UNIVERSIDADE FEDERAL DO PARANÁ

NASIR ALI

DEVELOPMENT OF A RAPID AND COST-EFFECTIVE SAMPLEPREPARATION PROTOCOL FOR DNA EXTRACTION FROM *Mycobacterium tuberculosis* WITH APPLICATION TO POINT OF CARE TESTING

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Tese apresentada como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia no Curso de Pós-Graduação do Setor de Tecnologia da Universidade Federal do Paraná.

Orientador: Prof. Dr. Marco Aurelio Kreiger
Co-Orientador: Prof. Dr. Alexandre Dias Tavares Costa

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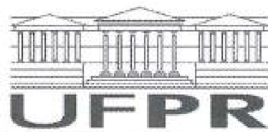

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I dedicate this work to my whole family specially my mother and sister.

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I am just a child who has never grown up. I still keep asking these ‘how’ and ‘why’ questions. Occasionally, I find an answer.

(Stephen Hawking)

RESUMO

Segundo a Organização Mundial de Saúde (OMS), “a tuberculose (TB) é a nona principal causa de morte no mundo e a principal causa de um único agente infeccioso, acima do HIV / AIDS”. As metas estabelecidas pela estratégia End TB incluem redução de 80% na incidência de TB e redução de 90% na mortalidade associada à TB entre 2015 a 2030. O diagnóstico do ponto de atendimento é um dos aspectos que podem ter o potencial para ajudar nessa epidemia mundial. O preparo rápido da amostra e a racionalização da extração de DNA é um dos desafios para o diagnóstico molecular atualmente disponível para detecção de *Mycobacterium tuberculosis* (Mtb), que impede seu uso em áreas com recursos limitados. Este trabalho apresenta um protocolo prova de conceito para liquefação e desinfecção de escarro, seguido de um procedimento para extração de DNA baseado em papel, que foi subsequentemente usado em um equipamento de PCR Tempo Real portátil (o sistema Q3-Plus) para amplificar e detectar alvos genômicos específicos de Mtb. Diversos compostos químicos e diferentes matrizes foram testadas para substituir os componentes dos protocolos comerciais tradicionais usados para tratamento, estocagem e extração de DNA. O protocolo completo otimizado (liquefação da amostra, extração de DNA, e determinação usando PCR portátil) foi testado com 17 amostras de pacientes. Os resultados foram comparados com os obtidos com um termociclador padrão (ABI7500), assim como com o GeneXpert e a cultura, que são considerados os métodos padrão-ouro tradicional e molecular. Foi observado que o protocolo desenvolvido nesta tese mostrou concordância para a maioria das amostras, como com o GeneXpert e a cultura. Embora o número de amostras usado foi baixo, o alto grau de concordância entre os protocolos e plataformas certamente dá suporte a estudos futuros com coortes maiores. Mais importante, o tempo total desde a coleta da amostra até o resultado final do PCR foi de menos de 3 horas, que é muito mais rápido que a cultura e aproximadamente o mesmo tempo do GeneXpert. Entretanto, o custo total por amostra é muito menor com nosso método, o que é sempre o objetivo dos agentes que desenvolvem políticas públicas. Em resumo, o método para preparação de amostra apresentado aqui traz um procedimento rápido e simples, com um número baixo de passos assim como baixo uso de reagentes e equipamentos, resultando num sistema fácil de usar que é apropriado para ambientes POC.

Palavras-chave: Point of care 1. Preparação de amostra 2. PCR 3.

ABSTRACT

According to the World Health Organization (WHO), "Tuberculosis (TB) is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS". The targets set by the End TB strategy include 80% reduction in TB incidence and 90% reduction in TB associated mortality between 2015 to 2030. Point of care diagnosis is one of the aspects that might have the potential to help this worldwide epidemic. Smooth sample preparation and streamlining DNA extraction is one of the challenges for the currently available molecular diagnosis of *Mycobacterium tuberculosis* (Mtb), which hinders their use in resource-constrained areas. This work presents a proof of concept protocol for sputum liquification and disinfection followed by a paper-based DNA extraction procedure, which was used by a portable real time PCR instrument (the Q3-Plus system) to amplify and detect specific Mtb genomic targets. Several chemicals (SDS, NP-40, Urea, Triton X-100 and Guanidine Thiocyanate) and matrices (3M paper and disposable pipette extraction) were tested to substitute the components of traditional commercial protocols used for sample treatment, storage, and DNA extraction. The optimized full method (sample liquification, DNA extraction, and portable PCR determination) was tested with 17 patient samples. Results were compared to those obtained with a standard thermocycler (ABI7500), as well as with GeneXpert and culture, which are considered as molecular and traditional gold standard methods. It was found that the protocol developed in this thesis showed an agreement for the majority of the samples as with the GeneXpert and culture. Although the sample number was low, the high agreement between the protocols and platforms certainly warrants further studies with a higher cohort. More importantly, total time from sample collection to final PCR result was less than 3 hours, which is much faster than culture and at a similar rate as GeneXpert. Moreover, the overall cost per sample extraction is smaller (5 R$) than Roche's kit (20 R$), which is always a goal for public health policy makers.

In summary, the sample preparation method described here provides a rapid and easy procedure with a reduced number of steps, as well as minimal use of reagents and equipments, which results in an easy-to-use system suitable for POC settings.

Key-words: Point of care 1. Sample preparation 2. PCR 3.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	- Adenine
AFB	- Acid-fast bacili
AIDS	- Acquired Immune Deficiency Syndrome
AG	- Arabinogalactans
bp	- Base pair
BSA	- Bovine serum albumin
C	- Cytocine
CDCT	- Centro de Desenvolvimento Científico e Tecnológico
CFU	- Colony forming units
CFP 10	- Culture filtrate protein 10
CPqAM	- Centro de Pesquisas Aggeu Magalhães
CSF	- Cerebro spinal fluid
CT	- Cycle Threshold
CXR	- Conventional chest X-ray
DST	- Drug-susceptibility testing
ELISA	- Enzyme-linked immunosorbent assay
ELISPOT	- Enzyme-linked immunosorbent spot
ESAT 6	- Early secreted antigenic target 6
G	- Guanine
GE	- Genome equivalents
GSCN	- Guanidine Thiocyanate
IBMP	- Instituto de Biologia Molecular do Paraná
ICC	- Instituto Carlos Chagas
IGRA	- Interferon gamma release assay
INH	- Isoniazid
IU	- International units
LAMP	- Loop-mediated isothermal amplification
LF-LAM	- Lateral flow lipoarabinomannan
LMIC	- Low and middle-income countries
LOC	- Lab on chip

LOD	- Limit of detection
LPA	- Line probe assay
M	- Molar
MDR-TB	- Multidrug- resistant tuberculosis
MOTT	- Mycobacteria other than tuberculosis
Mtb	- Mycobacterium Tuberculosis
NAAT	- Nucleic acid amplification tests
NALC	- N- Acetyl L-Cysteine
NTM	- Non-tubercle mycobacterium
NTC	- Non-template control
MA	- Mycolic acids
MGIT	- Mycobacterial growth indicator tube
MGIT	- Mycobacterial growth indicator tube
MTC	- Mycobacterium Tuberculosis Complex
PBS	- Phosphate buffered saline
PG	- Peptidoglycans
POC	- Point of care
POCT	- Point of care testing
PPD	- Purified protein derivative
PVP	- Polyvinylpyrrolidone
QFT-GIT	- QuantiFERON-TB Gold In-Tube
RIF	- Rifampicin
R&D	- Research and development
RR-TB	- rifampicin resistant tuberculosis
SDS	- Sodium dodecyl sulfate
T	- Thymine
TB	- Tuberculosis
T. cruzi	- Trypanosoma Cruzi
TE	- Tris-EDTA
TST	- Tuberculin skin test
WHO	- World Health Organization
XDR-TB	- Extensively drug resistent tuberculosis

LIST OF SYMBOLS

@ - At the rate of

© - copyright

® - Registerd mark

ɣ - Gamma

µL - Microlitre

µg - Microgram

ng - Nanogram

µM - Micromolar

mM - Milimolar

SUMMARY

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1 INTRODUCTION

The United Nations Sustainable Development Goals have set targets for the elimination of tuberculosis and its related deaths by 2030 (GLOBALTUBERCULOSIS REPORT, 2017). Delay in disease diagnosis due to increased turnaround time affects disease control strategies and have been the focus of much research. Furthermore, misidentification of the disease and ineffectiveness of disease therapy make the situation more critical due to continued disease transmission from the infected person(s).

Institute of Molecular Biology Parana (IBMP - www.ibmp.org.br) came into existence almost eighteen years ago, as a result of joint venture between Government of the state of Parana and Oswaldo Cruz Foundation (FIOCRUZ), aims for research and innovation by applying molecular technique as well as industrial manufacturing of various medical products, including molecular diagnostic kits. HIV/HCV/HBV NAT Kit is one of the amplification modules developed at IBMP and has been tested for more than 10 million bags for safe blood transfusion all over Brazil. Besides this, the institute is also working on various ongoing projects like Sepsis diagnostic, Neglected tropical disease diagnosis and Biopharmaceuticals as well. In order to work on a diagnostic aspect of this infectious disease, IBMP has started an initiative with collaboration with other partners under the project “Research and development in new solutions for diagnosis and treatment of neglected diseases”. This project aims for the development of a rapid and cost-effective sample preparation, streamlined with easy DNA extraction methods, and integrated with a bench top point of care diagnostic. Also, the rapid and less expensive diagnostic test, fulfilling the minimum ASSURED (Affordable, sensitive, specific, user-friendly, rapid, robust, equipment free and deliverable to end user) criteria of World Health Organization (WHO).

Sample preparation is the initial step in all nucleic acid amplification tests (NAAT). Previous research has focused on the later steps (i.e. amplification and detection). As a consequence, the available NAAT protocols are not only expensive and time-consuming but also require a dedicated laboratory space, which makes these tests less useful for resource-limited areas. Point of care diagnosis provides as an alternative and is less expensive and easier to perform as compared to NAAT based assays. The main challenge in translating these NAAT tests to POC testing is the lack of a smooth and easy sample preparation protocol, which hinders the whole procedure.

The overall goal of this thesis was to develop a rapid sample preparation technique with minimum use of reagents and equipments for Mtb diagnosis, which could be implemented for point of care diagnosis. Such sample preparation protocol should be streamlined to a DNA extraction procedure compatible with molecular diagnostics by means of real time PCR so that it could be used in resource-limited areas without the requirement for special operation, storage, or transportation facilities.

1.2 OBJECTIVES

1.2.1 GENERAL OBJECTIVE

The general objective of this thesis is to develop a rapid and cost-effective sputum sample preparation protocol for effective DNA extraction *Mycobacterium tuberculosis*, suitable for application for tuberculosis diagnosis at the point of care.

1.2.2 SPECIFIC OBJECTIVES

The specific objectives of this work are as follows:

a) To develop a solution to liquify and decontaminate sputum;
b) To develop a procedure to extract DNA from the mixture produced in (a), validating the procedure with
    1. *Trypanossoma cruzi* (model organism), and
    2. *Mycobacterium tuberculosis* (target organism);
c) To integrate the procedure developed in (b) with a portable PCR
    1. *Trypanossoma cruzi* (model organism), and
    2. *Mycobacterium tuberculosis* (target organism);
d) To validate the complete protocol using patient samples.

1 REVIEW OF LITERATURE

WHO has devised a post-2015 global tuberculosis strategy named as —the End TB Strategy—all WHO Member States have endorced the strategy and applies to all for the period of 2016–35. The End TB strategy is a two-pronged strategy; i.e. “Integrated, patient-centred care and prevention” and “Bold policies and supportive systems”. The first prong consists of early diagnosis of TB, treatment of all TB patients and preventive measures of persons at high risk.

2.1 GENERAL BACKGROUND

Human tuberculosis is one of the ancient diseases which have afflicted human beings for millennia (DONOGHUE et al., 2010), and still remains a major threat to global health. Different names have been used in old treaties for this disease such as white plague, phthisis and consumption (associated with weight loss) (DANIEL, 2006).

Like many other countries in the world, Brazil has also been tormented with this disease especially during the earlier twentieth century where half of the infected patients died globally due to insufficient treatment facilities all over the world. In 1899, two important institutions have been established in Brazil to deal with TB epidemics. One was called Liga Brasileira Contra a Tuberculose (Brazilian League against Tuberculosis) – recently known as Fundação Ataulpho de Paiva (Ataulpho de Paiva Foundation), while the other one was named as Liga Paulista Contra a Tuberculose (São Paulo’s League against Tuberculosis). Both institutions have played very important roles by extending branches throughout the whole country and focusing on various aspects of TB control, not only in the area of TB prevention and treatment but also promoting philanthropy and encouraging public-private partnership (HIJJAR et al., 2007).

In 1882, Robert Koch, a German physician and microbiologist, reported *Mycobacterium* as the etiological agent of tuberculosis. Before Koch, French scientist Jean-Antoine Villemin has already presented the germ theory for tuberculosis, which provided the basis for Koch’s work. New staining techniques and development of solidified culture media also contributed to this important discovery. In 1905, Robert Koch was awarded the Nobel Prize in Physiology or Medicine for his remarkable work

(CAMBAU; DRANCOURT, 2014). Moreover, he presented his remarkable work on the 24th of March 1882, which is now celebrated as "World TB day".

TB is not only a disease that affects the infected individuals, but also a social stigma, which has serious socio-economic consequences on patients as well as the family. Although there is no difference in contagion between men and women, women face serious social distancing and stigmatization. This phenomena of social stigmatization in many cases lead to delay, in diagnosis, which results in further complication of the disease (COURTWRIGHT; TURNER, 2010).

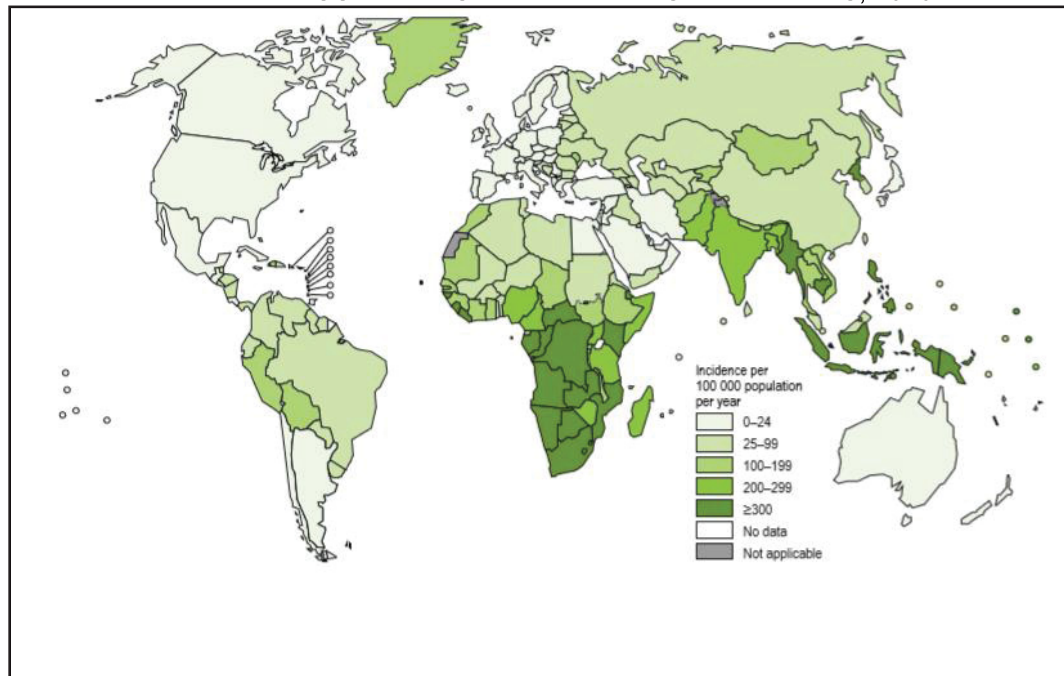
2.2 GLOBAL TB EPIDEMIOLOGY

TB ranks ninth among top ten death-causing diseases in general, and stand first amongst infectious disease, surpassing HIV/AIDS (Human immunodeficiency virus/ acquired immunodeficiency syndrome) (GLOBALTUBERCULOSIS REPORT, 2017). TB is one of the most common and major infectious cause of death at a global level. It is estimated that one-third of the world's population is infected with Mtb. Despite the fact that the disease is largely curable, millions of people die every year due to Mtb, especially in low and middle-income countries (LMIC). According to, World Health Organization (WHO), is one of the 30 high TB burden countries, Brazil has been showing a decreasing trend in TB incidence since 2000. In 2016 WHO estimated 10.4 million people were infected with Mtb (FIG) and 1.3 million HIV-negative people died due to TB. Moreover, 374 000 deaths happened in HIV co-infected patients (while TB is the leading cause of death in HIV co-infected patients and the majority of co-infected cases are reported as smear-negative) (THERON et al., 2011). (GLOBALTUBERCULOSIS REPORT, 2017). Furthermore, 56% of the global TB incidence is restricted to five countries including India, Indonesia, China, the Philippines and Pakistan.

Today, TB has become a global threat due to the emergence of strains that are resistant to various drugs. These strains can be categorized into multidrug-resistant (MDR) and extensively drug resistant (XDR) strains. MDR-TB can be resistant to two anti-TB drugs namely rifampin and isoniazid, while XDR-TB is a more complex form which not only shows resistance to rifampin and isoniazid as well as to fluoroquinolone. Moreover, it shows resistance to at least one of the second-line injectable drugs including capreomycin, amikacin and kanamycin (MIGLIORI et al., 2008). It is

estimated that in 2016, 490 000 of multidrug-resistant TB (MDR-TB) and 110 000 of rifampicin-resistant (RR-TB) cases have occurred, of which 47% were found in China, India and the Russain Federation.

FIGURE 1 - ESTIMATED TB INCIDENT RATES, 2016



FONT: Adapted from WHO report (2017)

2.3 *MYCOBACTERIUM TUBERCULOSIS*

Tuberculosis (TB) is a contagious disease which is caused by *M. tuberculosis* complex (MTC), a genetically related group of slow-growing bacteria (GAGNEUX, 2013). The most frequent causative agent of TB in humans is known as *Mycobacterium tuberculosis* which was named in 1886 (GRANGE, 1982; COLE et al., 1998).

Domain: Bacteria; **Phylum**: Actinobacteria; **Class**: Actinobacteria; **Order**: Actinomycetes; **family**: Mycobacteriaceae; **Genus**: *Mycobacterium*

Species: The *Mycobacterium tuberculosis* complex (MTC) comprised of Mycobacterium tuberculosis (Mtb), *Mycobacterium bovis*, *Mycobacterium africanum, Mycobacterium microti, Mycobacterium canettii*.

Mtb is a non-motile, non-spore-forming, obligate aerobe, a facultative intracellular bacterium (CRUZ-KNIGHT; BLAKE-GUMBS, 2013). Furthermore, Mtb is a rod-shaped bacterium which measures as 0.2-0.5 X 2-4 µm in size. Mtb is a slow-

growing microorganism with doubling time of about 18-24 h (GENGENBACHER; KAUFMANN, 2012; BACON et al., 2004).

The first complete sequence of the genome of Mtb (H37Rv) was reported by Cole and colleagues in 1998 (COLE et al., 1998). Mtb genome comprises of a circular chromosome made up of 4,411,532 base pairs (bp) and 4,000 genes (CAMUS et al., 2002). As compare to adenine (A) and thymine (T), the genome has high content (65%) of guanine (G) and cytosine (C).

One of the special features that make Mtb distinguished from other bacteria is its unique lipid-rich cell wall, which provides a strong impermeable barrier to different compounds such as dyes and drugs, and thus play an important role in its virulence. Mtb is also called Acid-fast bacilli (AFB) as it retains the red basic fuchsin dye after rinsing with acid. Mtb cell wall consists of two layers, inner and outer. The inner layer further consists of 3 components (BRENNAN, 2003):

1) Mycolic acids (MA)
2) Arabinogalactans (AG)
3) Peptidoglycans (PG)

These molecules covalently linked together, giving rise to a large complex known as MA-AG-PG complex. Rising from the base of the plasma membrane, The MA-AG-PG complex grows outwards and attach to the outer layer of cell wall i.e. the capsule which is made of different proteins and capsule polysaccharides (ALDERWICK et al., 2015).

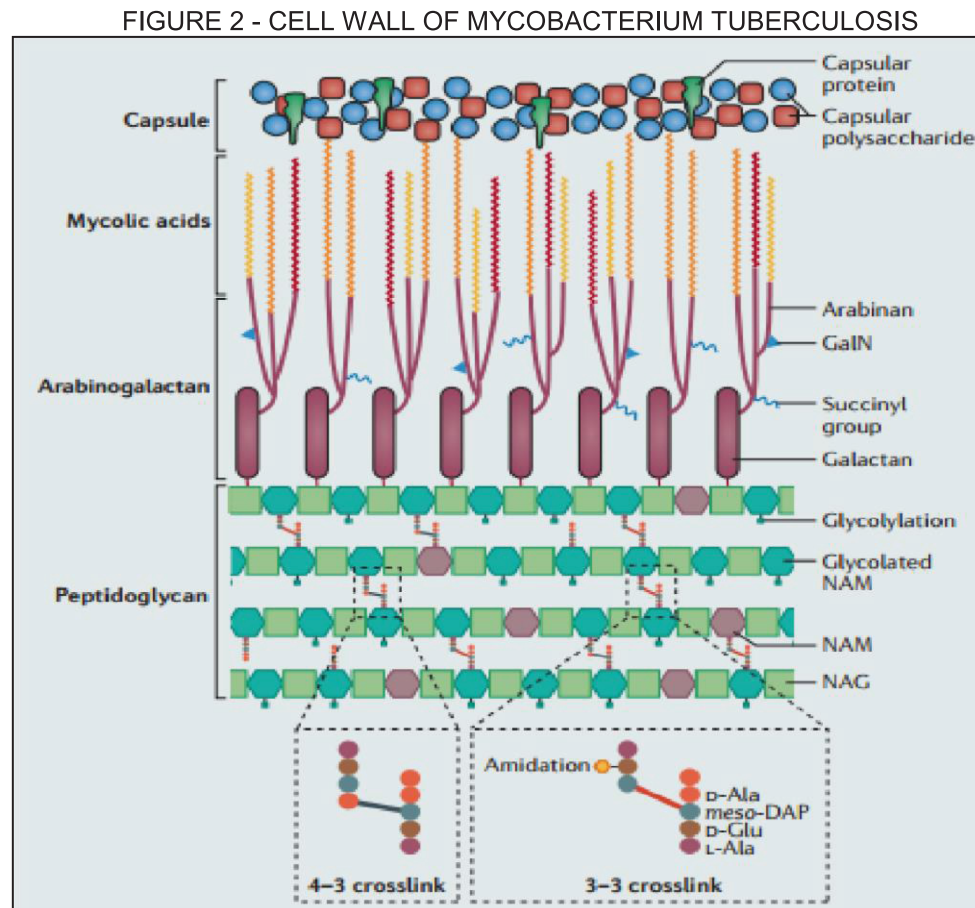
Although the genus *Mycobacterium* consists of more than 190 species, it is known mainly for two pathogenic species: *M. tuberculosis* which causes tuberculosis and *M. leprae* which causes leprosy. Genus *Mycobacterium* is divided into different groups:

(1) *M. tuberculosis* complex (MTC); (2) *M. leprae* and; (3) Nontuberculous mycobacteria (NTM).

Some of the most important members of *M. tuberculosis* complex (MTC) includes *M. africanum* which causes 50% of human TB in the western African region (JONG; ANTONIO; GAGNEUX, 2010). *M. bovis* is mainly responsible for tuberculosis in cattle. However, it also infects a wide range of mammalian hosts including humans. Due to the development of pasteurization techniques for dairy products, TB related death due *M. bovis* has been reduced. *M. microti* cause TB in voles (SMITH et al., 2009). NTM may be called environmental mycobacteria also known as “Mycobacteria

Other Than Tuberculosis" (MOTT) which means they do not cause neither tuberculosis nor leprosy.

FIGURE 2 - CELL WALL OF MYCOBACTERIUM TUBERCULOSIS



FONT: Adapted from KIESER; RUBIN. (2014)

2.4 THE DIAGNOSIS OF TB OVERTIME

2.4.1 Smear Microscopy

Smear microscopy is a commonly used technique for visualizing mycobacteria under a microscope after staining with Ziehl-Neelsen stain or Acid-fast bacilli (AFB) stain. This method has been used for many decades and is still used primarily in low and middle-income countries (LMIC) not only for initial diagnosis but also for disease monitoring as well (STEINGART et al., 2006). The limit of detection (LOD) of smear microscopy is estimated at 5×10^3 to 1×10^4 AFB/mL of the sputum. Smear-positive patients have high bacterial load and are considered to be ten times more infectious

than smear-negative patients (TOSTMANN et al., 2008). Smear microscopy is considered more effective in high prevalence areas.

Robert Koch presented more than two hundred slides stained with alkaline methylene blue-based technique to Berlin Physiological Society, which was invented by Koch himself. Different modifications and improvements have been made to the Koch's alkaline blue-based method. One of the modifications was the introduction of carbol-fuchsin by Franz Ziehl and Freidrich Neelsen (GILPIN et al., 2007). Carbolfuchsin is a basic dye made mainly from basic fuchsin and phenol. The Mycolic acid of the waxy wall of mycobacteria has the affinity for carbol-fuchsin and thus retain the dye in the cell wall, staining mycobacteria as red rods. On the basis of affinity for mycolic acid, Hagmann reported another dye (auramine) or combination of (rhodamine and auramine) as an alternative to carbol-fuchsin (LIENHARDT; KRAIGSLEY; SIZEMORE, 2016).

Smear microscopy is only 50% sensitive as compared to culture positive samples and this may be lower due to low bacterial load in cases of children and co-infection with HIV (HEPPLE; FORD; MCNERNEY, 2012). Likewise, in term of specificity, smear-positive for AFB cannot differentiate among different strains of mycobacteria. Although smear microscopy is an inexpensive and rapid test, patients seldom get the results on the same day. Thus, methods that are more efficient have been developed to enhance the throughput and accelerate the diagnosis.

2.4.2 Tuberculin Skin Test (TST)

Tuberculin skin test (TST), is an immunological test initially developed in 1890 by Robert Koch.The test is also known as the Mantoux test, as the French scientist Charles Mantoux set the diagnostic criteria for the test. The test using purified protein derivative (PPD) injected intradermally for identification of previous exposure to *Mycobacterium*. The test is considered positive if swelling occurs in the skin of the forearm after 48 to 72 h. The standard PPD is a precipitate which is composed of 1.2% nucleic acid, 5.9% polysaccharide and 92.9% protein, extracted from the culture of Mtb (YANG; KRUH-GARCIA; DOBOS, 2013). Although the test is considered to be very simple, administration and reading of the test results need experience and well-trained personnel. Moreover, the test is associated with high false positive and false negative results due to previous BCG vaccination or non-mycobacterial infection (FARHAT et al., 2006). BCG vaccination in infancy and associated false positive results in the TST

test is reported as 8.5% before ten years and 1% after ten years (ZWERLING et al., 2011). Therefore TST result should be interpreted carefully in disease burden countries like India, China and Brazil as in these countries infants are vaccinated with BCG vaccine. Furthermore, the test is less sensitive in HIV-infected patients due to the immunocompromised state of the patient. These findings suggest TST as low potential for point-of-care applications (WANG et al., 2013).

2.4.3 Interferon-Gamma (IFN- γ) Release Assays (IGRA)

Interferon-gamma release assays (IGRAs) are *ex vivo* blood tests based on measuring the T-cell release of IFN-γ after exposure to various *Mycobacterium tuberculosis* complex specific antigens which attributes specific features. These antigens include TB7.7 peptide antigen, culture filtrate protein 10 (CFP-10) and early secreted antigenic target 6 (ESAT-6) (PAI et al., 2014). IGRAs comprise two commercial tests: QuantiFERON-TB Gold In-Tube (QFT-GIT) (Cellestis Ltd., Carnegie, Australia) assay, which is an enzyme-linked immunosorbent assay (ELISA) based assay; and T-SPOT.TB (Oxford Immunotec Ltd, Oxford, UK), which is an enzyme-linked immunosorbent spot (ELISPOT) assay. Although IGRAs offers high specificity as compared to TST skin test, the test is characterized with modest predictive value as TST (RANGAKA et al., 2012). Moreover, with a turnaround time of one day, the need for specific lab equipments and trained personnel make IGRA a low potential test for point-of-care settings (WANG et al., 2013).

2.4.4 Conventional and Digital Chest Radiography

Conventional chest X-ray (CXR) has been used for many decades for diagnosis of pulmonary TB, both in high and low resource settings. CXR is a very sensitive tool for disease diagnosis as compared to other conventional methods. The high sensitivity of this tool was reported in a prevalence survey by VAN’T HOOG et al., (2012), where they reported 100% sensitivity in HIV-negative people while 92% sensitivity in HIV-positive patients. In contrast, specificity was as low as 73%. CXR is a very effective technique which is used not only for smear-negative patients of pulmonary TB but also considered beneficial as a screening pre-molecular test (Xpert MTB/RIF), as shown in a survey conducted in 22 high TB burden countries (PANDE et al., 2015). Due to high sensitivity, CXR meets the criteria of a screening test. Furthermore, CXR has the ability to identify 87% to 94% of TB related abnormalities and 94% to 98% other abnormalities

(HOOG et al., 2012). Moreover, CXR is not efficient in smear-negative but culture-confirmed, HIV-coinfected cases (DAVIS et al., 2010).

Digital radiography has revolutionized diagnostic imaging because of its intrinsic features such as decreased exposure, fine handling and good quality images. Moreover, storage, transferring and robust processing procedures have made this technique easier and efficient (SCHALEKAMP et al., 2014). Modern X-ray system eliminated the need for an experienced person. Also, these systems are assisted with software which facilitates reading the results. This makes CXR as one of the techniques with the potential to be used as POC (HOGEWEG et al., 2015).

2.4.5 Culture

Culture is still widely accepted as the gold standard test for diagnosis of tuberculosis. Unlike smear microscopy, culture is not only more sensitive but also important for the identification and differentiation of drug-resistance strains (ASSAEL, 2013). The lower limit of detection for culture is 100 bacilli per mL of the sputum (TIRUVILUAMALA; REICHMAN, 2002). Although culture is considered to be a gold standard there are some shortcomings of the test as well of being expensive as compare to smear microscopy, time-consuming due to slow growth of bacteria, need a specialized laboratory and trained personnel.

Generally, two types of media are used for culturing *Mycobacterium*; Solid (egg-based culture medium e.g. Löwenstein-Jensen (LJ) and liquid (Middlebrook media). As compared to solid media, liquid media is more sensitive. With the introduction of the BACTEC mycobacterial growth indicator tube (MGIT) 960, the technique is able to detect less than 10 colonies forming unit CFU/mL (ZYL-SMIT et al., 2011).
However, the long turn around time of 2-3 weeks of the culture technique limits its utility especially in high prevalence and low resource areas.

2.4.6 Phenotypic/Biochemical Identification of Mycobacteria

Contrary to the conventional diagnostic method, various biochemical tests have been developed on the basis of different characteristics of mycobacteria. Pigment production test is developed on the basis of different types and amounts of carotenoid pigment. On this basis, mycobacteria can be divided into photochromogenic (pigment production in the presence of light), Scotochromogenic (Pigment production in dark) and non-chromogenic.

On the basis of growth rate, mycobacteria can be divided into slow-growing (cause turbidity in medium culture in >7 days) and rapidly-growing (cause turbidity in medium culture in < 7 days). Niacin reduction test is based on the conversion of niacin to ribonucleotides by the enzyme produced by different mycobacteria such as *M. simiae and M. tubeculosis.* Various mycobacteria can be differentiated by nitrate reduction test which can reduced nitrate to nitrite. *M. tuberculosis, M. fortuitum, M.szulgai* and *M. kansasii* are some of the species which are nitrate positive. Iron uptake test and potassium tellurite reduction tests are used for the identification of rapidly growing mycobacteria. While the Tween 80 hydrolysis test is used for the diagnosis of non-chromogenic and scotochromogenic mycobacteria.

Other tests such as the catalase test and arylsulfatase test are also used for the identification of mycobacteria. Arylsulfatase test differentiates between non-photochromogenic and fast-growing mycobacteria (AZIZ et al., 2007; AZADI et al., 2018).

2.4.7 Nucleic Acid Amplification Testing (NAAT)

Nucleic acid amplification tests (NAAT) is a molecular technique which has the ability to amplify the target sequence present in clinical specimen via various ways, the most common of which is real-time PCR (DAVIES; PAI, 2008). Limited sensitivity of smear microscopy and increased turnaround time of culture technique lead to the development of NAAT assays (PAI; KALANTRI; DHEDA, 2006). Alternatively, NAAT offers faster turnaround time as compared to culture technique and high sensitivity as compared to sputum smear-positive microscopy (GRECO et al., 2006). However, in smear-negative cases, NAAT have a comparatively low sensitivity(80%) (LARAQUE et al., 2009).

All NAAT tests have their own specific features including reaction time, species identification and amplification format. Some of the representative assays may include; GenoType Mycobacteria Direct (Abbreviation) (Hain Life science), INNO-LiPA Mycobacteria (Innogenetic), COBAS AMPLICOR MTB test (Roche), GeneXpert system (Cepheid) and Real Art M. *tuberculosis* TM PCR reagent (Abbott). Despite being an efficient diagnostic method, the majority of NAATs rely on highly expensive instrumentation and multistep procedures. Furthermore, it also requires well-trained personnel with sophisticated infrastructure. These requirements limited the full

potential of NAATs in a resource-limited setting where patients cannot afford an expensive test.

2.4.8 Xpert MTB/RIF

Xpert MTB/RIF is a cartridge-based PCR assay, with the ability to detect Mtb as well as rifampicin resistance simultaneously (HELB et al., 2010). After endorsement from WHO in 2011 as a primary test for various TB groups including pulmonary, extrapulmonary, TB/HIV co-infection and pediatric TB, by the end of 2016 around 25 million cartridges and 25000 modules has been procured (Steingart et al., 2013; DENKINGER et al., 2014; DETJEN et al., 2015). Xpert has shown promising results in terms of sensitivity and specificity for both pulmonary and extrapulmonary TB (THERON et al., 2011; DENKINGER et al., 2014).

Xpert MTB/RIF is a multifeatured test which use molecular beacon technology for identifying and amplification of MTC target sequence as well as binding with the 81 bp-core region of rpoB gene for the identification of rifampicin resistance gene using multi-hybridization probes labelled with colored fluorophores in less than 2h (HELB et al., 2010; LAWN; NICOL, 2012).

On the basis of the limit of detection in a spiked sputum sample, Xpert was able to detect 131 CFU/mL (HELB et al., 2010), while culture and microscopy have the sensitivity of 10 to 50 CFU/mL and 10,000 CFU/mL respectively (LAWN et al., 2013). Although the feasibility of Xpert has been suggested by various studies for POC in sophisticated clinics due to the lack of requirement for skilled or technical person (THERON et al., 2014; HANRAHAN et al., 2015) However, Xpert depends on continuous power supply, controlled environment with relevance to temperature, heat, humidity and protection from the dust (SCHNIPPEL et al., 2012). Another aspect of the technique is inability to differentiate between live and dead bacterium makes Xpert unsuitable for treatment monitoring (FRIEDRICH et al., 2013).

Overall, GeneXpert has open the door for new generation of technologies and in the coming years, we will observe more sensitive and capable of POC instruments in the form of Xpert MTB/RIF Ultra and GeneXpert Omni respectively.

2.4.9 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a molecular-based technique that performs DNA amplification in an isothermal condition. The technique was developed

in 2000 by (NOTOMI et al., 2000) using a minimum two set of primers; outer and inner, which amplify six specific regions in the target sequence in less than 1 h with increased specificity. Unlike real-time PCR and various other molecular based-techniques, LAMP does not rely on sophisticated detection systems. Instead, it uses a DNA polymerase that does not require denaturation of DNA prior to extension, which abrogates the need for a specialized thermal cycler. The LAMP can be considered as an alternative tool to smear microscopy and Xpert MTB/RIF in high-burden and low-income areas (NLIWASA et al., 2016; BOJANG et al., 2016). Furthermore, the technique is simple, cheap, rapid, highly efficient and have the potential to be used in point of care settings (LEE et al., 2017). Although this technique has various benefits over other molecular-based techniques, there are some important limitations such as a high number of false positives due to primer-primer interaction in the multiplex reaction. Moreover, designing of a high number of primers is one of the difficulties of this technique.

2.4.10 Lateral Flow Lipoarabinomannan Assay (LF-LAM)

Lipoarabinomannan (*LAM*) is one of the constituents of a mycobacterial cell wall, which not only plays a structural role but is also important in many biological activities (LAWN, 2012). Detection of LAM in urine has paved the way for POC testing of TB. There are specific advantages to a urine sample over a sputum sample. Primarily, urine samples can be collected, processed, and stored easily as compared to sputum samples. Secondly, it is less hazardous because it does not generate bioaerosol. Despite the fact that there are various antigens present in the urine of pulmonary TB patients, LAM seems to be the most important one (MINION et al., 2011).

The test is based on binding of LAM to gold-labelled colloidal antibodies. As the sample moves along the strip, already immobilized antibodies on nitrocellulose membrane capture LAM. The development of a purple line shows a positive result (LAWN, 2012).

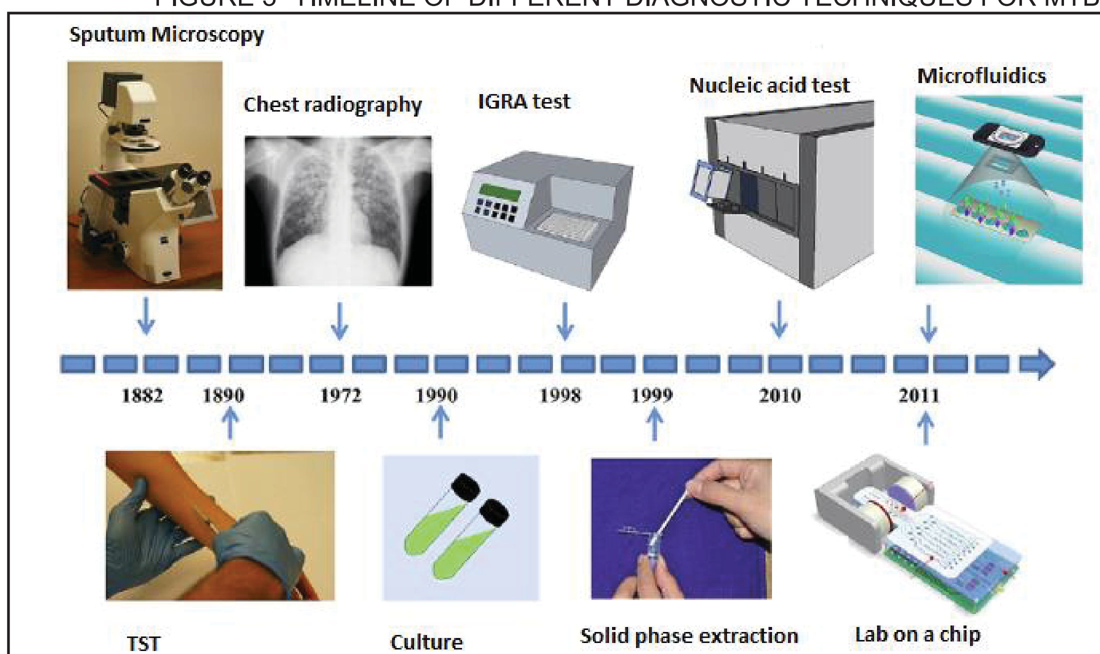
2.4.11 Line Probe Assay (LiPA)

Line probe assays (LPAs) are designed for detection for both *Mycobacterium tuberculosis* and drug susceptibility for Isoniazid (INH) and Rifampicin (RIF). Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are posing serious challenges, not only for public health but for medical and scientific communities as well. Rifampicin and isoniazid are the most effective, first-line, drugs

against TB. Conventional diagnostic procedures like culture and drug-susceptibility testing (DST) delay the initiation of adequate treatment due to which patients are prone to initiate inadequate remedies which lead not only to drug resistance but also poses a serious threat of continuous source of TB transmission as well. According to NATHAVITHARANA et al., (2017) only 25% of multidrug-resistant cases are diagnosed while 75% goes undiagnosed due to non-availability of POC testing.

In 2008, WHO endorsed LPAs in smear-positive samples for Mtb detection and RIF resistance testing. The first generation of LPAs namely INNO-LiPA Rif.TB assay (Innogenetics, Ghent, Belgium) and Genotype MTBDR assay (Hain Life science GmbH, Nehren, Germany) are no longer in use in clinical settings. Alternatively, the new generation of LPAs; **Geno**Type **MTBDR***plus* V1 or “Hain1” and **Geno**Type **MTBDR***plus* V2 or “Hain2”, and Nipro NTM+MDRTB Detection Kit 2 or “Nipro" is in use. Mutation in three genes (*rpoB, KatG and inhA*) give rise to resistance to RIF and INH, and LPAs target these mutations. LPAs are able to detect 95% of RIF related drug resistance by targeting the core region of *rpoB* gene (TELENTI et al., 1997). On the contrary, INH drug-resistance is due to multi-gene mutations residing 80 to 90% on *kat-G* and *inhA* genes, while 5 to 10% mutations occur in the ahpC-oxyR intergenic region (SEIFERT et al., 2015). Although LPAs have the advantage over Xpert MTB/RIF for diagnosing isoniazid resistance. However, LPAs are restricted to only reference labs and take longer turnaround time as compare to Xpert MTB/RIF (NATHAVITHARANA et al., 2017).

FIGURE 3- TIMELINE OF DIFFERENT DIAGNOSTIC TECHNIQUES FOR MTB



FONT: Adapted from WANG et al. (2013)

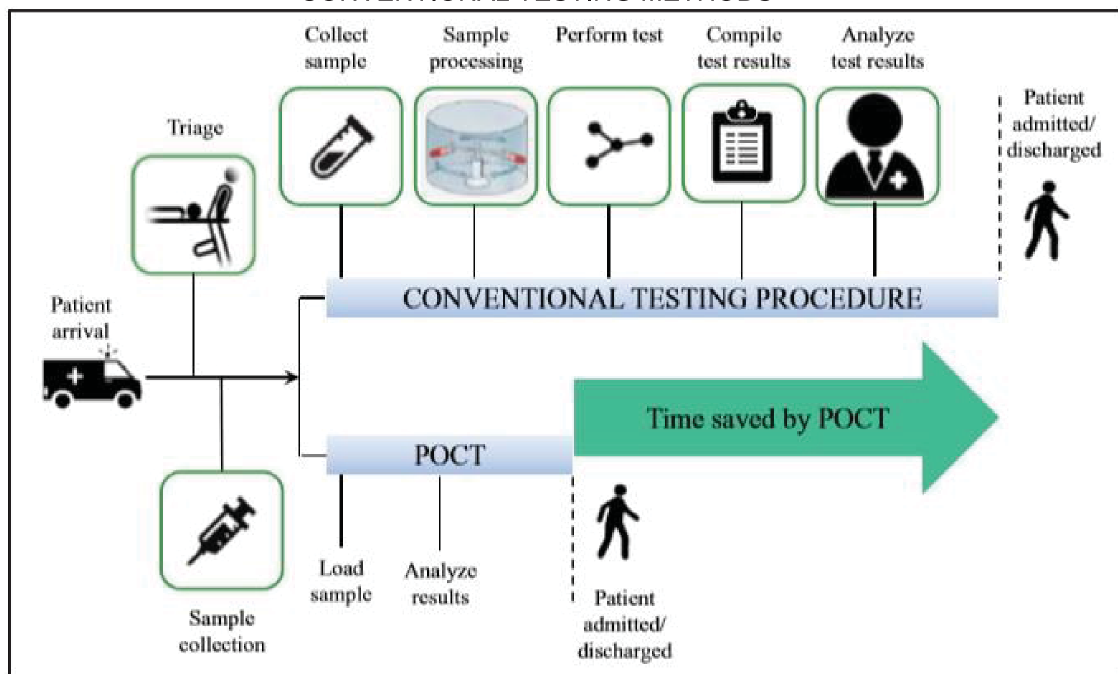
2.5 POINT OF CARE TESTING (POCT)

Diagnosis is one of the most important aspects of healthcare facilities in the developed world, providing standardized diagnostics results to the patients. Similarly, it is an important area in low-resource and under-developed countries, but due to poor health infrastructure and lack of access to standardized reference labs, the patients in many cases are unable to get better disease diagnosis and management. Keeping this in mind, a low cost and rapid diagnostic technology is not only much needed for these low-resource areas of the world but also have potential utility for the developed world as well. Due to specific economic reasons, research, and development (R&D) in the field of diagnostics did not get the due attention just like other areas such as vaccine development and drug discovery from private and public sponsors (YAGER; DOMINGO; GERDES, 2008). However, the growing concerns of antibiotic resistance, the high cost of important life-saving drugs and rapid transitional change from epidemic-to-pandemic of various diseases have compelled public/private partners and medical communities to invest in this important medical area (YAGER; DOMINGO; GERDES, 2008).

Point-of-care testing, which is also known as ‘near-patient’ or ‘bedside’ testing, is one of the emerging areas of diagnostics worldwide (WILLMOTT; ARROWSMITH,

2010). POCT may be defined as “any diagnostic test performed at or near the location of the patient” (LARSSON; GREIG-PYLYPCZUK; HUISMAN, 2015) or, more explicitly, “Patient specimens assayed at or near the patient with the assumption that test results will be available instantly or in a very short time frame to assist caregivers with immediate diagnosis and/or clinical intervention” (EHRMEYER; LAESSIG, 2007). Contrary to conventional diagnostic procedures, POCT is emerging healthcare approaches where patients cannot rely on a centralized medical laboratory where a patient sample is being collected, analyzed and then the patients have to wait for reports for hours and in many cases for days. The main objective of the POCT is to reduce the time (pre-analytical, analytical and post-analytical) required to get from sample to results and, thus, lead to early disease diagnosis enabling informed decision making for treatment initiation as mentioned in Figure.4 (SRINIVASAN; TUNG, 2015).

FIGURE 4 - COMPARISON OF TURNAROUND TIME BETWEEN POCT AND CONVENTIONAL TESTING METHODS



FONT: Adapted from DING; SRINIVASAN; TUNG. (2015)

POCT has been revolutionized by recent advances in mobile phone technology, Lab on chip platforms and paper-based assays, individually or in combination, which is paving the ways for a low-cost point of care testing especially in the developing and resource-constrained areas (VASHIST et al., 2015). Moreover, recent developments

in miniaturization such as microfluidics, nanotechnology and combination of different bio-sensing platforms and smartphone-assisted readers of different chemical and biological molecules have pushed the frontier of POCT nearer towards WHO "ASSURED" criteria of Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to end user. Furthermore, the management of various infectious diseases like HIV and malaria have been transformed due to the use of modern POC diagnostic tools especially in those areas where health facilities are limited (YAGER; DOMINGO; GERDES, 2008). These tests are simple enough that can be used in primary care centres as well as in homes with privacy which allows patients to self-test and make informed decisions (PEELING; MABEY, 2010).

Altogether, these developments have revolutionized the existing centralized or reference lab facilities and led to a new Lab-on-chip or Lab-on-a-card technology providing sensitive and specific diagnostic facilities at POC level to both developed and underdeveloped countries and low resource settings (YAMADA et al., 2017; MAHATO; SRIVASTAVA; CHANDRA, 2017). Technological advances have made a paradigm shift in clinical diagnosis from traditional diagnostic procedures to molecular level analysis (MAHATO; SRIVASTAVA; CHANDRA, 2017). Diagnostic procedures such as PCR or ELISA are limited to centralized laboratories where trained personnel are needed to operate hi-tech equipments. POC diagnostics promise the same results as provided by hi-tech equipments in terms of sensitivity and provide lowest turnaround time with the easier operating system. The development and combination of different technologies like nanotechnology, microfluidics, modern mobile phones and different kind of polymers will give rise to portable and robust POC devices (ZAREI, 2017).

2.6 EXISTING AND EMERGING POINT-OF-CARE TESTING (POCT)

Since its inception forty years ago, POCT has seen a gradual increase with the passage of time (JOHN; PRICE, 2014). POC has been divided largely into two categories. A variety of POCT technologies are being used such as POCT analyzers, biosensor devices, lab-on-chip (LOC), test strips, and lateral flow assay (LFA) cartridges. Such cost-effective technologies offer rapid analysis in just a few minutes using minimal sample volumes (www.clinlabint.com).

2.6.1 Cell Phone (CP) Based Technologies

Recently various commercially available smartphones are featuring a long range of applications for monitoring different physical activities including blood pressure, heart activity, physical activity, pulse rate, weight and sleep (VASHIST; SCHNEIDER; LUONG, 2014). A modern-day cell phone can be used as computational microscope equipped with different modes featuring dark field, bright field, transmission, fluorescence, and microscopy modes. Furthermore, by using various technologies for imaging, different blood cells, water born parasites and human pathogens in water can be screened by CP (BRESLAUER et al., 2009; VASHIST et al., 2015).

2.6.2 Paper Based Diagnostics

Paper-based assays (PBA) are providing an easy and cost-effective solution to complex diagnostic processes. PBA provides new promising assays including Lateral flow Assays (LFAs), dipstick assays and microfluidic paper-based analytical devices (μPADs) using filter paper, nitrocellulose, and paper/polymer or paper/nanomaterial (MAO; JUN, 2012; HU et al., 2014a). The Commonly used pregnancy test is an example of paper-based LFA which detects human chorionic gonadotropin (hCG) hormone in urine. This model has been replicated for various other genetic and infectious diseases including HIV and various primary hepatic carcinoma biomarkers. Moreover, these LFAs have also been used for nucleic acid testing. Furthermore, integration of CP and PBAs based technologies giving rise to new solutions in a qualitative analysis as well as CP-based image technology has also been used for precise quantification analysis (BERK et al., 2003; YANG et al., 2011).

Various options have been used for increasing the sensitivity of traditional PBAs by applying signal enhancement strategies of nanomaterials and enzymes. However, these manipulations are associated with increased cost and lower shelf life (MERKOC; PAROLO; ESCOSURA-MUN, 2013). Another promising application of paper-based assays is an integration of fluidic μPAD batteries into the microfluid channels providing power to on-chip devices like electrochromic displays and LEDs. Moreover, by changing the passive capillary transport in paper substrates of μPADs to active transport mechanisms such as electric or acoustic fields have enhanced reproducibility, uniformity and accuracy(YEO; FRIEND, 2014).

2.6.3 Lab-on-a-Chip Platforms

Lab-on-a-chip platforms are promising technologies which integrate single or multiple analysis functions in a single integrated circuit commonly known as a chip. The chips are made of silicon, glass, polymers, papers, or various other materials including ceramics. Different commercial product based on LOC platforms have been developed including blood glucose analyzer (CHIN; SIA, 2012). Other LOC based products include whole blood chemistry analyzer (VASHIST et al., 2015). LOC has various potential benefits including low fluid volume consumption which in turn give rise to less waste, less volume requirement for sample and reagents which further reduce the cost of the test. However, a majority of LOCs are in developmental or invalidation stage so are unavailable for common practice (CHIN; SIA, 2012; NGE; ROGERS; WOOLLEY, 2013; VASHIST et al., 2015).

2.6.4 Xpert MTB/RIF Ultra

TB diagnostics have been flourished in the last decade with the introduction of several novel assays including Xpert MTB/RIF, urine lateral flow lipoarabinomannan (LF-LAM) and loop-mediated isothermal amplification (TB-LAMP) which were briefly discussed earlier in this section. Here some of the emerging diagnostic tests which are specific to TB POC diagnosis is going to discuss.

Xpert MTB/RIF Ultra is a more advanced version of the Xpert with the enhanced limit of detection of 16 CFU/ml as compared to Xpert 114 CFU/ml. Xpert Ultra provide 5% more sensitivity than Xpert (GARCÍA-BASTEIRO et al., 2018). However, it performs poorly in patients with a previous history of TB, showing 3.2 % lower specificity. Therefore, the result should be interpreted carefully, taking both the physical examination and the clinical history into consideration. In contrast to conventional Xpert reporting categories of high, medium, low, and very low. Xpert Ultra provides another extra reporting category of "trace". However, at this level, it cannot interpret resistance to an antibiotic, reporting just "MTB detected, trace, RIF indeterminate" (GARCÍA-BASTEIRO; SAAVEDRA; COBELENS, 2017; GARCÍA-BASTEIRO et al., 2018).

2.6.5 GeneXpert Omni

GeneXpert Omni is a small handheld module and has resolved two important concerns associated with traditional GeneXpert. Firstly, Omni provides 4 h battery life with an extra supplemental battery of 12 h. Secondly, Omni use the same cartridge

used in GeneXpert thus makes Omni applicable in rural areas. Omni has the ability to perform the single assay in 110 min. However, Omni is in the initial stage and has not been endorsed by WHO as well as there is no evidence to support its use (BOYLE, 2017).

2.7 CHALLENGES FOR IMPLEMENTATION IN POINT OF CARE DIAGNOSTIC TESTING

One of the big challenges for Point of care diagnostic testing is the lack of an ideal nucleic acid extraction method (NAE) which is hindered by various factors. Two of the traditional extraction methods (solid-phase and magnetic bead-based extraction) are available choices for implementing in POC-Dx devices. However, the need for centrifugation (in case of solid-phase extraction) and external magnetic source for mixing (in case of magnetic-bead extraction) are hindering their widespread implementation in POC-Dx devices. In this regard, the magnet-bead method has an edge on solid phase method as an implementation of magnetic stirring is somewhat easier than membrane separation through the stationary phase. However, the major challenges which are hampering the widespread implementation of POC-Dx in resource-limited areas are non-availability of proper infrastructure, expensive equipments and reagents, periodic maintenance support and provision of constant temperature (for equipments and reagents). Additionally, proper disposal of medical waste generated after analyses is another concern (ALI et al., 2017).

Paper-based assays (PBAs) is one of the platforms which seems to have the potential for ASSURED (Affordable, sensitive, specific, user-friendly, robust, and deliverable to end user) criteria of an ideal POC outlined by WHO (MARTINEZ et al., 2010; HU et al., 2014b; YAMADA et al., 2017). PBAs are able to provide affordable, rapid, and minimally equipment-free POC devices. Additionally, have the ability to provide a sensitive and specific diagnosis as compared to traditional diagnostic technique. Contrary to metabolic and protein-based POC assays, the major challenge for POC is to integrate all the three steps including (1) Sample preparation and extraction (2) amplification and (3) detection (CUI et al., 2015).

3 MATERIALS AND METHODS

3.1.1 Chemical Reagents and Equipments

Chemicals were purchased from different companies such as Sigma-Aldrich, Merk, Vivantis, Biosolve and Lumiprobe. DNA extraction was performed using a High Pure PCR Template Preparation Kit (Roche Applied Science), FTA micro elute Card (Cat No. WB120401), litmus paper was used for pH determination. All the chemicals used are listed below.

TABLE 1- LIST OF CHEMICALS AND REAGENTS USED IN THE STUDY

No#	Chemical	Company	Lot	Stock concentration and solution (10% in water/ 1% in TE buffer)
1	Mucin	Sigma-Aldrich	SLBG4336V	20%/5-10%
2	Guanidine Thiocyanate	Sigma Aldrich	BCBB3309	6M/2-3M
3	NP-40	Sigma-Aldrich	74385-1L	10%/1%
4	SDS	Vivantis	G7001	10%/1%
5	Triton X-100	Biosolve	20182332	10%/1%
6	Urea	Merk	K43108887240	8M/1M
7	NaOH	Merk	B0764495	6%/0.5%
8	N- Acetyl-L-Cysteine	Sigma Aldrich	SLBJ1054V	0.5%
9	Sybr green 100X	Lumiprobe	51010	100X/1X
10	Taqman Oligomix	Applied Biosystems	P170804-006F09-250917	20X/1X
11	Polyvinylpyrrolidone	Sigma-Aldrich	35H122515	10%/0.5%
12	TE Buffer (Solution 2)	Ambion	1501001-120716	N/A

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Master mix (IBMP/Fiocruz-PR, Brazil), containing a 3X concentrated Mix along with *Taq* DNA Polymerase and dNTPs, except DNA template and primers were from IBMP. Some of the probe and primers of Mtb were synthesized at IBMP (IS6110) while others were ordered as they are commercially available as Taqman gene expression assay such as IS6110-A. All these probes and primers are listed in Table 2.

PCR was performed on Applied Biosystem 7500 (ABI7500). Extracted DNA and oligonucleotide were measured using Denovix spectrophotometer. A portable

thermocycler named Q3-plus was used for amplification and detection parallel to ABI7500 (RAMPAZZO et al., 2014; ZAHRA et al., 2016; GUARNACCIA et al., 2017).

TABLE 2- LIST OF PROBES AND PRIMERS USED IN THE EXPERIMENTS

Gene	Category	Primer sequence (5'-3')	Product	Reference
T cruzi	Cruzi 1	ASTCGGCTGATCGTTTTCGA	166	(RAMPAZZO et al., 2014)
	Cruzi 2	AATTCCTCCAAGCAGCGGATA		
	Cruzi 3	FAM CACACACTGGACACCAA MGB		(MEJIA et al., 2011)
IS6110	IS6110-FP-01	CTGTGGGTAGCAGACCTCACCTA	67	(ROCCHETTI et al., 2016)
	IS6110-RP-01	CGGTGACAAAGGCCACGTA		
	IS6110-Pb-01	FAM TGTCGACCTGGGCAGGGTTCG BHQ1		
	IS6110-FP-02	AGACGTTATCCACCATAC	123	(BARLETTA et al., 2014)
	IS6110-RP-02	AGTGCATTGTCATAGGAG		
	IS6110-Pb-02	FAM TCTCAGTACACATCGATCCGGT BHQ1		
	TAQM 3	AGGCGAACCCTGCCCAG	122	(BROCCOLO et al., 2003)
	TAQM 4	GATCGCTGATCCGGCCA		
	TAQM_P	TGTGGGTAGCAGACCTCACCTATGTGTCGA		
IS6110-A	Forward	CAGGACCACGATCGCTGAT	73	(ALMEIDA et al., 2013)
	Reverse	TGCTGGTGGTCCGAAGC		
	Probe	TCCCGCCGATCTCG		

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3.1.2 Biological Materials

Epimastigote *Trypanosoma* cruzi DNA strain (Dm28c)(CONTRERAS; MOREL; GOLDENBERG, 1985) was kindly donated by Institute Carlos Chagas (ICC), FIOCRUZ-PR, Brazil. Mtb extracted DNA (H37Rv) was received from our collaborators from Centro de Desenvolvimento Científico e Tecnológico (CDCT)

Porto Alegre-RS, Brazil (Dr. Maria Lucia Rosa Rossetti). Mycobacterium DNA was obtained in high concentration (503ng/µL and 535 ng/µL), so for working stock, these were diluted 25ng/µL or 50ng/µL.

A total of 30 sputum samples from 30 TB patients (Non-Treated) were collected in three aliquots for each patient, the diagnosis of TB was established by clinical findings, molecular GeneXpert test and culture. The samples include 10 Gene Xpert positive and 20 Gene Xpert negative samples properly labelled with the ID number of individual patients. All these samples were received from LBM-ULBRA (Universidade Luterana do Brasil) is a research laboratory that collects and test clinical specimens

from various health units located in Canoas, the Rio Grande do Sul, Brasil. Additionally, 12 treated Mtb samples were received from Centro de Desenvolvimento Científico e Tecnológico (CDCT), Porto Alegre, Brazil. Sample collection/study was approved by the Ethical Committee of Universidade Luterana do Brasil (ULBRA/Canoas-RS) under the registration number (CAAE: 70697116.7.0000.5349).

3.2 LIQUIFICATION AND DECONTAMINATION STUDIES

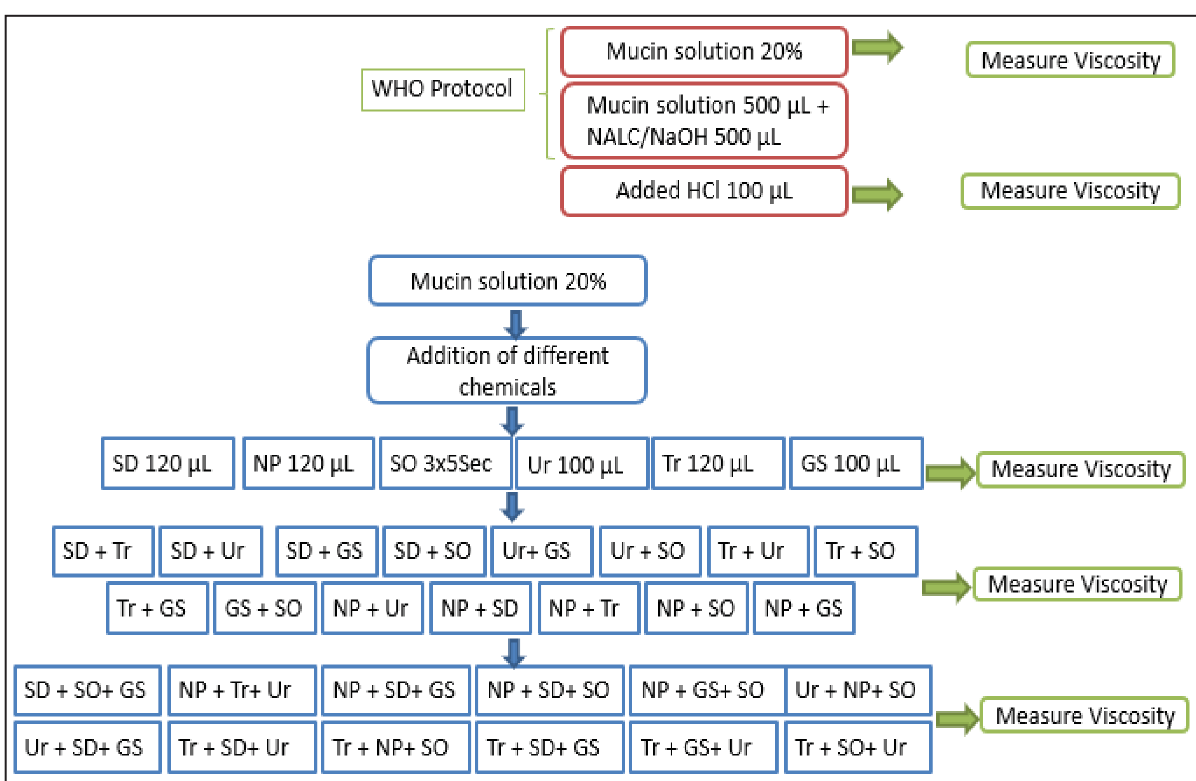
Since liquification and decontamination of Mtb samples is the prerequisite for sample preparation according to WHO (ALLEN; NICOL; TOW, 2016). NALC (N-acetyl-L-cysteine) works as a mucolytic agent for rapid digestion of sputum sample while NaOH helps in decontamination of flora present in the specimen. This part of the study was designed to identify chemical(s) that could be used safely outside the lab to liquify and decontaminate a sample. After treating each chemical or combination(s) of the chemicals, measurement of the viscosity was part of the study, so after each treatment of mucin with chemical(s) viscosity or liquification was measured visually as well as with pipette. Figure 5 shows an overview of the protocol tested.

Pig mucin (20% w/v) was used to simulate a sputum matrix, prepared in nuclease-free H2O, mimicking natural human sputum in colour, consistency, and viscosity (GOVINDARAJAN et al., 2012). For treatment of this sputum-like mucin sample, a set of different chemicals were prepared and tested alone and in combination: (1) NP-40 10%, used at 1%a nonionic surfactant as a part of cell lysis buffers (2) SDS 10% sodium dodecyl sulfate (SDS), used at 1% as an anionic surfactant as a part of cell lysis buffer (3) Triton X-100 10%, used at 1% as a nonionic detergent as a part of cell lysis buffer (4) Urea 8M, used at 4M as a chaotropic agent (5) Guanidine Thiocyanate 6M, used at 3M as a denaturing agent. All these chemicals were selected on the basis of their chemical properties and their presence in commercial lysis buffer.

A sample volume of 500 µL of porcine mucin was chosen as a realistic volume to obtain from a patient. For the standard sample preparation, NALC/NaOH was added to the standard sample in the same volume i.e. 500 µL as we know that NALC acts as a mucolytic agent for samples with high protein content and in combination with NaOH act as decontaminant for sputum samples (BRADNER et al., 2013). Next, 100 µL of

25% of HCl (0.75M) was added for adjusting the pH back to neutrality tested by pH paper. In WHO standard protocol a digestant is prepared from 4% NaOH and 2.9% sodium citrate in equal volume and 0.5 g of NALC is added per 100 mL of sodium hydroxide-sodium citrate solution. An equal volume of NALC/NaOH solution is added to a sputum specimen in 50 mL centrifuge tube and briefly vortexed (5-20 s) and allowed for 15-20 min incubation (Mycobacteriology Laboratory Manual, 2014). The initial viscosity of porcine mucin was rated as (++++) and after treatment with chemicals, it was rated from (++++) to (+). After each treatment, the viscosity was observed and estimated with: (1) the naked eye, (2) gentle agitation of the tubes, and (3) gentle pipetting of the mixture. To avoid repetition all the details with results are mentioned in the results section No. 4.1.1 while below the addition of single, double, and triple chemicals with mucin has been shown.

FIGURE5 – GENERAL STUDY DESIGN FOR MUCIN LIQUIFICATION



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NOTE: NP=NP-40, SD=SDS, Tr =Triton X-100, GS= Guandine thiocyanate, Ur= Urea, SO=Sonication NALC= N-acetyl-L-cysteine

TABLE 3- ADDITION OF SINGLE CHEMICAL

1. Addition of NP-40	**2. Addition of SDS**	**3. Addition of Triton X-100**
1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100 µL of HCL 3. Add NP-40 120 µL 4. Measure viscosity	1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100 µL of HCl 3. Add SDS 120 µL 4. Measure viscosity	1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100 µL of HCl 3. Add TritonX100 120 µL 4. Measure viscosity
Total volume= 1220 µL	**Total volume= 1220 µL**	**Total volume= 1220 µL**
4. Addition of Urea	**5. Addition of Guanidine**	**6. Sonication Bath**
1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100µL of HCl 3. Add Urea 800µL 4. Measure viscosity	1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100µL of HCl 3. Add GSCN 800µL 4. Measure viscosity	1. Mucin 500 µL +NALC/NaOH 500 µL 2. 100µL of HCl 3. Sonication Bath (3X 30 s at 50% power) 4. Measure viscosity
Total volume= 1900µL	**Total volume= 1900µL**	**Total volume= 1100µL**

FONT: The author

TABLE 4- ADDITION OF TWO CHEMICALS

1. Addition of NP-40+SDS	**2. Addition of NP-40+TritonX-100**
1. Mucin 500 µL+NALC/NaOH 500 µL 2. 100µL of HCl 3. Add NP-40 120µL 4. Add SDS 120 µL 5. Measure viscosity	1. Mucin 500 µL+NALC/NaOH 0500 µL 2.100µL of HCL 3. Add NP-40 120µL 4. Add Triton 120 µL 5. Measure viscosity
Total volume= 1340µL	**Total volume= 1340µL**
3. Addition of NP-40+Urea	**4. Addition of NP-40+GSCN**
1. Mucin **500 µL**+NALC/NaOH **500 µL** 2. 100µL of HCL 3. Add NP-40 120µL 4. Add Urea 100 µL 5. Measure viscosity	1. Mucin 500 µL+NALC/NaOH 500 µL 2. 100µL of HCL 3. Add NP-40 120µL 4. Add GSCN 100 µL 5. Measure viscosity
Total volume= 1320µL	**Total volume= 1320µL**

FONT: The author

TABLE 5- ADDITION OF THREE CHEMICALS

1. **Addition of NP-40+SDS+Triton-X**	**2. Addition of SDS+ Urea+ GSCN**
1.Mucin 500 µL+NALC/500 µL	1.Mucin 500 µL+NALC/NaOH 500 µL
2.100uL of HCl 3. Add NP-40 120µL 4. Add SDS 120µL 5. Add Triton X 120µL 6.Measure viscosity	2.100µL of HCl 3. Add SDS 120µL 4. Add Urea 8M 350µL 5. Add GSCN 8M 350µL 6.Measure viscosity
Total volume=1460µL	**Total volume=1920µL**
3. Addition of NP-40+Urea+SDS	**4. Addition of NP-40+GSCN+SDS**
1.Mucin 500 µL+NALC/NaOH 500 µL	1.Mucin 500 µL+NALC/NaOH 500 µL
2.100µL of HCl 3. Add NP-40 120µL 4. Add Urea 100 µL 5. Add SDS 10% 120µL 6.Measure viscosity	2.100µL of HCl 3. Add NP-40 120µL 4. Add GSCN 100 µL 5. Add SDS 10% 120µL 6.Measure viscosity
Total volume=1440µL	**Total volume=1440µL**

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3.3 EXTRACTION STUDIES

3.3.1 DNA Extraction and Dilution Curve for *T. cruzi*

Epimastigote cells (10^7) of *Trypanosoma cruzi* (strain Dm28c) was collected from ICC and washed in Phosphate buffer saline (PBS 1X, pH 7.4). High Pure PCR Template Preparation Kit (Roche Applied Science) was used for total DNA extraction and was chosen as a gold standard method (SCHIJMAN et al., 2011). Extracted DNA from 10^7 cells was considered as representative of 10^7 genome equivalents (GE). 100 µL of extracted DNA was taken and dilutions were made ranging from 10^5 to 10^{-1} in TE Buffer. Real-time PCR was performed using 5 µL of extracted DNA, 0.3 µM of each primer (cruzi1 and cruzi2) and 0.2 µM of a probe (cruzi3, FAM-BHQ1) in a final volume of 20 µL. Conventional qPCR was performed on the ABI7500 (Life Technologies), with cycling condition as follows: 95˚C/10 min, and 40 cycles of 95˚C/15 s and 60˚C/1 min (PIRON et al., 2007)(SCHIJMAN et al., 2011)(RAMPAZZO et al., 2014).

3.3.2 Evaluation of Different Sample Preparations Protocol for *T. cruzi* DNA Extractions

3.3.2.1 Comparison of Standard and In-house Sample Preparation

200 µL of mucin was distributed to 4 tubes and 50µL of DNA was added to (Tube 2, 3, 4) and tube1 was left without DNA. 200µL of NALC/NaOH was added to tube 2 and tube 4 and incubated for 15min and then 40 µL of HCl was added and mixed for

3-5 s. Extraction was performed according to Roche High pure extraction kit with some modification such as, not adding lysis and binding buffer while 40µL of Proteinase K was added to all 4 tubes respectively. The rest of the protocol was followed as described in the kit. Protocol tested were:

(1) Standard 200 µL of mucin only as a no-DNA and no-chemical"

(2) NALC tube: 200 µL mucin spiked with 50 µL *T. cruzi* DNA. Additionally, 200 µL of NALC/NaOH and 40 µL of HCl was added. This tube was considered as the standard control ("no chemicals").

(3) GSCN tube: 200µL of mucin was spiked with 50 µL of *Trypanosoma cruzi* (*T. cruzi*) DNA and added with 200µ of GSCN.

(4) GSCN + NALC tube: 200 µL of mucin spiked with 50 µL of T cruzi DNA and also added with 200 µL of GSCN. Additionally, 200 µL of NALC/NaOH and 40 µL of HCl was added.

3.3.2.2 Evaluation of In-house Sample Preparation

Two tubes (1 and 2) were selected and 200 µL of mucin was added to each tube. Both the tubes containing 200 µL of mucin was spiked with 20 µL of 10^7 Trypanosoma cruzi (*T. cruzi*) cells. Both the tubes were extracted according to standard High pure Roche extraction kit without any modifications. A 7-point dilution of 1:10 was prepared and PCR was carried out.

3.3.2.3 Extraction of live T cruzi: A Comparison and Evaluation of Different Sample Preparations

200µL of mucin was added to five tubes (1, 2, 3, 4, 5). 100µL of *T. cruzi* cells were added to each tube and mixed thoroughly. All the tubes were added 200µL of NALC/NaOH and 50µL of HCl. 200µL of GSCN was added to tube 2,3,4,5.100 µL of Triton X-100 was added to tube 3 and 5. 120 µL of Urea was added to tube 3 and 5 and Proteinase K was added Tube 1, 2 and 3 while the binding buffer was added to tube 1 only. Following were the conditions used.

(1) 200 µL mucin,100 µL *T. cruzi* parasite, 200 µL of NALC/NaOH, 50 µL of HCl

(2) 200 µLmucin,100 µL *T. cruzi* parasite, 200 µL of NALC/NaOH, 50 µL of HCl and 200 µL of GSCN

(3) 200 µLmucin,100 µL *T. cruzi* parasite, 200 µL of NALC/NaOH, 50 µL of HCl, 200 µL of GSCN, 100 µL of Triton X-100 and 20 µL Urea

(4) This tube composition is same as Tube.2 but without Proteinase K from Roche protocol

(5) This tube composition is same as Tube.3 but without Proteinase K from Roche protocol

3.3.3 Mtb (H37Rv) Reference Strain Dilution Curve

A 7 point, 10-fold dilution series was performed using reference strain Mtb (H37Rv) DNA (25ng/ µL) in human negative blood. Six points were 100pg, 10pg, 1pg,100fg,10fg and 1fg. A single-plex and duplex real-time PCR was performed using 20X Oligomix of Taqman gene expression assay in three different formats; ABI7500, Q3 liquid reaction and Q3 gelified reaction.

3.3.4 FTA Based DNA Extraction

FTA elute micro cards are elute matrix which utilizes FTA technology for lysis of the cells resulting in the release of nucleic acids from various biological samples including blood, saliva, buccal swabs, and culture cells. A series of DNA extraction was carried out with a different set of conditions using FTA card for *T. cruzi* and Mtb which is mentioned in the next sections.

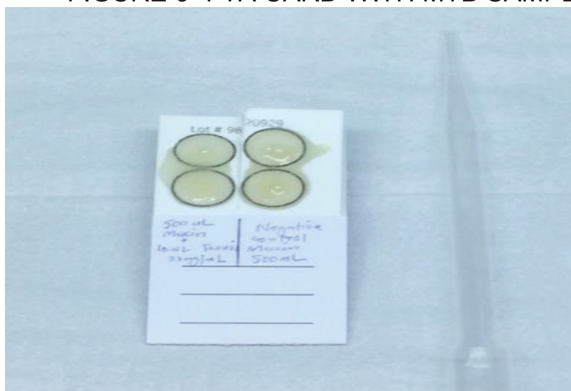
3.3.4.1 FTA extraction with live *T. cruzi* and dilution curve

T. cruzi epimastigote cells (10^7) were collected in 1 mL PBS (1X) from ICC and a 10-fold dilution was made. Next, four tubes of mucin were taken while each tube containing 500 µL mucin and 50 µL of four dilution points (10^4,10^3,10^2,10^1) were added and mixed to the mucin tubes which give rise to dilution 10^3,10^2,10^1 and 10^0. All the samples were applied to FTA cards using a sterile filter tip and dried at room temperature for 1 h. For DNA extraction, 6-9 punches (Ø 2mm) were cut down from different places of FTA card using a sterile hole puncher and placed in respective tubes. 600 µL of H_2O was added and vortexed 3X for 5 s. All the tubes were incubated at 95℃ for 10 min. DNA was extracted in 200 µL of H_2O.

3.3.4.2 FTA Card Extraction with Mtb Samples

Firstly, Sample was homogenized with Solution 1(Guanidine thiocyanate 6M) with the ratio of sputum/solution 1 as 1.0mL/0.4mL (i.e. added 0.4mL Solution1 to 1.0 mL of sputum) and vortexed vigorously for 20-30 s. Secondly, 700 µL to 1mL of diluted sputum sample was applied directly to a 3.5cm×3.5cm area of FTA card using a Pasteur pipette. The FTA card was then allowed to dry at room temperature for 1 h shown in the Figure (1).

FIGURE 6- FTA CARD WITH MTB SAMPLE



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One disc of 6mm from the spotted area was punched using a sterilized hole puncher and placed in 1.5 mL PCR tube. The hole puncher was sterilized/disinfected with 70% alcohol after every punch and between cuts from different samples.

This DNA extraction is mainly based on (GUIO et al., 2006), with some modifications(change in punch size and Elution buffer). 500 µL of Solution 2 was added to the tube to containing disc and vortexed three times for 5 s with 5 s interval and Incubated at 95℃ for 5 min. The temperature was brought down to 25℃, waited 5 min and gave a short spin. The supernatant was collected in a new tube.

3.3.4.3 FTA-Based Different DNA Extraction Protocols with Modifications

Six different extraction protocols were designed with modification in the manufacturer´s protocol, such as punch (size/number) and incubation time.

TABLE6 – SIX DIFFERENT DNA EXTRACTION PROTOCOLS

Protocol 1	**Protocol 2**	**Protocol 3**	**Protocol 4**	**Protocol 5**	**Protocol 6**
1. Take the whole circle and put in clean tube	1. Take the whole circle and put in clean tube	1. Take 3 punches	1. Take 3 punches	1. Take the whole circle and put in clean tube	1. Take 3 punches
2. Add 200µL H_2O or TE pH8	2. Add 200µL H_2O or TE pH8	2. Add 200µL H_2O or TE pH8	2. Add 200µL H_2O or TE pH8	2. Add 200µL H_2O or TE pH8	2. Add 200µL H_2O or TE pH8
3. Vortex 3X for 5 s	3. Vortex 3X for 5 s	3. Vortex 3X for 5 s	3. Vortex 3X for 5 s	3. Bath sonication 3X 15-20 s	3. Bath sonication 3X 15-20 s
4. Incubate at 95°C/5min	----	4. Incubate at 95°C/5min	----	----	-----
5. Collect extract	5. Collect extract	5. Collect extract	5. Collect extract	5. Collect extract	5. Collect extract
6. Run PCR	6. Run PCR	6. Run PCR	6. Run PCR	6. Run PCR	6. Run PCR

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3.3.5 Extraction with Disposable Pipette Device

This extraction method was designed according to (PAWLOWSKI; KARALUS, 2012) with some modifications according to our requirements. Sample preparation and lysis was performed by adding 300µL of epimastigote *T. cruzi* cells (suspended in 1X PBS were mixed) with 300µL of mucin and mixed with 500µL of 6M GSCN. In order to expel the ambient air from the pipette, the bulb of the pipette was depressed and the sample was pulled into the pipette, passing through the silica bed placed in the pipette. In order to drain all the bead and sample into the bulb, the extraction device was upside down for 2-3 time. For complete mixing of sorbent beads and sample, the bulb was rubbed carefully by not expelling out the sample from the device.

After complete mixing, the device was inverted and expelled all the sample into the original 1.5mL tube. Repeating the same step, 1mL of the sample was pulled into the extraction device with the moderate rate and again by depressing the bulb, the whole sample was expelled out into a 1.5mL tube. This step was repeated for a total of 5 times by passing the sample through the sorbent. For washing 1mL of 95% ethanol

was passed over silica inside the device for a total of three times. Completely wiped out ethanol from the device by pressing and releasing the bulb for 5 minutes and recovered nucleic acid by passing over 250 µL of 10mM Tris (pH 7) 5 times.

3.3.6 Optimization of FTA based extraction

For optimization of FTA based extraction two different strategies were adopted; (1) using inhibitor removal chemicals/buffer (PVP and BSA) and (2) using different volumes of the Elution buffer. For this purpose different concentrations (10%, 2.5%, 1%, 0.5% and 0.1%) of PVP was experimentd. On the other hand, two different volumes i.e. 200 µL and 500 µL of Elution buffer was used.

3.4 DNA QUANTIFICATION

DNA quantification was performed on "Denovix DS-11/DS-11+ spectrophotometer" and by electrophorese extracted DNA on 3% agarose gel. Denovix spectrophotometer provides direct and easy measurements within a 5 s only by using just a pipette and wipe. DNA quantification is performed according to manufacturer protocol.

3.5 REAL-TIME PCR

The qPCR reactions were performed on ABI7500 equipment (Life Technologies, USA) in 96-well plates. Reactions were performed using the Universal PCR Master mix 3x and dNTPs produced and/or supplied by IBMP/Fiocruz- PR. While 20X TaqMan an Oligomix (Assay name: IS6110-A; Assay ID: AIPACNK) was used. Reactions were standardized to the final volume of 20 µL, considering the addition of 5 µL of sample (extracted DNA). The following cycling conditions were used for the target(s): 50˚C/2 min, 95˚C/10 min, and 40 cycles of 95˚C/15 s and 60˚C/1 min. All the Cts were set manually for each PCR.

3.6 Q3

On-chip qPCR was performed on a prototypal equipment, developed by STMicroelectronics under the name "Q3". On-chip reactions contained 1 µL of extracted DNA in a final volume of 5 µL (ZAHRA et al., 2016). The cycling conditions were as follows: 70˚C/10 , 80˚C/10 s and 97˚C/60 s while 45 cycles of 97˚C/ 20 s and

62˚C/60 s. Settings for Q3-Plus optical parameters were: for the FAM channel, led power of 3, camera gain of 15 and image exposure of 1 s, and for the HEX channel power of 8, camera gain of 14 and image exposure of 2 s.

3.7 STATISTICAL ANALYSIS

The raw data was analyzed using Microsoft Excel 2016 and GraphPad Prism® version 7 was used.

4 RESULTS

The scheme below is intended as a guide for the reasoning of the sequence of experiments described in this section.

No.	TRADITIONAL METHOD	THIS THESIS PROPOSAL/ PROTOCOL	SECTION#
1	Lysis of sample with lysis buffer (GSCN)	Solution 1 and FTA card Denaturation and lysis tests	4.1.1 4.2.1 4.2.2 4.2.3
2	Binding to silica matrix (beads or column)	FTA card: Binding tests	4.3 4.4
3	Washing	Definition of punch size as well as the volume and time of the wash	4.5.3
4	Elution	Volume: time and temperature of elution	4.5.3
5	Integrity and activity	OD and PCR *T. cruzi* whole cells (model) TB samples (inactivated and intact)	4.6

4.1 SAMPLE PREPARATION AND LIQUIFICATION STUDY

4.1.1 Chemical Liquification of Mucin

The basic approach for sample preparation used in this investigation is similar to that used by other researchers (Govindarajan et al, 2012). The initial viscosity of porcine mucin was rated as (++++) with the naked eye and gentle agitation of the tube (Figure 7).

FIGURE 7- 20% (W/V) PIG MUCIN AS SIMULATING SPUTUM

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Table 7 describes the volumes and combinations of chemicals tested: GSCN, GSCN+UREA, GSCN+TX100, and GSCN+UREA+TX100. After each subsequent treatment, the viscosity was observed and measured by visualizing, gentle agitation, and pipetting of the tubes. Tube #1 (NALC/NaOH, GSCN, Urea and Triton X-100) was considered as standard protocol, with all the chemical added. Tube #7 (Triton X-100) showed liquification as (+++) while tubes #2 (GSCN, Urea and Triton x-100), #3 (GSCN, Urea and Triton x-100), #4 (GSCN, Urea and Triton x-100), #6 (Urea) and #8 (GSCN, Urea and Triton x-100) showed (++), and tube #5 (only GSCN) showed (+). When liquification was tested after treatment with chemical(s) for tube #4, #5 and #6 with the naked eye and gentle agitation all seemed to have similar results. However, when tested with the pipetting, the upward flow of mucin for tube #5 showed better liquification as compared to tube #4 and tube #6. Taken together, these results suggest that GSCN alone has better liquification potential as compared to other chemicals.

TABLE 7 - LIQUIFICATION OF MUCIN WITH DIFFERENT CHEMICALS

No.	Mucin	NALC/NaOH	HCl	GSCN	Urea	Triton X-100	Total	Result Before/After
1	500µL	500µL	120µL	400µL	400µL	100µL	2000µL	++++/ +
2	500µL	-	-	200µL	200µL	100µL	1000µL	++++/ ++
3	500µL	-	-	500µL	500µL	200µL	1700µL	++++/ ++
4	500µL	-	-	250µL	250µL	100µL	1100µL	++++/ ++
5	500µL	-	-	500µL	-	-	1000µL	++++/ +
6	500µL	-	-	-	500µL	-	1000µL	++++/++
7	500µL	-	-	-	-	200µL	700µL	++++/ +++
8	500µL	-	-	300µL	100µL	50µL	1000µL	++++/++

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NOTE: (++++) classified as most viscous and (+) indicated as the least viscous (i.e., liquid-like).

4.2 COMPARISON OF DIFFERENT SAMPLE PREPARATIONS

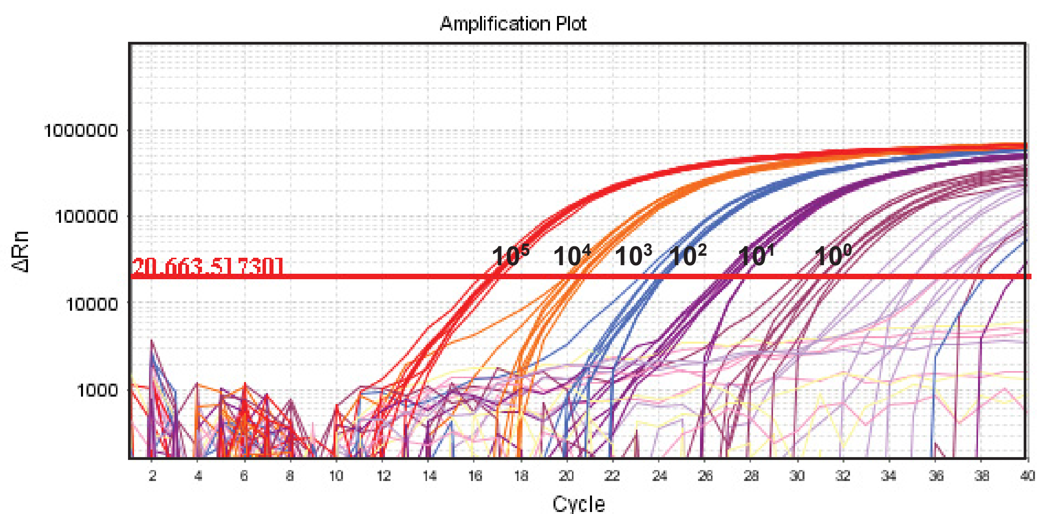
4.2.1 Comparison of In-house and Other Protocols

In order to evaluate if the addition of GSCN alone to mucin containing the known amount of extracted DNA would allow for DNA extraction from the sample protocols to the same extent as commercial kits, we prepared three different sample protocols along with a “no-treatment control”. A 7-point dilution was prepared using the DNA extracted by each protocol and PCR was carried out.

All the samples were extracted using Roche's High Pure PCR Extraction kit but with minor modification; not adding lysis buffer, which was substituted by GSCN. Extracted DNA was tested for the presence of *T. cruzi* in a 1:10 dilution curve. All the sample preparations show positive amplification except for “no-DNA and no-chemical” tube. Mean Cts are shown in TABLE 4 and representative traces are shown in Figure 8. The Cts obtained from all protocols, showed no significant difference which suggests that GSCN can be used in extraction method for non-cellular as well as a liquifying agent for sputum.

Overall, these results suggest that GSCN (a natural chaotropic agent) can be used as lysis buffer alone. Also, suggest high GSCN concentration does not interfere with the efficiency of DNA binding and recovery from the column.

FIGURE 8– REPRESENTATIVE DILUTION CURVE AMPLIFICATION OF DIFFERENT SAMPLE PREPARATION PROTOCOLS FOR *T. cruzi.*



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NOTE: Here it is shown DNA extracted from Protocol (2, 3 and 4). Traces are superimposed.

TABLE 8 - COMPARISON OF THREE DIFFERENT LYSIS BUFFER SUBSTITUTES

[DNA]	MUCIN (NTC)	NALC	GSCN	GSCN+NALC
10^5		17.02 ± 0.23	16.90 ± 0.11	16.68 ± 0.19
10^4	38.30	20.77 ± 0.17	20.53 ± 0.31	20.18 ± 0.11
10^3	39.43	24.28 ± 0.03	24.17 ± 0.11	23.54 ± 0.20
10^2	37.75	27.45 ± 0.40	27.47 ± 0.33	26.83 ± 0.08
10^1	36.73	31.61 ± 0.41	30.58 ± 0.59	31.08 ± 0.66
10^0	ND	36.88 ± 0.84	34.57 ± 0.76 ND	35.25 ± 1.86
10^{-1}	ND	36.45 ±0.00	ND	ND
NTC	ND	ND	ND	ND

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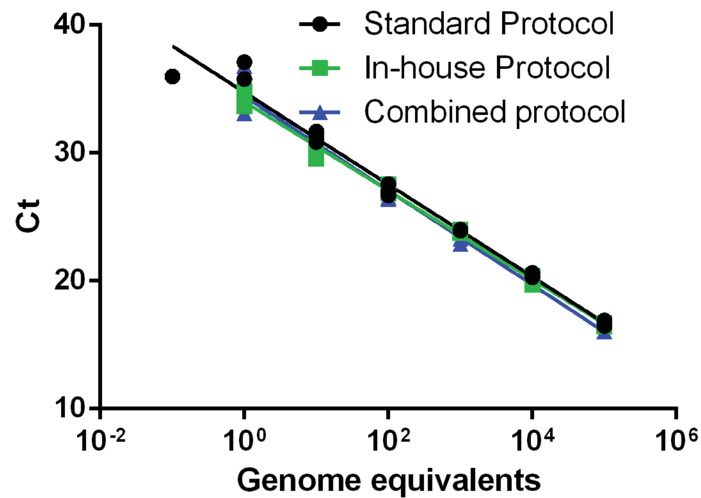
FIGURE 9 - COMPARISON OF THREE DIFFERENT SAMPLE PREPARATIONS



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4.2.2 Comparison between In-house and Commercial Extraction Methods using GSCN as Substitute for Lysis Buffer

This experiment was designed to compare the difference between commercial extraction (without any modification in sample preparation and extraction) and in-house (GSCN used as a substitute for lysis buffer) extraction method. The Cts obtained from both methods showed no significant difference (Table 9) which confirms previous results. Taken together, these results suggest that GSCN can be used as mucin liquefying agent and as lysis buffer to allow DNA extraction from intact cells.

FIGURE 10- COMPARISON OF DILUTION CURVE OF *T. cruzi* DNA WITH TWO EXTRACTION METHODS

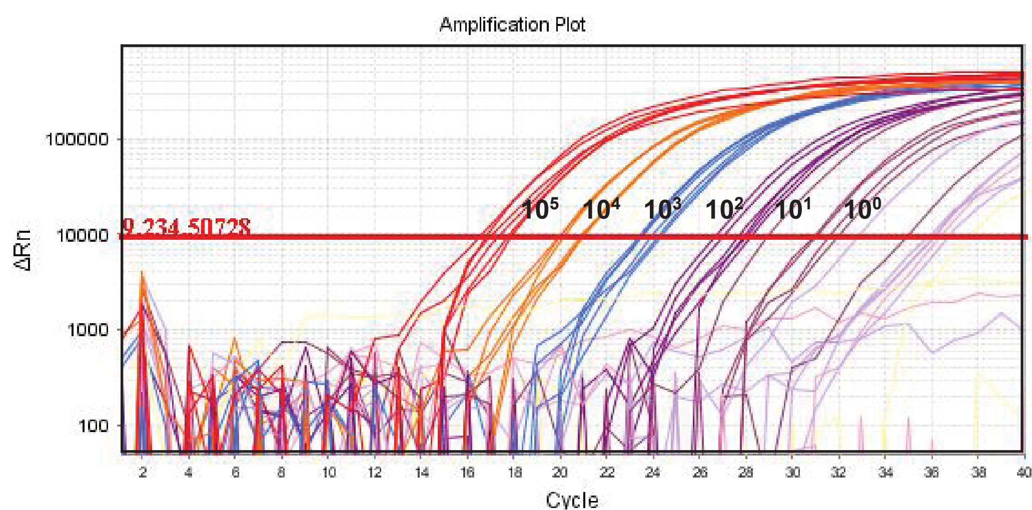

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TABLE 9 - COMPARISON OF IN-HOUSE AND COMMERCIAL EXTRACTION METHOD

[DNA]	Roche Commercial	GSCN (In-house)
10^5	17.71 ± 0.23	18.57 ± 0.22
10^4	20.92 ± 0.09	21.89 ± 0.04
10^3	24.31 ± 0.09	25.08 ± 0.19
10^2	28.02 ± 0.53	28.92 ± 0.20
10^1	31.39 ± 1.35	33.55 ± 2.05
10^0	36.18 ± 2.06	36.98 ± 0.24
10^{-1}	ND	37.26
NTC	ND	ND

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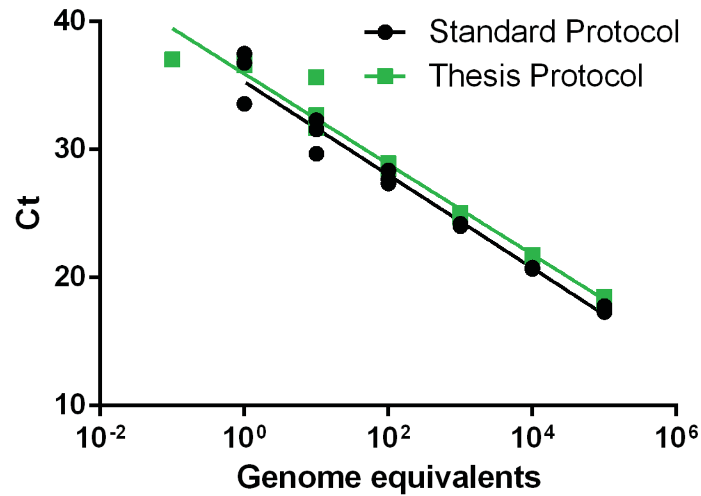
Figure 11- COMPARISON BETWEEN COMMERCIAL AND IN-HOUSE EXTRACTION METHOD



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4.2.3 Extraction of *T. cruzi* Epimastigotes DNA using Guanidine Thiocyanate (GSCN) as Substitute for Lysis Buffer: Effect of Proteinase K

In order to evaluate GSCN as a lysis and binding buffer and the need for Proteinase K in the presence of GSCN, epimastigote cells of *T. cruzi* were treated with different conditions. As we know that Proteinase K is normally used for digestion of proteins in nucleic acid extraction and have a high substrate specificity. For this purpose, a 10^7 *T. cruzi* epimastigote cells (Dm28c), which can be considered as 10^7 genome equivalents (GE)(RAMPAZZO et al., 2014) were collected in PBS and extracted.

The Initial composition was same for all tubes where 200 µL mucin, 100 µL of *T. cruzi*, 200 µL of NALC/NaOH and 50 µL of HCl was added to all five tubes. Tube #1 is considered as standard and extracted according to Roche protocol without any modification. Tube #2 and Tube #3 was not added lysis buffer but instead it was added GSCN, Urea and Triton X-100 as lysis buffer. Tube #4 (which have the same composition as tube #2) and Tube #5 (which have the same composition as tube #3) were not added Proteinase K during the extraction procedure. Table (6) illustrates mean of the Cts extracted with each different set of conditions. It is apparent from the table that there is no significant difference between the Cts obtained from the five different conditions.

FIGURE 12- COMPARISON OF FIVE DIFFERENT EXTRACTION CONDITIONS

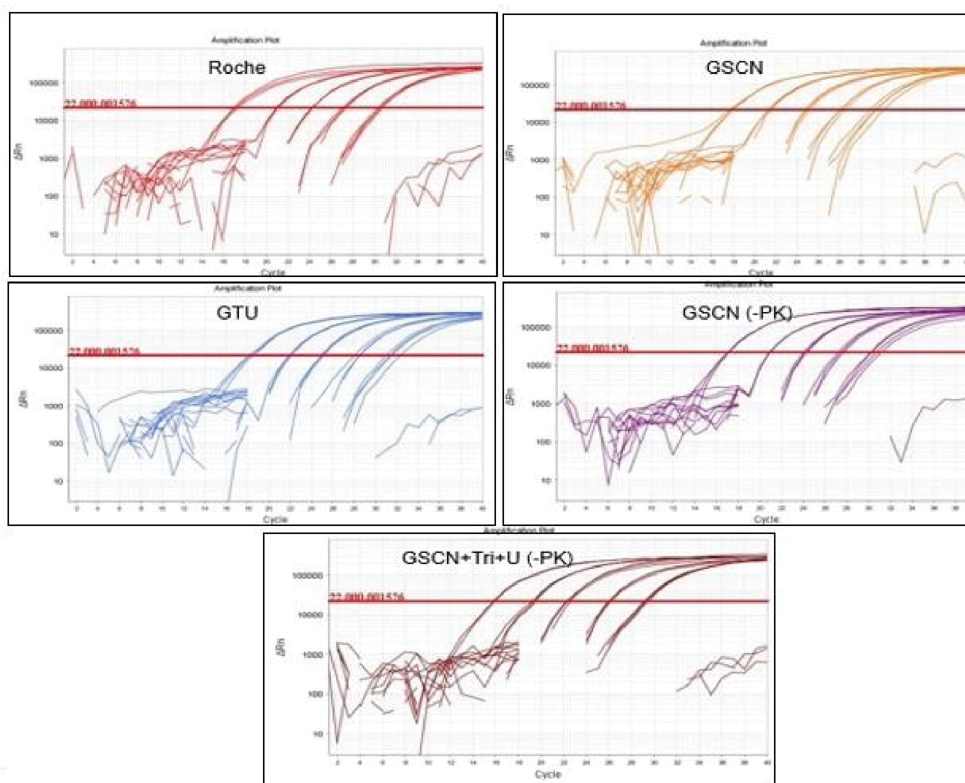

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TABLE 10 - COMPARISON OF DIFFERENT CHEMICALS USED IN DIFFERENT SET OF CONDITIONS WITH STANDARD ROCHE EXTRACTION PROTOCOL

	Roche	GSCN	GTU	GSCN (PK -)	GSCN+Tri+Urea (PK-)
10^4	15.75 ± 0.12	17.34 ± 0.27	16.52 ± 0.24	15.80 ± 0.08	15.05 ± o.11
10^3	18.52 ± 0.10	20.54 ± 0.21	19.59 ± 0.09	19.54 ± 0.02	17.52 ± 0.21
10^2	21.74 ± 0.03	23.88 ± 0.17	22.80 ± 0.09	22.72 ± 0.16	20.77 ± 0.20
10^1	25.13 ± 0.19	27.30 ± 0.25	26.25 ± 0.34	26.25 ± 0.24	24.37 ± 0.08
10^0	28.41 ± 0.16	30.88 ± 0.42	29.11 ± 0.67	29.60 ± 0.28	27.60 ±0.19

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NOTE: GSCN= Guanidine Thiocyanate; GTU= Guanidine Thiocyanate+Triton X-100+ Urea; (PK -) = without Proteinase K.

FIGURE 13 - *T. cruzi* EPIMASTIGOTE EXTRACTION WITH DIFFERENT PROTOCOLS

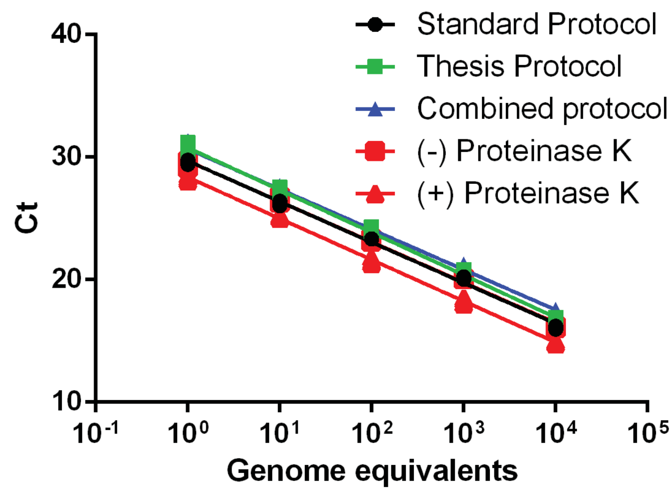

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FIGURE 14- DIFFERENT EXTRACTION PROTOCOL: LINEAR REGRESSION OF *T. cruzi* TARGET 10^4 to 10^0. THE EXPERIMENTS WERE RUN IN TRIPLICATE USING STRAIN Dm28c.

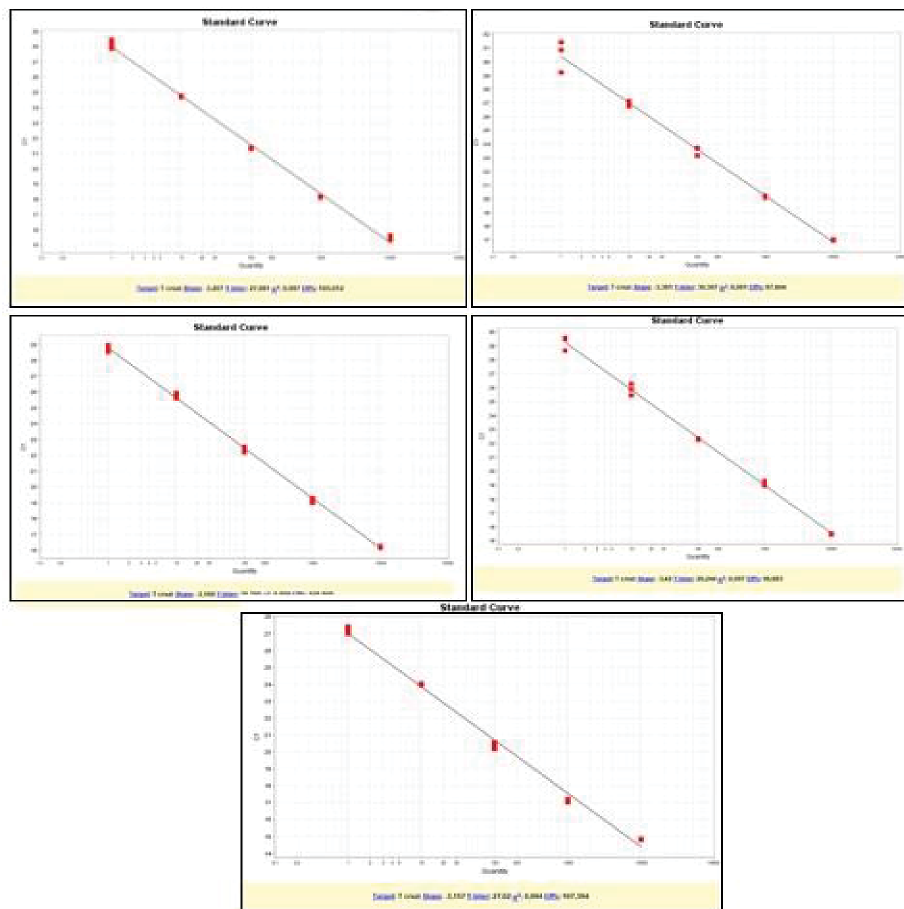

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TABLE 11- DIFFERENT EXTRACTION PROTOCOLS WITH EFFICIENCY AND R^2

No.	Method	Efficiency	R^2
1	ROCHE STANDARD PROTOCOL	105%	0.99
2	GUANIDINE THIOCYANATE	97%	0.99
3	GUANIDINE THIOCYANATE+TRITON X-100+ UREA	106%	0.99
4	GSCN PK (-)	96%	0.99
5	GUANIDINE THIOCYANATE+TRITON X-100+ UREA; (PK -)	107%	0.99

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4.2.4 Evaluation of Disposable Pipette Extraction Method

The approach used in this extraction methodology is based on the work by (PAWLOWSKI; KARALUS, 2012), where they used an extraction tool (Disposable pipette) incorporated with silica-based technology or Boom technology (BOOM et al., 1990). The solid phase silica material is fitted inside the pipette, which does not need any spin column. The extraction tool is neither dependent on electricity nor centrifugation and can be easily adapted to point of care testing. A mixture of 500 µL of sample and 500 µL of GSCN (6M pH=6.5) is pulled in to the extraction pipette over the silica material. The sample is grinded with the sorbent beads by hand carefully. The complete details are mentioned in the method section (3.3.3). In our case, different conditions were tested with *T. cruzi* and minor modifications were made in term of sample volume (300 µL and 500 µL) and concentrations of GSCN (6M and 8M). However, the method did not show any satisfactory results for our experiments.

4.3 Evaluation and optimization of FTA extraction protocol with *T. cruzi*

For the purpose of evaluation and optimization of FTA based extraction different protocols were tested. As a sample preparation, 500 µL of mucin was mixed with 500 µL of GSCN, spotted on FTA card and dried for 1 h at room temperature.

For extraction, several experimental conditions were tested with different numbers of punches (1 punch was equal to 3mm in diameter) (1, 2, 3 and whole circle) and two eluting solutions (water and TE buffer). Moreover, incubation and sonication conditions were also tested. The initial experiment did not show any satisfactory results; most probably due to unknown inhibitors. However, after diluting the sample into 1:10 and 1:100, they showed positive amplification.

A total of six different protocols were designed and tested individually. Protocol 3 and 4 proved to be the best for our purpose, as seen in Figure 15 and 16 below and other protocols are mention in Figure 17. All the six protocols are mentioned in Table.6 of method section (3.3.4.3) as well as here below.

FIGURE 15_PROTOCOL3 (1:10 AND 1:100 DILUTION)

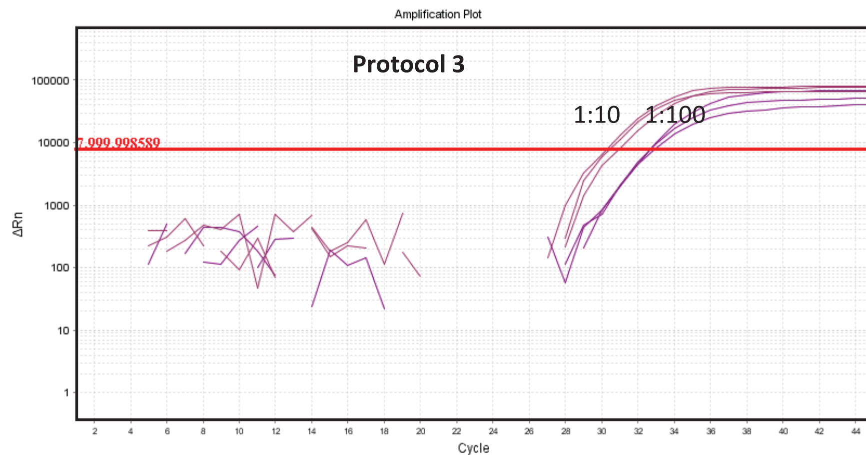

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FIGURE 16- PROTOCOL4 (1:10 AND 1:100 DILUTION)

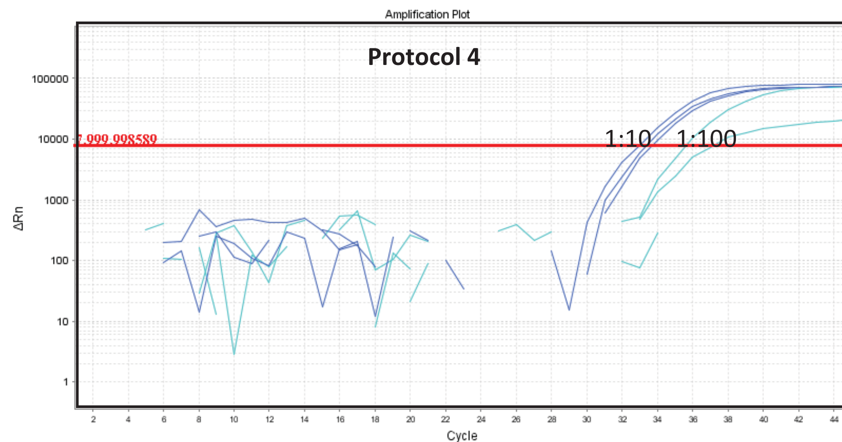

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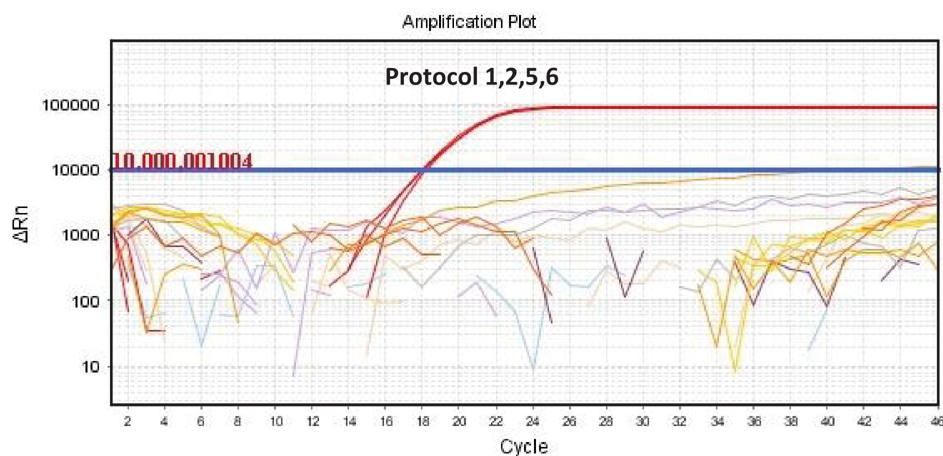
FIGURE 17- PROTOCOLS 1, 2, 5, 6



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4.4 ESTABLISHING A DUPLEX PCR FOR DETECTION OF MTB DNA IN HUMAN SAMPLES

4.4.1 Dilution Curve of MtbDNA on ABI 7500

These experiments were performed in collaboration with Dr. Maria Lucia Rossetti (CDCT-RS). To evaluate the limit of detection of Mtb H37Rv, a PCR using IBMP mastermix and a 20X Taqman oligomix a PCR was performed. A 7-point dilution curve shows amplification up to 1fg prepared with the reference strain of Mtb H37Rv (25ng/µL) diluted in human negative blood using customized oligos (Custom TaqMan Gene Expression Assay from Applied Biosystems. The PCR was run for single-plex (Figure 18) and duplex (Figure 19) with 18S as an internal control. It was observed that ABI7500 can detect the target Mtb up to 1 fg which amplified at threshold cycle 34 (RED ARROWS) for both singleflex and duplex reactions. ABI 7500 PCR curves for the detection of an Mtb DNA target (IS6110) in a ten-fold dilution series from 100 pg down to 1 fg. The singleflex and duplex representative curves are shown in triplicates for 100 pg 10 pg, 1pg,100fg and sextuplicate for 10 fg and 1 fg respectively.

FIGURE 18 - SINGLE PLEX AMPLIFICATION THE 10-FOLD DILUTION OF STANDARD DNA (H37Rv)

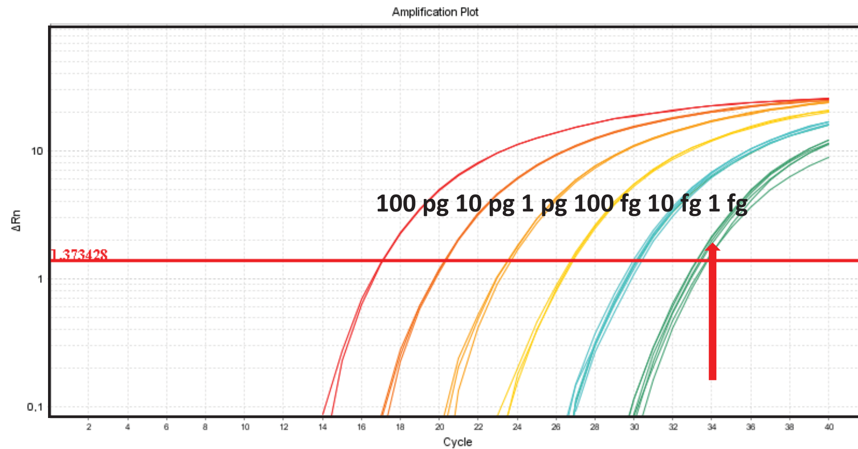

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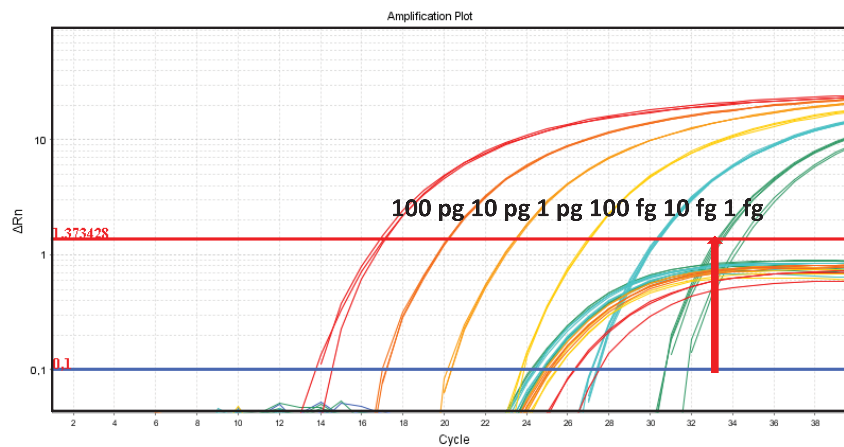
FIGURE 19- DUPLEX: AMPLIFICATION THE 10-FOLD DILUTION OF STANDARD DNA (H37RV)



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4.4.2 Evaluation of Strain M. tuberculosis (H37Rv) Dilution Curve with a Portable Instrument

These experiments were performed in collaboration with Dr. Maria Lucia Rossetti (CDCT-RS). The aim of this experiment was to analyze and compare a portable instrument using two reaction storage formats; liquid or gelified, using Mtb DNA dilution curve. For this purpose, PCR on the standard ABI7500 and the portable instrument Q3 and later Q3 gelified was performed. Comparison of threshold cycles is shown in Table. 12 for the detection of dilution curves amplifying target IS6110 on the ABI7500, Q3 liquid and Q3 gelified format. Results shown are averages ± SD for each dilution point. PCR efficiency was calculated for both Q3 liquid reaction and Q3 Gelified

reaction. The ABI7500 showed 99% efficiency while Q3 liquid and Q3 gelified reaction showed 92.11% and 91.64% PCR efficiency respectively.

TABLE 12- COMPARISON OF THREE FORMATES FOR DILUTION CURVE OF Mtb

	ABI7500	Q3	Q3 Gelified
100 pg	16,72 ± 0.6	13,5 ± 0.5	16,55 ± 0.4
10 pg	20,26 ± 0.0	18,1 ± 0.3	21,7 ± 0.1
1 pg	23,53 ± 0.1	21,4 ± 0.1	25,75 ± 2.3
100 fg	26,77 ± 0.1	24,3 ± 0.7	28,65 ± 0.5
10 fg	30,13 ± 0.1	28,6 ± 0.3	36,7 ± 1.7
1 fg	32,97 ± 1.3	32 ± 0.3	40,25 ± 1.6
Y-intercept	23.478	17.49	15.33
R^2	0.998	0.99	0.97

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NOTE: values indicated are mean ± S.D (n = 3)

FIGURE 20 - LINEAR REGRESSION OF MEAN OF THE THRESHOLD CYCLES FOR DETECTION OF IS6110 USING Q3-PLUS WITH LIQUID REACTION. SLOPE = -3.27; R^2 = 0.997

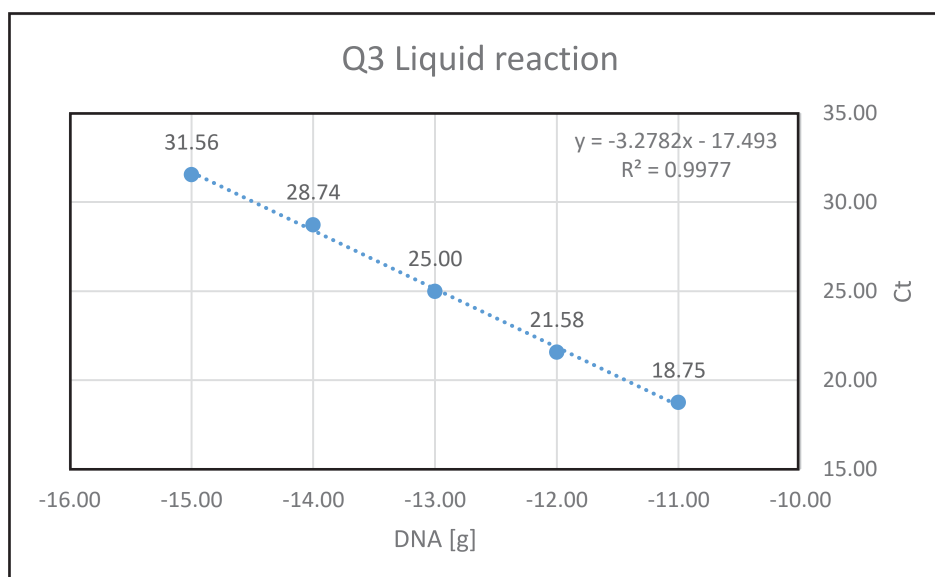

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FIGURE 21 - LINEAR REGRESSION FOR THE MEAN OF THE THRESHOLD CYCLES FOR DETECTION OF IS6110 USING Q3-PLUS WITH THE GELIFIED FORMAT. SLOPE = -3.54; R^2 = 0.975

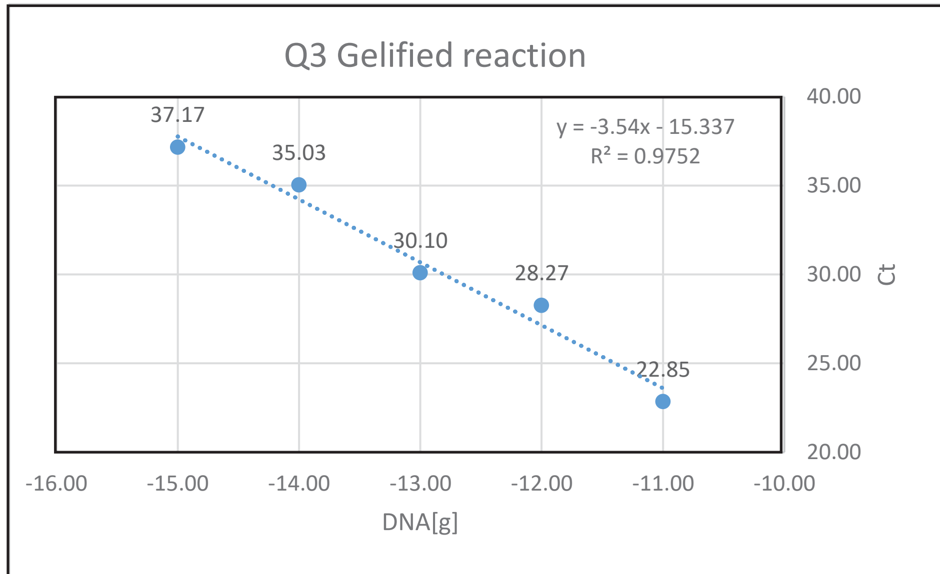

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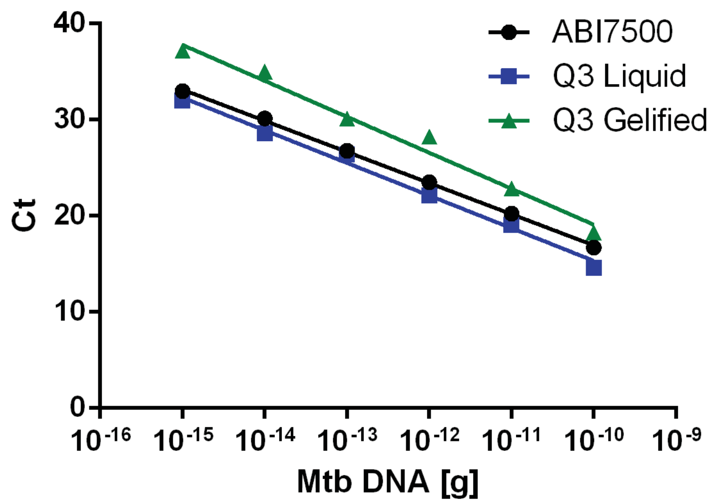
FIGURE 22- COMPARISON OF THREE FORMATES FOR THE AMPLIFICATION OF Mtb (H37Rv)



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4.4.3 Evaluation and Analysis of FTA Based Extraction Protocol for Mtb Target Spiked in Mucin

After defining substitutes for the lysis and binding buffer, we sought to substitute the silica material used for DNA retention (usually in membrane or beads form). FTA cards are paper-based, and thus binds DNA. So, we added *T. cruzi* DNA/cells to mucin and added GSCN. This mixture added to the FTA cards and extracted with protocol 3 and 4. Not surprisingly, the first direct extract did not show any PCR amplification while diluting both the samples further for 1:10 and 1:100 showed positive amplification, which may be due to some contaminants and inhibitors. These results suggest that this FTA card, which also contain an embedded detergents; Tris, EDTA and SDS (BURGOYNE, 1996) is suitable for DNA binding and retention after the GSCN protocol developed here.

TABLE 13 - COMPARISON OF ABI7500, Q3 LIQUID AND Q3 GELIFIED WITH PROTOCOL3 AND PROTOCOL4 FOR DETECTION OF MTB DNA

	ABI7500	**Q3**	**Q3 Gelified**
Sample Name	**Ct mean**	**Ct mean**	**Ct mean**
P3A1 (No Dilution)	Undetermined	-	-
P3A2(1:10)	22,3 ± 0.01	22,1 ± 0.3	28 ± 19.7
P3A3(1:100)	25,69 ± 0.05	24,4 ± 0.1	29,4 ± 0.14
P4B1(No Dilution)	Undetermined	-	-
P4B2(1:10)	24,85 ± 0.07	24,85 ± 0.8	31,05 ± 0.21
P4B3(1:100)	28,13 ± 0.09	28,15 ± 1.1	35 ± 24.75

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NOTE: Cts are presented as mean ± S. D P3=Protocols3 and P4= Protocols4 where A1=Undiluted, A2= 1:10 dilution and A3=1:100 dilution

Threshold cycles comparison for the detection of 3 points dilution curves of extracted Mtb DNA on the ABI7500, Q3 liquid reaction and Q3 Gelified format. Results are shown as Ct mean for each dilution point.

4.5 OPTIMIZATION STUDIES

4.5.1 Evaluation of PVP or BSA on the Quality of Extracted DNA

Since direct detection of the target DNA in the extract was not possible due to the possible presence of contaminants, we tried using two chemicals known to remove PCR inhibitors during nucleic acid extraction protocols. Bovine serum albumin (BSA) also enhance PCR amplification and specificity by combating contaminants (FARELL;

ALEXANDRE, 2012). PVP and BSA were selected to add to extraction buffer and observe the effect of the chemical by getting amplification in direct extract without increasing the volume of elution buffer. PVP is known to bind phenolic compounds from plant extracts (REZADOOST; KORDROSTAMI; KUMLEH, 2016), while BSA is known for its ability in unspecific binding, which ultimately improves PCR amplification. Additionally, TE buffer and ultrapure water were evaluated as eluting buffer alternatives. Table 14 summarizes these results. The combinations “PVP+water”, “TE buffer alone”, and “TE+BSA” show significant improvement, allowing the detection of Mtb DNA in the undiluted DNA resulting from the simplified extraction protocol. Overall, these results suggest that TE buffer and water should be tested with different concentrations of PVP and BSA.

TABLE 14 - EVALUATION OF THE EFFECT OF PVP AND BSA ON EXTRACTION

Experimental condition	Ct mean± SD	No. of replicates detection	A_{260}/A_{230}
Water +BSA	38.11 ±27,24	0⁄2	1.56
1:10	22.76 ± 0,12	2⁄2	
1:100	26.28 ± 0,19	2⁄2	
Water+PVP	19.82 ± 0,02	2⁄2	0.87
1:10	23.15 ± 0,00	2⁄2	
1:100	26.36 ± 0,07	2⁄2	
Water alone	ND	0⁄2	0.96
1:10	22.52 ± 0,02	2⁄2	
1:100	26.25 ± 0,04	2⁄2	
TE+BSA	20.59 ± 0,30	2⁄2	0.57
1:10	22.73 ± 0,10	2⁄2	
1:100	26.19 ± 0,22	2⁄2	
TE+PVP	29.43 ± 21,07	0⁄2	1.71
1:10	22.47 ± 0,01	2⁄2	
1;100	25.67 ± 0,03	2⁄2	
TE alone	21.39 ± 0,53	2⁄2	0.69
1:10	22.91 ± 0,04	2⁄2	
1:100	26.49 ± 0,16	2⁄2	

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4.5.2 Evaluation of Different Concentration of PVP and BSA in Elution Buffer

Different concentrations of PVP (10%, 2.5%, 1%, 0.5% or 0.1%) or BSA (2% or 0.1%) both in TE buffer, were used as elution buffer in the simplified protocol developed in this thesis. However, no significant difference was observed by using PVP and BSA at different concentrations for the detection of Mtb target.

TABLE 15 - EVALUATION OF DIFFERENT CONCENTRATION OF PVP AND BSA

Sample Name	(%)	C_T Mean
TE+PVP	10	24.05 ± 0.07
TE+PVP	2.5	21.48 ± 0.01

TE+PVP	1	20.59 ± 0.04
TE+PVP	0.5	20.57 ± 0.14
TE+PVP	0.1	20.44 ± 0.02
H_2O+PVP	10	25.25 ± 0.04
H_2O+PVP	2.5	20.98 ± 0.04
H_2O+PVP	1	20.53 ± 0.02
H_2O+PVP	0.5	20.27 ± 0.03
H_2O+PVP	0.1	20.81 ± 0.49
TE+BSA	2	20.17 ± 0.01
TE+BSA	0.1	21.29 ± 0.29

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4.5.3 Evaluation and Optimization of Elution Buffer Volume

Based on the previous results mentioned in section (4.5.1) and (4.5.2), here we tested different volumes (200 µL and 500 µL) of TE buffer with and without the use of 0.1% PVP and 0.1mg/ µL of BSA. As can be seen from the table 16, there is no significant improvement shown by the addition of PVP and BSA. Contrary to this, increase in the volume of TE buffer shown significant improvements in amplification in the direct extract by increasing the volume from 200 µL to 500 µL, we were able to get amplification in the direct extract as well as from 1:10 and 1:100 dilution. At this point, we were able to perform nucleic acid extraction without using a commercial kit.

TABLE 16- EVALUATION OF DIFFERENT VOLUMES OF ELUTION BUFFER

200µL		**500µL**	
Sample Name	C_T Mean	Sample Name	C_T Mean
200TE	ND	500TE	20,92 ± 0.04
200TE 1:10	22,90 ± 0.04	500TE 1:10	24,59 ± 0.11
200TE1:100	26,28 ± 0.03	500TE 1:100	28,03 ± 0.10
200TE+PB 0.1%	ND	500TE+PB 0.1% 1:1	21,02 ± 0.03
200TE+PB 0.1% 1:10	23,77 ± 0.04	500TE+PB 0.1% 1:10	24,89 ± 0.02
200TE+PB 0.1% 1:100	27,08 ± 0.03	500TE+PB 0.1% 1:100	28,12 ± 0.15

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ND= Not detected; PB= PVP and BSA

4.5.4 Evaluation of Limit of Detection of FTA Based Extraction

A 7-point dilution was made from Mtb DNA (25 ng/µL) into negative human DNA and 10 µL of diluted DNA of each point was spiked with 500 µL of mucin. Next, 200 µL of GSCN was added to each tube and applied to 7 different cards. The card was allowed to dry for one hour. One punch (6 mm) was performed in each card, and DNA was extracted using the developed protocol (with and without PVP and BSA) and 500 µL elution buffer. It can be seen from the Table 17 taht the ABI7500 platform is able to detect up to 4th point of dilution (2.5 pg/ µL) and this was considered until this point as a standard for future experiments. PCR efficiency was calculated (for 2.5 ng/µL, 0.25 ng/µL, 25 pg/µL, and 2.5 pg/µL) and obtained 102% efficiency which is mentioned in below Figure 23.

TABLE 17- COMPARISON BETWEEN EXTRACTIONS WITH OR WITHOUT PVP AND BSA

Without PVP and BSA		With PVP and BSA	
Sample Name	**C_T Mean**	**Sample Name**	**C_T Mean**
2.5 ng/µL	24.31 ± 0.06	2.5 ng/µL	24.95 ± 0.08
0.25 ng/µL	27.72 ± 0.04	0.25 ng/µL	28.45 ± 0.11
25 pg/µL	30.88 ± 0.04	25 pg/µL	31.85 ± 0.20
2.5 pg/µL	33.78 ± 0.18	2.5 pg/µL	35.39 ± 1.16
250 fg/µL	36.38 ± 0.50	250 fg/µL	39.38 ± 0.0
25 fg/µL	38.04 ± 0.0	25 fg/µL	36.78± 0.0
2.5 fg/µL	36.81 ± 0.48	2.5 fg/µL	ND

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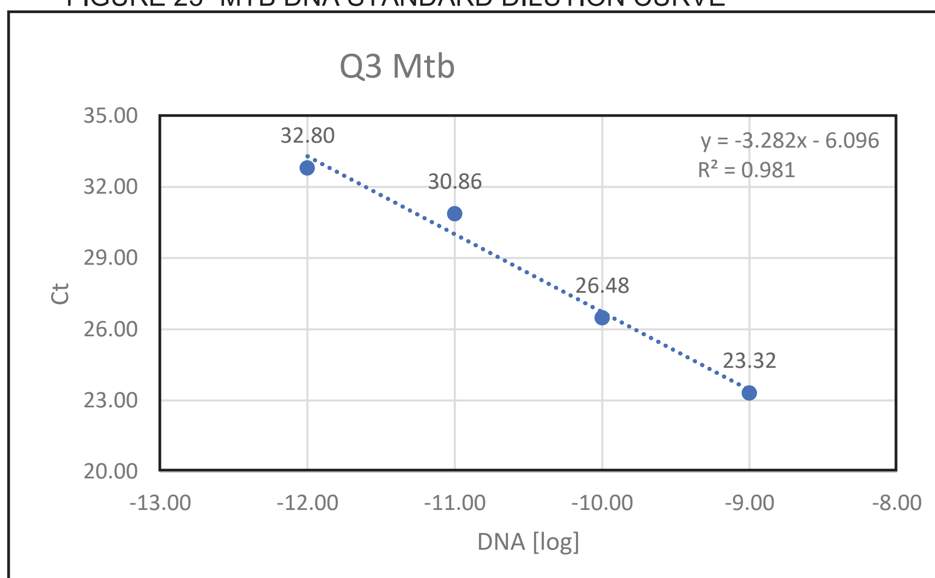
FIGURE 23- MTB DNA STANDARD DILUTION CURVE



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4.6 COMPARISON OF AMPLIFICATION AND DETECTION BETWEEN STANDARD AND POINT OF CARE PROTOCOLS

4.6.1 NALC-Treated Samples

After successful extraction and amplification with the in-house protocol for the Mtb standard curve (4.5.4) we decided to test our protocol on real Mtb samples. For this purpose, the experiment was conducted with patients' samples kindly donated by our collaborators in Porto Alegre (Dr. Maria Lucia Rossetti CDCT-RS). These samples were treated with NALC, according to the standard procedure proposed by WHO. Initially, three random patient samples (GPII-3, GPII-9 and GPII-12) were selected for a preliminary experiment. 500 µL was taken from each of the three, applied to an FTA card and extracted according to the simplified protocol developed in this thesis. Next, the remaining 9 samples (GPII1, GPII2, GPII4, GPII5, GPII6, GPII7, GPII8, GPII10, GPII11) were extracted and tested the same way. All the extracted DNA were run along with a 1:10 dilution of the respective eluted DNA. Result obtained from both platforms (i.e. ABI7500 and Q3) are mentioned in the form of averages and SD in Table 18.

Table 18 shows that the results were satisfactory, with detection of an Mtb target in the raw and in the diluted eluate.

4.6.2 Sample Extracted using PVP and BSA

As the previous experiments were facing some inhibition in the direct extract, we decided to add some chemicals with known inhibition removal ability. For this purpose, PVP and BSA were chosen to add to samples. The same twelve samples (GPII1, GPII2, GPII3, GPII4, GPII5, GPII6, GPII7, GPII8, GPII9, GPII10, GPII11 and GPII12) from Porto Alegre were used. However, a modification from our previous experiment (section 4.5.1) was made using 0.5% PVP and 0.5 mg/mL BSA in the elution buffer. The representative data obtained from both platforms (i.e. ABI7500 and Q3) are mentioned in the form of averages and SD in Table 18. No significant improvement was observed. This is another evidence that indeed PVP and BSA are not needed for PCR-grade DNA extraction with the simplified protocol.

TABLE18- COMPARISON OF THRESHOLD ON THE ABI7500 AND THE Q3. RESULTS SHOWN ARE AVERAGES ± SD.

	WITHOUT PVP AND BSA		**WITH PVP AND BSA**	
Sample Name	ABI7500	Q3	ABI7500	Q3
2.5 ng/µL	24.19 ± 0.13	22.58 ± 1.04	24.09 ± 0.12	23.41 ± 0.83
0.25 ng/µL	27.65 ± 0.10	26.70 ± 1.04	27.56 ± 0.06	26.74 ± 0.84
25 pg/µL	30.78 ± 0.18	30.32 ± 1.00	30.57 ± 0.04	30.85 ± 0.99
2.5 pg/µL	33.88 ± 0.69	33.10 ± 0.46	32.8±0.45	33.4 ± 1.77
GPII-1	32.29 ± 0.11	31.5 ± 0.42	32.31 ± 0.10	33.2 ± 1.27
GPII-1 1:10	36.38 ± 3.59	22.85 ± 12.66	35.41 ± 1.55	34.70
GPII-2	28.18 ± 0.12	28.25 ± 0.35	29.38 ± 0.07	29.95 ± 0.35
GPII-2 1:10	31.10 ± 0.06	31.9 ± 0.00	32.45 ± 0.04	31.55 ± 0.78
GPII-3	24.83 ± 0.02	24.1 ± 0.28	25.4 ± 0.1	25.7 ± 0.57
GPII-3 1:10	28.45 ± 0.01	31.45 ± 0.35	28.3 ± 0.1	27.6 ± 0.57
GPII-4	35.49 ± 0.64	33.3 ± 0.28	34.46 ± 0.22	33.25 ± 0.92
GPII-4 1:10	38.56	ND	36.45	36.65 ± 5.30
GPII-5	24.98 ± 0.00	24.65 ± 0.49	25.96 ± 0.03	26.45 ± 0.49
GPII-5 1:10	28.20 ± 0.02	27.9 ± 0.85	28.91 ± 0.06	28.4 ± 0.14
GPII-6	33.07 ± 0.02	33.4 ± 1.13	34.56 ± 0.64	32.75 ± 0.78
GPII-6 1:10	36.41	34.3	36.12	20.95 ± 4.45
GPII-7	35.96	33.2	36.42 ± 0.46	35.8
GPII-7 1:10	ND	37.6±4.10	39.49	ND
GPII-8	28.63 ± 0.06	28.05 ± 0.35	29.90 ± 0.00	30.3 ± 0.14
GPII-8 1:10	32.14 ± 0.22	30.45 ± 0.07	33.40 ± 0.08	32.25 ± 0.92
GPII9	32.47 ± 0.15	33 ± 1.13	32.8 ± 0.2	33.7 ± 0.14
GPII9 1:10	35.67 ± 0.78	41.7	35.9 ± 0.2	33.2 ± 0
GPII-10	37.54	41	37.98 ± 0.01	14
GPII-10 1:10	ND	ND	ND	ND
GPII-11	ND	ND	36.93	ND
GPII-11 1:10	ND	ND	ND	ND
GPII12	29.25 ± 0.22	27.95 ± 0.07	29.0 ± 0.0	33.7 ± 0.14
GPII12 1:10	32.41 ± 0.16	31.05 ± 0.64	32.6 ± 0.5	33.2 ± 0

FONT: The author ND = Not Detected

4.6.3 Mtb Samples without Treatment

These experiments were performed in collaboration with Dr. Maria Lucia Rossetti (CDCT-RS). A total of 30 untreated sputum Mtb samples were received from Laboratório de Biologia Molecular (LBM), Universidade Luterana do Brasil, Canoas-RS, Brazil, which included 10 positive and 7 negative samples. These samples were not treated with the WHO procedure (i.e. they were not previously decontaminated with NALC/NaOH). All the samples were tested in parallel by Gene Xpert PCR and culture. Untreated samples were submitted to the simplified protocol described in this thesis. The extracted DNA was then evaluated on the standard (ABI7500) and the portable (Q3) instruments, using commercial Taqman oligomix and IBMP mastermix.

The data collected for all samples are mentioned in Table 19. As can be seen from the table the samples tested by our protocol showed significant results as compare to GeneXpert and the gold standard culture technique both in terms of sensitivity and specificity. However, in some cases, our protocol showed a false positive result. Overall, these results indicate that in-house protocol has the potential to be used in future for detection of Mtb. Table 19 shows that our in-house protocol is in good agreement with GeneXpert’s results; only one sample gave a different result (#10 UHU20). All other, negative and positives, gave the same results. Not surprisingly, when GeneXpert results differ from culture, ours differed too.

TABLE 19 - COMPARISON AMONG DIFFERENT PLATFORMS FOR DETECTION OF MTB IN PATIENT'S SAMPLES

		REFERENCE LAB RESULTS		**THIS THESIS PROTOCOL (EXTRACTION AND DETECTION)**		
#	**Sample Name**	**GENEXPERT**	**CULTURE**	**ABI7500**	**Q3**	**Status of Molecular Diagnosis**
1	UHU2	Positive	Negative	Positive	Positive	Positive
2	UHU3	Positive	Positive	Positive	Positive	Positive
3	UHU8	Negative	Negative	Negative	Negative	Negative
4	UHU12	Positive	Positive	Positive	Positive	Positive
5	UHU13	Negative	Negative	Negative	Negative	Negative
6	UHU14	Negative	Negative	Negative	Negative	Negative
7	UHU15	Positive	Positive	Positive	Positive	Positive
8	UHU18	Positive	Positive	Positive	Positive	Positive
9	UHU19	Positive	Positive	Positive	Positive	Positive
10	UHU20	Positive	Negative	Negative	Negative	Negative
11	UHU21	Positive	Negative	Positive	Positive	Negative
12	UHU22	Positive	Positive	Positive	Positive	Positive
13	UHU23	Negative	Negative	Negative	Negative	Negative
14	UHU31	Positive	Positive	Positive	Positive	Positive
15	UHU32	Negative	Negative	Negative	Negative	Negative
16	UHU33	Negative	Negative	Negative	Negative	Negative
17	UHU40	Negative	Negative	Negative	Negative	Negative

FONT: The author

5 DISCUSSION

According to the WHO Report 2017, TB is the number one cause of death among infectious diseases worldwide, surpassing HIV/AIDS and the ninth leading cause of death in-general (GLOBALTUBERCULOSIS REPORT, 2017). The End TB strategy has come up with the ambitious goal of 80% reduction in TB incidence and 90% reduction in TB associated mortality between 2015 and 2030 (HARRIES et al., 2018). It is reported that TB still poses a serious health issue in resource-limited and less developed countries due to the lack of new solutions for its control (HARRIES et al., 2018). In order to achieve these goals, the point of care diagnosis is one of the potential solutions to this worldwide epidemic, i.e. other strategies include prevention and treatment (FLOYD et al., 2018). Smooth sample preparation and streamlining of DNA extraction to an efficient detection method is one of the challenges for currently available molecular diagnoses of *Mycobacterium tuberculosis* (ALI et al., 2017), which results in their limited use in resource-constrained areas. In this study, our aim was to demonstrate a rapid, simple, and cost-effective sample preparation and DNA extraction protocol for *Mycobacterium tuberculosis,* integrating paper-based DNA extraction and specific target amplification/detection by a portable and easy-to-use real time PCR platform.

Sputum is the most common biological specimen used for the diagnosis of pulmonary TB disease, the most common form of TB in adults (ALLEN; NICOL; TOW, 2016). As compared to urine and blood, sputum is thicker and more viscous but provides better insight into TB and other respiratory related diseases (ALTINER et al., 2009). To disrupt the complex network of interlinked mucin matrix, which is the main culprit for trapping the target microorganism, a mechanism is needed to liquify the sputum thus releasing the pathogen (ALLEN; NICOL; TOW, 2016). Different strategies (chemical, mechanical or combination of both), can be applied for liquefaction of the sputum sample, from viscous heterogenous to a homogenous/solubilized liquid state (ALI et al., 2017). In the current study, several chemicals were screened for homogenization of sputum sample such as GSCN, NP-40, Urea, Triton-X 100 etc. based on their natural tendency as a detergent and/or chaotropic agent. After experimenting all these chemicals, GSCN showed efficient results in terms of liquification as compared to other chemicals. In a previous study, PAWLOWSKI and KARALUS, (2012) have shown that GSCN (6M) can be used in sample preparation for

the extraction of DNA and protein for a variety of samples (including liquid samples and in case solid samples such as food and stool must be suspended in PBS or water).

The most widely used mucolytic agent for sputum sample is NALC, which break the disulphide bonds between the mucin molecule via reduction of sulfhydryl groups (PERES et al., 2009). On the other hand, mechanical digestion can be performed via magnetic stir bar, glass beads and/or sonication, usually assisted with a chemical mucolytic agent since mechanical liquefaction alone is not efficient (FERGUSON et al., 2016). The sensitivity of *M. tuberculosis* diagnosis is largely dependent on the efficiency of the sputum processing protocol. GSCN (6M pH 6.5) is a known chaotropic agent and already used in various commercial extraction kits. More importantly, sputum sample can be stored in GSCN because it kills viable Mtb (CLINGHAN et al., 2013) Although this treatment impares further use of the sample for culturing, the protocol described here is intended to be used as a screening tool. Positive samples would be referred to the hospital programme, but the patient can start the treatment immediately, based on the epidemiology of the area. Nowadays, the heterogenous sample has to be divided into three very distinct techniques. We propose that our screening protocol can be used to allow immediate beginning of treatment while other samples are collected for a more thorough diagnosis.

FTA elute card™, based on Whatman FTA technology (Whatman, Inc.), are chemically treated papers that enable cell lysis upon contact and release nucleic acids (WOLFGRAMM et al., 2009). Integrating liquefaction and decontamination of the sputum sample to FTA-based extraction is another approach for TB diagnosis adopted in this protocol. Our preliminary test using *T. cruzi* showed inhibition of the qPCR in the direct extract, possibly caused by excess of proteins, detergent (SDS), protease, salts or other contaminants (KATCHER; SCHWARTZ, 1994). For this purpose, different inhibitor removal chemicals were used such as PVP and BSA. Different concentrations of PVP and BSA were used to analyze the effect on test reaction. However, no significant difference was observed. Another approach used was increase in volume of Elution buffer which showed improved results by mitigating the effects of inhibitors that can interfere with PCR. Some other techniques such as high-pressure liquid chromatography and calorimetric assaysuse use the same approach for eradicating inhibitors from reaction (AL-SOUD; RÅDSTRÖM, 2001). Another evidence in our case was a relatively low A_{260}/A_{230} ratio, which is also another indicator of the presence of inhibitors.

SANTOS et al (2012) used FTA cards for detection of human papillomavirus in cervicovaginal samples. They compared the manufacturer protocol with an in-house modified protocol and concluded that the direct use of FTA card in PCR is more efficient (91.7%) than using elution steps (54.2%). Here, we applied elution approach for Mtb in sputum samples tested with different size of punches and elution buffers modifying the manufacturer's protocol. Similarly, GOVINDARAJAN et al., (2012) have demonstrated a rapid and cost-effective sample preparation using a microfluidic "origami", using spiked mucin with *E. coli* as a model*.* As compared to the microfluidic "origami", the FTA card is more efficient in cell lysis and disintegration of the viscous matrix of a sputum sample because of the dry reagents already stored on the paper. Furthermore, our protocol takes 1h 30min from raw sample to DNA extraction with a little need for a heat block and with minimal use of liquid reagents as compared to microfluidic origami procedure.

The use of FTA cards for storage, transport and preservation of sputum samples has been performed successfully for diagnosis of Mtb by (GUIO et al., 2006), who have shown that Mtb DNA was stabilized with FTA card for six months at room temperature. These findings provide not only a simple and economically favourable solution for collection, storage and transportation of the sputum but also suggest the use of these cards for further sample preparation and extraction.

GeneXpert and culture are both considered as reliable Mtb diagnostic assays for sputum samples. However, both techniques present challenges for application in POC-Dx. When compared to our in-house protocol with the early results obtained with GeneXpert and culture, 1 out of 10 positive GeneXpert samples were negative and the rest of the sample was found positive in accordance with GeneXpert (while the same 1 negative sample were also Culture negative as well). similarly, out of seven GeneXpert negative samples, all the samples were found negative. In contrary to GeneXpert, all the 7 culture-positive samples were found positive. While in 10 culture negative samples 2 sample was found positive (this sample was also positive for GeneXpert). Although the low number of samples analyzed in this study is a limiting factor, these results are comparable to gold standard techniques in case of detection with the hands-on time of around 3h and can be applied in the future as a triage test in resource-limited areas. Also, our results are in accordance with the culture and GeneXpert results. Thus, it has the potential to be used in high TB burden countries like Brazil, after testing with more characterized samples not only with Q3 liquid

reaction but also in a gelified format as well. The gelified reactions performed similarly to conventional reactions and could be implemented in laboratories and small medical centres. The insertion of an internal control of the reaction, the human 18S rRNA, guarantees us the functionality of the system as a whole, with respect to sample extraction, nucleic acid integrity, the absence of inhibitors in the reaction, and correct functioning of equipment and software.

Turnaround time can be considered as an important aspect of any diagnostic assay. The culture technique, which is the gold standard for Mtb disease diagnosis, can take 4 to 16 weeks (AZIZ et al., 2007), and such a long period can result in further complexities such as delays in initiation of the treatment or loss of disease follow up. We have estimated 3 hours from raw sample to result in assay, starting from sample collection to result analysis. This feature not only provides a rapid diagnostic test but also has the potential to provide an accurate diagnosis which leads to an early start of the treatment.

Our assay reagents are workable at room temperature without the need for any storage facility, which makes tuberculosis diagnosis feasible in poorly equipped areas with a high degree of sensitivity. Furthermore, access to quality diagnosis at POC will not only allow timely initiation of treatment but also reduce the dropout rate of the patients which is one of the key factors for the spread of TB disease.

The comparison of the cost of DNA extraction is R$ 20.00 for the Roche's kit while can be rougly estimated at R$ 5 (GSCN+FTA paper). The cost comparison of the PCR reaction per patient between a Q3-plus instrument and ABI 7500 is almost same ($10-15) while there is a huge difference in cost of the instrument (Q3-plus < $10,000 while ABI7500 > $40,000) (ZAHRA et al., 2016). In general, ABI7500 needs skilled professionals, sophisticated lab environment and requires time specific maintenance and calibration while Q3-Plus platform relies on minimum personal training may be one day and does not require maintenance/calibration or other lab infrastructure.

TABLE 20 - COMPARISON OF COST OF COMMERCIAL AND AND THESIS PROTOCOL

DNA Extraction	**Amplification and Detection**
Roche's Kit vsThesis Protocol	ABI7500 vs Q3-Plus
R$ 20.00 vs R$ 5	> $40,000 vs < $10,000

FONT: The author

In terms of sensitivity, this in-house method provides better results than traditional microscopy techniques, with less turnaround time as compared to culture.

Our developed protocol uses the minimum number of equipment as compared to commercial assays. The reagents used in this sample preparation are more easily available as compared to commercially available DNA sample pre-analytic reagents (e.g. DNA Genotek) and extraction kits (Roche) which are expensive and require extra storage and transportation facilities. The same model has been demonstrated by (SHATZKES; TEFEREDEGNE; MURATA, 2014) by developing an inexpensive alternative to commercial reagents for influenza virus. NAATs such as GeneXpert are considered highly specific in molecular diagnosis, However, high cost for sample preparation, makes them less effective in resource-limited areas (GOVINDARAJAN et al., 2012). In contrast, our protocol provides almost the same specificity with the use of comparatively inexpensive reagents. Further optimization of the protocol (by using just 6 mm of FTA paper fitted in a portable device) can cut down the cost significantly without compromising the quality.

Furthermore, FTA card technology is not only providing an extraction tool but also provide a storage and transportation medium for sputum and can be used for record keeping for future queries. Although this technique provides better results when compared to conventional diagnostics procedures, overcoming the false positive issue needs further research. This assay also provides rapid results as compared to conventional molecular diagnostic assays. Theoretically, this assay can provide a molecular analysis for any infectious disease with changing the corresponding probes and primers. Finally, this assay is inexpensive, portable and – to a certain extent – disposable. Thus, it can be applied in resource-limited areas ensuring assistance to a number of patients that are deprived of standard diagnosis. Although the number of samples analyzed in this study is small, these findings could contribute to large-scale screening facility for the prevention of TB in the future. In our study, samples were obtained from a reference laboratory in southern Brazil area, where culture positive cases from various geographical regions are referred. The validation of the reactions with clinical samples demonstrates that the test can be implemented in several medical centres and small hospitals, and our protocol is very effective, with performance comparable to qPCR and culture test.

6 CONCLUSIONS

As a result of this project, it was possible to develop a simple and inexpensive sample preparation protocol for the detection of TB, integrated with an easy-to-use portable real time PCR system.

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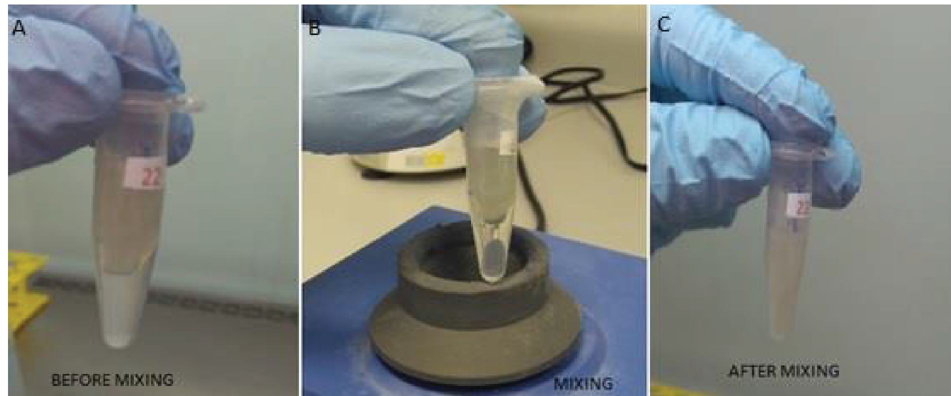
ANNEXES–

FIGURE 8- SOULTION 1 ADDITION TO MTB SAMPLE



NOTE: A) TUBE AFTER ADDITION OF GSCN TO SAMPLE

B) MIXING OF THE SAMPLE ALONG WITH GSCN

C) AFTER MIXING THE SAMPLE IS LYSED

FONT: THE AUTHOR

1. **DNA extraction with 3M paper combining 5mL syringe**
   - **Sample preparation**

500μL of mucin was taken and mixed with 500μL of GSCN and 31.5μL of HCl was added and thoroughly mixed. Next, 5μL of *T. cruzi* DNA (25ng/μL) was added and mixed, for extraction purpose, a 3M paper was folded into 4 layers according to the column area and placed in a 5 mL syringe column. The column was placed in a 15mL falcon tube

- **DNA extraction**

Binding buffer was prepared by taking 800μL of GSCN and 31.5μL HCl (pH=6.5).200μL of binding buffer was added for wetting purpose to 4 layered 3M paper. Waited for 3 minutes and then the whole sample was added to the 3M paper. Wait for filtering out all the sample while in the last stage the sample from the paper was expelled carefully with the plunger of the syringe. After 5 minutes, 300μL of binding buffer was added as washing step (in following

experiments number of washes were increased) 100μL of TE buffer (0.1M, pH=7.5) was added as an elution buffer, and eluted the DNA O.D was measured by nanodrop

2. Experimentation of 3M paper with Roche reagents (Binding, washing and elution buffer)

- **Sample preparation**

10μL of *T. cruzi* (25ng/μL) was spiked to 500μL of mucin, mixed and spin for 30 s.

- **DNA extraction**

Added 500μL of binding buffer, mixed for 30 s and short spin. Filtered the whole specimen from 3M paper and waited for 3 minutes, the remaining traces of the sample in the 3M paper was expelled carefully with plunger. 500μL of wash buffer (W1) was added to extraction column and filtered. For complete filtration, the buffer was expelled carefully by plunger. The second wash was done same like the previous. 200μL of elution buffer was added and filtered while the filtrate was collected in falcon tube. On every step filtrate was collected for O.D measurements so all the filtrate was measured for O.D.

3. Experimentation with syringe column using reagents of Roche and comparison with GSCN as binding buffer.

- **Sample preparation and DNA extraction**

Roche	GSCN
(1) 200μL of mucin was spiked with 10μL of *T. cruzi* (25ng/μL) and 200μL of binding buffer was added and mixed (2) Incubated for 10minutes at 70°C. (3) After filtration and gentle plunger expulsion, 500μL of wash buffer was added and filtered (4) Step 3 was repeated (5) 200μL of elution buffer was added and filtered through air pressure (6) Reading was taken of O. D	(1) 200μL of mucin was spiked with 10μL of *T. cruzi*(25ng/μL) and 200μL of GSCN+HCl as the binding buffer was added (2) Incubated for 10minutes at 70°C. (3) After filtration and gentle plunger expulsion, 500μL of the binding buffer as wash buffer was added and filtered (4) Step 3 was repeated (5) 200μL of elution buffer was added and filtered through air pressure (6) Reading was taken of O. D

4. **Comparison of Bubble pipette extraction vs Roche extraction with modifications**
   - **Sample preparation and DNA extraction**

The lower layer of silica column of Roche extraction kit was taken out and placed in 3mL syringe while 18 layers of 3M paper were added upon that layer. The rest of the protocol was according to the kit protocol. Bubble pipette extraction method was followed according to (Pawlowski and Karalus, 2012). Previously mentioned in detail for the extraction of *T. cruzi*, only sample preparation is mentioned here.

Roche extraction	Bubble pipette extraction
(1) 500μL of mucin was spiked with 35μL of *T. cruzi* (25ng/μL) and 200μL of binding buffer was added and mixed (2) Roche extraction was followed (3) O.D reading was taken	(1) 500μL of mucin was spiked with 35μL of *T. cruzi* (25ng/μL) and 500μL of GSCN+ 7μL of HCl was added (HCl was added to get 6.5pH) (2) The rest of the protocol was according to (Pawlowski and Karalus, 2012). (3) O.D reading was taken