COMPARISON OF CROSS PRIMING AMPLIFICATION AND LOOP MEDIATED AMPLIFICATION FOR TUBERCULOSIS DETECTION IN AN INTEGRATED DIAGNOSTIC DEVICE

By

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

December, 2013

Nashville, Tennessee

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ACKNOWLEDGEMENTS

I would like to acknowledge the Bill and Melinda Gates Foundation for their funding of this research. I would like to thank my advisor Rick Haselton and collaborator David Wright. I would also like to thank our collaborators at Ustar Technologies, Hangzhou, Ltd, particularly Sam Pickerill and Qimin You for their help with this research. I would also like to thank PATH for generously providing surrogate sputum samples. I would also like to thank members of the Haselton and Wright lab for their help and valuable advice.

Finally, I would like to thank my family for their support.

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LIST OF ABBREVIATIONS

BCG	Bacilli Calmette-Guerin
CPA	Cross priming amplification
Ct	Cycle threshold
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
dsDNA	double stranded deoxyribose nucleic acid
GuSCN	Guanidine thiocyanate
HIV	Human immunodeficiency virus
IFN-γ	interferon-gamma
IGRA	interferon-gamma release assay
INH	Isoniazid
LAM	Lipoarabinomannan
LAMP	Loop mediated amplification
ManLAM	Mannose-lipoarabinomannan
mg	Milligram
mL	Milliliter
PCR	Polymerase chain reaction
PZA	Pyrazinamide
RFU	Relative fluorescence unit
RIF	Rifampin
ssDNA	single stranded deoxyribose nucleic acid
TB	Tuberculosis
TST	Tuberculin skin test
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Global Health Impact of Tuberculosis

Tuberculosis (TB) is an infection caused by the bacteria *Mycobacterium tuberculosis*. It generally occurs within the lungs (pulmonary tuberculosis), though it can occur elsewhere in the body such as in the lymphatic system or central nervous system (extrapulmonary tuberculosis) (Frieden 2003). Disease symptoms include fever, night sweats, weight loss, persistent cough, and the production of blood-tinged sputum(Frieden 2003). The disease occurs in two states: latent and active tuberculosis. In the latent state of tuberculosis, the bacteria are contained within granulomas of the host and are not replicating or being disseminated. This state may persist throughout the host's life without active TB ever developing; however, 10% of patients with latent tuberculosis will develop active tuberculosis within their lifetime. In the active state, the bacteria start replicating and the infection can spread to other parts of the body and to other people. According to the World Health Organization (WHO) Annual Report, one out of every three people is infected with the latent form of tuberculosis(WHO 2011). The risk of active tuberculosis disease increases dramatically for those infected with human immunodeficiency virus (HIV), from 10% over their lifetime to a 10% chance of developing active tuberculosis each year (Millet 2012). In HIV-infected individuals, the weakened immune system is not able to effectively isolate and contain the bacteria within granulomas. TB is the direct cause of death for half of those who die with HIV. In 2010, 350,000 people died from HIV coinfection with TB. There were also 1.1 million tuberculosis deaths in HIV-negative individuals, making it the second leading

cause of death worldwide (WHO 2011). However in the United States, only 3.2% of the population is estimated to have latent tuberculosis (CDC 2010). The low rates of tuberculosis in the United States are partly due to the prevention programs in place (Gordin 2012) and greater access to healthcare (Millet 2012). TB more frequently occurs among those living in impoverished conditions, the elderly, and those who are immunocompromised (Millet 2012). The disease burden falls most heavily on developing countries that lack the healthcare infrastructure to properly diagnose and treat the disease.

Mycobacterium tuberculosis

The primary cause of tuberculosis is the bacterium *Mycobacterium tuberculosis*(Willey 2008). The bacteria *Mycobacterium bovis* and *Mycobacterium africanum* also cause the disease, but this is less common and occurs mainly in animals. *M. tuberculosis* is related to other mycobacterial species such as *Mycobacterium leprae*, the causative agent of leprosy, and the nonpathogenic *Mycobacterium smegmatis*. *M. tuberculosis* is a rod-shaped, gram-positive bacteria (Brennan 2003). Like other gram-positive bacteria, *M. tuberculosis* contains peptidoglycan, a cross-linked polymer of amino sugars and amino acids. However, the mycobacterial species are unique in that the peptidoglycan in their cell envelope is usually attached to lipids instead of to proteins, as is found in other gram-positive bacteria. The bacterial envelope is composed of two layers: a plasma membrane, and a cell wall that surrounds it (Brennan 2003). The cell wall contains mycolic acids such as lipoarabinomannan (LAM) or mannose-lipoarabinomannan (ManLAM), trehalose dimycolate, and phthiocerol dimycocerosate. These lipids are toxic to eukaryotic cells and are thought to form a hydrophobic barrier around the bacterium. This barrier inhibits the activity of anti-microbial agents by preventing their diffusion across the plasma membrane. It

also makes bacterial cell lysis difficult, as the wall is robust and inhibits the diffusion of lysis chemicals. Overall, the unique cell-wall lipids compose about 60% of the cell wall (Kolattukudy 1997). Mycobacteria also contain a large amount of different very long-chain saturated (C18-C32) and monosaturated n-fatty acids. A major cell-wall polysaccharide is arabinogalactan. The unique lipids and polysaccharides of *M. tuberculosis* give the species its robustness.

Host Response to Bacteria

Once *M. tuberculosis* enters the host via aerosolized droplets formed during coughing, the bacteria will activate the mannose receptors on macrophages causing the macrophages to phagocytose the bacteria. The ManLAM component of the cell wall is thought to play an important role in causing phagocytosis (Torrelles 2012). Inside the macrophage, the bacterium inhibits phagosome-lysosome fusion by altering the phagosome membrane (Willey 2008). Thus it is able to exist within the macrophages without being destroyed by the macrophage's normal physiological processes. Once the bacteria cells are phagocytosed, the macrophages release cytokines and hypersensitivity results in small hard nodules being formed. These are called tubercles, and may show up on chest X-rays as lung infiltrates. The tubercles contain bacteria, macrophages, T-cells and human proteins. The disease usually stops at this stage, existing as latent tuberculosis. The tubercles may eventually develop a cheese-like consistency, called a caseous lesion, or harden into a Ghon complex. The tubercle lesions can liquefy and form pulmonary or tuberculous cavities within the lungs, which allow the bacteria to spread throughout the body. This is classified as miliary tuberculosis (Willey 2008).

The immune response to tuberculosis is not fully understood and a unique antibody or host biomarker has yet to be found. Much research has been devoted to this area because finding a unique biomarker would enable the development of an accurate diagnostic test and could lead to a reliable vaccine. Currently, the only vaccine is the bacilli Calmette-Guerin (BCG) vaccine, but this has demonstrated variable effectiveness. It is more commonly used outside the US where exposure is more likely. In specific cases, such as with children who cannot avoid exposure to an adult with a drug resistant TB or with health care workers who have frequent exposure to those with tuberculosis the vaccine may be administered.

Spread of the Disease

Once the bacteria begin to reproduce and spread within the host, the disease is classified as active tuberculosis. At this point, the disease is contagious and is spread through coughing which produces aerosolized droplets. The bacteria travel in these aerosolized droplets. They may persist for 30 minutes outside of the host and can travel through air ducts in building ventilation systems. This is particularly a problem in confined areas, such as prisons or hospitals. This affects low resource areas as well, as overcrowding is a problem (Millet 2012). It has been shown the disease is less likely to be transmitted in outdoor settings (Gordin 2012). When left untreated a person with active TB will infect 10-15 people per year on average (WHO 2002). Most commonly, the infection is contained initially by the host immune response and becomes latent. In rare cases, the disease progresses directly to the active form.

Treatment of Tuberculosis

The current standard treatment for the active form of tuberculosis is a six month regimen using isoniazid (INH), rifampin (RIF), ethambutol (EMB) and pyrazinamide (PZA)

for an initial two month period to stop the actively dividing bacteria. After this, a consolidation phase occurs for the subsequent four months where INH and RIF are continued. This is to ensure that the slow dividing bacteria are killed as well. Dosing frequency can vary depending on patient history and response, but in the initial phase daily or twice or thrice weekly treatments is common and in the consolidation phase twice or thrice weekly treatment continues (Gordin 2012). INH, RIF, EMB and PZA are considered the first-line of anti-tuberculosis treatment drugs (Blumberg 2003). Other drugs may be used subsequently if the patient does not respond to this initial pharmaceutical combination. The long treatment time of six months is a disadvantage. Patient compliance is low for completing the regiment, especially when symptoms of the active disease are relieved within the first few months. This is particularly noticeable in low resource settings, where access to medical facilities and pharmaceuticals are limited. Lack of treatment compliance has led to an increase in drug-resistant bacteria because treatment is often stopped prior to completing the six month regimen.

Current Diagnostics for Tuberculosis

Detection of the Active Form of Tuberculosis

The most common form of detection for active tuberculosis is the use of sputum smear microscopy (Lawn 2011). A sputum sample is taken from the patient, is stained with a Ziehl-Neelsen stain, then analyzed under the microscope after being stained. A lab technician will examine the sputum sample for the presence of tuberculosis colonies. This technique has been used for detection of tuberculosis since the 1880s (Willey 2008). Sputum smear microscopy is most commonly used in low resource settings where hospitals and clinics have the infrastructure that supports this test. They have the resources to run the test, and many

technicians in low resource areas are trained to perform the technique. The patient is considered positive for tuberculosis when a concentration of 10,000 bacilli/mL is found (Willey 2008). However, this test is subject to interpretation by the technician and its accuracy can depend on how experienced the technician is. It is also inaccurate for those with a HIV and tuberculosis co-infection. The areas with high prevalence of tuberculosis also have a high prevalence of HIV. More than half of patients with a HIV and tuberculosis co-infection receive a negative result using sputum smear microscopy (Lawn 2011). The amount of bacteria in sputum is directly proportional to pulmonary cavitation and in immunocompromised patients, there is less pulmonary cavitation. The test is also labor intensive and slow, taking about a week to perform (Frieden 2003).

Detection for the Latent Form of TB

There are multiple methods currently utilized for the detection of tuberculosis. For the latent form, the most common test is the tuberculin skin test (TST) (Gordin 2012). Tuberculin is a partially purified protein derivative isolated from the bacteria (Willey 2008). It consists of a variety of proteins found in the bacteria. The TST is administered intradermally. T-cells will migrate to the injection site, causing an increase in the redness of the area that correlates to the degree of hypersensitivity of the patient (Willey 2008). Thus, a person who has been exposed to tuberculosis will develop a hard, red area at the site of injection. Those who have not been exposed to tuberculosis will have naïve T-cells do not recognize the pathogen and as a result there will not be any migration or redness. This diagnostic test presents problems with patient compliance, because the patient must return within two days to have the test read. The skill of the administrator also affects the accuracy of the test. If the tuberculin is not injected properly, the patient may present with a false

negative. Furthermore, the test only demonstrates that a person has been exposed to tuberculosis at some point. A person may or may not have the active or latent form of the disease. Another test used for the detection of latent TB is the interferon-gamma release assay (IGRA). IGRAs measure the IFN-γ responses to ESAT-6 and CFP-10, two antigens that are relatively specific for TB (Lawn 2011). This test will generate results within two hours, ensuring higher patient compliance. The WHO has recommended that hospitals switch to using IGRA instead of TST (Gordin 2012).

New Methods for Diagnostics

New methods for diagnosing tuberculosis are needed, particularly in the form of accurate, point-of-care devices. Several groups have reported immunochromatography methods for tuberculosis detection. These tests use antibody-antigen binding reactions that when combined with enzymes will produce colorimetric changes in the presence of a specific substrate. These tests are limited by the accessibility of the target analyte and the strength of the bond between the antibody and antigen (McNerney 2011; Niemz 2011). The limited knowledge of biomarkers unique to tuberculosis also inhibits this technology (Wallis 2010). Detection methods that operate based on using an enzyme-linked immunosorbent assay (ELISA) that detects LAM (Lawn 2012) and optical methods such as Raman spectroscopy are also being investigated (McNerney 2011). Another method of detection involves the use of isothermal amplification technologies such as loop-mediated amplification (LAMP) or cross priming amplification (CPA) to amplify a target tuberculosis gene and thus enable detection. These methods operate at a constant temperature and do not require a thermal cycler, making them ideal for low resource settings (Fang 2009; Niemz 2011). Nucleic acid amplification tests using either isothermal amplification tests or traditional polymerase chain

reaction (PCR) have been studied for detection of tuberculosis as well as for detection of specific target sequences that indicate drug resistance. As PCR requires a thermal cycler and a trained technician, it is not ideal for point-of-care diagnosis.

WHO has recently endorsed the use of the GeneXpert MTB/RIF assay system for the diagnosis of tuberculosis. This system, produced by Cepheid, uses real time PCR to detect *Mycobacterium tuberculosis* and markers for rifampin resistance simultaneously. It is a self-contained automated system. However, the sputum must be processed beforehand. The GeneXpert generates results within two hours. A study by Lawn et al (Lawn 2011) showed the sensitivity of the GeneXpert MTB/RIF to be 73.3% compared to the 28% sensitivity of sputum smear microscopy (Boehme 2010; Helb 2010). It is not a point-of-care detection system because it requires hospital or laboratory infrastructure. However, its use has become more common.

Table 1: Lysis Methods

Method of Lysis	**Mechanism**
Physical	
Glass Beads/Mini Bead Beater	Use of beads to mechanically disrupt cell walls (Belisle 1998; Lanigan 2004; Aldous 2005; Rezwan 2007)
French Press	High pressure (Rezwan 2007; Gill 2008)
Sonicator/ ultrasonic horn	Acoustic energy (Lanigan 2004; Rezwan 2007)
Freeze/thaw cycles	Breakage of cell wall due to formation of ice crystals (Gonzalez-y-Merchand 1996; Amita 2002)
Chemical	
Detergents	Chemical disruption of cell membranes (Amita 2002; Lanigan 2004; Aldous 2005)
Enzymatic degradation	Use of enzymes such as lysozyme, proteinase K, or lipase to degrade linkages in cell walls (Belisle 1998; Amita 2002; Aldous 2005)
Chaotropic agents	Interferes with intramolecular forces to disrupt cell membranes (Gonzalez-y-Merchand 1996)

Lysis of Mycobacterium tuberculosis

In order to perform a nucleic acid amplification test, the target DNA must first be isolated from the bacteria by lysing the bacteria and extracting the DNA genome. As the cell wall of *Mycobacterium tuberculosis* is particularly tough, lysing the bacteria can be difficult. There are various methods for lysing mycobacterium. Several involve using physical forces such as mechanical force, high pressure or ultrasonic waves. Others use chemicals to disrupt or enzymatically degrade the walls. A brief description of commonly used techniques is given in **Table 1**. There have been many studies to determine the most effective cell lysis process (Amita 2002; Lanigan 2004; Aldous 2005; Rezwan 2007). However, different applications require different standards, either needing a large quantity of DNA or high quality DNA. Methods must be chosen to be compatible with the needs of the subsequent diagnostic assay. In general, mechanical methods have been seen as more effective at releasing DNA from bacteria than chemical methods. Following lysis, extraction can eliminate contaminants from the patient sample.

Table 2: Principles of Isothermal Amplification (Gill 2008; Niemz 2011; Craw 2012)

Technology	**Basic Principle**	**Reference**
NASBA	cDNA formed amplified by T7RNA Polymerase	(Compton 1991; Niemz 2011)
HDA	Helicase unwinds DNA, forward and reverse primers extended by polymerase	(Vincent 2004)
RPA	Recombinase filament formed by recombinase enzyme and primer, which inserts primer into target, after disassembly of filament, extension occurs by strand displacing polymerase	(Piepenburg 2006)
LAMP	Using strand displacing polymerase (Bst) and specially designed primers, form structures of loops (Notomi)	(Notomi 2000)
SDA	Bifunctional primers which have both target recognition and endonucleases target region, which incorporate restriction target. Knocked off by bumper primers. dsDNA produced has restriction sites, which when nicked allow polymerase to displace strand and amplify	(Walker 1992)
NEAR	Similar to SDA, uses nicking	(Maples 2009)
RCA	Using Phi29 polymerase on circular DNA targets. Primer anneals to circular ssDNA, goes in circle with new strand displacing old strand (concatenated)	(Demidov 2002)
ICAN	Chimeric primers bind and elongated by BcaBEST DNA polymerase, RNaseH nicks strand, which allows strand displacing DNA polymerase to release the new strand	(Uemori 2007)
SMAP2	Similar enzymes to LAMP, assymetric primers	(Mitani 2007)
SPIA	Chimeric RNA/DNA primers bind target, RNase H degradation of RNA portion exposes binding site which let another primer anneal, strand displacing polymerase	(Kurn 2005)
CPA	Multiple primers including a cross primer which incorporate another priming site. Strand displacing polymerase (Bst) able to knock off products, as primers anneal next to each other. Favorable reaction for hairpin structure to be removed from template over reannealing	(Fang 2009)

Table 3: Comparison of Isothermal Amplification Methods **(**Niemz 2011; Craw 2012)

Technology	**Template**	**Product**	**Amplification kinetics**	**Fluorescence Detection**	**Temperature of Operation (˚C)**	**Time to Detection (min)**
NASBA	RNA/ssDNA	RNA amplicons		Possibly, if used SYBR Gold	41	105
HDA	dsDNA	dsDNA	exponential	Yes	65	75-90
RPA	dsDNA	DNA	Geometric	Use of special fluorescent probes	30-42	20
LAMP	dsDNA	dsDNA (in form of increasing loops)	exponential	Yes, works with intercalating dyes	60-65	60-90
SDA	ssDNA (could denature dsDNA)	dsDNA		Must use specific probes	37	120
NEAR	dsDNA	ssDNA	geometric		55	10
RCA	Circular ssDNA, can add reagents to work with dsDNA	ssDNA, concatenated	Linear, can be modified to have geometric		65	60
ICAN	dsDNA if heat denatured	dsDNA		yes	55	60
SMAP2	ssDNA	dsDNA (concatenated)		yes	60	15-30
SPIA	RNA/DNA	DNA	linear	no	45-50	240
CPA	dsDNA	dsDNA	exponential	yes	63	60-90

Isothermal Amplification

The development of polymerase chain reaction (PCR) revolutionized the field of molecular biology and subsequently diagnostics. Being able to easily amplify small amounts of DNA has enabled accurate detection of pathogens and the detection of genetic sequences which give information about drug resistance, such as mutations in the *rpoB* gene (Walter 2012) for RIF resistance in *M. tuberculosis*. However, PCR still requires quality facilities, well-trained technicians, and a thermal cycler. In the developing countries that need detection of infectious diseases the most, these facilities are not always available. Thus, there has been a focus on developing technologies that have the potential to be taken out into the field and can easily detect pathogens by their DNA. Within the past two decades, several different methods for isothermal amplification of DNA have been developed. **Table 2** and **Table 3** outline the principles and basic requirements of some common isothermal amplification methods. Isothermal amplification technologies are able to amplify a target DNA sequence at a constant temperature, eliminating the need for a thermal cycler. These technologies are compatible with point-of-care diagnostic tests. In this study, loop-mediated amplification and cross priming amplification were chosen because they can be detected with a simple intercalating dye, have exponential amplification, and can give results within an hour. These methods are similar, operating using a strand-displacing polymerase and specially designed primers.

Loop-mediated Amplification

This technique developed by Notomi et al in 2000 (Notomi 2000), has been widely studied for uses in isothermal amplification. It operates using primers which form cauliflower like structures when extended by a strand displacing polymerase. Two outer primers amplify the sequence and then two inner loop primers proceed with amplification as shown in **Figure 1**. LAMP is favored for its simplicity, robustness and ease of use. Amplification is measured with intercalating dyes or turbidity.

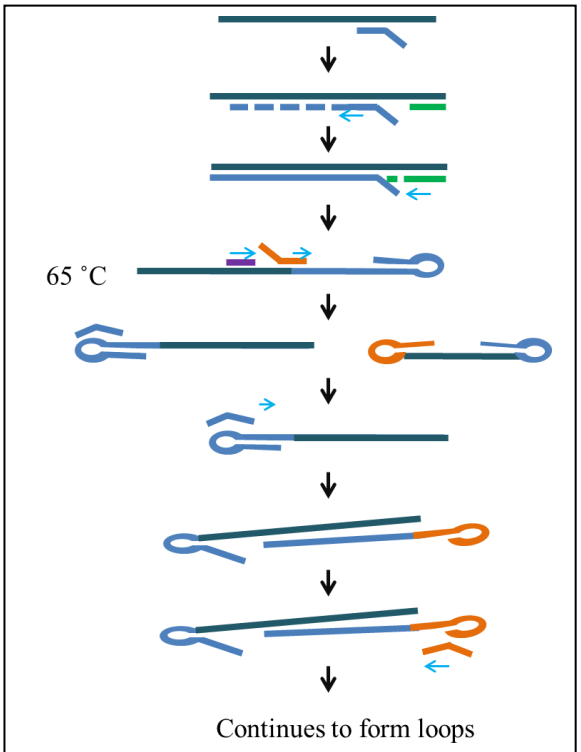

Figure 1: Amplification schematic for LAMP. Primers F3 (green) and B3 (purple) release strands extended from FIP (blue) and BIP (orange) to form initial loops. FIP and BIP continue to anneal and extend to form loop structures.

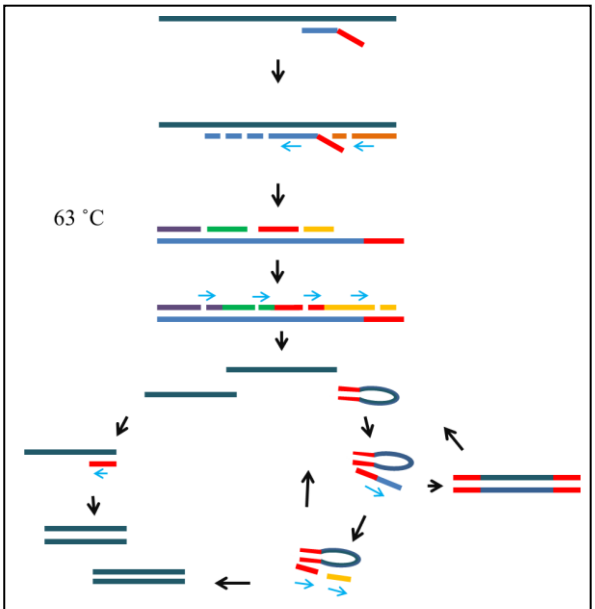

Figure 2: Amplification schematic for CPA. Cross primer (red/blue) anneals first and when displaced by 4s (orange) forms a strand to which primers 2a (red), 3a (yellow), 5a (green), and F Bumper (purple) anneal in tandem. Formation of hairpin and continued annealing of primers forwards amplification.

Cross Priming Amplification

Cross priming amplification (CPA) relies on specially designed primers and a strand-displacing polymerase to amplify DNA. **Figure 2** outlines the basic process of CPA. In CPA, a cross primer is created with two sides: one being complementary to the DNA sequence and another non-complementary end. The cross primer will anneal and extend. Simultaneously, a primer upstream of the cross primer will

anneal and extend, displacing the cross primer strand. The non-complementary end on the cross primer adds another annealing site for other primers. Another set of primers will anneal in tandem on this strand containing the cross primer. When extension occurs, these strands displace each other. This creates several shorter fragments which can then be amplified. A hairpin structure is created that energetically prefers the hairpin structure to reannealing, thus increasing open sites available for replication (Xu 2012). CPA demonstrates exponential amplification at a constant temperature.

There are multiple factors to consider when choosing the most appropriate isothermal amplification method. The sensitivity or limit of detection, specificity, and time to amplification are important when determining if a technique is appropriate for detection. Limit of detection of the reaction refers to the lowest number of copies that the reaction amplifies. Specificity refers to the reaction accurately amplifying only target DNA. Nonspecific amplification may be due to the primers inaccurately amplifying non-target DNA or due to spontaneous amplification of primers.

Overall Goals for Point-of-Care Diagnostics of Tuberculosis

Point-of-care diagnostics are designed to operate simply and robustly in low resource settings. They should be easy to use and interpret and have repeatable results. In this study, we combine an easy-to-use lysis and extraction method (Bordelon 2011) with isothermal amplification to detect a specific target sequence for *M. tuberculosis*. This will enable easy detection of tuberculosis at the point-of-care. The easier and earlier tuberculosis is detected, the more likely the patient is to receive treatment. This helps to prevent the spread of the disease and lower the overall incidence and disease burden. In this study, we examine a

chemical lysis protocol with different nucleic acid amplification methods for their use in a point-of-care diagnostic in conjunction with a low resource extraction cassette.

CHAPTER II

COMPARISON OF CPA AND LAMP FOR TUBERCULOSIS DETECTION IN AN INTEGRATED DIAGNOSTIC DEVICE

Abstract

Tuberculosis infects one out of three individuals worldwide. In order to control tuberculosis infection, accurate diagnostics are needed at the point-of-care. An ideal point-of-care diagnostic for tuberculosis would include an integrated system for lysis of the bacteria, extraction of the DNA from the bacterial lysate and the sputum, and detection of a specific biomarker. We compared the use of two different isothermal amplification methods, cross priming amplification (CPA) and loop mediated amplification (LAMP), for the detection of tuberculosis within a previously developed extraction cassette designed for low resource areas. Under ideal laboratory conditions, CPA and LAMP had a limit of detection of 500 copies and 50 copies respectively. As part of an integrated system, CPA and LAMP detected a concentration of bacteria at $1X10^3$ cells/mL at 46 ± 5.8 minutes and 57 ± 4.6 minutes. For the integrated system of tuberculosis detection, CPA generates faster results. However, LAMP was shown to have a lower limit of detection and more specificity under ideal conditions. Overall, this study supports the continued investigation of using isothermal amplification methods combined with a low resource extraction cassette as a point-of-care diagnostic test.

Introduction

According to the World Health Organization, tuberculosis infects one out of every three individuals and is the second leading cause of death worldwide (WHO 2011). When left untreated a person with active TB will infect 10-15 people per year on average (WHO 2002). Thus, early detection is crucial to preventing the spread of the disease. The standard method for diagnosis of active TB in low resource areas is sputum smear microscopy (Willey 2008). This test is far from ideal. Its accuracy depends on the experience of the technician and it takes about a week to have results (Frieden 2003). An ideal diagnostic would reliably provide accurate results within an hour at the point-of-care. Isothermal amplification is one promising simple biomarker amplification method which may be integrated into a simple and sensitive TB test for point-of-care.

There are multiple factors to consider when comparing isothermal amplification methods. The limit of detection, specificity, and time to amplification are important when determining if a technique is able to be used for detection. Limit of detection of the reaction refers to the lowest number of copies that the reaction amplifies. Specificity refers to the reaction accurately amplifying only target DNA. Nonspecific amplification may be due to the primers inaccurately amplifying non-target DNA or due to spontaneous amplification of primers.

In this study, we combine an easy to use lysis and extraction method (Bordelon 2011) with isothermal amplification to detect a specific target sequence for *M. tuberculosis* as a simple integrated diagnostic for TB. This will enable easy detection of tuberculosis at the point-of-care. We examine CPA and LAMP for their use in a point-of-care diagnostic integrated with a chemical lysis protocol and low resource extraction cassette.

Materials and Methods

Surrogate Sputum Samples

Surrogate sputum samples were generously provided by Program for Appropriate Technology in Health (PATH), a nonprofit global health organization based in Seattle. As outlined by the Bill & Melinda Gates Foundation, these samples contained artificial sputum composed of 47 mg/mL of Type II porcine mucin, 6 mg/mL of salmon sperm DNA, 3.6 mg/mL phosphatidylcholine, 33 mg/mL bovine serum albumin, 114 mM sodium chloride and 2 mM sodium azide. These concentrations are based on the component concentrations of sputum determined by Sanders et al (Sanders 2001). Artificial sputum was mixed overnight with known amounts of chemically inactivated *Mycobacterium tuberculosis* (Rif sensitive, clone H37Rv Johannesburg) at 4˚C to obtain a uniform slurry. Bacteria were provided to PATH from Drs. Wendy Stevens, Bavesh Kana and Lesley Scott at the University of Witwatersrand. The bacteria were chemically inactivated using SR Buffer from Cepheid. No growth was confirmed for 42 days before being shipped to PATH. Bacteria were counted by a Guava Easycyte mini microcapillary flow cytometer after being gently rocked with 400 µm glass beads to disperse large aggregates. Surrogate sputum samples were spiked at three different concentrations of cells with high at $1X10^5$ cells/mL, medium at $1X10^4$ cells/mL and low at $1X10^3$ cells/mL. Surrogate sputum without bacteria was used as a negative sample, giving a total of four different concentrations.

Chemical Lysis

Chemical lysis was performed to release the bacterial DNA. 500 µL of the four individual concentrations were mixed with 500 µL of GuSCN at 4 M, sodium citrate at 25 mM, Triton X-100 at 4.9% and sodium dodecyl sulfate at 0.2% and 0.8 mg of MyOne Silane

Dynal beads. This mixture was agitated for 10 minutes on a Fisher Vortex Genie 2 at speed 4 in Fisherbrand 2 mL tubes. After agitation, samples with the chemical lysis solution were introduced into the low resource extraction cassette.

Low Resource Extraction Cassette

A low resource extraction cassette technique previously developed in the lab (Bordelon 2011) was used to extract DNA from chemically lysed surrogate sputum samples. Extraction solutions were pipetted into fluorinated ethylene propylene (FEP) tubing with an inner diameter of 0.23 cm and an outer diameter of 0.31 cm. The GuSCN in the lysis solution enabled the binding of DNA to the silica-coated Dynal beads through a salt bridge. A large magnet was used to pull the DNA-bound magnetized beads from one solution to another. Extraction solutions after the lysis solution were a wash solution containing GuHCl at 4 M and sodium citrate at 25 mM, a precipitation solution of 80% ethanol and 5 mM KPO_4, a wash solution of 70% ethanol, and an elution chamber. The elution chamber contained either 50 µL of water, 40 µL of CPA reaction mix, or 50 µL of LAMP reaction mix. Ends of the FEP tubing were sealed with clay sealant to keep the solutions in place once the lysis/binding solution was introduced. The beads were thoroughly dispersed in each chamber of the extraction cassette before magnetically moving them to the next chamber. In the final chamber DNA on the beads was released in the elution solution. The DNA was subsequently amplified by PCR, CPA, and LAMP as described below.

Table 4: Primer designs for PCR, CPA, and LAMP amplification of IS6110

Primer	**Sequence**
CPA	
CP	TAGCAGACCTCACCTATGTGTCTTCGGTGACAAAGGCCACGT
2a	TAGCAGACCTCACCTATGTGTC
3a	CTGGGCAGGGTTCGCCT
4s	TGGCCATCGTGGAAGCGA
5a	ACAGCCCGTCCCGCCGAT
F bumper	AGGACCACGATCGCTGATC
LAMP	
F3	TGATCCGGCCACAGCC
B3	TCGTGGAAGCGACCCG
FIP	GCTACCCACAGCCGGTTAGGTGTCCCGCCGATCTCGT
BIP	TCACCTATGTGTCGACCTGGGCGCCCAGGATCCTGCGA
PCR	
Forward	ACCAGCACCTAACCGGCTGTGG
Reverse	CATCGTGGAAGCGACCCGCCAG
IS6110 Target Genetic Sequence	
CTGATGACCAAACTCGGCCTGTCCGGGACCACCCGCGGCAAAGCCCGCAGGACCACGATCG CTGATCCGGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGCCGCTTCGGACCACCAGCACCT AACCGGCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGGCAGGGTTCGCCTACGTGGC CTTTGTCACCGACGCCTACGTCGCAGGATCCTGGGCTGGCGGGTCGCTTCCACGATGGCCA	

Nucleic Acid Amplification Reactions

CPA primers were designed by USTAR (Xu 2012). CPA reactions were performed in a final volume of 40 µL in 10 mM Tris HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 1 M Betaine, 0.6 mM dNTPs, 0.1% Tween 20, and 12 units Bst 2.0 DNA polymerase from New England Biolabs. Primers were at concentrations of 0.6 µM for CP, 0.5 µM for 2a, 0.3 µM for 3a and 5a, and 0.06 µM for 4a and F bumper. CPA reactions were amplified at 63˚C. For LAMP, primers were designed using PrimerExplorer v4, available online (http://primerexplorer.jp/e/). Sequences for all primers are shown in **Table 4**. Reactions were performed for LAMP at a final volume of 50 µL in 10 mM Tris HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 1 M Betaine, 0.6 mM dNTPs, 0.1% Tween 20, and 12 units Bst 2.0 DNA polymerase . LAMP primers were at concentrations of 0.2 µM for F3 and B3, and 1.6 µM for FIP and BIP. Reactions were amplified at 65˚C. PCR was performed using primers from Cannas et al (Cannas 2008) and a QIAGEN QuantiTect Sybr Green PCR kit. Primer concentrations were 0.2 µM for forward and reverse primers.

Isothermal Detection of Surrogate Sputum Samples with ESE Quant TubeScanner

Isothermal reaction mixes were placed as the final chamber of the low resource extraction cassette. Chemical lysis was performed on surrogate sputum samples provided by PATH. After pulling through the extraction buffers, Dynal beads were placed within isothermal reaction mix for approximately 30 seconds to elute off DNA. The FEP tubing was then cut before the isothermal reaction mix and placed within a PCR tube, covered with parafilm and amplified in a Qiagen ESE Quant TubeScanner for 100 minutes as seen in **Figure 3**. The scanner took optical readings every minute. Fluorescence values were taken from the TubeScanner, normalized by dividing by the average of the first 5 scans, and Ct was

calculated with a threshold of 2 RFU. The threshold value was chosen to be within the linear region of the normalized amplification curves when plotted on the log scale. The scan at which the normalized fluorescence was first above 2 relative fluorescence unit (RFU) was determined to be the Ct.

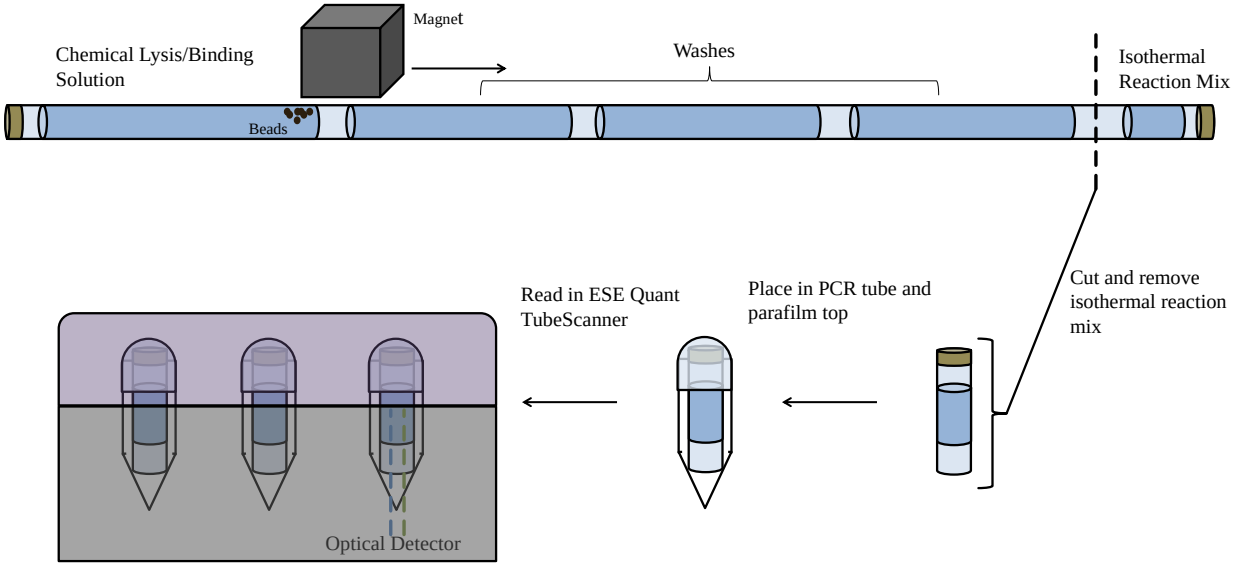

Figure 3: Diagram of integrated extraction and isothermal amplification detection using ESE Quant TubeScanner.

Limit of Detection and Specificity of CPA and LAMP under Ideal Conditions

PCR, CPA, and LAMP reactions were amplified using a Qiagen RotorGene Q thermal cycler instrument with the protocol described in **Table 5.** The limit of detection of the reaction was determined by running reactions on a pGEM-T Easy Vector plasmid (Promega) with an insert of the IS6110 genetic sequence (gift from USTAR) at concentrations of $5X10^8$ copies, $5X10^6$ copies, $5X10^4$ copies, $5X10^2$ copies, 50 copies and 5 copies. Three runs of each nucleic acid amplification technique were performed. A threshold of 0.18 RFU was used to calculate the Ct for each run. The threshold was chosen to be within the linear range of the amplification curves and above the background fluorescence seen with

the isothermal amplification primers. Time to amplification was determined from Ct by multiplying the Ct value by 75/60 in the case of PCR, because there were 75 seconds in the cycle. An additional 15 minutes was added to include the initial hold step at 95˚C. For LAMP and CPA, Ct was multiplied by 85/75. This ratio was determined to account for the transition time between cycles were it remained at the amplification temperature.

Table 5: RotorGene Instrument Set Up

	PCR		CPA		LAMP	
Step	Temperature	Time	Temperature	Time	Temperature	Time
Hold	95 °C	15 min	N/A	N/A	N/A	N/A
Cycles	40		80		80	
	95°C	15 sec	63°C	20 sec	65°C	20 sec
	62°C	30 sec	63°C	20 sec	65°C	20 sec
Read on Green	72°C	30 sec	63°C	20 sec	65°C	20 sec
Melt Curve Analysis	55-95°C		50-95°C		50-95°C	

Specificity was determined by running reactions with $5X10^5$ copies of pGEM-T Easy vector without IS6110 target sequence, 1.25 µg of salmon sperm DNA, and 50 ng of salmon sperm DNA as nontarget DNA can cause amplification in nucleic acid amplification reactions. Amplification was determined to have taken place if the detection threshold was reached and the melt curve analysis showed a peak from amplification product because large amounts of background DNA can cause fluorescence to be over the threshold without

amplification having occurred. To determine limit of detection and specificity of the reaction, three trials were performed with each trial containing duplicate samples.

The time to nonspecific amplification for PCR, CPA, and LAMP reactions without DNA was determined by running the reactions for an extended number of cycles until reactions without DNA amplified. PCR was run for 144 cycles and CPA and LAMP were run for 180 cycles. Again, a threshold of 0.18 RFU was used. Six replicates were performed on n=3 trials for PCR, CPA, and LAMP.

Statistical Analysis

In order to determine statistical significance between different data sets, an ANOVA test was performed. A p value of 5% was used to determined significance. If data sets were found to be significantly different, student T tests were performed between the different data sets. A two tailed distribution with unequal variances was performed on sample sets. A p value of 5% was used to determine significance.

Results

Limit of Detection and Specificity of CPA and LAMP under Ideal Conditions

In order to compare the sensitivity of CPA and LAMP, isothermal amplification reactions were performed on DNA concentrations ranging from 5 copies to $5X10^8$ copies/reaction. The limit of detection was determined and compared with PCR. As seen in **Figure 4**, the limit of detection for LAMP was about 50 copies/reaction whereas the limit of detection for CPA was 500 copies/reaction. The limit of detection of PCR was 5 copies/reaction. This concentration amplified at 56 ± 2.6 minutes. LAMP amplified 50% of the 50 copies/reaction samples at 63 ± 9.1 minutes. CPA only amplifies one of the 50 copies/reaction samples. At 500 copies, CPA amplifies

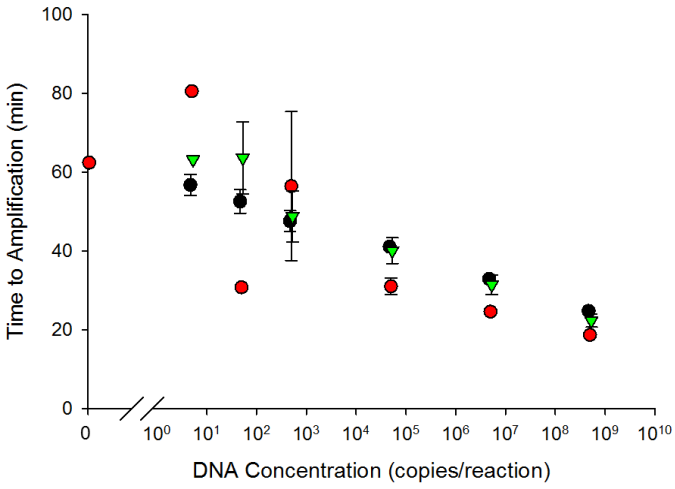

Figure 4: Time to amplification of DNA concentrations ranging from 5 copies to $5X10^8$ copies for PCR (black), CPA (red), and LAMP (green). Data points offset by shifting the x variable.

at 56 ± 18 minutes. Its time to amplification varies greatly at this point. In general, as the concentration of DNA decreases, time to amplification increases and occurs over a wider range of time for all nucleic acid amplification techniques. All three techniques for nucleic acid amplification amplify 500 copies/reaction. For 500 copies/reaction, CPA amplifies at 56 ± 18 minutes, LAMP amplifies at 48 ± 6.4 minutes, and PCR amplifies at 47 ± 2.6 minutes. Thus, at lower concentrations on average LAMP amplifies faster than CPA.

To determine the specificity of CPA and LAMP, salmon sperm DNA at 1.25 µg and

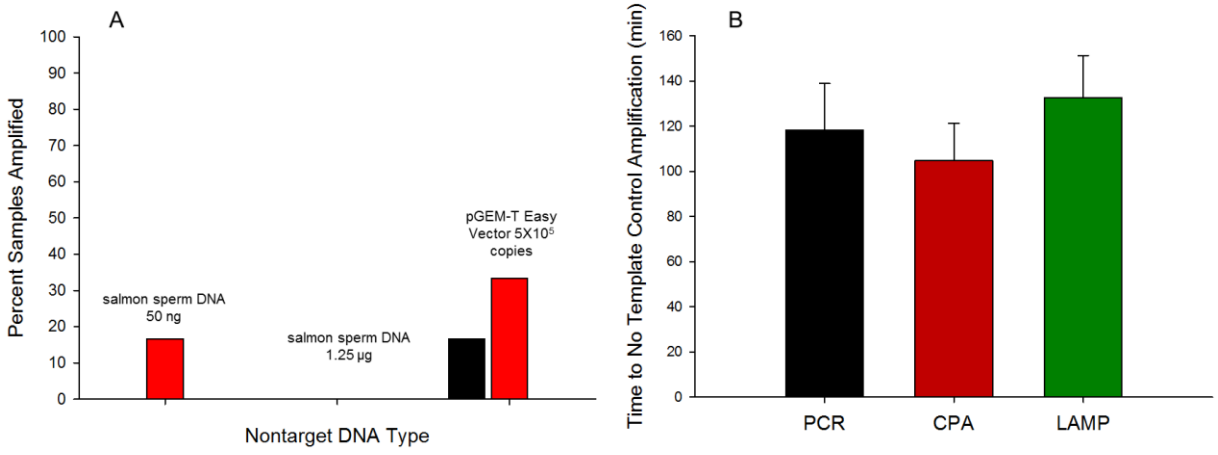

Figure 5: Nonspecific amplification of PCR (black), CPA (red) and LAMP (green) for nontarget background DNA (N=6) (A) and for no template controls run for 144 cycles for PCR and 180 cycles for isothermal amplification (N=18) (B).

50 ng, and pGEM-T Easy Vector at $5X10^5$ copies were tested. Reactions without DNA were also run for an extended time to determine when nonspecific amplification would occur. As seen in **Figure 5A**, nonspecific amplification occurs for CPA and PCR, but not for LAMP. PCR shows nonspecific amplification for 16% of the pGEM-T Easy Vector samples at $5X10^5$ copies/reaction. This is the only type of nontarget DNA tested that amplified for PCR. CPA shows nonspecific amplification for 33% of the pGEM-T Easy Vector samples, and for 16% of the salmon sperm DNA samples at 50 ng. Nonspecific amplification occurs for reactions without DNA for PCR on average at 118 ± 20 minutes, for CPA at 104 ± 16 minutes, and for LAMP at 132 ± 18 minutes as shown in **Figure 5B**.

Chemically Lysed Surrogate Sputum Samples Detected with Nucleic Acid Amplification under Ideal Conditions

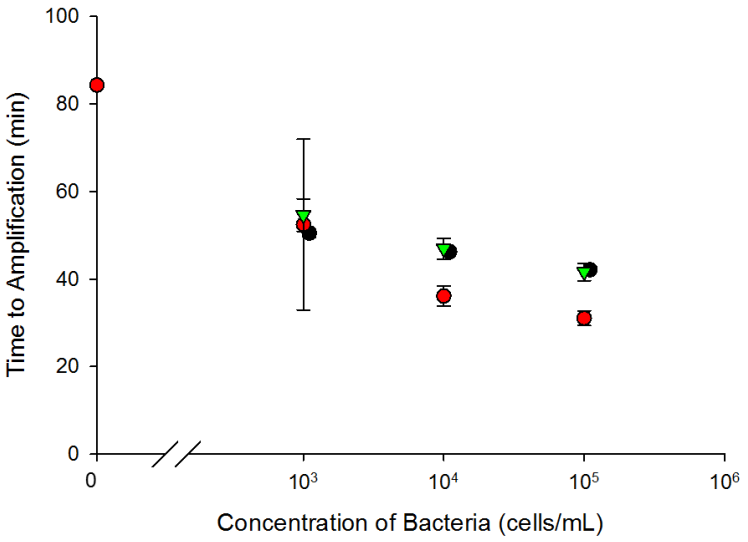

Figure 6: Time to amplification for chemical lysis of surrogate sputum samples into a water elution for PCR (black), CPA (red) and LAMP (green). Data points offset by shifting the x variable.

In order to ensure that the chosen chemical lysis and extraction protocol extracts DNA from sputum samples, surrogate sputum samples were chemically lysed and extracted with water as the final elution chamber. DNA from the elution was then amplified with all three nucleic acid amplification methods. As seen in **Figure 6**, all three nucleic acid amplification methods detected the three concentrations of bacteria tested. PCR amplified high, medium, and low samples at 42 ± 0.4 minutes, 46 ± 0.4 minutes, and at 50.0 ± 0.9 minutes respectively. This corresponds to calculated concentrations of 3.2 ± 1.6X10^5 cells/mL, 2.9 ± 0.8X10^4 cells/mL and 3.0 ± 2.0X10^3 cells/mL. CPA amplified high, medium, and low samples at 31 ± 1.6 minutes, 36 ± 2.3 minutes, and 52 ± 19 minutes. It also amplified one negative sample at 84 minutes. LAMP amplified high, medium, and low samples at 41 ± 2.0 minutes, 46 ± 2.3 minutes, and 54 ± 3.7 minutes. Neither LAMP nor PCR amplified any of the negative samples.

Isothermal Detection of Surrogate Sputum Samples in TubeScanner

Chemical lysis and extraction were combined with isothermal amplification detection to have an integrated system for TB diagnostics. When DNA from chemically lysed and

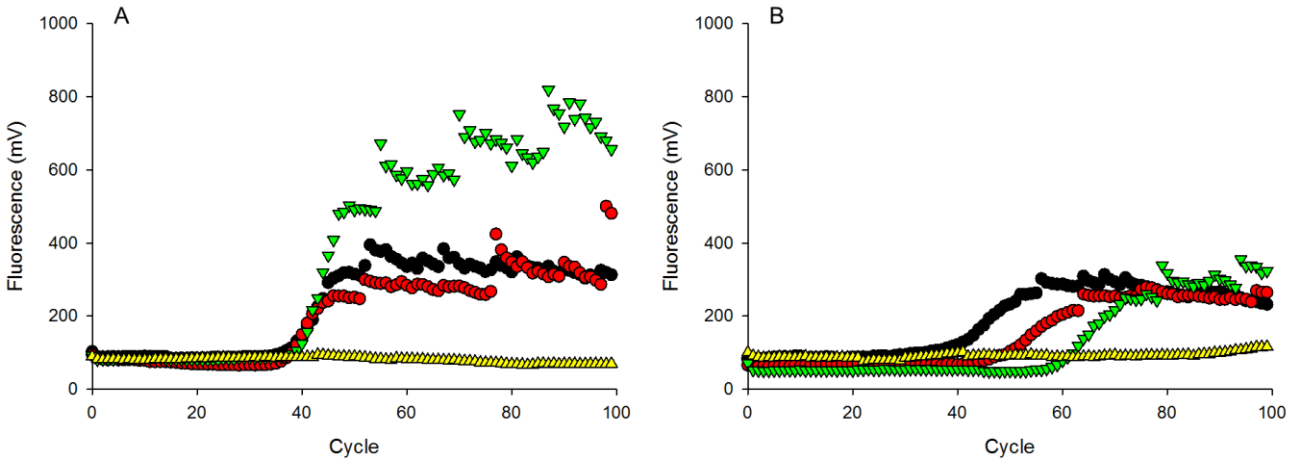

Figure 7: Fluorescence amplification curves from TubeScanner for CPA (A) and LAMP (B) for high (black), medium (red), low (green) and negative (yellow).

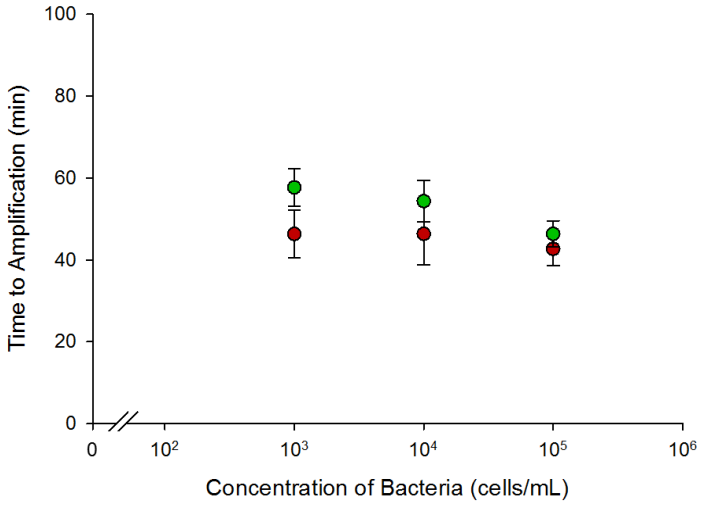

Figure 8: Time to amplification for chemical lysis of surrogate sputum samples into isothermal reaction solution for CPA (red) and LAMP (green).

extracted surrogate sputum samples was eluted directly into isothermal amplification reaction solution, high, medium, and low samples all amplified. The negative sample did not amplify. This was true for both CPA and LAMP. The fluorescence curves for one set of samples amplified by CPA and LAMP are shown in **Figure 7A** and **Figure 7B** respectively. When all of the data was analyzed for cycle threshold, CPA amplified on average at 42 ± 4.0 minutes for the high, 46 ± 7.5 minute for the medium, and 46 ± 5.8 minutes for the low as seen in **Figure 8.** LAMP amplified at 46 ± 3.2, 54 ± 5.1 and 57 ± 4.6 minutes for high, medium, and low.

Discussion

An integrated system of lysis and extraction of DNA from surrogate sputum samples and amplification for detection is required for a point-of-care test. This study demonstrates the viability of such a test using the low resource extraction cassette and isothermal amplification. When DNA from lysed surrogate sputum samples was pulled into the final chamber of isothermal amplification reaction solution, it eluted off of the beads and amplified as seen in **Figure 7** and **Figure 8**. This occurred with both LAMP and CPA for the

lowest level of bacteria tested. Importantly, neither technique amplified the patient negative control. Being able to correctly diagnose a negative result is as important as correctly diagnosing a positive result as an incorrect positive diagnosis will result in a patient being given a lengthy treatment regimen for no purpose. It will also put a strain on the resources of an area if too many patients are being needlessly treated for a disease they do not have.

In **Figure 8**, it is shown that overall, CPA and LAMP were both able to detect the highest and lowest concentrations of the surrogate sputum samples tested. For the low concentration, CPA detected at 46 ± 5.8 minutes and LAMP detected at 57 ± 4.6 minutes. On average, it was shown that CPA amplified faster than LAMP for this application. CPA amplified faster because either the final concentration of DNA in the isothermal reaction solution was higher than the limit of detection of CPA or LAMP was more susceptible to negative effects from inhibitors from the lysis and extraction. As it was only near the CPA limit of detection under ideal conditions that CPA did not amplify before LAMP, it would be expected for CPA to amplify before LAMP at DNA concentrations higher than the CPA limit of detection.

For point-of-care detection of *Mycobacterium tuberculosis* from sputum samples, the expected lowest concentration for bacteria is $1X10^3$ cells/mL. As H37Rv has 17 copies of the IS6110 gene and 0.5 mL is required for chemical lysis, this suggests the final copy number will be about 8,500 copies eluted into 40 or 50 µL if 100% of the DNA is extracted from the cells. This is unlikely, as both lysis efficiency and extraction efficiency would have to be 100% in order for this to occur. However, if only 10% of the available DNA was extracted from the cells, there would still be approximately 850 copies to detect. Thus, for our current system, either CPA or LAMP should detect the lowest concentration of bacteria expected.

An ideal system for TB detection would have results as soon as possible to decrease patient wait time. CPA and LAMP amplified 500 copies of target at 56 ± 18 minutes and 48 ± 6.4 minutes respectively under ideal conditions. Thus, both processes will detect the low concentration within an hour, but in ideal conditions LAMP will be faster. CPA will still be within the 80 minute cutoff determined by when nonspecific amplification is likely to occur.

Chemical lysis of surrogate sputum samples followed by extraction and elution into water demonstrates the viability of the chemical lysis protocol as seen in **Figure 6**. All three amplification techniques were able to detect the lowest level of bacteria in the surrogate sputum samples. The final concentration for the lowest level of bacteria was calculated by PCR to be 3.0 ± 2.0 $X10^3$ cells/mL. Amplification shows that the chemical lysis and extraction method were able to extract DNA from the surrogate sputum samples. It also shows that potential inhibitors from the lysis and extraction solutions do not prevent isothermal amplification from occurring if diluted. Under ideal conditions, CPA amplified at 52 ± 19 minutes and LAMP amplified at 54 ± 3.4 minutes for the low concentration. These are not statistically different from one another. However, CPA had a much larger variation in its time to amplification.

Each of the isothermal methods had strengths and weaknesses. Under ideal conditions, CPA had a limit of detection of 500 copies/reaction. At this copy number, CPA varies greatly in its time to amplification. While CPA amplified one reaction at 50 copies, it was not consistent. Also, CPA amplified the one 50 copies sample sooner than it amplified samples at higher concentrations of DNA. Its limit of detection does not include the lower concentrations it detected as these were inconsistent and in some cases occurred in the same time range that reactions without DNA amplified. As the concentration of DNA becomes

lower, CPA is not able to consistently amplify and when it does it takes a greater length of time.

CPA demonstrated nonspecific amplification for salmon sperm DNA at 50 ng and for the pGEM-T Easy Vector at $5X10^5$ copies/reaction. It is possible that CPA would have demonstrated nonspecific amplification for salmon sperm DNA at 1.25 µg if more samples were tested. CPA amplifies without DNA at 104 ± 16 minutes. In theory, the multiple unique primers should give CPA a greater amount of specificity as the primers will anneal to a greater percentage of the target sequence. However, the primers of CPA are specifically designed to form a hairpin structure when extended which forwards the amplification process. This design also causes an increase in the amount of secondary structures seen in the primers and thus increases the amount of primer dimers formed. Primer dimers can lead to nonspecific amplification. Also, as there are more unique primers, there are more ways for the primers to anneal to one another in primer dimers.

While CPA requires a greater number of unique primers, the overall concentration of primers within the reaction is lower than LAMP. It thus will have a lower level of initial fluorescence. If the detection modality used requires a low level of background fluorescence, CPA may be preferable. Ease of primer design is another factor to consider. CPA primers must be designed manually.

On the other hand, under ideal laboratory conditions, LAMP has a better limit of detection than CPA of about 50 copies/reaction. On average, this concentration amplified at 63 ± 9.1 minutes. 50% of the samples amplified at this concentration. At the lower concentration of 5 copies/reaction, only one sample amplified at 63 minutes. As with CPA, the time to amplification varies more as the concentration of DNA decreases.

A strength of LAMP was that it was shown to be very specific. It did not amplify any of the nontarget DNA samples tested. LAMP amplified without DNA present at 132 ± 18 minutes. The multiple primers used meant more of the target sequence was covered and thus LAMP was specific. Like CPA, the primers of LAMP are designed to form secondary structures when extended. This would imply that there should be more primer dimers and thus more nonspecific amplification. However, there were not as many primers as seen in CPA, meaning there were less possible ways for the primers to anneal with each other in primer dimers. The combined concentration of all the primers of LAMP was 3.6 μM. This would give a high background fluorescence, making LAMP less suitable for detection modalities that require low background fluorescence. Primer design for LAMP is simple. There is software readily available online that will design the four essential LAMP primers for a given genetic sequence.

When compared to PCR, neither isothermal amplification method was as sensitive. PCR consistently amplified at the lowest concentration tested of 5 copies/reaction. Amplification occurred for 5 copies at 56 ± 2.8 minutes. Therefore, PCR was shown to amplify faster than CPA or LAMP. PCR amplified 16% of the pGEM-T Easy Vector samples at $5X10^5$ copies, but it was more specific than CPA as it did not amplify either of the DNA concentrations of salmon sperm DNA. Therefore, PCR was shown to be more specific than CPA, but less specific than LAMP. As shown in **Figure 5B**, its overall time to nonspecific amplification without DNA was 118 ± 20 minutes. At this time, nonspecific amplification was caused by the primers forming primer dimers, generating a false positive.

When the times to nonspecific amplification without DNA were analyzed with an ANOVA test it was shown that the reactions had significantly different times to

amplification. Individual T tests showed each reaction was different from the other. However, though statistically different from one another, they show nonspecific amplification within the same time range. Generally, after an hour and a half, nonspecific amplification began to occur for all reactions. When used in the field, amplification should not be carried out for more than 80 minutes to ensure that all amplification that occurs is due to the presence of target DNA. Nonspecific amplification can lead to false positives and incorrect diagnosis of the disease. Therefore, carrying out amplification for only about 80 minutes will limit false positives.

Overall, when choosing which type of nucleic acid amplification to use, the application must be considered. Requirements for the limit of detection, time to amplification and specificity will vary for different applications. While CPA is amplifies high concentrations of DNA sooner than LAMP, it has a higher limit of detection. CPA is less consistent in its time of amplification near its limit of detection. PCR has the lowest limit of detection, but it is not suitable for point-of-care diagnostics. The anticipated amount of target DNA must be taken into account. For applications with a high amount of target DNA and where faster results are needed, CPA may be preferable. This is especially true if background nontarget DNA is thought to be low. On the other hand, for applications with a low anticipated amount of target DNA, LAMP may be the better choice. It should be noted that CPA was less specific than LAMP under ideal conditions for this set of primers. However, when tested with the surrogate sputum samples, neither technique amplified the negative control. It should also be noted that primer design has a great influence on specificity. While the primers in this study were more specific with LAMP than with CPA, this may not hold true with a different primer design.

In **Figure 7**, the raw fluorescence detected by the TubeScanner is shown. Towards the end of the run, the fluorescence fluctuates. The fluorescence profiles also have different initial and final fluorescence. The fluctuation of fluorescence can be partly explained by imperfections in the detection system. Pieces of FEP tubing were placed within PCR tubes and detection occurred from beneath. The FEP tubing pieces had the ability to shift during the run, which could lead to fluctuations. It was also shown that the height of the isothermal reaction mix had an effect on the fluorescence. If the reaction mix was lower, the fluorescence detected would be at a higher amplitude. The isothermal reaction mix height level was somewhat controlled by cutting at a certain length below where the isothermal amplification mix ended. However, this system was susceptible to small changes that could result in the differences in the overall fluorescence as seen above. Normalizing the data removed the effect of the different initial fluorescence values on the analysis for Ct.

CPA did not demonstrate distinguishable differences among the high, medium, and low concentrations from PATH. LAMP only showed a significant different between high and low samples. This could be due to a variety of factors. After chemical lysis, reactions were left on ice while the other concentrations were being extracted. While the ice should have prevented most amplification from occurring, there could have still been some reacting occurring which gave the different times. Also, it is possible that only a certain amount of DNA was eluted into the isothermal reaction solution due to the salts and primers. Also, it is possible that trace amounts of inhibitors prevent the higher concentrations from reacting as soon as possible. LAMP was less affected by these factors a difference between high and low. For the purpose of this test, it is more important to give a consistent yes or no answer

rather than to distinguish the varying levels of bacterial concentration. Therefore, CPA is better for producing rapid results.

Thus far, it has been demonstrated that after chemical lysis and extraction of a surrogate sputum sample, isothermal amplification detects the anticipated range of bacteria within an integrated system. It can also distinguish between positive samples and negative samples. To fulfill the requirements of a point-of-care system, only a portable heating and optical detection system is required. For the integrated system, CPA gives faster results for detection of a positive result. However, overall LAMP has been shown to be more specific. This study supports the continued exploration of using isothermal amplification methods combined with a low resource extraction cassette as a point-of-care diagnostic test.

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