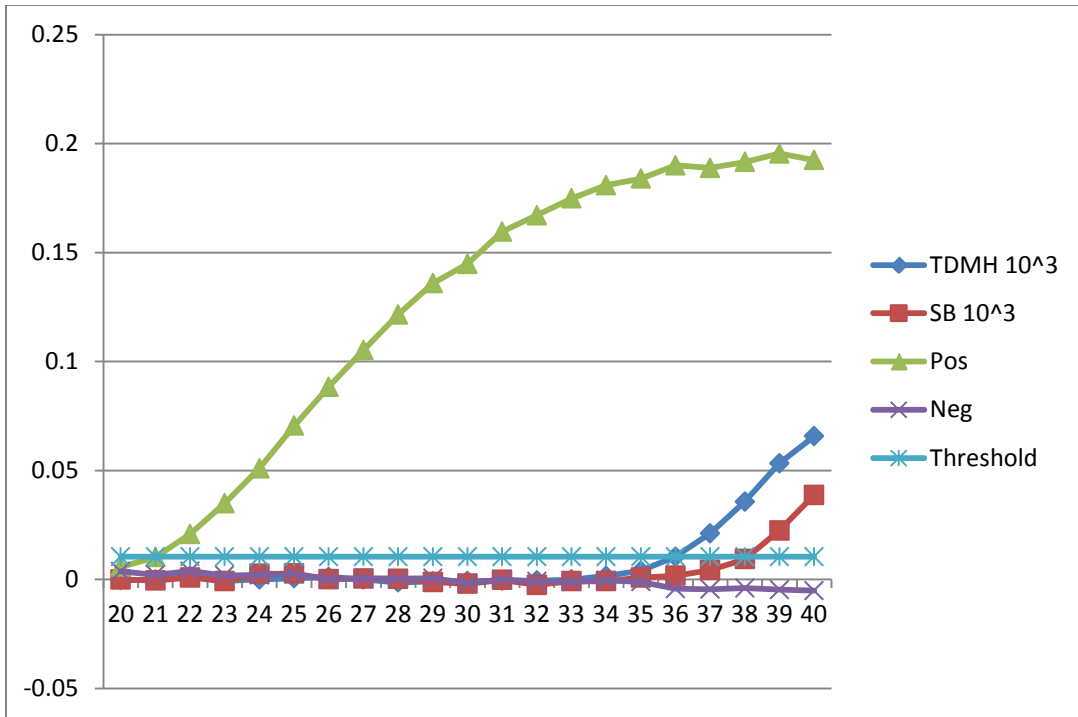


Figure 25: 10^3 virulent bacteria per RT-PCR reaction

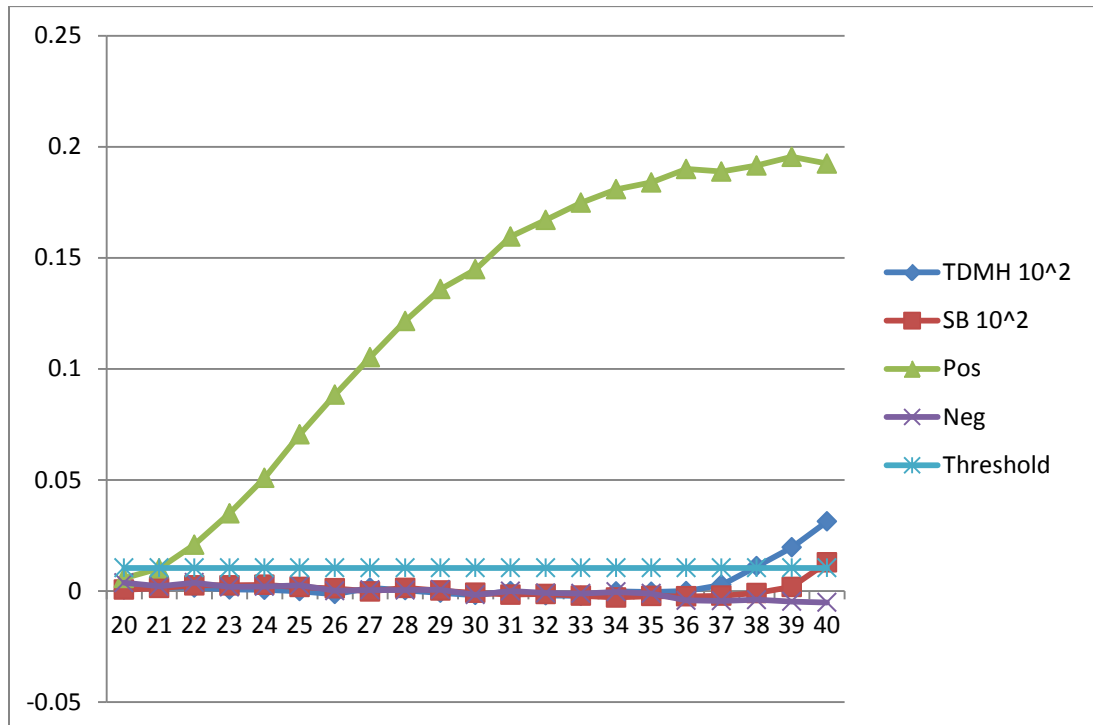


Figure 26: 10^2 virulent bacteria per RT-PCR reaction

Tables 5 and 6 depict the different Ct values for the samples and their associated average. Once again the rTDMH treated samples were producing lower Ct values than those treated with storage buffer. Even at 10 bacteria per a RT-PCR reaction which is the lowest concentration, there still is 1.4 cycle difference. Once again take into consideration that an undetermined value means that the sample never reached the threshold and the subsequent average Ct value is not truly reflective of the average.

Table 5: Storage Buffer Treated Sputum Samples with Virulent *M.tb*

Storage Buffer	C _T	C _T 2	C _T Mean	C _T SD
10 ⁴	35.22482	36.27316	35.74899	1.817498
10 ³	37.53482	38.08072	37.80777	1.817498
10 ²	37.89077	Undetermined	37.89077	1.817498
10	33.5439	35.58306	34.56348	1.817498

Table 6: rTDMH Treated Sputum Samples with Virulent *M.tb*

rTDMH	C _T	C _T 2	C _T Mean	C _T SD
10 ⁴	35.62404	34.17641	34.900225	1.817498
10 ³	38.07567	35.94001	37.00784	1.817498
10 ²	34.58217	38.00724	36.294705	1.817498
10	33.14672	33.20918	33.17795	1.817498

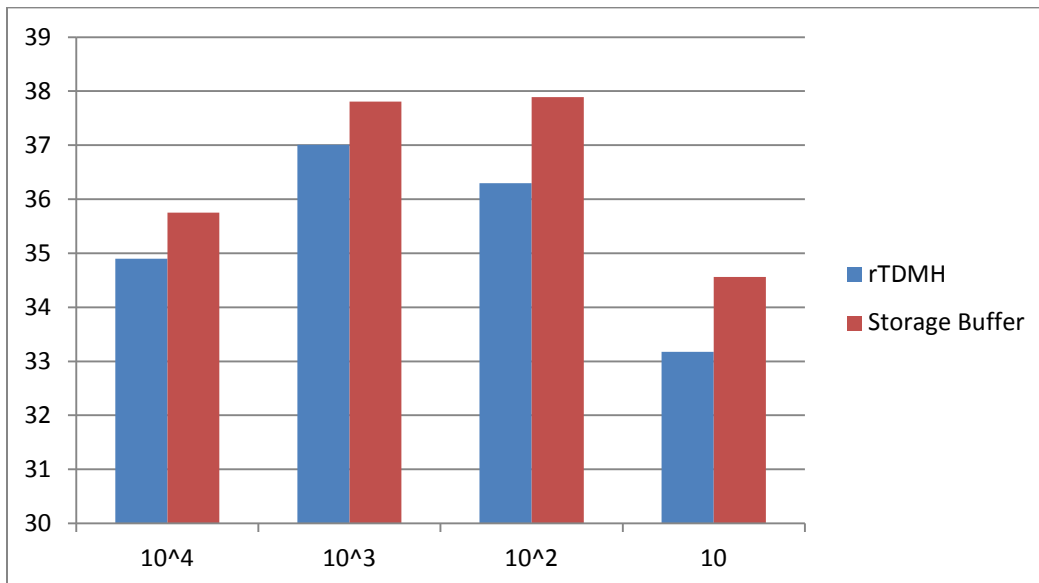


Figure 27: virulent *M. tb* spiked sputum samples

Results show an earlier amplification in bacteria that were treated with rTDMH versus those that were not treated. Once again an electrophoresis gel result, seen in Figure 25, was run to compare results seen in the amplification plot. Results here show a clear difference between samples treated rTDMH and those with storage buffer. As the concentration of bacteria decreases, a difference is also seen. At 10^2 bacteria there is no longer a visible band present in the storage buffer treated samples; however, one still is seen, and rather brightly, in samples treated with rTDMH.

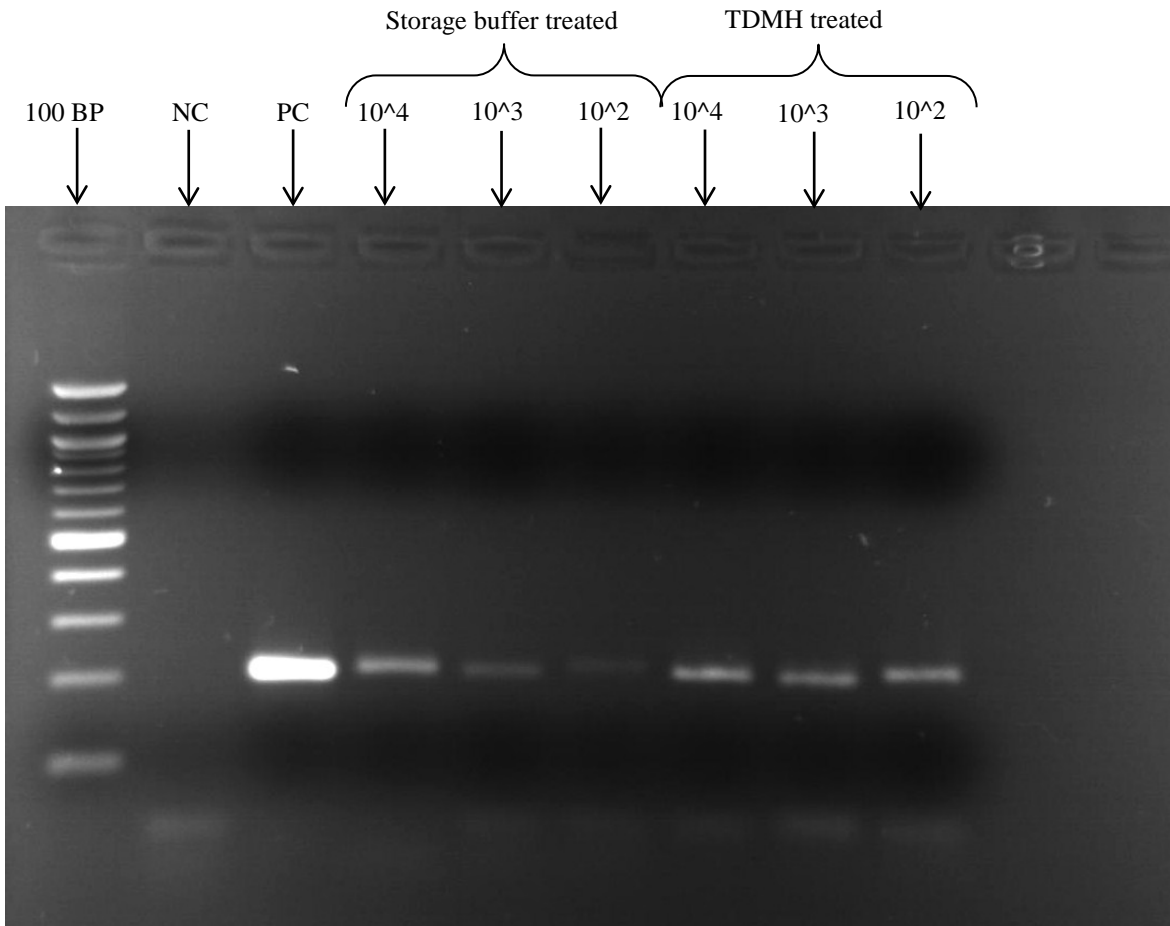


Figure 28: Electrophoresis Gel of virulent bacteria in sputum

6.0 CONCLUSION

Tuberculosis is a disease that affects our entire global community; although it does disproportionately affect resource limited areas more, resource rich countries are not excluded from the reach of this disease. This is why it remains an integral part of the World Health Organization's, Millennium Development Goals in number 6 part C [18]. Despite advancements, in technology it is one of the most prominent diseases in the world. WHO estimates that over one-third of the world's population is infected with this disease and this fact in and of itself is reason enough to allocate attention to it. But more than this, an increasing number of people are being diagnosed with infectious tuberculosis each year, approximately 9 million, and of those infected with the pathogen, three million die directly because of the disease [3]. Moreover, it is the number one killer of HIV infected individuals, with one out of every three deaths of HIV infected individuals being attributable to tuberculosis.

This disease has been present for thousands of years, and current treatments and diagnostic tools are not sufficient to significantly decrease the presence this disease has in our global community [1]. The diagnostic tools currently used not only take weeks to yield results but in HIV infected patients, sometimes result in false negatives, putting them and others at risk for contracting infectious tuberculosis. Real-time Polymerase Chain Reaction, RT-PCR, is the preferred method of disease diagnosis because of its specificity and rapid nature. The pathogen, *M. tuberculosis*'s, unique membrane structure makes it particularly difficult to use RT-PCR

effectively in current real-world diagnosis [12]. Its robust membrane makes it such that insufficient DNA is usually obtained to elicit the proper amplification that would be necessary to use as a diagnostic tool.

The purpose of this study was to develop an improved diagnostic tool that could potentially be applicable in real-world scenarios, yielding faster, more sensitive and accurate results than currently seen in tuberculosis diagnosis today. Results showed that it is possible to use a hydrolase, TDMH, for the glycolipid, TDM, which is found on the cellular membrane of *M. tuberculosis* to disrupt the cellular membrane, lyse it, thus releasing genomic DNA and then use that lysate in the RT-PCR tool. This would solve the problem of insufficient genomic DNA that is currently attributed to using RT-PCR.

Results were consistent in the *in-vitro* culture samples with MC² 7000, sputum samples spiked with MC² 7000 and sputum samples spiked with virulent *M. tuberculosis*. In each of these scenarios, the TDMH treated samples showed earlier amplification than those that were treated with storage buffer. The difference between the three samples was in the amount of amplification seen. *In-vitro* samples showed a much higher yield and amplification than those in sputum; however, sputum samples are the more real-world environment. Between sputum samples spiked with MC² 7000 and those with virulent *M. tuberculosis*, the results were more similar, although there was a slight earlier amplification and yield in those with the MC² 7000.

The inconsistency seen in sputum samples could be due to limitations of recovering same number bacteria after every clarification process. Furthermore, its consistency alone may make it more difficult to ensure that bacteria are thoroughly mixed. Also it is difficult to attain precise volumes of sputum.

In addition to this, the reason why the amplification may be later in sputum samples versus *in-vitro* culture could be due to bacterial loss in the clarification process. Because of the number of steps involved, every time the sample was washed and the supernatant removed, some bacteria could have been lost.

Due to lack of *Mtb*-infected sputum, we chose to use *Mtb*-spiked sputum. Although spiking is just one step away from clinical samples, it is noteworthy that spiking doesn't reproduce the embedding of bacteria in the sputum matrix. However, results of the spiked sputum are promising and pave the road for further work with clinical samples. The results show that it does successfully yield increased amplification in comparison to those simply treated with storage buffer. This suggests that it has the potential to be used in clinical settings as a diagnostic tool for tuberculosis. Having a simple, quick, sensitive and accurate tool such as this to use in diagnosis of tuberculosis could be significant in the fight against the tuberculosis epidemic. If such a tool were used in real-world settings, earlier diagnosis could occur, preventing the spread of infectious tuberculosis and reducing the mortality associated with tuberculosis. In addition, HIV infected individuals could be accurately tested for tuberculosis, preventing the false negatives that occur. These false negatives subsequently result in a higher risk of attaining active tuberculosis. If this occurred, tuberculosis associated death in HIV infected individuals would decline and given it is the leading cause of death in HIV infected individuals, the overall number of deaths of HIV infected individuals would correspondingly decrease. Thus the public health significance of this study is substantial. Using TDMH with RT-PCR could potentially be used as a better diagnostic tool which could result in reducing the spread of disease; reducing the mortality associated with disease, especially in in HIV infected individuals; and on a broader scale, reduces the economic burden associated with the disease.

6.1 FUTURE WORK

There are more experiments that must be done before it can be definitively said that this optimized RT-PCR tool that was developed actually can be applied in real-world settings. At this point it can be successfully used on spiked samples of sputum. The next step in studying its effectiveness is to test it in actual clinical blood, tissue, and sputum samples. These samples would not be artificially spiked with *M. tuberculosis*, rather the samples would already contain it. This would be the ultimate test to see if it is a viable option for an improved diagnostic tool.

APPENDIX A

GLOSSARY OF TERMS

BSL-2 Laboratory: suitable for work involving agents of moderate potential hazard to personnel and the environment. It includes various bacteria and viruses that cause only mild disease to humans, or are difficult to contract via aerosol in a lab setting. (MC² 7000 is used here).

BSL-3 Laboratory: This level is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease after inhalation. It includes various bacteria, parasites, and viruses that can cause severe to fatal disease in humans, but for which treatments exist. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets, specially designed hoods, or other physical containment devices, or by personnel wearing appropriate personal protective clothing and equipment. (Virulent *M. tuberculosis* is used here).

Carbenicillin: a bacteriolytic antibiotic preferred as a selecting agent.

Clarification: the process by which the sputum is decontaminated.

Competent Cells: competence is the ability of a cell to take up extracellular DNA from its environment.

CTAB: Cetrimonium bromide, used in the buffer solution for the extraction of DNA. It is a cationic surfactant.

Ethidium Bromide: an intercalating agent. It is used in agarose gel electrophoresis to identify DNA when under ultra-violet light.

Gel Electrophoresis: (Agarose Gel Electrophoresis): The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA, it will move toward the positive pole. Larger molecules will be slowed down more than smaller molecules, since the smaller molecules can fit through the holes easier. As a result, a mixture of large and small fragments of DNA that has been run through an agarose gel will be separated by size.

mc² 7000: the non-pathogenic, genetically attenuated form of the virulent *M. tuberculosis*. Approved for BSL-2 laboratory usage.

Lysis: The disintegration of a cell by rupture of the cell wall or membrane

Lysate: the product of the lysis. The fluid containing the contents of the lysed cells.

RT- PCR: a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification.

Sodium dodecyl sulfate polyacrylamide: It can be used to aid in lysing cells during DNA extraction and for unraveling proteins in SDS-PAGE. It is commonly used in preparing proteins for electrophoresis in the SDS-PAGE technique. This compound works by

disrupting non-covalent bonds in the proteins, denaturing them, and causing the molecules to lose their native shape (conformation).

SDS- PAGE: a protein separation technique that uses sodium dodecyl sulfate. It is a method of separating proteins according to their electrophoretic mobility.

Sonication: the act of applying sound energy to agitate a sample. For example, it is used in this study to disrupt cell membranes and release cellular contents. Sonication is also used to fragment molecules of DNA, in which the DNA subjected to brief periods of sonication is sheared into smaller fragments.

Transformation: is the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s). In this study, the cell lines used were competent *E. coli* bacteria.

APPENDIX B

ACRONYMS

100BP: 100 Base Pair ladder, used in gel electrophoresis

AFB: Acid Fast Bacilli microscopy

CB: Carbenicillin

CTAB: Cetrimonium bromide

MDR-TB: multiple drug resistant tuberculosis

NC: negative control

O.D.: optical density measured in spectrophotometer

PC: positive control

RT-PCR: Real- Time Polymerase Chain Reaction

SDS: sodium dodecyl sulfate polyacrylamide

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TDM: trehalose 6,6'-dimycolate

TDMH: hydrolase of trehalose 6,6'-dimycolate

XDR-TB: extremely drug resistant tuberculosis

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