Isolation and characterization of novel aptamers against the CFP-10/ESAT-6 heterodimer for the development of TB diagnostic tools

By

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DEDICATION

This thesis is dedicated to my family and the late Prof. Dewalt Roode. Without the constant support and wise words this thesis would never have come into being. I will be forever grateful for the constant encouragement and the little pushes here and there despite my reluctance.

DECLARATION

I, Lia Suzanne Rotherham, declare that the thesis, which I hereby submit for the degree Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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ABSTRACT

Tuberculosis (TB) is one of the biggest killers among infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. There are an estimated eight million new cases per year and two million deaths annually, which are compounded by the emergence of drug resistance TB and co-infections with HIV. As of 2004, it was estimated that more than 4% of the world's infected people living with active TB are in South Africa. During this period, South Africa accounted for about 2% of the world's new TB cases; and approximately 3% of the total deaths due to TB.

Despite the enormous burden of TB, conventional approaches to diagnosis used today continue to rely on tests that have major drawbacks. Many of these tests are slow and lack both sensitivity and specificity or require expensive equipment and trained personnel. For example, sputum smear microscopy is insensitive; the culture method is technically complex and slow; chest radiography is non-specific; the tuberculin skin test is imprecise and the results are non-specific; nucleic acid amplification tests and phage display are rapid but specificity and sensitivity are low; and the GeneXpert™ addresses the problems of time and sensitivity but the machine required is extremely expensive. Clearly, TB diagnostics need to be improved and a more specific diagnostic assay should preferably be based on antigens that are present exclusively in *Mycobacterium tuberculosis* (Mtb) and not in Bacillus Calmette-Guérin (BCG).

To help improve TB diagnostics, a systematic evolution of ligands by exponential enrichment (SELEX) process was used to select aptamers that can specifically bind to the CFP-10/ESAT-6 heterodimer, which is an early marker of TB. Anti-CFP-10/ESAT-6 aptamers were screened using an enzyme-linked oligonucleotide assay (ELONA) for binding affinity and specificity. A total of 24 aptamers had significant binding to the CFP-10/ESAT-6 heterodimer. One aptamer, named EA10, which bound to the heterodimer with a dissociation constant (K_D) of 8 nM, as determined by surface plasmon resonance (SPR), was used for a proof-of-principle in a TB diagnostic test. Evaluation of 80 clinical sputum samples from TB patients revealed that aptamer EA10 has a specificity of 68% and a sensitivity of 80-100%. These data bode well for the development of the aptamer for accurate diagnosis of TB.

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LIST OF ABBREVIATIONS

AFB	acid-fast bacilli
ATP	adenosine triphosphate
AAA	ATPases associated with diverse cellular activities
ACN	acetonitrile
ADA	adenosine deaminase assay
AuNP	gold nanoparticles
BCG	Bacillus Calmette-Guérin
bp	base pair
CFP-10	culture filtrate protein-10
CFP-10/ESAT-6	culture filtrate protein-10 and early secreted antigenic target, 6 kDa heterodimer
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDC	N-ethyl-N-(3,-dimethylaminopropyl)-carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
ELONA	enzyme-linked oligonucleotide assay
ESAT-6	early secreted antigenic target, 6 kDa
Esx	ESAT-6 system
FA	formic acid
gp120	120 kDa glycoprotein
HBS-N	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium chloride buffer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
IFA	information-dependent acquisition
IgG	immunoglobulin G
IGRA	interferon-γ-release assay
IPTG	isopropyl-1-thio-β-D-glactopyranoside
K_D	dissociation constant
kDa	kilodalton
KOH	potassium hydroxide
LAM	lipoarabinomannan
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani broth
MALDI	matrix-associated laser desorption/ionization
M. bovis	*Mycobacterium bovis*
MDM	monocyte-derived macrophages
MS	mass spectrometry
M. smeg.	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
NAATs	nucleic acid amplification tests
NaOAc	sodium acetate
Ni-NTA	nickel nitrilotriacetic acid
NHS	N-hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis

PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing 0.005% Tween-20
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PhaB	phage-amplified biological assay
PE	proline-glutamic acid
PPE	proline-proline-glutamic acid
PPK2	polyphosphate kinase 2
RD	region of difference
RNA	ribonucleic acid
rpm	revolutions per minute
RU	response units
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELEX	systematic evolution of ligands exponential enrichment
SPR	surface plasmon resonance
ssDNA	single-stranded deoxyribonucleic acid
TB	Tuberculosis
TMB	tetramethylbenzidine
TST	tuberculin skin test
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

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Chapter 1.　LITERATURE REVIEW

1.1 – MYCOBACTERIUM TUBERCULOSIS (MTB)

1.1.1 - A description of Mtb

Tuberculosis (TB) is one of the biggest killers among infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. *Mycobacterium tuberculosis* (Mtb) causes the disease and latent infection in much of the world's population (Beresford et al., 2010), largely due to the fact that the pathogen is extremely successful in evading the human immune system by forming granulomas, and partly because it is so poorly understood. Despite the high global burden of tuberculosis, the molecular details of its pathogenesis are poorly understood (Beresford and Sadoff, 2010). It is known that virulence and pathogenesis of Mtb are tightly associated with the ability of the bacteria to survive in host macrophages, which are the primary target of Mtb in the lungs (Russell, 2001, Tan et al., 2006, Vergne et al., 2004). After phagocytosis of the TB bacilli, macrophages express several antimicrobial mechanisms to limit the growth of the intracellular infection. Among these innate defence mechanisms is apoptosis, which has been linked to the killing of intracellular mycobacteria (Oddo et al., 1998, Derrick et al., 2007, Thoma-Uszynski et al., 2001).

Most TB infections are initiated by the respiratory route as Mtb spreads from individual to individual via the aerosolisation of infectious nucleus droplets. These infectious particles are released from the lungs of patients with cavitary pulmonary tuberculosis (Kaplan et al., 2003). TB in many cases follows a general pattern. Progression and resolution of the disease is divided into four stages: In the first stage, lasting 3 to 8 weeks after Mtb contained in inhaled aerosols becomes implanted in alveoli, the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary or Ghon complex. At this time, conversion to tuberculin reactivity occurs. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung. At this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis or miliary (disseminated) TB. During the third stage pleurisy or inflammation of the pleural surfaces can occur, lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. The last stage or resolution of the primary complex, where the disease does not progress, may take up to 3 years. In this stage, more slowly developing extra-pulmonary lesions can appear in some individuals. However, most humans who are infected with TB do not exhibit progression of the disease (Smith, 2003).

1.1.2 - Epidemiology of Mtb

Estimates of the TB epidemic suggest that there are roughly 8.8 million new cases per year, 1.45 million deaths annually (1 death every 15 s), and that one-third of the world's population is infected with Mtb (Who, 2011). The estimated case-detection rate for new smear-positive TB cases increased from 40% in 2000 to 62% in 2008 (Who, 2009). While there were some improvements in Africa, less than 50% of TB cases were reported in this region in 2008 (Who, 2009). Without treatment, up to 60% of people with the disease will die (Kaye et al., 1996).

TB is an enormous global public health problem with much of the burden felt by developing countries in south-east Asia, Africa and Eastern Europe. The prevalence of TB ranges from less than 10 per 100,000 in North America to 100 - 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa (Kaye and Frieden, 1996). Cases in the developing countries suggest that poverty and the lack of healthy living conditions and adequate medical care contribute to the spread and high infection rates of Mtb (Smith, 2003). Multidrug-resistant TB and human immunodeficiency virus (HIV)-associated TB pose considerable challenges, as there were an estimated 0.5 million new cases of multidrug-resistant TB in 2007 globally (Who, 2009).

1.1.3 - The rising concern of Mtb in Africa

The alarming statistic of TB infection have been compounded in recent years by the emergence of drug-resistant strains of TB and co-infections with HIV (Alderson et al., 2000). TB is now the most prevalent cause of morbidity and mortality for HIV-infected adults in many developing countries. An estimated 15 million people are co-infected with TB and HIV (Mcshane, 2005). Approximately 8–9 million new TB cases and 2–3 million deaths occurring each year (Dye et al., 1999, Frieden et al., 2003, Zumla et al., 2000). Although TB is curable, it is estimated that an individual with active TB infects at least three other individuals before receiving a diagnosis and treatment. It is therefore challenging to control the disease and reduce the spread of TB. This is of particular concern to developing countries that do not have the resources to invest in expensive TB diagnostics (Beresford and Sadoff, 2010).

1.2 – DIAGNOSTIC TOOLS AVAILABLE FOR MTB

Timely diagnosis of active TB cases is important for the control of the disease. Despite the enormous burden of TB, conventional approaches to diagnosis used today continue to rely on tests that have major drawbacks. Many of these tests are slow and lack both

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specificity and sensitivity. The problems with current diagnostics are further compounded by the HIV pandemic, as it increases the incidence of smear-negative and sputum-scarce cases.

A variety of methods are currently used for TB diagnostics (Table 1-1). The current gold standard is a combination of smear microscopy and culture. Brief descriptions of the available methods are listed below.

Microscopy:

Microscopy is used to examine clinical specimens or cultures for acid-fast bacilli (AFB). A variety of different stains are available, but the three commonly used ones are Ziehl-Neesen, auramine-rhodomine fluorochrome 2 (Gordin et al., 1990) and Kinyoun (Attorri et al., 2000) stains. Microscopy is capable of identifying AFB in a sample, but does not indicate if the bacteria are viable or what species the bacilli are. Only 40-50% of patients with pulmonary TB are smear-positive and it is estimated that 10% of smear-negative patients will also be culture-negative (Dye et al., 1999). Even though microscopy is not very accurate it still remains the most rapid technique, and is of particular importance in developing countries.

Solid culture-based techniques:

Culture of Mtb in clinical specimens is more sensitive than smear microscopy. Culture is performed on solid media, containing a cocktail of antimicrobial agents that only allow mycobacteria to replicate. The solid media used are either egg-based such as Lowenstein-Jensen (Gil-Setas et al., 2004, Hanna et al., 1999) and Ogawa media (Abe et al., 1992) or agar-based such as Middlebrook 7H9, 7H10 and 7H11 (Hanna et al., 1999). This is the most sensitive of current diagnostic methods, however, it requires 3-8 weeks of solid culture for accurate diagnostic. There is a downfall in that in 10-20% of the cases the bacillus cannot be cultured (Andersen et al., 2000). Culture is relatively expensive and is therefore not routinely used in resource-poor settings.

Rapid liquid culture:

Faster culture methods for mycobacteria isolates can be achieved through liquid cultures. These liquid cultures can either be in an automated or semi-automated system. The time to detection is significantly reduced from 3-8 weeks to just 10-14 days. Mtb often exhibits a very characteristic morphological pattern when grown in liquid media, allowing a rapid indication of Mtb and other mycobacterial species (Attorri et al., 2000, Kaminski et al., 1995).

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Nucleic acid amplification tests (NAATs):

NAATs are molecular systems that allow the detection of small amounts of RNA or DNA, and are based on amplification of the target sequences. The methods used rely on polymerase chain reaction (PCR) or amplification of a nucleic acid probe (ligase chain reaction). Detection of the amplified products are then analysed by agarose gel electrophoresis, DNA sequencing, enzyme immunoassay using probes for colorimetric detection or by fluorescent emission technology (Louie et al., 2000). A recent improvement of the NAAT is the GeneXpert™, which is a fully automated molecular test for TB case detection, as well as drug resistance testing. Currently, the test is designed to detect rifampin resistance but the principle could be applied to other genes that indicate drug resistance. The automated test relies on a heminested PCR to amplify Mtb-specific sequences and the *rpoB* gene, which is probed with molecular beacons for mutations in the rifampin-resistance determining region (Boehme et al., 2010). Testing is carried out in a disposable cartridge that contains all the reagents needed for bacterial lysis, nucleic acid extraction, PCR and amplicon detection (Boehme et al., 2010).

Mycobacteriophage-based methods:

Mycobacteriophage tests are an alternative to PCR tests and are useful in resource-poor settings where PCR is impractical. Mycobacterial cultures are infected with mycobacteriophage and the signal is amplified biologically by replication of the phage within the mycobacteria. There are two methods of detection, the simplest being the phage-amplified biological assay (PhaB). The phage is plated onto a lawn of rapidly growing *Mycobacterium smegmatis (M. smeg.)*, the phage lyses the bacteria and the number of viable bacteria is compared to the original sample (Eltringham et al., 1999, Wilson et al., 1997). The second method uses a luciferase reporter phage. Cultures are infected with luciferase reporter phage, which results in viable mycobacteria producing quantifiable amounts of light (Banaiee et al., 2001).

Tuberculin skin test (TST):

TST is currently the standard for detecting latent TB infection, although it is not an ideal test. It is based on the detection of delayed-type hypersensitivity to purified protein derivative (PPD). PPD is a mixture of antigens shared by several mycobacteria that gives rise to a skin reaction. Two visits are required for the test, the first for inoculation and the second visit is 48 hours after the first to determine the results based on the size of the skin reaction. TST is relatively cheap and can be performed without the need of a specialised laboratory; however, interpretation of results are subjective and can lead to false results (Chaisson et al., 1996).

Interferon-γ release assays (IGRAs):

IGRAs were initially developed to replace TST. Blood samples are taken from patients and incubated with antigens specific for TB-causing strains of mycobacteria. T-lymphocytes in the blood produce interferon-γ, which is a marker for infection or active TB (Janssens et al., 2007). Currently, there are two forms of IGRAs: either IFN-γ secretion is measured in whole-blood by ELISA (Mori et al., 2004); or IFN-γ-secreting T-cells are directly enumerated by an enzyme-linked immunospot (ELISpot) assay (Lalvani et al., 1997). The two assays are commercially licensed as QuantiFERON-TB Gold In-Tube (Cellestis Inc, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK), respectively.

Adenosine deaminase and cytokine assays:

Adenosine deaminase (ADA) and several cytokines have been evaluated for their potential as a TB diagnostic test. Even though they are simple and relatively inexpensive, neither assay can be used alone as they both lack accuracy (Lein et al., 1997).

Serological tests:

A wide range of serological tests have been developed that use a variety of antigens to detect antibodies in the blood (Bothamley, 1995). They are less expensive, rapid and simple (Gounder et al., 2002). As antibody responses are directed against a broad set of antigens, responses between individuals vary depending on the amount of antigen present, which results in low sensitivity. It has been suggested that serological tests be used in combination with other tests in the diagnosis of smear-negative TB cases in resource-poor settings (Al Zahrani et al., 2000).

Loop-mediated isothermal amplification (LAMP):

LAMP amplifies DNA with high efficiency under isothermal conditions using six sets of primers. The large amount of DNA generated, along with the high specificity of the reaction, makes it possible to detect amplification by visual inspection of fluorescence without the need of specialised equipment like thermal cyclers or gel electrophoresis instruments (Boehme et al., 2007). LAMP assay studies are underway in South Africa to determine their efficiency in high burden-settings.

Lipoarabinomannan (LAM) antigen detection assay:

The LAM assay uses urine, which is a sterile biological fluid, and can be obtained in sputum-scarce patients. The assay has the potential of being applied to other biological fluids (Dheda et al., 2009, Tessema et al., 2001). Recent studies have shown that the urine LAM test may be useful in HIV-positive patients (Lawn et al., 2009, Mutetwa et al.,

2009, Reither et al., 2009, Shah et al., 2009) but not in HIV-negative patients (Daley et al., 2009). The test is most useful in HIV-positive individuals that have a CD4 count of ≤ 200 cells/mm^3 (Getahun et al., 2007).

In light of several limitations with current TB diagnostics (see Table 1-1) there is a need for a rapid, reliable, cost-effective and user-friendly TB diagnostic. Aptamers have properties that can potentially fulfill these requirements.

1.3 – APTAMERS AS A DIAGNOSTIC TOOL FOR MTB INFECTION

1.3.1 - Definition of aptamers
Aptamers are artificial oligonucleotide ligands with the capacity to bind virtually any class of target molecule with high affinity and specificity. Numerous experiments have shown that aptamers can practically be selected against any target including proteins, peptides, nucleic acids, polysaccharides, small organic molecules (amino acids, nucleotides, and other metabolites), virus particles, whole cells, and even tissues (Lee et al., 2006, Kulbachinskiy, 2007, Gold et al., 1995). This means that aptamers can be useful in both therapeutic and diagnostic applications.

1.3.2 – How aptamers are selected
Selection of aptamers has been made possible by the development of the systematic evolution of ligands by exponential enrichment (SELEX) process (Robertson et al., 1990, Ellington et al., 1990, Tuerk et al., 1990). The SELEX process is quite simple and consists of several rounds of selection of sequences that bind to a target molecule. Each round consists of three main stages: (1) incubating the oligonucleotide library with the target of interest, (2) separating bound oligonucleotides from unbound oligonucleotides using the desired partitioning matrix, and (3) amplifying the bound oligonucleotides (Figure 1-1). Recently, a method for automated aptamer selection has been developed, which allows the selection of aptamers against a large number of different targets simultaneously (Cox et al., 2002, Eulberg et al., 2005).

Both single-stranded DNA (ssDNA) and RNA libraries are used in different selections. These libraries typically consist of a random region of nucleotides that range from 20 to 60 nucleotides. It was thought that the longer the random region the more efficient the selection would be as there is a larger variety of different structures (Conrad et al., 1996). At the same time, though the amount of single-stranded library used in a selection never

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Table 1-1: Advantages and disadvantages of current TB diagnostics

Method	Disease and site of disease	Advantages	Disadvantages	Commercial products available	References
Smear microscopy	Pulmonary TB	Requires moderate training, minimal infrastructure and minimal equipment	Low sensitivity (35 - 70%), sputum samples need to be processed, cannot distinguish between mycobacterial pathogens at species level.	Clinical smear microscopy	(Corbett et al., 2003, Perkins, 2000, Shah et al., 2006, Wedlock et al., 2002)
Solid culture	Pulmonary TB	Good sensitivity (up to 98%)	Time for diagnosis is 3-6 weeks, high technical competence required. Also requires implementation of biosafety practices and equipment. Relatively expensive.	Many commercialized media and reagents.	(Attorri et al., 2000, Banaiee et al., 2001, Drobniewski et al., 2003, Kaminski and Hardy, 1995)
Liquid culture	Pulmonary TB	High sensitivity	Time to detection is slow but faster than solid cultures (10 - 14 days), high contamination rates, technically difficult, requires biosafety practices and equipment. Extremely expensive.	MGIT (Becton Dickinson); BacT/ALERT (BioMerieux), BACTEC 9000MB, ESP Myco and Accumed/Difco ESPII	(Attorri et al., 2000, Banaiee et al., 2001, Drobniewski et al., 2003, Kaminski and Hardy, 1995)
Chest radiograph	Pulmonary TB	Use not restricted to TB	Low sensitivity and specificity, expensive equipment required and trained personnel. Subjective interpretation of results.	Clinical radiography	(Perkins, 2000)
Nucleic acid amplification tests (NAAT)	Pulmonary and extrapulmonary TB	Highly specific for Mtb, sensitivity between that of smear and culture (60-100%), rapid detection (3-6 hours)	Expensive, requires equipment and trained personnel, labour intensive, and potential for cross-contamination among specimens, inconsistent results between different tests	Amplified *Mycobacterium tuberculosis* direct test (Gen-Probe), Amplicor *M. tuberculosis* test (Roche), BD ProbeTec	(Drobniewski et al., 2003, Mchugh et al., 2004)
Phage-amplification assays	Pulmonary TB	Simple, robust, high specificity, can be used for drug susceptibility testing simultaneously	High cost, and variable sensitivity	FASTPlaque, PhageTek MB® (Biotec Laboratories Ltd., UK)	(Banaiee et al., 2001, Eltringham et al., 1999, Perkins, 2000, Wilson et al., 1997, Albert et al., 2001)
Tuberculin skin test (TST)	Latent TB infection	Cheap	Sensitivity decreases in immuno-compromised individuals, cross-reaction with BCG	Mantoux test, Heaf test	(Fine et al., 1999, Floyd et al., 2002, Huebner et al., 1993, Kwamanga et al., 1995, Stuart et al., 2000).

Method	Disease and site of disease	Advantages	Disadvantages	Commercial products available	References
Interferon-γ release assays (IGRA)	TB pleuritis, Latent TB infection	Highly specific for Mtb, responds to multiple antigens simultaneously.	Requires equipment and trained personnel, low sensitivity, detects latent and active infection, can cross react with BCG, does not discriminate between Mtb and atypical mycobacteria	QuantiFERON-TB Gold (Cellestis), T-SPOT.TB (Oxford Immunotec), ELISPOT	(Brock et al., 2001, Chapman et al., 2002, Pai et al., 2008, Van Pinxteren et al., 2000, Lalvani et al., 1997, Mori et al., 2004)
Adenosine deaminase assay	TB pleuritis, pericarditis, peritonitis and TB meningitis	High sensitivity and specificity in pleural and pericardial fluids. Rapid, simple, non-invasive and can be used in any clinical laboratory	Lacks accuracy	ADA assay kit (BioSupply, UK)	(Dinnes et al., 2007, Pai et al., 2008)
Serological antibody detection tests	Pulmonary and extrapulmonary TB	Rapid, simple and inexpensive	Highly inconsistent estimates of sensitivity (16 - 57%) and specificity (62 - 100%), affected by exposure to atypical mycobacteria, BCG vaccination and HIV infection	Anda-TB (Anda Biologicals), Detect-TB (Biochem Immunosystems), ICT TB Test (ICT Diagnostics) MycoDot (Mossam Associates)	(Pai et al 2008), (Dinnes et al 2007)
Loop-mediated isothermal amplification (LAMP)	Pulmonary TB	Sensitivity and specificity similar to conventional PCR (60 -100%), closed tube system therefore little cross-contamination	False negative results based on smear-negative culture-positive samples being misdiagnosed.	LAMP assay	(Boehme et al., 2007).
Lipoarabinomannan (LAM) antigen detection assay	Extrapulmonary TB	Useful in HIV-infected people with CD4 counts of < 200 cells/mm^3, modest cost and high specificity	Not as efficient in HIV-negative patients, in general low sensitivity (13-21%)	ClearviewH TB ELISA (Inverness Medical Innovations)	(Dheda et al., 2010)
GeneXpert™	Pulmonary and extrapulmonary TB	Rapid, little human intervention, specificity of 60-80%, simultaneous drug resistance testing	Need for annual calibration, the machine and the cartridges are currently expensive	Xpert™ MTB/RIF (Cepheid)	(Boehme et al., 2010)
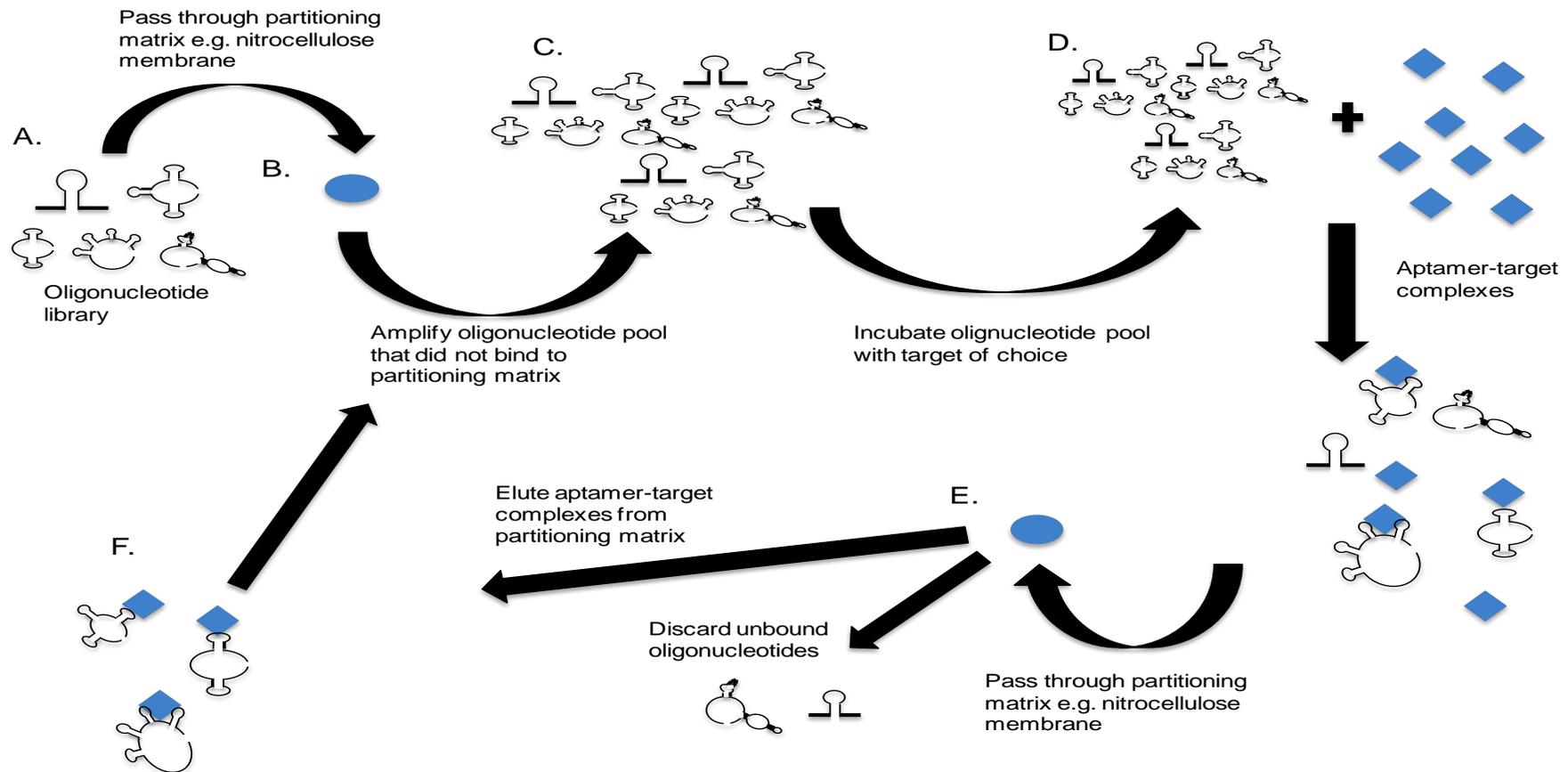
Figure 1-1: An illustration of the SELEX process.
The process typically starts by passing the oligonucleotide library (A) through the partitioning matrix (B) to expel any partition matrix binders from the pool. The oligonucleotide pool that does not bind to the partitioning matrix is amplified (C). The amplified pool is then incubated with the target to form aptamer-target complexes, as well as free target and free oligonucleotide sequences (D). This entire pool is passed through the partitioning substrate (E) where aptamer-target complexes are captured (F) and free oligonucleotide sequences are discarded. The aptamer-target complexes are then separated by a phenol-chloroform extraction after which the oligonucleotide pool is amplified for the next round of selection.

exceeds 10^{16} molecules, which corresponds to the total number of different sequence variants for libraries with a random region less than 25 nucleotides. Therefore, using libraries with random regions larger than 25 nucleotides are likely to be underrepresented and contain the same number of different variants as shorter libraries.

1.3.3 – Structural characteristics of aptamers

A distinguishing characteristic of all aptamers is their ability to form distinct and well-defined secondary and three-dimensional structures, respectively. It has been found in numerous experiments that the unpaired oligonucleotides are most important for specific interaction with their targets, whereas the stable secondary structures are necessary to maintain a proper spatial arrangement of the recognition element (Ringquist et al., 1995). Usually there are only one or two structural motifs that comprise the basis of an aptamer. There are three secondary motifs that occur most frequently, namely: (1) hairpins, (2) pseudoknots, and (3) quadruplexes. Hairpins are the most widespread and occur in both RNA and ssDNA. Pseudoknots are a result of complementary interactions of sequences, located to the left or right of a hairpin, with the sequence of the hairpin. Pseudoknots are most characteristic of RNA aptamers but can occur in DNA sequences as well (Pleij et al., 1985, Ringquist et al., 1995, Tuerk et al., 1992, Schneider et al., 1995). Quadruplexes are four-stranded structures. As a rule the quadruplex cross-section is formed by four guanine nucleotides, where each guanine base in the G-quartet forms hydrogen bonds with two adjacent bases. Usually two or three G-quartets will occur in succession in a quadruplex. Quadraplex formations are ion-specific and preferentially form in the presence of K^+ and Na^+ ions (Balagurumoorthy et al., 1994, Jin et al., 1990, Jing et al., 1997, Kang et al., 1992, Lu et al., 1992, Panyutin et al., 1990, Rando et al., 1995, Ringquist et al., 1995, Schultze et al., 1994, Sen et al., 1990, Smith et al., 1992, Sundquist et al., 1989, Williamson et al., 1989). The quadruplex structures are more common in ssDNA structures but can occur in RNA structures (Andreola et al., 2001, Jing et al., 1997, Shi et al., 1997, Tasset et al., 1997, Weiss et al., 1997, Wen et al., 2002).

1.3.4 – Affinity and specificity of aptamers

Most selection experiments produce aptamers that interact with their target with very high affinity. The best targets are proteins and their dissociation constants (K_D) are usually in the nanomolar to sub-nanomolar ranges (10^9 – 10^{11}) (Gold et al., 2010, Khati et al., 2003, Schneider et al., 1995). The main reason for the high affinity is due to the relative rigidity of nucleic acids compared to peptides and other polymeric structures (Eaton et al., 1995).

The K_D for low molecular weight targets is usually in the micromolar to sub-micromolar ranges (10^6 – 10^7) (Geiger et al., 1996). This can be explained by the fact that low

molecular weight molecules are likely to have smaller areas of contact with aptamers as compared to protein targets, and small molecules are less rigid than proteins (Eaton et al., 1995).

Aptamers also exhibit high specificity to their target molecule, as selection for high affinity to a target usually results in highly specific aptamers (Eaton et al., 1995). This is due to the fact that aptamers that have a high affinity have larger areas of interaction with their targets. Thus, even minimal alterations in the target surface will weaken the interaction with the aptamer. However, one needs to consider that the ability of aptamers to distinguish similar proteins depends on the site in the protein that the aptamer binds to. For example, an aptamer will be more specific if it binds to a variable region of a protein as opposed to a conserved region of a protein (Biroccio et al., 2002, Conrad et al., 1994, Fisher et al., 2002, Tsiang et al., 1995, Yamamoto et al., 2000).

1.3.5 - Current use of aptamers

1.3.5.1 - Basic fields of aptamer usage

Aptamers can be used to investigate the mechanisms of interaction of proteins and nucleic acids. These studies involve proteins that recognise nucleic acid sequences *in vivo*. Selection of these aptamers allows one to find the natural sequences of RNA or DNA that are recognised by the protein of interest (Convery et al., 1998, Giver et al., 1993, Huang et al., 2003, Jaeger et al., 1998, Kettenberger et al., 2006, Rowsell et al., 1998, Schneider et al., 1995, Shtatland et al., 2000, Tuerk et al., 1992). Aptamers can be used as specific inhibitors of function of target proteins (such as gp120) (Khati et al., 2003). Several aptamers with potential therapeutic applications have been isolated against a variety of targets (Khati et al., 2003, Bock et al., 1992, Brody et al., 2000, Burgstaller et al., 2002, Cerchia et al., 2002, Gold et al., 1995, Nimjee et al., 2005, Pestourie et al., 2005, Proske et al., 2005, Thomas et al., 1997, Tuerk et al., 1992, Zhang et al., 2004). Aptamers can also be used for affinity purification and identification of target proteins (Javaherian et al., 2009). Aptamers are also widely used as detection agents for different target molecules (Bruno et al., 1999, Burgstaller et al., 2002, Charlton et al., 1997, Fitter et al., 2005, Hesselberth et al., 2000, Homann et al., 1999, Morris et al., 1998, Proske et al., 2005). These aptamers are potentially useful in diagnostic applications.

1.3.5.2 – Diagnostic applications

It has been shown that aptamers could successfully replace antibodies in ELISA, *in situ* fluorescent hybridization, Western blotting, etc. making them desirable diagnostic tools (Bruno and Kiel, 1999, Burgstaller et al., 2002, Charlton et al., 1997, Fitter and James,

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2005, Hesselberth et al., 2000, Homann and Goringer, 1999, Morris et al., 1998, Proske et al., 2005). For instance, aptamers have been used for measuring concentrations of various metabolites and protein factors (Charlton et al., 1997), for detection of toxins (Bruno and Kiel, 1999), revealing specific types of cells and tissues (Hesselberth et al., 2000, Proske et al., 2005), and cells of pathogenic microorganisms (Homann and Goringer, 1999, Bruno and Kiel, 1999, Charlton et al., 1997, Hesselberth et al., 2000, Proske et al., 2005). Aptamers have also been used in multiplexing to simultaneously detect different target molecules (Kulbachinskiy, 2007, Gold et al., 2010).

In 2006, colorimetric sensors specific for adenosine, cocaine, thrombin and PDGF, using aptamer-modified gold nanoparticles (AuNPs), was introduced (Huang et al., 2005, Liu et al., 2005, Pavlov et al., 2004). In the absence of the target molecule, the aptamer-modified AuNPs remained aggregated, appearing red; in the presence of the target, the AuNPs disassembled, resulting in a blue color, which occurred in less than a minute. Wang and colleagues demonstrated that a labelled aptamer-AuNP colorimetric system could be used for detection of thrombin with extremely high sensitivity (with a detection limit of 14 fM) using dot-blot arrays (Wang et al., 2008). One can also use an unlabelled method where the aptamer is covalently bound to the AuNPs. In the presence of its target the aptamer will dissociate from the AuNPs, resulting in aggregation of nanoparticles and a change in colour from blue to red (Xia et al., 2010). Xia and colleagues demonstrated that an aptamer-AuNP colorimetric system similar to the one used for ATP, potassium and cocaine detection could be used for detection of thrombin with a detection limit of 0.6 µM (Figure 1-2) (Xia et al., 2010).

Although the use of AuNP-based colorimetric sensors provides a way of detection without the need for analytical equipment, it requires careful handling of microliter-scale solutions, which is an advantage although difficult to handle. Therefore, a more user-friendly device is required. One such device is a lateral flow device based on aptamers, which is based on a dipstick method and uses slightly larger, easy to handle volumes. For example, the lateral flow developed for cocaine uses biotin-labelled anti-cocaine aptamers conjugated to AuNPs to detect the presence of cocaine (Liu et al., 2006b).
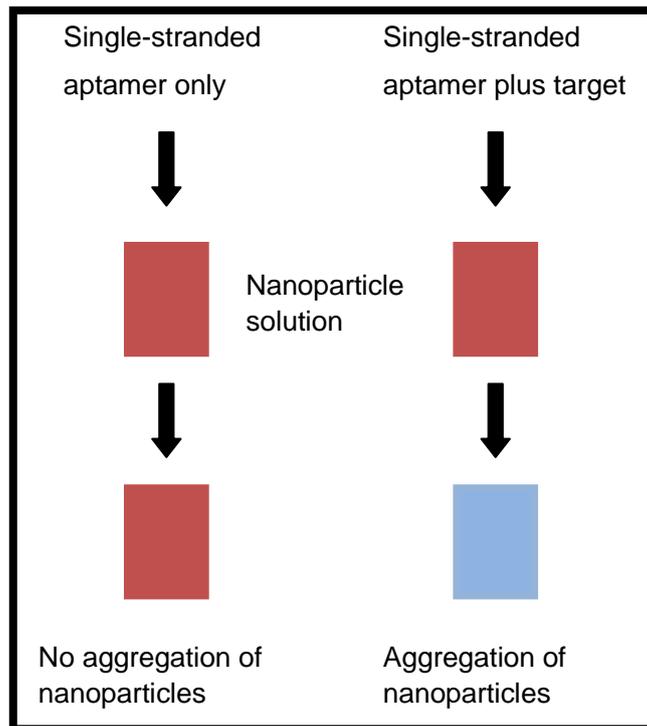
Figure 1-2: Colorimetric readout system based on aptamers-AuNP conjugates.
A colour change from red to blue indicates the presence of the target (adapted from (Xia et al., 2010).

1.3.6 – Advantages and disadvantages of aptamers to conventional diagnostic tools

It has been demonstrated that modified bases of ssDNA or RNA aptamers help to form stable three-dimensional structures that are resistant to nucleases found in body fluids, and also allows aptamers to be used in a wider range of diagnostic applications (Gold et al., 2010). This, coupled with the fact that aptamers are more stable to heat, pH and organic solvents than antibodies, makes aptamers suitable for diagnostic purposes. Aptamers can be obtained in large amounts through chemical synthesis resulting in little batch to batch variation, thus making aptamers cost-effective (Lee et al., 2007). However, the characteristics that are most important to the success of aptamers in diagnostic assays are the affinity, sensitivity and specificity of the ligand that provides molecular recognition. These properties give aptamers an advantage over conventional molecules and antibodies as diagnostic reagents. A disadvantage of aptamers for diagnostics was the use of radio-labeling for detection, due to specialized labs and time for detection (Dougan et al., 1997). This problem was addressed by the insertion of a detectable reagent during chemical synthesis on the 5′ or 3′end of the aptamer (Davis et al., 1998, Huang et al., 1996, Rosemeyer et al., 1995). However, one still needs to ensure that the modification does not alter the properties of the aptamer (i.e. binding kinetics or structure). This has been achieved in recent years by the use of nanoparticles and biosensors (Xia et al., 2010, Xu et al., 2009, Zhang et al., 2010).

1.3.7 - How aptamers can be useful as novel diagnostic tools for detecting Mtb

Though it remains to be demonstrated, more sensitive, rapid, and user-friendly diagnostic tools might have a significant impact on disease control by shortening the diagnostic delay and reducing the period of transmission.

Diagnostic needs in most underdeveloped countries are those that support elimination of TB. These needs include tools for identification of latent infection in high-risk individuals, and detection of disease in immigrant and other high-risk populations through active case finding. A diagnostic test is needed that can replace chest radiographs and smear microscopy. The test should be able to detect early disease in patients; identify outbreaks and characterisation of nosocomial and community transmission; as well as discriminate between patients whose mycobacterial infections are due to non-tuberculosis species (Perkins, 2000).

Aptamers by virtue of their specificity and sensitivity would serve as essential tools for the early and specific detection of active TB infection, thus meeting the needs of a diagnostic tool that is required in underdeveloped countries. CFP-10 and ESAT-6 are potent T-cell antigens recognised by over 70% of tuberculosis patients, which has led to their proposed use as a diagnostic reagent for tuberculosis in both humans and animals (Brodin et al., 2005).

1.4 – CFP-10/ESAT-6 HETERODIMER

There are 23 ESAT-6-like genes in the Mtb H37Rv genome. These genes are located in 11 genomic loci and are named as EsxA-W (Maciag et al., 2009). Inspection of the genetic diversity revealed that in five out of eleven cases blocks of conserved genes flank the Esx genes. Besides the Esx genes, the other conserved regions encode proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) proteins, adenosine triphosphate (ATP)-dependent chaperones of the ATPases associated with diverse cellular activities (AAA) family, membrane-bound ATPases, transmembrane proteins and serine proteases, which are known as mycosins (Maciag et al., 2009). The 11 genomic regions are clustered into five regions, namely: region 1 spanning the genes *Rv3866-Rv3883c*; region 2 spanning genes *Rv3884c-Rv3895c*; region 3 spanning genes *Rv0282-Rv0292*; region 4 containing the genes *Rv3444-Rv3450c*; and region 5 containing the genes *Rv1782-Rv1798* (Maciag et al., 2009). The genomes of Mtb H37Rv, *M. bovis* and *M. bovis* BCG have been compared, and various regions of difference (RD) have been identified. One of these regions, designated as RD1, is a 9500-bp region that is absent in all *M. bovis* BCG strains (Maciag et al., 2009). This deletion entirely removes the

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genomic fragment from *Rv3872 to Rv3879c*. Among the lost genes are *esxB* (*Rv3874*) and *esxA* (*Rv3875*), which respectively encode the CFP-10 and ESAT-6 proteins. This deletion is thought to be responsible for the primary attenuation of *M. bovis* to *M. bovis* BCG (Maciag et al., 2009).

1.4.1 – Function of the CFP-10/ESAT-6 heterodimer

1.4.1.1 - Functions of the monomer and the heterodimer

ESAT-6 alone or in combination with CFP-10 enhances the permeability of artificial membranes (Hsu et al., 2003) by disrupting the lipid bilayers and acts as a cytolysin, while the exposed C-terminal region of CFP-10 may be involved in interactions with a host cell target protein resulting in stabilisation of the helical conformation (Renshaw et al., 2002).

Both proteins are important in both pathogenesis and virulence of Mtb as the CFP-10/ESAT-6 secretion system contributes to the arrest of phagosome maturation and promotes survival of mycobacteria within macrophages. The knock-out of any of the RD1 genes shows a similar phenotype to the vaccine strain *M. bovis* BCG. This has been illustrated in both human and mouse macrophages and has been found to be due to autophagosome formation (Zhang et al., 2012, Lewis et al., 2003, Guinn et al., 2004). This provides a novel link between the CFP-10/ESAT-6 secretion system and mycobacterial virulence and pathogenesis (Tan et al., 2006). However, it is unclear as to whether it is ESAT-6, CFP-10 or the complex that is responsible for the arrest of phagosome maturation.

1.4.1.2 - Biochemical pathway of the heterodimer

Both CFP-10 and ESAT-6 are secreted by the ESAT-6 system-1 (ESX-1), a dedicated secretion apparatus encoded by genes flanking *esxA* and *esxB* in the extended RD1 region. Among the proteins predicted to be involved in this process is a member of the AAA-family of ATPases (*Rv3868*) (Stanley et al., 2003), which may perform chaperone-like functions by assisting in the assembly and disassembly of protein complexes and several putative membrane proteins or ATP binding sites. This could be involved in forming a transmembrane channel for the translocation of the effector molecules (Brodin et al., 2005).

1.4.1.3 - Why the heterodimer is important

The expression characteristics of both proteins, together with their structural properties, have led Renshaw *et al.* (2002) to propose that the biologically active form is the heterodimer. This implies that ESAT-6, without its partner CFP-10, might not be active

(Brodin et al., 2005). The virulence of Mtb is reduced by the knockout of either ESAT-6 or CFP-10 (Renshaw et al., 2002); therefore, the heterodimer is very important in the virulence of Mtb. It has also been reported that the CFP-10/ESAT-6 complex acts as a signalling molecule (Renshaw et al., 2005), which is likely to lead to the heterodimer being a key target in diagnostics.

1.5 – SCOPE AND OBJECTIVES

The scope of the project was to focus on generating aptamers that can specifically bind to early markers of active TB, namely the CFP-10/ESAT-6 heterodimer, which can then be used as rapid and reliable diagnostic tools for active TB. Thus, the objectives of this study were:

- To express and purify CFP-10 and ESAT-6 antigens.
- To isolate and characterize aptamers that bind to the CFP-10/ESAT-6 heterodimer.
- To test the utility of the aptamers to detect the CFP-10/ESAT-6 heterodimer in clinical samples of patients with active TB.

1.6 – STRUCTURE OF THESIS

The first result chapter of this thesis, Chapter 2, describes expression and purification of CFP-10 and ESAT-6, respectively. The chapter also describes the biological activity and dimerisation of the purified TB antigens. Expression, purification, biological activity and dimerisation of ESAT-6 and CFP-10, respectively, were necessary prerequisite for isolating the aptamers.

Chapter 3 focuses on optimisation of the SELEX protocol using different methods, and the actual isolation and characterisation of ssDNA aptamers against the CFP-10/ESAT-6 heterodimer.

Chapter 4 presents a proof-of-concept TB diagnostic study, in which the sensitivity and specificity of one aptamer, called EA10, was evaluated for detection of the CFP-10/ESAT-6 heterodimer or CFP-10 monomer in clinical samples of sputum from suspected and definitive active TB patients. Samples from apparently healthy donors were used as negative controls.

The final chapter, Chapter 5, provides a general discussion, summarising key findings and proposes further work beyond the scope of this thesis that needs to be done in order to develop a market-ready aptamer-based TB diagnostic tool of real utility.

Chapter 2.   EXPRESSION AND PURIFICATION OF CFP-10 AND ESAT-6

SUMMARY

CFP-10 and ESAT-6 of Mtb are not only promising antigens for use in the development of new tuberculosis diagnostics, but they are also key in TB pathogenesis. These two proteins are often expressed in an insoluble form in heterologous hosts, thus necessitating time-consuming and expensive methodologies to enable their purification. Purification protocols to date rely on metal ion affinity chromatography, followed by anion exchange chromatography in the presence of a denaturant to purify the desired protein.

In this chapter a simple and rapid protocol for the purification of soluble hexahistidine-tagged CFP-10 and ESAT-6 proteins, expressed in *E. coli,* is reported. The inclusion of either a refolding step or two imidazole wash steps to the standard purification protocol for CFP-10 and ESAT-6, respectively, obviates the need for additional chromatography steps to purify the two proteins. The methodology has the added benefit of removing histidine-rich host cell proteins, which unavoidably co-purifies with histidine-tagged recombinant proteins, and could therefore improve the purification of histidine-tagged recombinant proteins in general.

2.1 – INTRODUCTION

Two Mtb proteins that have attracted attention as desirable targets for new TB drugs and diagnostics are CFP-10 and ESAT-6 (Arend et al., 2000, Brodin et al., 2005, Dillon et al., 2000, Kulshrestha et al., 2005). The CFP-10 protein, encoded by the *Rv3874* (*esxB*) gene, and the ESAT-6 protein, encoded by the *Rv3875* (*esxA*) gene, can interact *in vitro* to form a tight 1:1 heterodimer with a molecular mass of 20.5 kDa (Renshaw et al., 2005, Renshaw et al., 2002). These two proteins are potent T-cell antigens recognised by over 70% of tuberculosis patients (Andersen, 1994, Skjot et al., 2000). Moreover, since CFP-10 and ESAT-6 are not present in BCG or other attenuated mycobacterium strains, they are considered to be useful in discriminating between infected and vaccinated individuals (Alderson et al., 2000, Andersen, 1994, Skjot et al., 2000).

Both CFP-10 and ESAT-6 have also been suggested to play an important role in the virulence of Mtb (Hsu et al., 2003, Pym et al., 2002, Tan et al., 2006). In fact, the CFP-10/ESAT-6 secretion system promotes survival of mycobacteria within macrophages, providing a link between the CFP-10/ESAT-6 secretion system and mycobacterial virulence and TB pathogenesis (Tan et al., 2006). Moreover, the virulence of Mtb has been shown to be reduced in mouse models by the inactivation of either ESAT-6 or CFP-10 (Pym et al., 2002, Renshaw et al., 2002). However, it is unclear as to whether ESAT-6, CFP-10, the CFP-10/ESAT-6 heterodimer and/or other factors secreted by the same pathway are directly responsible for increased Mtb virulence. It was also recently reported that the CFP-10/ESAT-6 complex acts as a signaling molecule that leads to modulation of host cell behaviour (Renshaw et al., 2005).

Various studies have reported on the expression and purification of histidine-tagged CFP-10 and ESAT-6 proteins. Expression of CFP-10 in *E. coli* as both soluble (Renshaw et al., 2002) and insoluble products has been reported. Insoluble CFP-10 requires metal ion affinity chromatography, followed by anion exchange chromatography in the presence of urea to purify the protein (Berthet et al., 1998). Alternatively, the recombinant CFP-10 can be purified using a three-step column chromatography procedure (affinity, anion exchange and gel filtration chromatography) without the use of urea or guanidine-HCl (Kulshrestha et al., 2005). ESAT-6 is expressed as an insoluble protein contained within inclusion bodies, requiring denaturation and renaturation, followed by affinity, anion exchange and size exclusion chromatographic steps for its purification. Although the protein has been solubilised with urea (Bai et al., 2008, Wang et al., 2005) and guanidine-HCl (Renshaw et al., 2002, Wang et al., 2005), it was reported that the yield of refolded protein is higher when urea is used compared to the use of guanidine-HCl (Wang et al., 2005). Despite the success of these elaborate methods to yield pure

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preparations of recombinant CFP-10 and ESAT-6 proteins, they are costly, time-consuming and can result in the co-purification of other histidine-rich proteins.

This chapter reports a rapid purification protocol for recombinant histidine-tagged CFP-10 and ESAT-6 proteins with no visible contaminants. This chapter will also demonstrate that the purification methods allow the purified proteins to both retain their native conformation and biological activity, as evidenced by their ability to dimerise and bind to human macrophages. The availability of purified soluble and biologically active CFP-10 and ESAT-6 should facilitate studies aimed at characterising their function, as well as their use in developing new TB diagnostics.

2.2 – MATERIALS AND METHODS

2.2.1 – Cells

HeLa cells (1×10^6 cell/ml) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) supplemented with 10% fetal calf serum (FCS; Lonza), 2 mM glutamine and 1% antibiotics (penicillin and streptomycin; Lonza), and incubated overnight at 37°C. Non-adherent cells were removed by washing with PBS (Lonza). The cells were then incubated for a further 24 hours in fresh DMEM. The cells were allowed to grow to a confluency of 75-80%.

Monocyte-derived macrophages (MDMs), for use in biological activity assays, were obtained as follows: Firstly, human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ PLUS (GE Healthcare) density gradient centrifugation from heparinised blood of healthy donors (South African National Blood Bank), as described previously (Khati et al., 2003). The PBMCs were suspended in RPMI medium containing 20% FCS, seeded into 150-cm^2 tissue culture flasks (Corning, Adcock Ingram) and incubated for 2 hours at 37°C. Subsequently, non-adherent cells were removed, and the remaining adherent cells (monocytes) were washed in PBS (Lonza) and incubated for a further 24 hours in fresh DMEM. The monocytes were then harvested, suspended in X-VIVO 10 (BioWhittaker) supplemented with 2% autologous serum and re-seeded into tissue culture flasks. Following seven days of differentiation, the resulting MDMs were typed by flow cytometry using an antibody directed against the CD68 cell surface receptor (R&D Systems) and detected with a secondary FITC-conjugated antibody (Kirkegaard and Perry Laboratories (KPL). Only cultures in which MDMs represented at least 95% of the cells were used.

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2.2.2 - Expression and purification of CFP-10

2.2.2.1 - Expression of CFP-10

E. coli BL21 (DE3) cells (Novagen) were transformed with the T7 promoter-based expression plasmid pMRLB46 (Megan Lucas, Colorado State University), expressing full-length CFP-10 fused to an N-terminal hexahistine tag. Transformed cells were plated onto LB agar plates containing 100 μg/ml ampicillin (Fermentas) and left to grow overnight. Thereafter, a single colony was picked and grown in 5 ml of LB medium and 100 μg/ml ampicillin at 37°C in a shaking incubator at 200 rpm overnight. The overnight cultures were inoculated into 2-L Erlenmeyer flasks, containing 1 L of LB medium and 100 μg/ml ampicillin, at 37°C in a shaking incubator at 200 rpm. Expression of the recombinant protein was induced in mid-exponential phase cultures, corresponding to an optical density (OD) at 600 nm of 0.6, by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sigma) to a final concentration of 0.4 mM. The cultures were harvested 4 hours after induction by centrifugation at 6 500 rpm for 20 minutes at 4°C. The cell pellets were stored overnight at -80°C.

2.2.2.2 - Solubilisation of CFP-10 with 6 M urea

Pellets obtained after harvesting cells from 1-ml cultures were suspended in 1 ml of Buffer A (6 M urea [Merck], 25 mM Tris-HCl [Sigma] [pH 7.4], 200 mM NaCl [Sigma], 10 mM imidazole [Merck]), and centrifuged at 14 000 rpm for 15 minutes. The recovered supernatant was dialyzed (Slide-A-Lyser, Pierce, 3-kDa MWCO) in Buffer B (25 mM Tris-HCl [Sigma] [pH 7.4], 200 mM NaCl [Sigma], 10 mM imidazole [Merck]) overnight at 4°C. The supernatant was then centrifuged at 14 000 rpm for 10 minutes to separate soluble and insoluble proteins. The solubility of the protein was assessed by 17% SDS-PAGE by loading both the pellet and the supernatant fractions after centrifugation.

2.2.2.3 - Purification and solubilisation of CFP-10

Cell pellets from induced cultures were suspended in 10 ml of Buffer A and lysed by sonication on ice at 60% power output for 3 minutes. This step was repeated three times, after which the lysate was centrifuged at 14 000 rpm for 15 minutes. The supernatant was recovered and loaded onto a Ni-NTA column (BioRad), which had been pre-equilibrated with Buffer A, by rotation overnight at 4°C. The Ni-NTA column was washed once with 100 ml of Buffer A. The urea was removed by six washes with 10 ml of Buffer A, in which the urea concentration was decreased from 6 M to 1 M, followed by two washes with 20 ml of Buffer B. Finally, CFP-10 was eluted in a single step with 10 ml of Buffer B containing 300 mM imidazole. The purity of the eluted protein was assessed by electrophoresis on a 17% SDS-PAGE gel and stained with Coomassie brilliant blue R-250 (0.25% Coomassie Blue R-250, 50% Methanol and 10% acetic acid).

2.2.3 – Expression and purification of ESAT-6

2.2.3.1 - Expression of ESAT-6

ESAT-6 was expressed in a similar manner to CFP-10, except for a minor modification in the temperature at which the cultures were induced. Previous reports regarding expression of different antigens of Mtb indicated that lowering of the growth temperature leads to enhanced solubility of the antigens (Chaudhary et al., 2005, Kulshrestha et al., 2005). Since ESAT-6 enters inclusion bodies when induced at 37°C (Renshaw et al., 2002), the cultures were therefore incubated overnight at 25°C to increase its solubility. After incubation, cells were harvested by centrifugation at 6 500 rpm for 20 minutes at 4°C. The cell pellets were stored overnight at -80°C.

2.2.3.2 - Purification and solubilisation of ESAT-6

ESAT-6 was purified in a similar manner to CFP-10, except that as a final purification step, the column was washed with two 20-ml washes of Buffer B containing 40 mM and 50 mM imidazole, respectively. ESAT-6 was eluted with Buffer B containing 300 mM imidazole. The purity of the eluted protein was assessed by 17% SDS-PAGE and stained with Coomassie brilliant blue R-250.

2.2.4 – Western blot analysis

The purified CFP-10 and ESAT-6 proteins were separated by electrophoresis on a 17% SDS-PAGE and electroblotted onto Hybond™-ECL nitrocellulose membranes (GE Healthcare). Each membrane was incubated for 1 hour in PBS supplemented with 0.005 % (v/v) Tween-20 and 5% (w/v) fat-free milk powder, followed by incubation at room temperature for 1 hour with a 1:15 000 dilution of either an anti-CFP10 polyclonal antibody (provided by Megan Lucas, Colorado State University) or an anti-ESAT-6 monoclonal antibody (Santa Cruz Biotechnology). The blots were washed three consecutive times with wash buffer (PBS containing 0.005% Tween-20), and incubated for 1 hour with the secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG, respectively, conjugated to horseradish peroxidase (diluted 1:15 000; Santa Cruz Biotechnology). After three successive washes with wash buffer, immuno-reactive proteins were detected with the enhanced chemiluminescence (ECL) Advance™ Western Blotting Detection kit (GE Healthcare) according to the manufacturer's instructions.

2.2.5 – Protein identification by mass spectrometry (MS)

Regions of Coomassie blue-stained SDS-polyacrylamide gels containing protein bands of interest were excised. In-gel digestion of proteins and peptide extraction were performed, as

described by Shevchenko *et al*. (2006). The samples were analysed on an Agilent 1100 HPLC system equipped with capillary and nano LC pumps coupled to a QSTAR ELITE mass spectrometer. Aliquots (1-2 µg) of each sample were desalted on a Symmetry C18 trap column (0.18 x 23.5 mm) with Buffer 1 (0.5% acetonitrile [ACN], 0.5% formic acid [FA]). Peptides were separated on a NanoEase XBridge C18 column (0.1 x 50 mm) connected to the trap column via a 6-port switching valve, and eluted in a gradient of Buffer 2 (98% ACN, 0.5% FA). Nano-spray was achieved using a MicroIonSpray head assembled with a New Objective PicoTip emitter and an electrospray voltage of 3 kV was applied to the emitter. The mass spectrometer was operated in Information Dependant Acquisition (IDA) using an Exit Factor of 2.0 and Maximum Accumulation Time of 2.5 seconds. MS scans were acquired from 400-1600 m/z and the three most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Protein identification was performed using the Paragon™ algorithm thorough Search in Protein Pilot (Protein Pilot v3.0 software). An identification confidence of 95% was selected during searches.

2.2.6 – Complex formation of CFP-10 and ESAT-6

Equimolar amounts (5 µM) of the purified recombinant CFP-10 and ESAT-6 proteins were dimerised in phosphate buffer (25 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) at room temperature to allow formation of the heterodimer, as described previously (Renshaw et al., 2002), and then analyzed by native PAGE and Western blot analysis.

2.2.6.1 – Surface Plasmon Resonance (SPR)

To confirm interaction between the two proteins, measurements were also performed at 25°C with a BIAcore® 3000 biosensor instrument (GE Healthcare). The carboxymethylated dextran surface of CM5 biosensor chips (Separation Scientific) was activated by injecting 50 µl of 0.5 M N-hydroxysuccinimide (NHS), 0.2 M N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) at a flow rate of 5 µl/minute in order to allow protein coupling. The CFP-10 protein, diluted in 10 mM sodium acetate (NaOAc) (pH 3.5) to a final concentration of 200 nM, was injected at 5 µl/minute, followed by blocking of the free activated carboxymethyl groups with 70 µl of 1 M ethanolamine-HCl (pH 8). An equimolar concentration of ESAT-6 protein (200 nM) was injected at 5 µl/minute over the flow cell in which CFP-10 was immobilised, followed by removal of unbound protein by injecting Buffer HBS-N (10 mM HEPES, pH 7.4, 150 mM NaCl). An uncoupled flow cell was included to evaluate non-specific binding of ESAT-6 to the ethanolamine-HCl blocked chip surface. The data was analysed using BIAevaluation 4.1 software (GE Healthcare).

2.2.6.2 - Fluorescent microscopy

Samples (500 µl) corresponding to CFP-10, ESAT-6 and the CFP-10/ESAT-6 heterodimer were labelled with Alexa Fluor 546 (excitation = 556 nm and emission = 573 nm; Molecular Probes, Invitrogen) by incubating a 10-fold molar excess of the succinimidyl ester derivative of the dye with the respective samples in a buffer consisting of 25 mM NaH_2PO_4 and 100 mM NaCl (pH 7.5) at room temperature overnight. Excess dye was removed by dialysis and the extent of labelling was determined by the absorbance of the labelled complex at 280 and 556 nm. The protein-label function on the NanoDrop® 1000 spectrophotometer was used to determine the amount of fluorescently labelled protein, as previously described by Renshaw and colleagues (Renshaw et al., 2005).

HeLa cells and MDMs were seeded at 50% confluency into Lab-Tek™ chamber slides (Nunc, AEC Amersham), and incubated for 24 hours at 37°C. The slides were then incubated for 15 minutes at room temperature in PBS with 1 µM of the Alexa Fluor 546-labelled CFP-10, ESAT-6 and the CFP-10/ESAT-6 heterodimer. After removal of unbound protein complexes by two washes with PBS, the cells were fixed with 4% (w/v) paraformaldehyde (Merck) and permeabilised with 0.2% (v/v) Triton X-100 (Sigma). Cover slips were mounted, using mounting media containing 4', 6-diamidino-2-phenylindole (DAPI, excitation = 350 nm and emission = 470 nm, Santa Cruz Biotechnology) for DNA counterstaining, and stored at room temperature in the dark until dry. As a control, the MDMs and HeLa cells were also incubated with 1 µM of the Alexa Fluor 546 dye only. The samples were analysed on an Olympus® BX41 fluorescent microscope, together with analySIS LifeScience® software (Wirsam Scientific and Precision Equipment).

2.3 – RESULTS

2.3.1 – Expression, solubilisation and purification of CFP-10

The CFP-10 protein was expressed in *E. coli* BL21 (DE3) cells at 37°C. The cells were disrupted by sonication, followed by centrifugation. For purification the supernatant was bound to a Ni-NTA column, washed and nine fractions were collected, of which five fractions contained CFP-10. SDS-PAGE of the collected fractions indicated the presence of a protein corresponding to the expected molecular weight of about 10 kDa for CFP-10 (Figure 2-1 A) as well as a high-molecular-weight contaminant. The identity of the 10-kDa protein was confirmed by Western blotting using an anti-CFP-10 antibody (Figure 2-1 B).
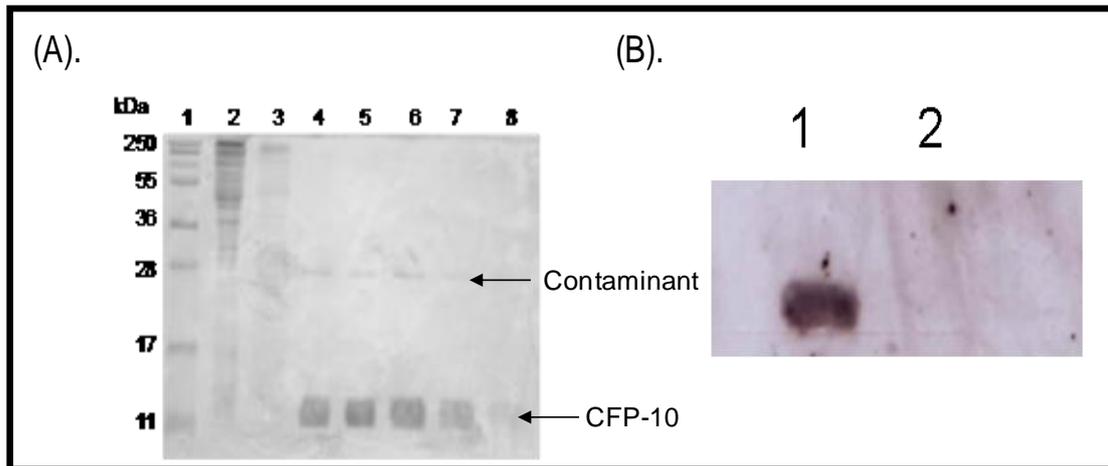
Figure 2-1: Analysis of purified fractions of CFP-10.
(A). SDS-PAGE of CFP-10 after Ni-NTA purification, in the absence of 6 M urea. Lane 1: Molecular weight marker (SM1811, Inqaba); lane 2: supernatant before purification; lane 3: flow through; lane 4: 50 mM imidazole wash; and lanes 5-8: purified CFP-10 fractions. (B). Western blot analysis of purified fractions identifies CFP-10. Lane 1: Purified fraction of CFP-10; lane 2: purified fraction of ESAT-6 (negative control).

Based on reports indicating that differences in the refolding of proteins under a given condition may be used to selectively purify proteins (Rudolph et al., 1996), we evaluated this approach as a means to purify the CFP-10 protein. To solubilise CFP-10, 6 M urea was added to the resuspended cell pellets, the urea was then washed out in a stepwise manner, and finally the sample was centrifuged to fractionate soluble and insoluble proteins. Both the supernatant and the resulting pellets before and after solubilisation were assessed for the presence of CFP-10. As CFP-10 was present in the supernatant after the urea was washed out (Figure 2-2) it is clear that CFP-10 can correctly refold in the absence of the denaturant. Furthermore, the high-molecular-weight contaminant was only present in the pellet after dialysis, indicating that the contaminant could not refold under these conditions (Figure 2-2).
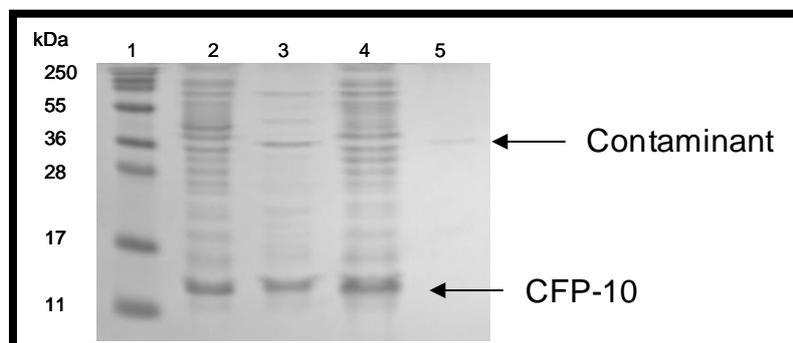
Figure 2-2: SDS-PAGE of CFP-10 using 6 M urea and dialysis membrane for purification.
Lane 1: Molecular weight marker (SM1811, Inqaba); lane 2: supernatant before dialysis; lane 3: pelleted cells before dialysis; lane 4: supernatant after dialysis; and lane 5: pelleted cells after dialysis.

Next, a similar denaturing-renaturing procedure was tested for its ability to remove the contaminant from the Ni-NTA resin without affecting CFP-10 binding to the resin. A 6 M

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urea buffer (Buffer A) was used during binding of the cell lysates to the resin. Urea was washed out in a stepwise manner using washes of decreasing urea concentrations. The addition of urea and subsequent stepwise removal of the urea resulted in a pure fraction of CFP-10 protein, without any visible contaminant, as evidenced by SDS-PAGE analysis (Figure 2-3). The protein concentration was measured by absorbance at 280 nm and 2.4 mg of highly pure (99%) monometric CFP-10 was obtained from 1 L of culture.
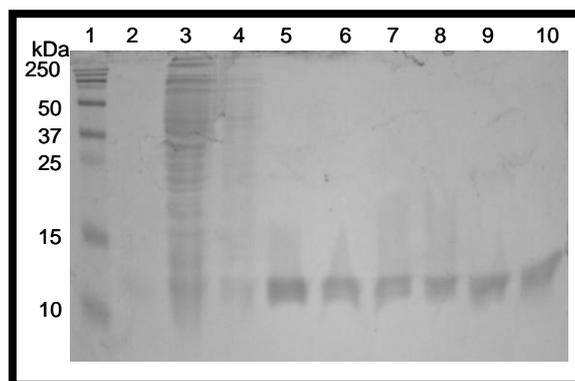
Figure 2-3: Purification and refolding of CFP-10 in the presence of 6 M urea.
CFP-10 was purified under denaturing conditions and refolded, while bound to Ni-NTA resin, by removing the denaturant through a series of washes. Lane 1: Molecular weight marker (Precision Plus Protein™ Standards kaleidoscope, BioRad); lane 2: CFP-10 that remained bound to the resin after refolding; lane 3: flow through; lane 4: 6 M urea wash; and lane 5-10: purified CFP-10 fractions.

During the purification protocol, CFP-10 had varying levels of purity. This was due to the contaminant present in the early stages of purification. However, on improvement of the purification methodology by the addition of urea the purity of the eluted protein was improved (Table 2-1).

Table 2-1: Yields in each step of purification of CFP-10

Purification step	Total purified protein [a, b] (mg/ml)	Yield of purified Fraction [b] (mg/ml)	Contaminant (mg/ml)	Purity [c] (%)
Purification in absence of 6 M urea	4	2.4	1.6	>60%
Purification in presence of 6 M urea	2.4	2.4	0	>95%

[a] Corresponding to 1 L of culture.

[b] Quantified using the Nanodrop® protein A_{280} assay.

[c] Purity estimated by SDS-PAGE.

2.3.2 – Expression and purification of ESAT-6

ESAT-6 was expressed in *E. coli* BL21 (DE3) cells at 25°C to increase its solubility by preventing the protein entering inclusion bodies. The supernatant was bound to the Ni-NTA column for purification. Fractions were collected during the column purification and analysed by SDS-PAGE. The fractions collected contained a 10-kDa protein, which is the expected molecular weight of ESAT-6, as well as a high-molecular-weight contaminant species, similar to the contaminant found during the purification of CFP-10 (Figure 2-4 A). A Western blot was performed and confirmed the 10-kDa protein as ESAT-6 (Figure 2-4 B).
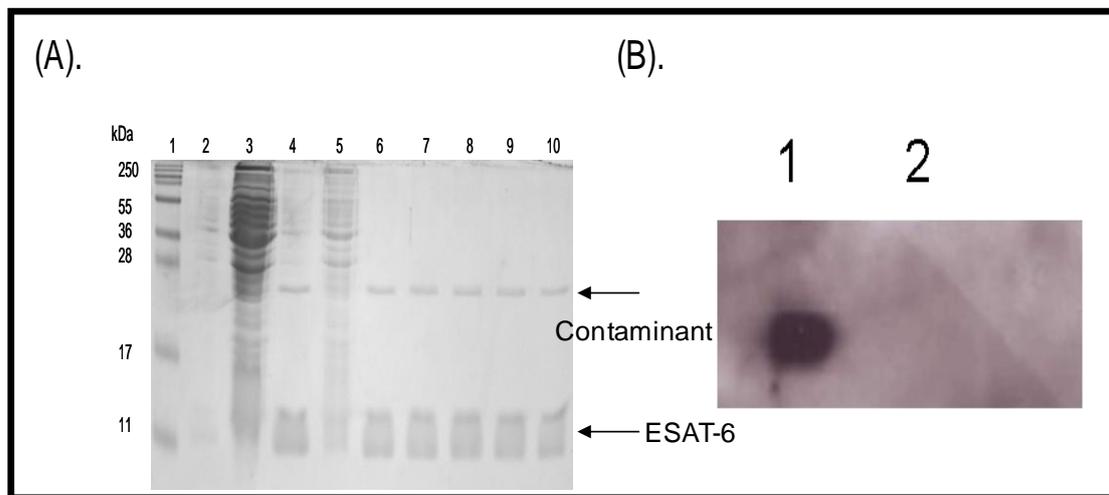
Figure 2-4: Analysis of purified ESAT-6 fractions.
(A). SDS-PAGE of ESAT-6 after Ni-NTA purification. Lane 1: Molecular weight marker (SM1811, Inqaba); lane 2: Ni-NTA resin after ESAT-6 purification; lane 3: flow through; lane 4: purified ESAT-6 fraction, lane 5: flow through of 6 M urea wash; and lanes 6-10: purified ESAT-6 fractions. (B). Western blot analysis of the ESAT-6 protein. Lane 1: ESAT-6 purified from Ni-NTA column and lane 2: CFP-10 purified from a Ni-NTA column (negative control).

However, with the addition of two imidazole washes (40 mM and 50 mM, respectively) the high-molecular-weight contaminant was absent from the purified ESAT-6 fractions (Figure 2-5).
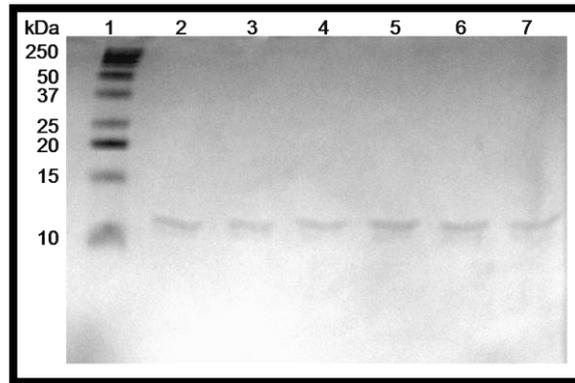
Figure 2-5: SDS-PAGE of purified ESAT-6 after the additional imidazole washes.
Lane 1: Molecular weight marker (Precision Plus Protein™ Standards kaleidoscope, BioRad); and lanes 2-7: purified ESAT-6 fractions.

Although inclusion of the additional wash steps was sufficient to completely remove the contaminant, it also resulted in a reduction in the final yield of purified ESAT-6. Nevertheless, this purification procedure yielded 1.3 mg of monomeric ESAT-6 from 4 L of culture, as measured by UV spectroscopy, and had a purity of 99% (Table 2-2).

Table 2-2: Yields in each step of purification of ESAT-6

Purification step	Total purified protein[a, b] (mg/ml)	Yield of purified fraction[a, b] (mg/ml)	Contaminant (mg/ml)	Purity[c] (%)
Expression at 37°C	0.02	-	-	-
Purification before imidazole washes	0.1	0.06	0.06	>50%
Purification after imidazole washes	0.06	0.06 (1.3)[d]	0	>95%

[a] Corresponding to 4 L of culture.

[b] Quantified using the Nanodrop® protein A_{280} assay

[c] Purity estimated by SDS-PAGE.

[d] Value in parenthesis is after concentration of protein using a Vivaspin-6 column (Satorius).

2.3.3 – Protein identification using mass spectrometry

Purification of the recombinant hexahistidine-tagged CFP-10 and ESAT-6 proteins through Ni-NTA affinity chromatography coincided with the purification of a high-molecular-weight contaminant protein (28-kDa). Interestingly, a similar sized protein has been noted previously in purified recombinant ESAT-6 protein preparations and was suggested to represent a multimeric form of ESAT-6 (Kulshrestha et al., 2005). However,

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our results above indicated that the high-molecular-weight protein did not interact with antibodies specific for CFP-10 or ESAT-6 (2.3.1 and 2.3.2, respectively). It was therefore of interest to determine the identity of the co-purifying protein. SDS-polyacrylamide gel slices containing this protein were subjected to nano-LC/MS/MS, the results of which identified the co-purifying protein as SlyD from *E. coli.* SlyD is a peptidoylproline *cis-trans* isomerase that is involved in the hydrogenase biosynthetic pathway of *E. coli* and aids the assembly of [Ni-Fe] clusters as a metallochaperone (Hottenrott et al., 1997, Zhang et al., 2005).

2.3.4 – Complex formation of CFP-10 and ESAT-6

The protein purification protocols described herein yielded highly pure CFP-10 and ESAT-6 recombinant proteins in soluble and monomeric forms. However, to be useful the purified recombinant proteins need to retain their native conformation and biological activity. To assess whether the proteins refolded efficiently, we performed a series of experiments aimed at determining whether the proteins formed a heterodimer with a functional conformation. These analyses were based on previous reports indicating that biologically active CFP-10 and ESAT-6 proteins form a heterodimer (Poulsen et al., 2010, Renshaw et al., 2005, Renshaw et al., 2002) that is capable of binding to Toll-like receptor 2 (TLR2) present on the surface of macrophages (Pathak et al., 2007).

Firstly, we determined whether the purified refolded recombinant CFP-10 and ESAT-6 proteins were capable of forming a heterodimer. Initially, the individual purified recombinant proteins were mixed in solution and the mixture was analysed by native PAGE. The results indicated that the protein complex migrated as a single band (Figure 2-6), even though there was bands that coincided with the monomeric proteins.
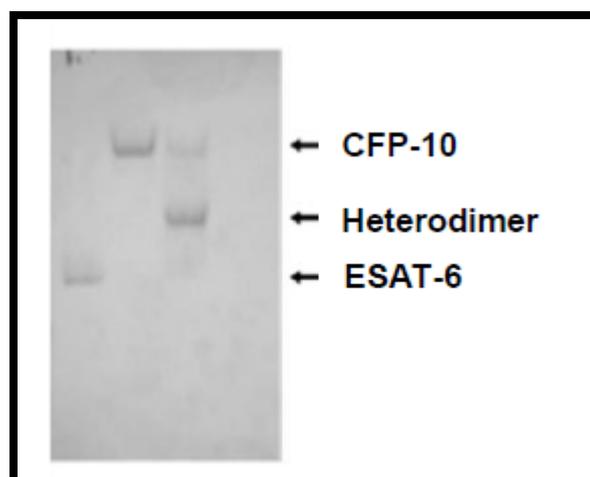
Figure 2-6: Native PAGE of the heterodimer.
CFP-10 and ESAT-6 were dimerised using a previously described protocol.

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2.3.5.1 - SPR

Further confirmation of the heterodimer formation was obtained using SPR (Figure 2-7). CFP-10 was immobilised on a CM5 chip and was used to capture ESAT-6 that was passed over the flow cell. An uncoupled flow cell was used as a control. The amount of ESAT-6 binding to the uncoupled flow cell was subtracted to determine specific binding to immobilised CFP-10. It was found that ESAT-6 could bind to immobilised CFP-10, thus forming the heterodimer on the chip surface. The heterodimer was, however, not completely stable on the chip surface as seen by the rapid dissociation of ESAT-6 from the immobilised CFP-10. This is likely due to the high flow rate of buffer over the chip surface once ESAT-6 had bound to immobilised CFP-10, and the high salt concentration of the buffer used that could result in the dissociation of the salt bridges between the two proteins.
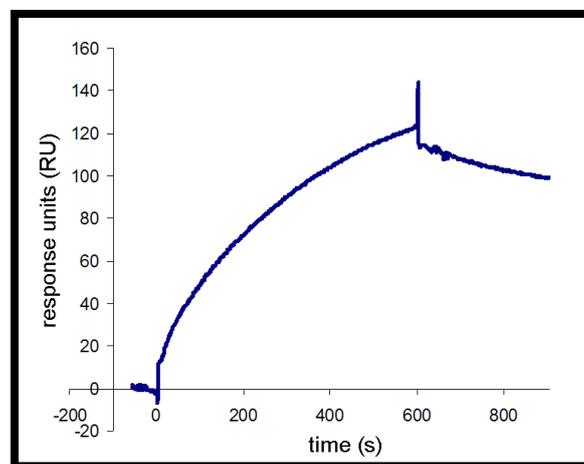
Figure 2-7: Real-time binding of ESAT-6 to immobilised CFP-10 using SPR.
Data was corrected by subtracting non-specific binding using a reference (empty) flow cell.

2.3.5.2 – Fluorescent microscopy

Having confirmed that the purified CFP-10 and ESAT-6 proteins interact to form a heterodimer, the binding of the CFP-10/ESAT-6 heterodimer to the surface of macrophage cells was assessed. For this purpose, both proteins in the heterodimer were labelled covalently with a fluorophore (Alexa Fluor 546), and the labelled monomers and heterodimer were incubated with macrophages and HeLa cells prior to examination by fluorescent microscopy. The macrophages showed intense fluorescence of the cell surface after incubation with the labelled heterodimer (Figure 2-8) and monomers (Appendix I). In contrast, no labelling of the surface of macrophages was detected when incubated with Alexa Fluor 546 only; indicating that the fluorescent localisation observed with the labelled CFP-10/ESAT-6 heterodimer is mediated by the heterodimer and not the attached fluorophore. Moreover, no florescence labelling was seen for the control HeLa cells (Appendix I), which lack the TLR2 receptor (Pathak et al., 2007), and thus provided

supporting evidence for macrophage-specific labelling with the CFP-10/ESAT-6 heterodimer.

Taken together, the results show that in biologically relevant assays the refolded ESAT-6 and CFP-10 proteins form a heterodimer capable of cell-type specific interaction, thus indicating that the purified recombinant proteins retained their native conformation and functionality. The results furthermore indicate that the short tags added to the N-terminus of the proteins do not alter the biological activity of the recombinant CFP-10 and ESAT-6 proteins produced in *E. coli*.

2.4 – DISCUSSION

CFP-10 and ESAT-6 are both members of a large family of secreted proteins that are found in low levels in Mtb culture supernatants (Berthet et al., 1998). However, owing to the slow growth of Mtb it is not practical to purify large amounts of the proteins from the culture medium for use in biochemical assays and diagnostics. This therefore necessitates high-level production of pure proteins in a soluble form. Unfortunately, expression of mycobacterial proteins in E. coli has proven to be problematic and a large proportion of the proteins are insoluble when over-expressed and purified (Bai et al., 2008, Bellinzoni et al., 2003). Specifically, several studies regarding expression and purification of CFP-10 and ESAT-6 have reported the production of these proteins as insoluble aggregates, thus requiring extensive denaturation and renaturing protocols for obtaining soluble proteins and several chromatographic steps to obtain pure protein preparations.
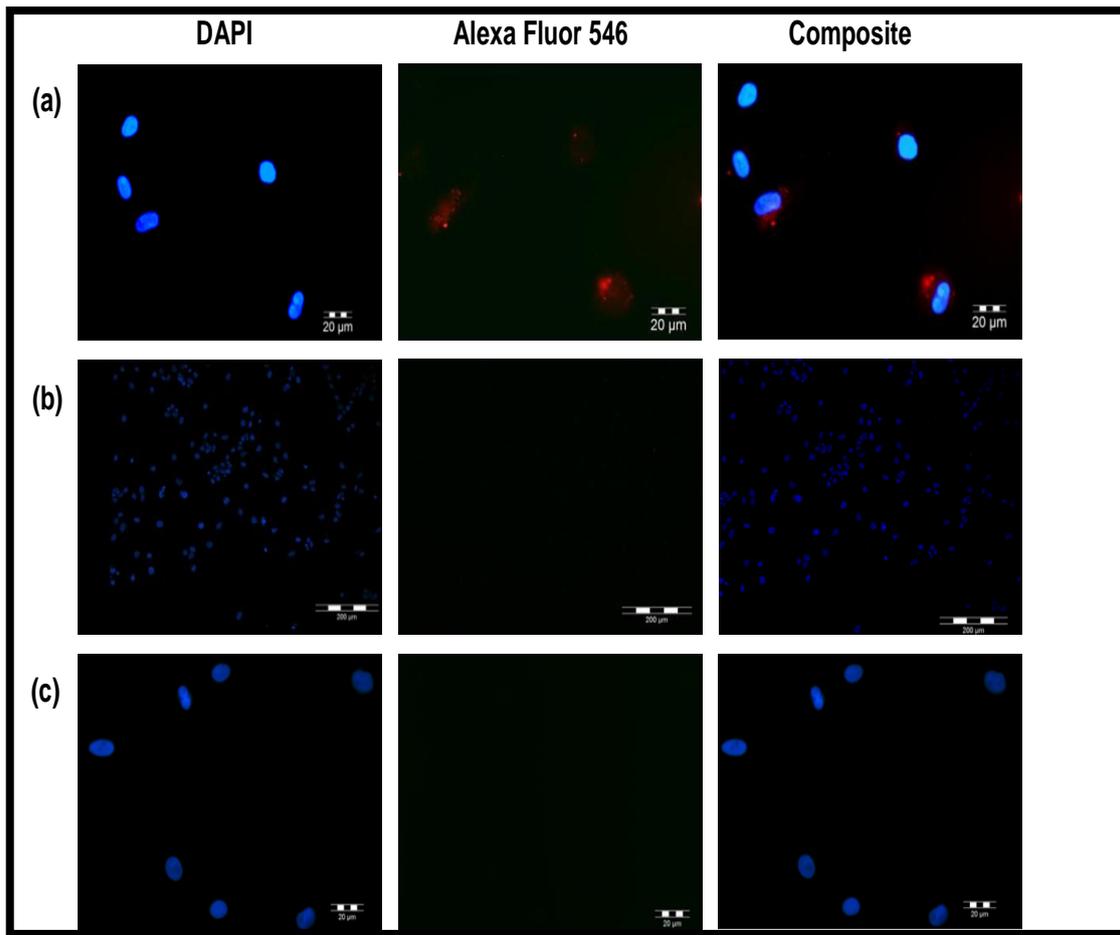
Figure 2-8: Fluorescently labelled protein binding to macrophages.
Macrophages (a) and HeLa (b) cells were incubated in the presence of Alexa Fluor 546-labeled CFP-10/ESAT-6, whereas macrophage cells incubated with Alexa Fluor 546 only (c) were included as a control. The right hand panels show microscope composite images of the same cells viewed separately for DAPI (blue) and Alexa Fluor 546 (red) fluorescence

In agreement with the results of Renshaw and colleagues (Renshaw et al., 2002), CFP-10 was expressed in a soluble form as was evidenced by its localisation to the supernatant following centrifugation of the cell lysate. In contrast to CFP-10, the ESAT-6 protein is expressed in *E. coli* predominantly as an insoluble protein and solubilisation of the protein requires exposure to a strong chaotropic agent such as guanidine-HCl or urea at concentrations from 6-8 M (Bai et al., 2008, Renshaw et al., 2002, Wang et al., 2005). This chapter illustrates an approach aimed at limiting *in vivo* aggregation of the recombinant protein. A popular approach consists of growing the cultures at reduced temperatures. Not only does this reduce the rate of protein synthesis thereby allowing the slower turnover of proteins to result in a greater yield of correctly folded soluble protein, but the activity and expression of a number of *E. coli* chaperones are decreased below 30°C (Ferrer et al., 2003, Schein, 1989).

As indicated above, purification of the recombinant hexahistidine-tagged CFP-10 and ESAT-6 proteins through Ni-NTA affinity chromatography coincided with the purification

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of SlyD. SlyD was originally discovered as a persistent contaminant of recombinant proteins that had been purified by immobilized metal affinity chromatography (Arnold, 1991, Wulfing et al., 1994). Three distinct domains can be identified in SlyD: an N-terminal domain (residues 1-95) that is similar to the FK binding proteins superfamily, the second domain (residues 148-179) is rich in cysteine and histidine residues, while the third domain (residues 149-196) corresponds to a metal-binding region (Bolanos-Garcia et al., 2006). Due to the histidine-rich C-terminal part of SlyD, it is capable of binding in native or denatured conditions to divalent cations such as Ni^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} (Mukherjee et al., 2003, Wulfing et al., 1994), as would histidine-tagged fusion proteins. Although many studies of SlyD have focused on how to purify the protein on its own for specific studies (Hottenrott et al., 1997, Kaluarachchi et al., 2009, Scholz et al., 2006, Wulfing et al., 1994), very few publications have reported on its removal from target proteins. However, *E. coli* NiCo21 (DE3) expression host cells have been engineered in which the SlyD protein has been tagged to enable its removal by chitin chromatography, following Ni-NTA purification of the histidine-tagged target protein (New England Biolabs).

This chapter provides a rapid protocol for the purification and renaturing of recombinant hexahistidine-tagged CFP-10 and ESAT-6 expressed in *E. coli*. The protocols described herein are two-step purification protocols that minimised the need for multiple chromatography steps described in previous protocols. An *E. coli* contaminant was also removed efficiently, resulting in 99% pure and biologically active recombinant CFP-10 and ESAT-6 proteins that can be used for functional studies and towards developing diagnostics specific to Mtb. The significance of these findings is that the described protein purification methods may be applicable to any recombinant protein system with a histidine-tag and does not require the use of specialized expression hosts or costly reagents to remove co-purifying histidine-rich host cell proteins.

Chapter 3.   ISOLATION AND CHARACTERISATION OF APTAMERS TARGETING THE CFP-10/ESAT-6 HETERODIMER

SUMMARY

CFP-10 and ESAT-6 are potent T-cell antigens and are thought to be one of the early markers of active TB. Moreover, since CFP-10 and ESAT-6 are not present in BCG or other attenuated mycobacterium strains, they are considered to be useful in discriminating between infected and vaccinated individuals. To help improve TB diagnostics; anti-CFP-10/ESAT-6 aptamers were isolated using the SELEX protocol. SELEX is a method in which single-stranded oligonucleotides are selected from a wide variety of sequences, based on the interaction of the sequences with a target molecule. As a result, 64 unique aptamers were isolated and screened for binding via an enzyme-linked oligonucleotide assay (ELONA). Of the 64 aptamers screened, 24 showed significant ($p < 0.05$) binding to the CFP-10/ESAT-6 heterodimer. An antibody competition assay revealed that some aptamers had either an enhanced or reduced binding to the CFP-10/ESAT-6 heterodimer when the antibody was present. A specificity test revealed that one of the 24 aptamers was a non-specific binder. The results aided in the selection of six aptamers that were studied further as potential TB diagnostic tools. The binding kinetics of the aptamers to CFP-10 was tested using surface plasmon resonance, to determine which ones had the best binding affinity; the experiment revealed that all six aptamers chosen had a high affinity for the CFP-10/ESAT-6 heterodimer.

3.1 – INTRODUCTION

The isolation of aptamers using the SELEX process (Ellington and Szostak, 1990, Robertson and Joyce, 1990, Tuerk and Gold, 1990) are beginning to rival antibodies in both therapeutic and diagnostic applications. The advantages of aptamers over antibodies is that aptamers are easy to synthesise; inexpensive; simple to chemically modify; not immunogenic; have low variability between batches; do not require animals for synthesis; have a relatively small size compared to antibodies, that allows tissue penetration; they are not sensitive to temperature and undergo reversible denaturation giving them a longer shelf-life than antibodies (Blank et al., 2001, Hamula et al., 2008).

Usually single-stranded DNA (ssDNA) aptamers are used for diagnostics due to the greater inherent stability of DNA and low cost of production compared to RNA aptamers. Currently, most studies on ssDNA aptamers for diagnostics focus on the detection of thrombin, adenosine or cocaine (Liu and Lu, 2005, Liu et al., 2006b, Wang et al., 2007, Wang et al., 2006, Wei et al., 2007). However, ssDNA aptamers have also been raised against (1-3)-b-D-glucans, which were the first aptamers developed for the detection of biotoxins in environmental respiratory diseases (Low et al., 2009). There are also reports on existing diagnostic and detection technologies that have been modified to include aptamers targeting bacteria and viruses. For example, the electrochemical detection of *Salmonella typhi,* which uses single-walled carbon nanotubes coated with aptamers specific for *Salmonella typhi* (Zelada-Guillen et al., 2009).

An important process in the selection of ssDNA aptamers is the generation of ssDNA for use in the selection process. There are many methods for the generation of ssDNA from double stranded DNA (dsDNA), which are applicable not only to SELEX but a variety of other molecular biology and biotechnology applications (e.g. sensors, microarrays and solid phase DNA sequencing). These methods include denaturing urea-polyacrylamide gel (Fitzwater et al., 1996, Pagratis, 1996, Stoltenburg et al., 2005, Zelada-Guillen et al., 2009), asymmetric PCR (Gyllensten et al., 1988, Wu et al., 1999), lambda exonuclease digestion (Higuchi et al., 1989, Jones et al., 2006) and magnetic separation with streptavidin-coated beads (Espelund et al., 1990, Hultman et al., 1989). For strand separation with denaturing PAGE, PCR is performed with a primer that contains a spacer as a terminator to produce strands with different lengths; these strands are then separated by denaturing PAGE electrophoresis. The desired ssDNA strand then has to be eluted from the denaturing-polyacrylamide gel. Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. Therefore, PCR is performed with an unequal molar ratio of forward and reverse primers resulting in an excess of ssDNA. However, asymmetric PCR products comprise not only single- but also

double-stranded DNA. Thus, the PCR products have to be separated by non-denaturing PAGE. The purification of small ssDNA fragments from polyacrylamide gels is very time-consuming and results in poor yields of ssDNA, which is undesirable during SELEX experiments due to massive loss of target binding aptamers. Therefore, these two methods are not recommended for SELEX experiments, especially in the first rounds of selection where binding sequences are not sufficiently amplified (Avci-Adali et al., 2010). The most widely used method for ssDNA generation is the immobilization of biotinylated dsDNA onto streptavidin-coated beads and the denaturation of dsDNA by alkaline treatment (Hamula et al., 2008, Avci-Adali et al., 2010, Paul et al., 2009). However, previous studies demonstrated the dissociation of streptavidin by alkaline treatment (Paul et al., 2009). Thus, undesired biotinylated strands enter into the eluate and can re-anneal to the complementary strands and lose their tertiary structure, which is important for the target binding ability. An alternative method to generate ssDNA is by lambda exonuclease digestion of the undesired strand. Lambda exonuclease is a highly processive 5´→3´ exodeoxyribonuclease that selectively digests the 5´-phosphorylated strand of dsDNA. For this, a 5´-phosphate group is incorporated into antisense strands of the dsDNA by performing PCR where one of the two primers has a 5´-phosphate group (usually this is the reverse primer). The phosphorylated strand is then removed by digestion with lambda exonuclease (Avci-Adali et al., 2010). A method that has not been used to date is the method of generating ssDNA through cDNA synthesis. This method uses RNA that has been transcribed from dsDNA, and then cDNA that is reverse transcribed from that RNA. The resulting cDNA is then used as a template for the next round of selection.

Currently, there are aptamers against other TB targets that have been isolated for both diagnostic and therapeutic purposes. These include ssDNA aptamers isolated against the Mtb polyphosphate kinase 2 (PPK2) for the inhibition of the PPK2 enzyme (Shum et al., 2011); aptamers raised against the whole bacterium for therapeutic use (Chen et al., 2007); and ssDNA aptamers against MPT64 for diagnostics of Mtb (Qin et al., 2009). To date there is no reports of aptamers against CFP-10 and ESAT-6. CFP-10 and ESAT-6 are potent T-cell antigens recognized by over 70% of tuberculosis patients (Andersen, 1994, Skjot et al., 2000), making them an ideal target for diagnostics. This chapter will report on the generation of ssDNA by widely used methods as well as by cDNA synthesis. This chapter also reports the first aptamers isolated against the CFP-10/ESAT-6 heterodimer, which will be further characterised and tested as a potential diagnostic for TB.

3.2 – MATERIALS AND METHODS

3.2.1 - Isolation of aptamers against the CFP-10/ESAT-6 heterodimer

3.2.1.1 - Aptamer libraries

The first step in the SELEX experiments was to create a pool of variant sequences from which ssDNA aptamers of relatively high affinity for the target proteins could be selected. A 90-mer ssDNA library, which had a random region of 49 nucleotides flanked by constant regions, was custom synthesised by Integrated DNA Technologies (IDT). The constant regions allowed for amplification by using them as primer annealing sites. The primer sequences were 5′ GCCTGTTGTGAGCCTCCTAAC 3′ (forward primer) and 5′ GGGAGACAAGAATAAGCATG 3′ (this reverse primer was either modified with a T7 promoter region (TAATACGACTCACTATA) at the 3' end; a biotin modification at the 3′ end; or a phosphate at the 5′ end). The library used was 5′ GCCTGTTGTGAGCCTCCTAAC(N49)CATGCTTATTCTTGTCTCCC 3′.

3.2.1.2 - In vitro selection of DNA aptamers

In the first round of selection, 500 pmol of ssDNA library was used to obtain a diversity of at least 10^{14} molecules of random sequences. The selection was done by a modification of the SELEX protocol in which the ssDNA-protein complexes are partitioned and purified using a nitrocellulose membrane (Jhaveri et al., 2001). Before selection, the ssDNA library was incubated with a nitrocellulose membrane to eliminate any membrane binders.

The ssDNA library was refolded by an initial denaturation at 95°C for 10 minutes, followed by immediately cooling on ice for 5 minutes and then left to reach room temperature in HMCKN binding buffer (20 mM HEPES, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl and 150 mM NaCl, pH 7.4). This was then either incubated with 1590 nM of CFP-10/ESAT-6 heterodimer for 1 hour at 37°C or immediately used in a no-protein control, which was directly filtered on the nitrocellulose membrane. The ssDNA-protein complexes were passed through a nitrocellulose membrane. Non-specifically bound ssDNA was removed with two washes of HMCKN binding buffer. Bound ssDNA was eluted by cutting the membrane and placing the pieces in an elution buffer (7 M urea, 100 mM sodium citrate and 3 mM EDTA), and heated to 100°C for 5 minutes. Then, phenol/chloroform was added and left to incubate for a further 25 minutes before extraction. This was followed by chloroform extraction and an ethanol precipitation.

Recovered ssDNA was amplified using polymerase chain reaction (PCR) under mutagenic conditions (increased $MgCl_2$ to a final concentration of 3.5 mM) to increase the diversity of the molecules. The PCR cycling conditions used for amplification were as

follows: 95°C for 3 minutes, 95°C for 1 minute, 54°C for 1 minute, 72°C for 90 seconds, steps two to four were repeated for as many cycles as needed, followed by a final extension of 72°C for 8 minutes. Three different methods were used to generate ssDNA from the dsDNA. The ssDNA generated from each method of ssDNA generation was used to isolate aptamers in three different selections.

The first method was to incorporate a biotin label during PCR using a biotin-labelled reverse primer (as described in section 3.2.1.1). The dsDNA was then added to streptavidin-coated magnetic beads (Dynabeads, Invitrogen) and the dsDNA was denatured using a 0.15 M NaOH solution or by heating to 95°C for 5 minutes. The resulting ssDNA was purified by use of an MN PCR purification kit (Separations) and used as template for the next round of selection (this method is referred to as 'biotin-based selection').

The second method used the dsDNA as a template for *in vitro* transcription to obtain RNA. For a 100-µl transcription reaction, 5 µg DNA template, a final concentration of 1 mM each of unmodified pyrimidine and purine nucleotides (Fermentas, Inqaba), a 1 x transcription buffer, 250 units of T7 RNA polymerase (New England BioLabs), 2 mM spermidine and 5 mM DTT were added, and the reaction mixture was incubated at 37°C for 2 hours. Transcription was terminated by addition of 1 unit RNase-free DNase I (Sigma) per µg of DNA template used, and the reaction was incubated for 30 minutes at 37°C, followed by purification through a Sephadex-G50 column and phenol/chloroform extraction. The RNA was used as the template for reverse transcription to obtain cDNA. For a 100-µl reverse transcription reaction, 1 µM of forward primer was added to 10 µg of RNA, incubated at 65°C for 5 minutes and then allowed to cool on ice. To the RNA-primer mix, 1 mM of dNTPs, a 1 x reverse transcription buffer and 500 units of Revert Aid reverse transcription enzyme (Fermentas) were added. The reaction mixture was then incubated at 42°C for 1 hour, followed by a 5-minute incubation at 70°C to inactivate the enzymes. The resulting cDNA-RNA complexes were treated with a 1 M NaOH, 0.5 M EDTA buffer to hydrolyse the RNA. The resulting cDNA was used as a template for the next round of selection (this method is referred to as 'T7-based selection').

The third method used to obtain ssDNA was through exonuclease treatment of the dsDNA. This was done by including a phosphate on the 5' end of the reverse primer. The digestion was performed using 6.6 µg of purified dsDNA and 25 units of lambda exonuclease enzyme (New England Biolabs) per 100-µl reaction and incubated for 4 hours at 37 °C. This results in the degradation of the reverse strand with the phosphate modification (Avci-Adali et al., 2010). The resulting ssDNA was then purified and used as

a template for the next round of selection (this method is referred to as 'exonuclease-based selection').

3.2.2 - Cloning and sequencing of ssDNA aptamers

The biotin-based selection was abandoned after the first round of selection due to difficulties in recovering ssDNA from the streptavidin-coated magnetic beads. The aptamer pool from the fifth (T7-based selection) and third (exonuclease-based selection) SELEX rounds were subjected to a negative selection (no target protein) against the nitrocellulose membrane alone. The pools recovered after negative selections were put through a final (sixth – T7-based selection and fourth – exonuclease-based selection) round of positive selection (with the target protein). After the last rounds of selection, both pools of ssDNA were amplified with the phosphate-modified reverse primer and ligated into pGEM-T Easy$^®$ vector DNA (Promega). Ligation was performed by adding 100 ng of double-stranded PCR product to 50 ng of pGEM-T Easy$^®$ vector and 3 units of T4 DNA ligase. The reaction was incubated at 37°C for 1 hour. *E. coli* TOP10 cells (Novagen) were transformed with the ligation reaction mixtures. After transformation, 244 colonies were picked and streaked onto duplicate LB agar plates containing 100 μg/ml of ampicillin and X-gal for blue-white colony screening. Colonies from one plate were used in colony-PCR screening using M13 primers (pUC/M13 primer forward: 5′ CCCAGTCACGACGTTGTAAAACG 3′ and pUC/M13 reverse primer: 5′ AGCGGATAACAATTTCACACAGG 3′) and to prepare overnight cultures for glycerol stocks. The other plate was sent to Inqaba Biotech for sequencing of the clones with the pUC/M13 forward and reverse primers. Sequence analysis and alignments were analysed using CLUSTAL W multiple alignment application in BioEdit V7.1.3.0 software (Hall, 1999). A full multiple alignment was generated by bootstrapped neighbour joining tree with the bootstrap value set to 1000. Default gap penalties for gap opening and gap extension were used. The aligned sequences were assessed for conserved motifs and were used to generate a maximum likelihood tree. *M-fold* (www.bioinfo.rpi.edu/applications/mfold/) was used to computationally identify secondary structures possible within the aptamer using the sequence information (Zuker, 2003). The nucleotide sequences were added in FASTA format as linear DNA molecules. Secondary structure predictions were made at 37 °C with 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl, 150 mM NaCl, 5 % suboptimality, an upper limit of 50 computed folding, a maximum interior / bulge loop size of 30 base pair and maximum asymmetry of interior / bulge loop of 30 base pair. No limits were defined for the distance between base pairs.

3.2.3 - Binding assay of ssDNA aptamers by ELONA

For determination of aptamer-protein interactions, the binding assay was modified from an ELISA protocol, and has been termed an ELONA (Drolet et al., 1996). Unique aptamer clones identified by sequencing were tested for their ability to bind to the CFP-10/ESAT-6 heterodimer using an ELONA. Each ssDNA aptamer was prepared using the exonuclease method (as described in 3.2.1.2) with minor modifications, in that all aptamers were prepared with the biotinylated forward primer. For each binding assay, 96-well micro-titre plates (Corning, Adcock Ingram) were coated with 500 ng of the CFP-10/ESAT-6 heterodimer in 50 µl of a 10 mM $NaHCO_3$ buffer (pH 8.5) and left overnight at 4°C. The plates were washed with 100 µl of 1 x phosphate-buffered saline (PBS) containing 0.005% (v/v) Tween-20 (PBS-T) (pH 7) and blocked with 100 µl of 5% (w/v) fat-free milk powder in PBS-T for 1 hour at 4°C. The plates were then washed three times with 100 µl of 1 x PBS-T, after which 50 µl of HMCKN buffer containing 150 nM biotinylated aptamer was added and incubated for 2 hours at room temperature. The plate was washed three times with 100 µl of 1 x PBS-T, followed by the addition of 50 µl of a horse radish peroxidase (HRP)-conjugated streptavidin (diluted 1:15000 in 1 x PBS-T) and incubated for 2 hours at 37°C. The plates were then washed four times with 100 µl of 1 x PBS-T, after which 50 µl of 3,3′,5,5′ - tetramethylbenzidine (TMB) detection substrate (Separations) was added. A change in colour to blue, which could be observed with the naked eye, indicated that the aptamers had bound to the CFP-10/ESAT-6 heterodimer. The reaction was stopped with 50 µl of a 2 M sulphuric acid solution, resulting in a colour change from blue to yellow. The plates were read on the MultiSkan Go plate reader (ThermoScientific, AEC-Amersham) at a wavelength of 450 nm. Each plate had a CFP-10/ESAT-6 and an aptamer-alone control, which were averaged and then subtracted from each well to eliminate background noise. Each aptamer was tested in triplicate, in two separate experiments. The repeats were averaged and the standard deviation calculated. All aptamers were then compared to an aptamer-alone control in a *t*-test to determine which aptamers had a significantly ($p < 0.05$) higher binding than the aptamer-alone control. The aptamer alone control was kept constant across all plates as all aptamer-alone controls had similar A_{450} values, this allowed for comparison of the plates.
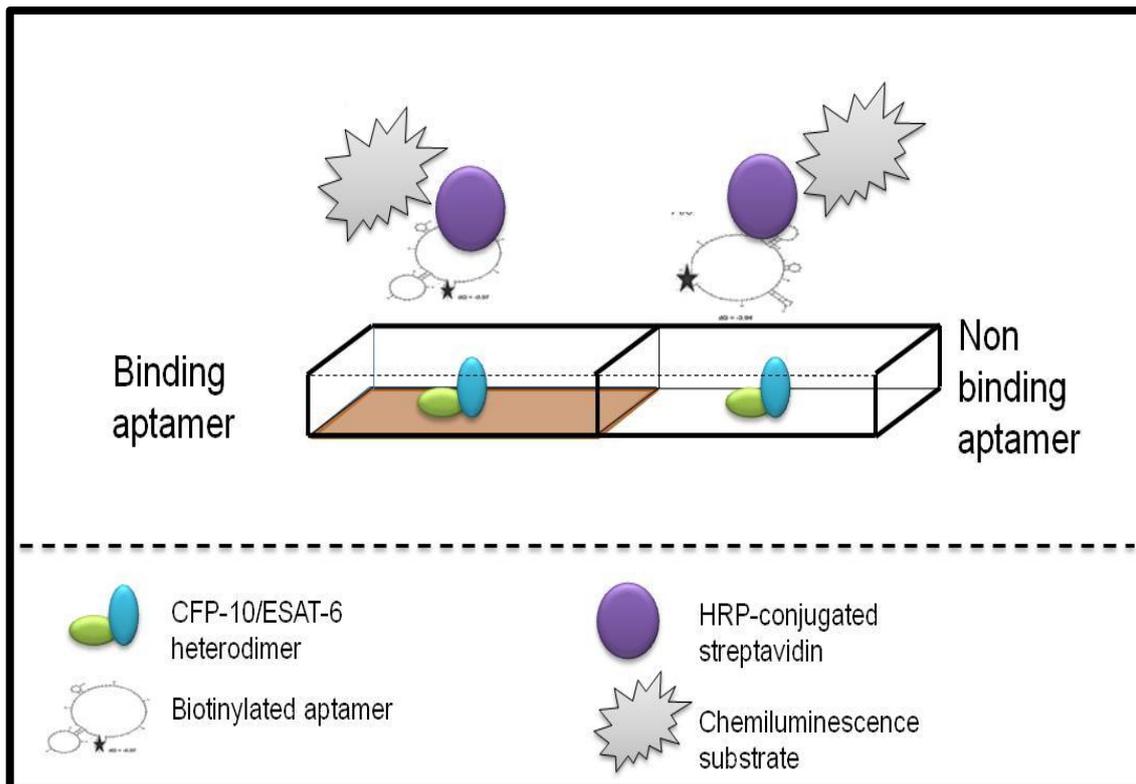
Figure 3-1: Schematic representation of the detection of the CFP-10/ESAT-6 heterodimer by aptamers conjugated to biotin using ELONA.

3.2.4 - Determination of antibody competition binding of individual ssDNA aptamers by ELONA

Only 24 aptamers that had significantly ($p<0.05$) higher binding than the aptamer-alone control to the heterodimer were used in further studies. The antibody competition binding was done using the ELONA described above (section 3.2.3) with minor modifications. The ESAT-6 antibody was bound to the plate by adding 500 ng of antibody per well in NaHCO$_3$ buffer. Following blocking with a 5% fat-free milk solution, 500 ng of the CFP-10/ESAT-6 heterodimer in PBS was added per well. The biotinylated aptamer was then added, followed by HRP-conjugated streptavidin. Each combination was done in triplicate and repeated in two independent assays. The antibody competition data was compared with data of the binding assay to identify differences in the binding capacity of the aptamers in the presence of the antibody. Although the competition assay and binding assay were done on different plates, the positive (CFP-10/ESAT-6 heterodimer and one aptamer, named EA10) and negative controls (aptamer-alone) were tested on both plates with similar results to normalise the data on the two plates.

3.2.5 - Determination of monomer binding and specificity of individual ssDNA aptamers by ELONA

Selected ssDNA aptamers were tested for binding to the ESAT-6 and CFP-10 monomers as well as specificity to the CFP-10/ESAT-6 heterodimer using an ELONA. Also included

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in the assay were an ESAT-6 family-related protein in the Esx-3 secretion system (EsxGH complex) (Lightbody et al., 2008, Maciag et al., 2009) and a HIV surface glycoprotein (gp120) that is unrelated to Mtb, to determine the specificity of the aptamers selected. An equal amount of protein was used to coat each well of the microtitre plate (500ng).

3.2.6 - Determination of the dissociation constant (K_D) of individual ssDNA aptamers using the BIAcore 3000

Based on aptamer binding to the CFP-10 monomer, it was decided to use the CFP-10 monomer for further studies instead of the heterodimer, as the heterodimer dissociated on the BIAcore (as shown in Chapter 2 of this thesis). All four flow cells on a CM5 chip (BIAcore) were activated with EDC:NHS. CFP-10 was immobilised on three of the four flow cells by injecting 50 µl of CFP-10 (50 µg/ml) over them. Ethanolamine-HCl was then injected over all four flow cells to quench any remaining active sites. Partially bound or unbound protein was removed by a wash with 10 µl of a 10 mM NaOH solution. Different concentrations of the aptamer (31 nM, 62 nM, 125 nM, 250 nM, and 500 nM) were randomly injected over the four flow cells at a flow rate of 10 µl/minute for 5 minutes; this was repeated for each of the six selected aptamers. The aptamer was then allowed to dissociate for 10 minutes. The flow cell that did not have CFP-10 immobilised was used as the blank to subtract non-specific binding. The evaluation was done using BiaEvaluation Software (BIAcore) to determine the K_D values for each flow cell. The average dissociation constant was then determined.

3.2.7 - Determination of whether aptamer folding affects binding to the target

It is important to determine if the aptamer needs to be refolded for further studies, as this has an impact on its downstream application. One aptamer, named EA10, was selected from the six selected aptamers, as this aptamer's binding was unaffected by antibody binding. One batch of EA10 was folded as described earlier in Section 3.2.1.2, and the other batch was used directly after thawing. An ELONA was preformed with both batches of the aptamer against the CFP-10 monomer.

3.3 – RESULTS

3.3.1 - Isolation of aptamers against the CFP-10/ESAT-6 heterodimer

To isolate aptamers against the CFP-10/ESAT-6 heterodimer, a modified SELEX protocol was used (Jhaveri and Ellington, 2001). A ssDNA library was used for the selection of aptamers. After each round of selection, a dsDNA PCR was done to optimise the number of cycles needed to convert ssDNA to dsDNA and amplify the selected pool (Figure 3-2).

It is important to optimise the number of cycles, as too few cycles will yield too little dsDNA template, and too many cycles yield diffuse bands at higher molecular weights (over-amplification) than the band of interest. The higher molecular weight products are likely due to mispairing of individual strands in the random region sequences.
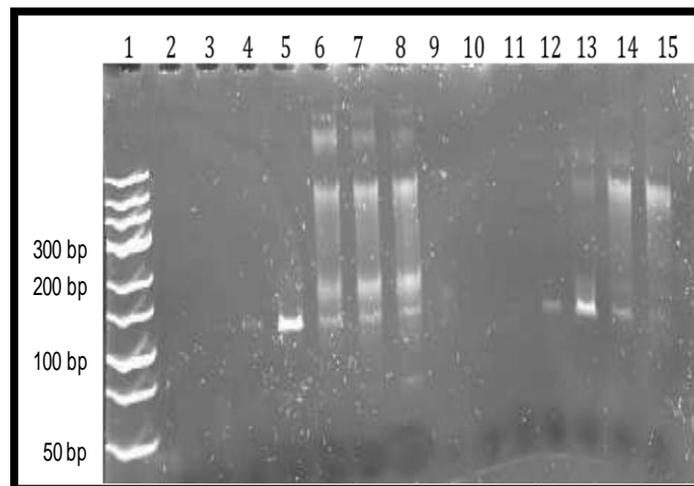
Figure 3-2: PAGE gel illustrating the optimisation of dsDNA PCR for the T7 and exonuclease selections.
Lane 1: DNA molecular weight marker (low range O'Gene ruler, Inqaba), lane 2: water control, lane 3: 4 cycles of exonuclease selection, lane 4: 8 cycles of exonuclease selection, lane 5: 12 cycles of exonuclease selection, lane 6: 16 cycles of exonuclease selection, lane 7: 20 cycles of exonuclease selection, lane 8: 24 cycles of exonuclease selection, lane 9: water control, lane 10: 4 cycles of T7 selection, lane 11: 8 cycles of T7 selection, lane 12: 12 cycles of T7 selection, lane 13: 16 cycles of T7 selection, lane 14: 20 cycles of T7 selection, lane 15: 24 cycles of T7 selection.

Based on the optimisation of the dsDNA PCR the number of cycles giving a single band at about 100 bp was used to produce dsDNA (in the example shown in Figure 3-2) the optimised number of cycles was 12). The dsDNA was then used to generate ssDNA using one of the three methods (biotin, T7- and exonuclease-based selections). The ssDNA obtained from each round was resolved on a native PAGE gel to ensure that the ssDNA obtained was pure, with no over-amplification, for use in the next round of selection (Figure 3-3).
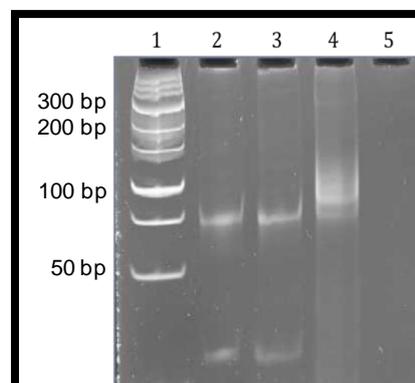
Figure 3-3: PAGE gel of the ssDNA for a round of selection.
The ssDNA generated was used in the next round of selection. Lane 1: DNA molecular weight marker (low range O'Gene ruler, Inqaba), lane 2: ssDNA T7 selection, lane 3: Exonuclease selection, lane 4: ssDNA library, lane 5: ssDNA biotin selection.

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The biotin-based selection failed as the two strands could not be separated and the dsDNA remained bound to the beads. Due to this, the biotin-based selection was abandoned after the first round of selection (Figure 3-4). The T7-based selection resulted in an enrichment of 54.7% after six rounds of selection and the exonuclease-based selection had an enrichment of 68% after four rounds of selection (Figure 3-4). The two pools from the two selections were then cloned and sequenced, respectively.
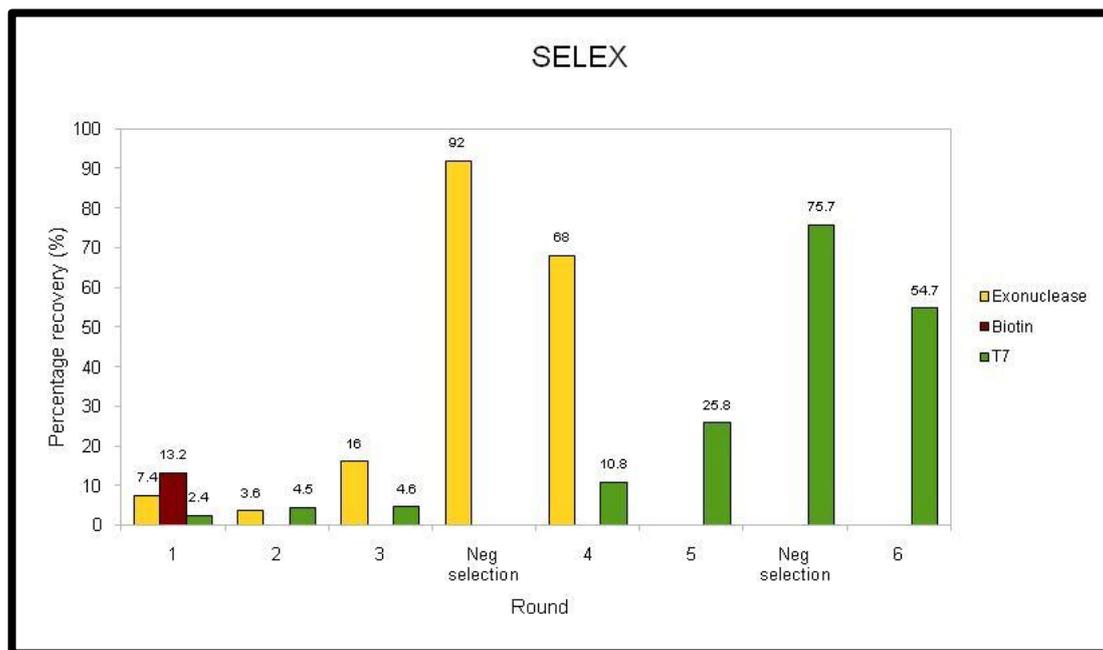
Figure 3-4: Graphical representation of the enrichment of binders for all three methods of ssDNA generation.

3.3.2 - Cloning and sequencing of ssDNA aptamers

To determine the sequences of the aptamers for further studies the aptamers were cloned into pGEM-T Easy®. Of the 244 colonies on the duplicate plates, 24 colonies were selected for colony-PCR screening. The universal pUC/M13 forward and reverse primers were used to determine if the clones had an insert or not. The T7-based selection clones all appeared to have an insert, whereas the clones from the exonuclease-based selection were all insert-negative (Figure 3-5).

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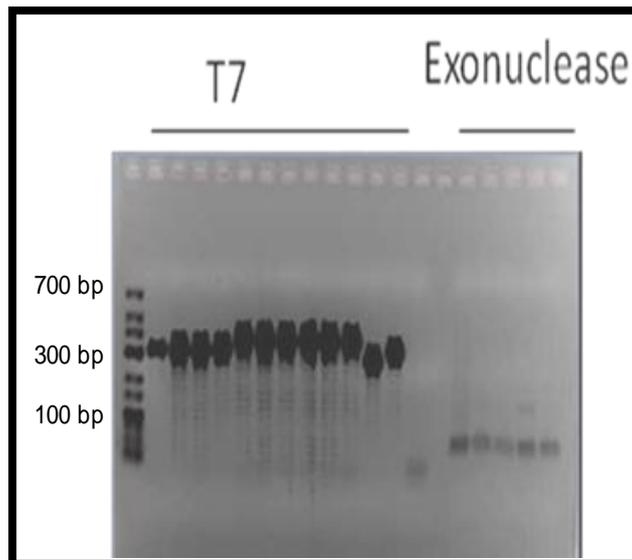
Figure 3-5: M13 PCR colony screening of individual clones picked for insert-positive clones.
24 clones were randomly picked from the duplicate colony agar plates and screened to see if the clones were insert-positive or -negative. The T7 selection clones had an insert, whereas the exonuclease selection clones had no insert.

All 244 clones were sent to Inqaba Biotech for sequencing and the sequences were analysed using BioEdit (Hall, 1999). Of the 244 clones, 104 were insert-positive (11 clones from the exonuclease-based selection and 93 clones from the T7-based selection). This confirmed the PCR screening results where most of the exonuclease-based selection clones were insert-negative. Of the 104 sequences that were analysed, there were 64 unique sequences; of the 64 unique sequences 15 sequences had two or more repeats (Table 3-1, Figure 3-6). Interestingly, only one identical sequence (EJ6 and TJ1) was common in both selections methods. Most of the aptamers seemed distantly related, as denoted by the length of the branches, which are drawn proportional to the number of sequence changes. As seen on the neighbour-joining tree (Figure 3-6), three families of two closely related aptamers were identified, namely: B4 and I8 that had two base pair differences between them, I1 and I2 with four base pairs differing between them and E4 and G12 with only one base pair difference between them. Based on majority of the aptamer sequences sharing no common motifs and no close relation to each other, all aptamers were included in subsequent assays. Both aptamers from the above three families of closely related clones were screened to determine if the single amino acid differences had any effect on the binding ability of the aptamer.
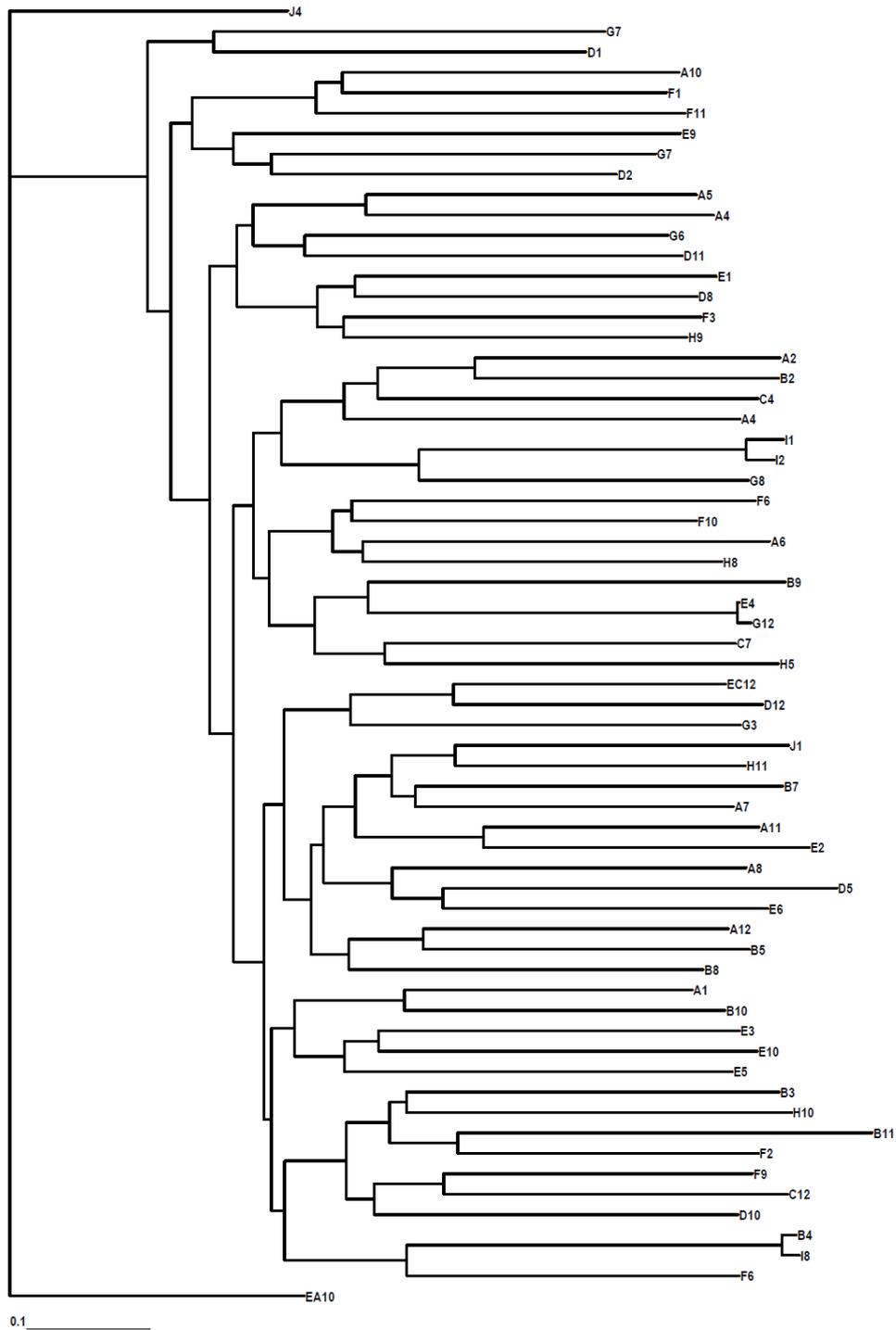
Figure 3-6: Neighbour joining tree for the 64 unique sequences.

Table 3-1: Sequences of the 64 unique aptamers isolated (5′–3′ direction).
The gaps in each sequence were inserted to highlight the random region and for alignment purposes using the flanking primer regions.

```
                   10        20        30        40        50        60        70        80        90
EA10   GCCTGTTGTGAGCCTCCTAAC~CCCATCTTATACGTATATGGACTCATCTCGACCCCCGATAGGCTTGGTA~CATGCTTATTCTTGTCTCCC
EC12   GCCTGTTGTGAGCCTCCTAAC~GTCTTGGGGGTGTGGTCTGGAGGAGTGGGGGCGAGGGGGGGGGTATCG~~CATGCTTATTCTTGTCTCCC
J1     GCCTGTTGTGAGCCTCCTAAC~TGTAGATTACCGCGTGTTTCCAGTTAGGTCAGAATTGTACTCCCGCGGT~CATGCTTATTCTTGTCTCCC
J4     GCCTGTTGTGAGCCTCCTAAC~CCGATGTTTTAGTTAAACTGACTGGTAAGTCCAGCCGATGGCAGGCATT~CATGCTTATTCTTGTCTCCC
A1     GCCTGTTGTGAGCCTCCTAAC~GGTCATAGGGTTTGCTCTGAAAATACAGTTGAACCTGAGCCGTGCAGTA~CATGCTTATTCTTGTCTCCC
A2     GCCTGTTGTGAGCCTCCTAAC~CCGTCTAACGAGATTGGGTCTTCATATGGCTCGAAGCGCGCGGTCAGTT~CATGCTTATTCTTGTCTCCC
A5     GCCTGTTGTGAGCCTCCTAAC~TGTAGATTCATGATTAACCAACCCATAGTCACACCGCGCTTCTTTCTAT~CATGCTTATTCTTGTCTCCC
A10    GCCTGTTGTGAGCCTCCTAAC~ACCGTAGGTTTTCAGGCCACGCGTGAGACATATCGTCCGATGCCCATTG~CATGCTTATTCTTGTCTCCC
A11    GCCTGTTGTGAGCCTCCTAAC~ACAGCAAATCTACCGTGCCGCCGTATATGCATGATCGACGATTTATGCA~CATGCTTATTCTTGTCTCCC
B3     GCCTGTTGTGAGCCTCCTAAC~TGGTGTACATGGTCTAGTCGTCTGCAGAGCCGGACCTCGTGGAACAAGG~CATGCTTATTCTTGTCTCCC
B7     GCCTGTTGTGAGCCTCCTAAC~ATTCGAATACAAACATTATAATACATATCTGAACCCCGCGGCCCAGTGTC~CATGCTTATTCTTGTCTCCC
D12    GCCTGTTGTGAGCCTCCTAAC~GGAACGTTAGGATGCTCCTTGTAAGTGGGTAGGTAGCGCGGTGAGTCGA~CATGCTTATTCTTGTCTCCC
E1     GCCTGTTGTGAGCCTCCTAAC~TCCGGTCTTCAACATGTCCAATCGAACTTCGGCGGAAATCCTTTACCTG~CATGCTTATTCTTGTCTCCC
E9     GCCTGTTGTGAGCCTCCTAAC~CGCTTATGGTCCGCCCAGAAGGTTCGGGTAAACCCTATTTGGATTGCGA~CATGCTTATTCTTGTCTCCC
F1     GCCTGTTGTGAGCCTCCTAAC~CCAATTGTCCTGATCACGAATGTGGAAGATTATGGTGTATGCCTTTCT~CATGCTTATTCTTGTCTCCC
F3     GCCTGTTGTGAGCCTCCTAAC~CACATGCACTTTTGGCAACAAGTAACCTTTAACCGGCAGTGCTTAGACG~CATGCTTATTCTTGTCTCCC
F9     GCCTGTTGTGAGCCTCCTAAC~TAATGTGGATGCTGCATCSTTAGTATTTCTAGCATGCAATATAGGCATG~CATGCTTATTCTTGTCTCCC
F11    GCCTGTTGTGAGCCTCCTAAC~CCCCCCGGCATTTTTGACAGAAGGGATAAGTCCTAAACCGTGGTACTCT~CATGCTTATTCTTGTCTCCC
G6     GCCTGTTGTGAGCCTCCTAAC~TTACAAACCTGCCTTTTATGATCGGATCTCATGCTGGATCCTATCGGGT~CATGCTTATTCTTGTCTCCC
G7     GCCTGTTGTGAGCCTCCTAAC~AACCTATTGTCGGGCATCTGTAATTCAAAGCTTTGCCGTAGGATTTAAA~CATGCTTATTCTTGTCTCCC
H9     GCCTGTTGTGAGCCTCCTAAC~TTCCAATAACGATACTTAAACCTGGTCTTTGCCAGTCAATGATAGCAT~CATGCTTATTCTTGTCTCCC
H10    GCCTGTTGTGAGCCTCCTAAC~TCATGTACATAATGGTTGATCAAGGTCGATTTGGCGTTAAGAGTCGAGC~CATGCTTATTCTTGTCTCCC
H11    GCCTGTTGTGAGCCTCCTAAC~ACACACGTAGCGCCTTGTTGAAGTATACTCAAAGCATTCTCCACGCGGG~CATGCTTATTCTTGTCTCCC
I1     GCCTGTTGTGAGCCTCCTAAC~TTTTAATATATCAATATTGCACAATACATGCGGAACAGATAGTATAATC~CATGCTTATTCTTGTCTCCC
A4     GCCTGTTGTGAGCCTCCTAAC~TATGGATGCATACTTGGTTTGCTCTCTCGCAGTTTTCAGTTCTGCGGGA~CATGCTTATTCTTGTCTCCC
B2     GCCTGTTGTGAGCCTCCTAAC~GGGTCTAATTAGTCACTACAATCGTGTCCATGGAAGCGAGGGTCAGTTC~CATGCTTATTCTTGTCTCCC
F6     GCCTGTTGTGAGCCTCCTAAC~GTGGGTCCGTTGAGGGGGGCGGGTGGAGTGAGACCCGGGGTCTGTGGGC~CATGCTTATTCTTGTCTCCC
G7     GCCTGTTGTGAGCCTCCTAAC~TCTGTATATATGACATGATCCTTTTTTAGAGTCATCTTAACAATCGCTA~CATGCTTATTCTTGTCTCCC
A4     GCCTGTTGTGAGCCTCCTAAC~AGTCTTACGCATTCGCGTCTAGTATGATTCACAAGATGAAACGTTATAA~CATGCTTATTCTTGTCTCCC
A6     GCCTGTTGTGAGCCTCCTAAC~CAGGAATACTCTTACTGTGGTCGCTGTTTGCGATCAGCAGCCCCCAGTG~CATGCTTATTCTTGTCTCCC
A7     GCCTGTTGTGAGCCTCCTAAC~ACAAAAATACAGGCGTGCTGTTGAAAAGAGAGGACGGGGCATCGGGGGG~CATGCTTATTCTTGTCTCCC
A8     GCCTGTTGTGAGCCTCCTAAC~GCTCAAAGTGTTCAGTCGGGACAAAGGTGCGCTCTTGTTGGAAGGCGGT~CATGCTTATTCTTGTCTCCC
A12    GCCTGTTGTGAGCCTCCTAAC~TGCGATATATGTGGTTGGTGTTAATGGGTGTTAGTGGTTGGTCAAAGTG~CATGCTTATTCTTGTCTCCC
B4     GCCTGTTGTGAGCCTCCTAAC~GAAAGTGCATTCGTAATTATTTCTCTGCCTTAGCCGCTGATTGAATTCA~CATGCTTATTCTTGTCTCCC
B5     GCCTGTTGTGAGCCTCCTAAC~ATCACGAGATTGAATTTAATATATCGGTTATCAGAGAGTGGTGGGGGTT~CATGCTTATTCTTGTCTCCC
B8     GCCTGTTGTGAGCCTCCTAAC~GGTTCACTAGGGTTTTTTTATTGAATGTCCGCAGATATCAGATGAAGC~~CATGCTTATTCTTGTCTCCC
B9     GCCTGTTGTGAGCCTCCTAAC~CGACAGGACGTGGACGACGACGGTTCATTTGATTGATCACTTGGACGCC~CATGCTTATTCTTGTCTCCC
B10    GCCTGTTGTGAGCCTCCTAAC~CTCGATGCTCCGTCCGCTCTTAAAACGTGCGAGCGGTAGCCTCGCGGTA~CATGCTTATTCTTGTCTCCC
B11    GCCTGTTGTGAGCCTCCTAAC~GTATGCGTTTGTAGAGCATACATGCATAACTCTTTTCTAAGTAGGTTAG~CATGCTTATTCTTGTCTCCC
C4     GCCTGTTGTGAGCCTCCTAAC~GGTAGGGACCATACACGTAGAGGATATCTGGAAAAGTGCTCGCGGGACC~CATGCTTATTCTTGTCTCCC
C7     GCCTGTTGTGAGCCTCCTAAC~CTTAATTTAGCACTGGGTGGTGCGGGCGGTTGTGGTTGGACGTGGGCCG~CATGCTTATTCTTGTCTCCC
C12    GCCTGTTGTGAGCCTCCTAAC~CAGTGTAGTAATTCTATTCATTGGATTGGATTCTTGGATTTGCATCGGG~CATGCTTATTCTTGTCTCCC
D1     GCCTGTTGTGAGCCTCCTAAC~TGTTTACGCTTCATAGATAACTTTATAAGGGCACGATATTCACCACACG~CATGCTTATTCTTGTCTCCC
D2     GCCTGTTGTGAGCCTCCTAAC~ACTTCAAGCACGAATAACGGTCTAGCACCTTATTTGGTTATAATTATAA~CATGCTTATTCTTGTCTCCC
D5     GCCTGTTGTGAGCCTCCTAAC~TTCGAACGTTACCTACAGAGGGCGACGTACTCGGATTTCGAGGCGTCGG~CATGCTTATTCTTGTCTCCC
D8     GCCTGTTGTGAGCCTCCTAAC~CATGGTTAGAGAACTCTCAAAATTATCCACTATGTGCAATCGATGGCTG~CATGCTTATTCTTGTCTCCC
D10    GCCTGTTGTGAGCCTCCTAAC~CTTTCTGCATGATATTGCTGTACGATGGGTGTTCATGATCCAGGTCAAG~CATGCTTATTCTTGTCTCCC
D11    GCCTGTTGTGAGCCTCCTAAC~TCGTATGATATAGTTAACTAGCATGGTCACGTAAAGAATGCAATAGGCG~CATGCTTATTCTTGTCTCCC
E2     GCCTGTTGTGAGCCTCCTAAC~ATTCTCGTTATTCGTTTACTCAGTATATAGAATGCCATTGAATTACACA~CATGCTTATTCTTGTCTCCC
E3     GCCTGTTGTGAGCCTCCTAAC~TGTTTGATAAGCTAGCGGTCATAGACACGTTACTCCGTGATTATGGCTA~CATGCTTATTCTTGTCTCCC
E4     GCCTGTTGTGAGCCTCCTAAC~AAACAATCCATATAAAGGGTAACCGCTGTTTGTAATTGAGGCGGTCAGA~CATGCTTATTCTTGTCTCCC
E5     GCCTGTTGTGAGCCTCCTAAC~ACCCCAACATGATCCAACCTAAAACCTTTCTTGCTTGTCAGTTACGCTA~CATGCTTATTCTTGTCTCCC
E6     GCCTGTTGTGAGCCTCCTAAC~GCCAAACGTGATCCGCTCAGAAATACATCTGGAAGGATATAGTACTATA~CATGCTTATTCTTGTCTCCC
E10    GCCTGTTGTGAGCCTCCTAAC~AGTCTTATACTGTGGTAGGTCGGCCTGCTCAACGTGAACGTTAATGCTT~CATGCTTATTCTTGTCTCCC
F2     GCCTGTTGTGAGCCTCCTAAC~CGCTGTGAGGCTGCCTTTGGCTTGAAAAGCTTTGTTTCAAACACTA~~~~CATGCTTATTCTTGTCTCCC
F6     GCCTGTTGTGAGCCTCCTAAC~TAAACTATAAGTATAGCTTTGTAGATAGCGACACAGGAGACCTCAGTTG~CATGCTTATTCTTGTCTCCC
F10    GCCTGTTGTGAGCCTCCTAAC~CGGTCAGTGCGGAAAGGTGGGTGGCACTGTGGGAGTGGTGCCGGCGGGG~CATGCTTATTCTTGTCTCCC
G3     GCCTGTTGTGAGCCTCCTAAC~AGAAAGCGATTGTGCCATTGAATTGAGAGGCTATAGGTGTACGACGACT~CATGCTTATTCTTGTCTCCC
G8     GCCTGTTGTGAGCCTCCTAAC~GCTCAACCTAGTGTCGTAACAAAAAGAATGCGAAAACGTCTAAACGCGA~CATGCTTATTCTTGTCTCCC
G12    GCCTGTTGTGAGCCTCCTAAC~AAACAATCCATATAAAGGGTAACCGCTGTTTGTAATTGACGCGGTCAGA~CATGCTTATTCTTGTCTCCC
H5     GCCTGTTGTGAGCCTCCTAAC~GAGTGCCTCGTAGAGGACACCGGACCCCGGTTGAGGGGGTTGGACATCCA~CATGCTTATTCTTGTCTCCC
H8     GCCTGTTGTGAGCCTCCTAAC~GTTTTTTATTGATTAAGTGTTTTCCTGTTGGGCCTGGCTATCGCCGAGC~CATGCTTATTCTTGTCTCCC
I2     GCCTGTTGTGAGCCTCCTAAC~TTTTAAGATATCAATATTGCAGAATACATGCGGTACGGATAGTATAATC~CATGCTTATTCTTGTCTCCC
I8     GCCTGTTGTGAGCCTCCTAAC~GAAAGTGCATTCATAATTATTTCTCTGCCTTATCCGCTGATTGAATTCA~CATGCTTATTCTTGTCTCCC
```

Once the aptamers had been sequenced it was important to determine their secondary structures, as this could give an indication of the exact sequence of the aptamer that interacts with the target. *M-fold* algorithms were used to predict the secondary structures of all the aptamers (Figure 3-7, Appendix II). Similar to previous studies on ssDNA aptamers the primer annealing regions were present in the bulge-loop region, whereas the random regions were found in the stem and bulge-loop regions. This is expected as the primer annealing regions are unlikely to interact with the target. Most of the aptamers have multiple secondary structures (Appendix II) but the free energy of the different structures are all similar, which means that all the structures predicted by *M-fold* are equally likely and the aptamer could be present in one or all of the predicted structures.
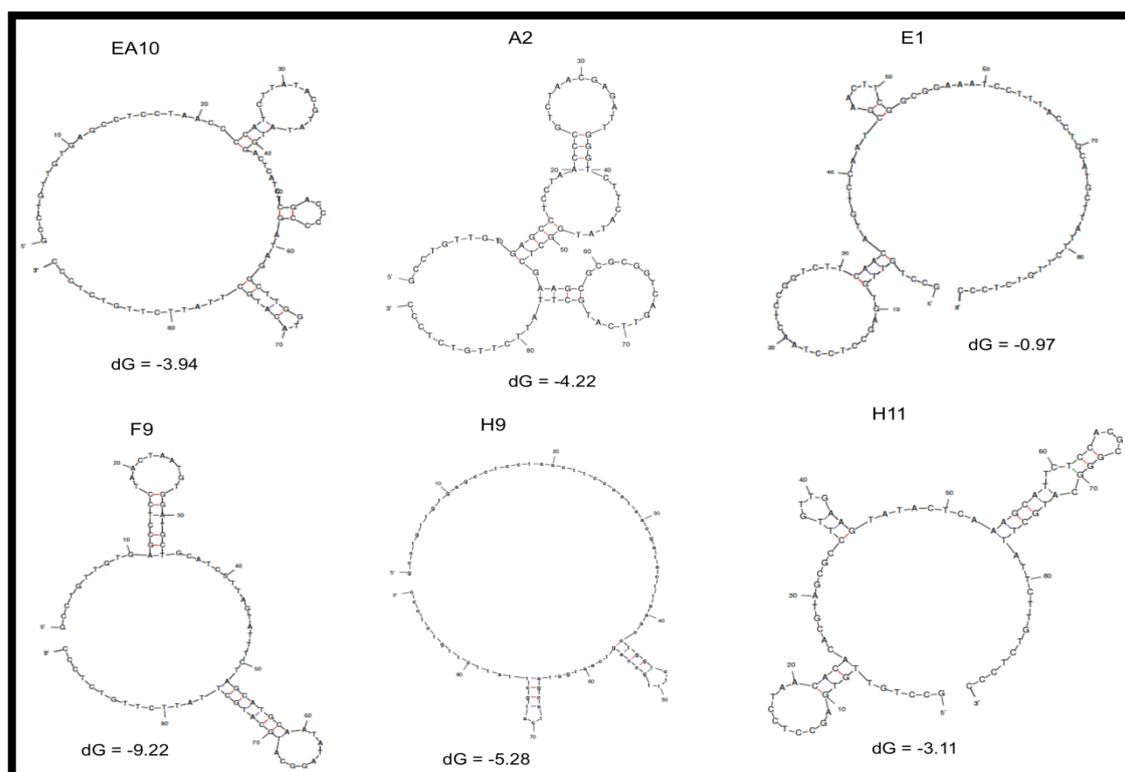
Figure 3-7: Predicted secondary structures for six of the ssDNA aptamers.
Predicted secondary structure for the six aptamers that have been further characterised, all predicted structures can be found in Appendix II. Free energy of the aptamers is shown by the dG values for each aptamer, the more negative the free energy, the more stable the structure is predicted to be.

3.3.3 - Binding assay of ssDNA aptamers to the CFP-10/ESAT-6 heterodimer by ELONA

To determine the binding ability of each individual aptamer to the CFP-10/ESAT-6 heterodimer, an ELONA assay was used. Screening of each individual clone allowed the elimination of non-specific binders, despite the negative selection. Out of the 64 biotinylated ssDNA aptamers screened by ELONA against the CFP-10/ESAT-6 heterodimer, 24 bound significantly ($p < 0.05$) (Figure 3-8).
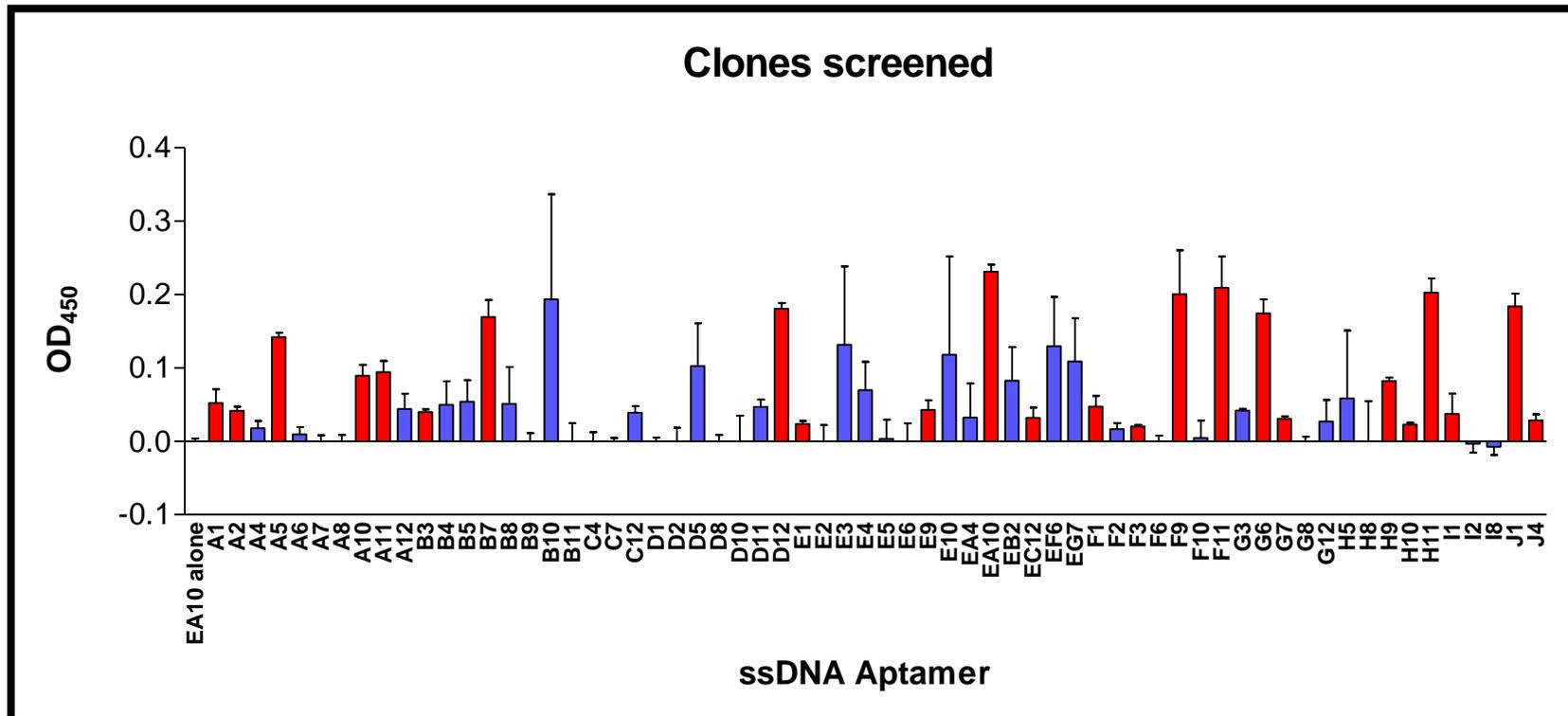
Figure 3-8: A graphical representation of the ELONA screening results.

A graphical representation of the screening of the anti- CFP-10/ESAT-6 ssDNA aptamers conjugated to biotin to the CFP-10/ESAT-6 heterodimer using an ELONA Red bars denote aptamers that significantly bind to the CFP-10/ESAT-6 target proteins with a $p<0.05$, and blue bars denote aptamers that non-significantly bind to the CFP-10/ESAT-6 target proteins, as determined by a two tailed t-test. The error bars denote standard deviations of the triplicate aptamers. Each aptamer was tested at least twice.

3.3.4 - Determination of antibody competition binding of individual ssDNA aptamers by ELONA

Based on the 24 aptamers that significantly bound to the CFP-10/ESAT-6 heterodimer further studies to characterise the aptamers were undertaken. One such experiment was a competition ELONA with the ESAT-6 monoclonal antibody to determine possible binding sites of the aptamers to the heterodimer. Based on the binding results shown in section 3.3.3, a positive control was selected. The aptamer selected as the positive control was EA10, and was included on both plates to normalise the results between the two ELONA experiments. Binding of some ssDNA aptamers (such as F9, E1 and H11) was abrogated by the presence of anti-ESAT-6 monoclonal antibody, while binding of other aptamers (such as A2 and EC12) was enhanced by the presence of the anti-ESAT-6 monoclonal antibody (Figure 3-9). Taken together, these data suggest that some aptamers (F9, E1 and H11) bind to a similar epitope on the heterodimer as that recognised by the anti-ESAT-6 monoclonal antibody, while others (A2 and EC12) bind to more distant and unique epitopes.
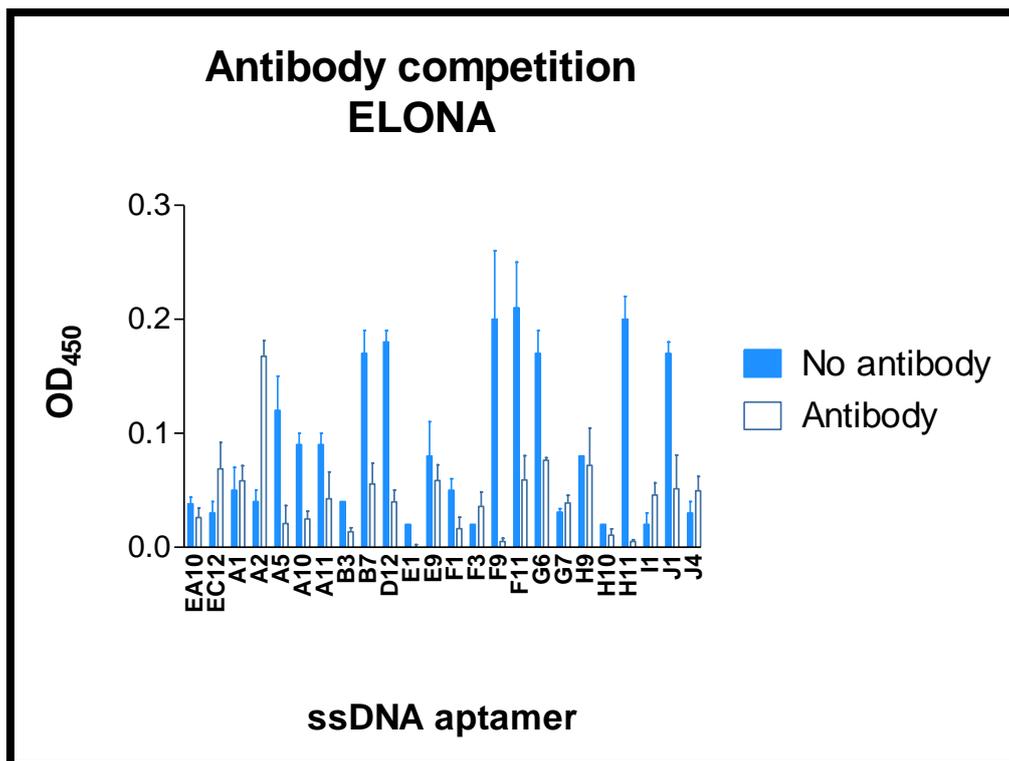
Figure 3-9: Aptamer-antibody competition assay.
Binding of anti-CFP-10/ ESAT-6 ssDNA biotinylated aptamers to the CFP-10/ESAT-6 heterodimer in the absence (blue) or presence (white) of anti-ESAT-6 monoclonal antibody. Error bars denote standard deviation of triplicates. Each aptamer was tested in two independent assays.

3.3.5 - Determination of monomer binding and specificity of individual ssDNA aptamers by ELONA

The next part of the study was to determine if the 24 aptamers were able to bind either of the monomers of the heterodimer (CFP-10 or ESAT-6), and to determine the specificity of each of the 24 aptamers that significantly ($p<0.05$) bound to the heterodimer (those shown in section 3.3.3). The proteins used for the study were the monomers (CFP-10 and ESAT-6), the heterodimer (CFP-10/ESAT-6), as well as the proteins used for specificity, namely an ESAT-6 family-related protein (EsxGH) and an unrelated protein (gp120). Two of the 24 aptamers (A1 and EC12) bound gp120 to a similar extent or better than the CFP-10/ESAT-6 heterodimer (Figure 3-10). None of the aptamers bound to EsxGH (Figure 3-10). It was interesting to note that none of the 24 aptamers screened recognised ESAT-6 (Figure 3-10).
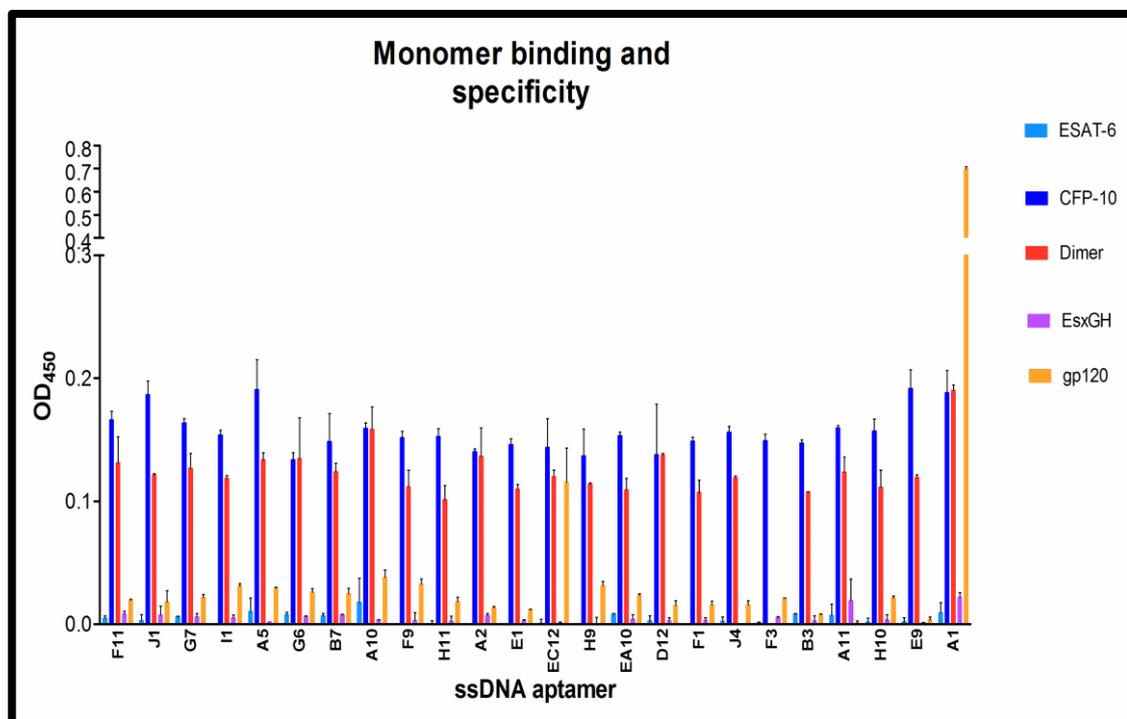
Figure 3-10: Monomer binding and specificity of the 24 ssDNA aptamers.

Binding of the anti-CFP-10/ESAT-6 aptamers to ESAT-6 (blue), CFP-10 (red), CFP-10/ESAT-6 heterodimer (green), EsxGH (purple) and gp120 (light blue) to show specificity of the aptamers.

Six aptamers were chosen for solid phase synthesis based on their specific binding to the CFP-10/ESAT-6 heterodimer and the CFP-10 monomer. The six aptamers chosen were A2, E1, F9, H9, H11 and EA10. The binding of these aptamers to the CFP-10/ESAT-6 heterodimer, as well as to CFP-10 and ESAT-6 was repeated to confirm that the aptamers could be chemically synthesised with a biotin modification and not affect the binding ability of the aptamers. Although the readings of all the synthesised aptamers gave higher readings (Figure 3-11) when compared to the *in vitro*-produced aptamers

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(Figure 3-10), there was no significant (p>0.05) difference between the in-house and the chemically synthesised aptamers. The results for both *in vitro*-produced and chemically synthesised aptamers were consistent in that none of the aptamers recognised ESAT-6.
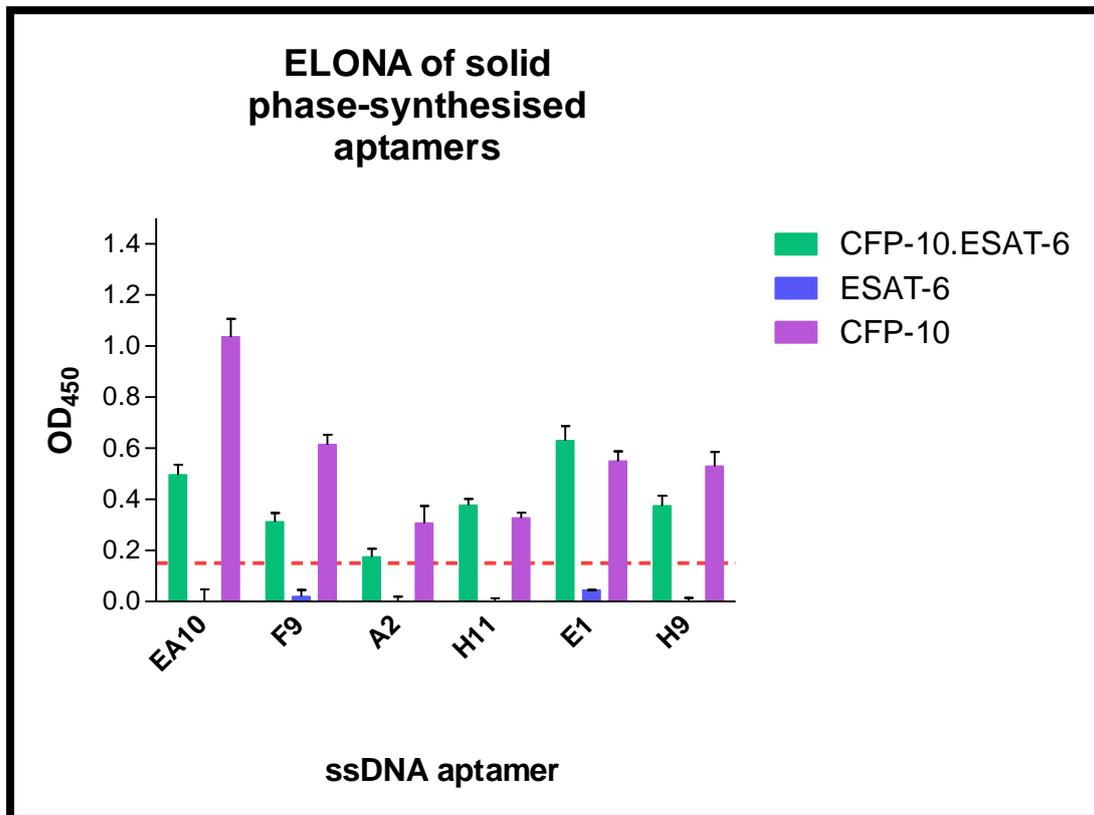
Figure 3-11: Binding of the chemically synthesised anti-CFP-10/ESAT-6 aptamers.
Binding of the chemically synthesized anti-CFP-10/ESAT-6 aptamers to no protein (blue), CFP-10/ESAT-6 heterodimer (red), ESAT-6 (green), and CFP-10 (purple). The cut-off of binding was determined by the signal of the no protein control and is indicated as a red dotted line in the graph.

3.3.6 - Determination of the dissociation constant (K_D) of an individual ssDNA aptamers

A further study to characterise the six selected aptamers was to determine the binding affinity of each aptamer to the CFP-10 target protein. The dissociation constant (K_D) of five selected aptamers, A2; EA10; F9; H9 and H11, were determined using BIAcore surface plasmon resonance technology (Figure 3-12). H9 had the lowest K_D at 1.6±0.5 nM as it associated the fastest while having a similar dissociation rate as the other aptamers (Table 3-2); while EA10 had a K_D of 8±1.07 nM and A2 had comparatively the highest K_D at 21.5±4.3 nM. The K_D rate of A2 is comparatively high due to the fast dissociation but a similar association compared to the other aptamers (Table 3-2). The kinetics of E1 could not be determined as the binding of the aptamer to the protein was in a concentration-independent manner. Charlotte Maserumule did the kinetic study of the A2; E1; F9; H9 and H11 aptamers.
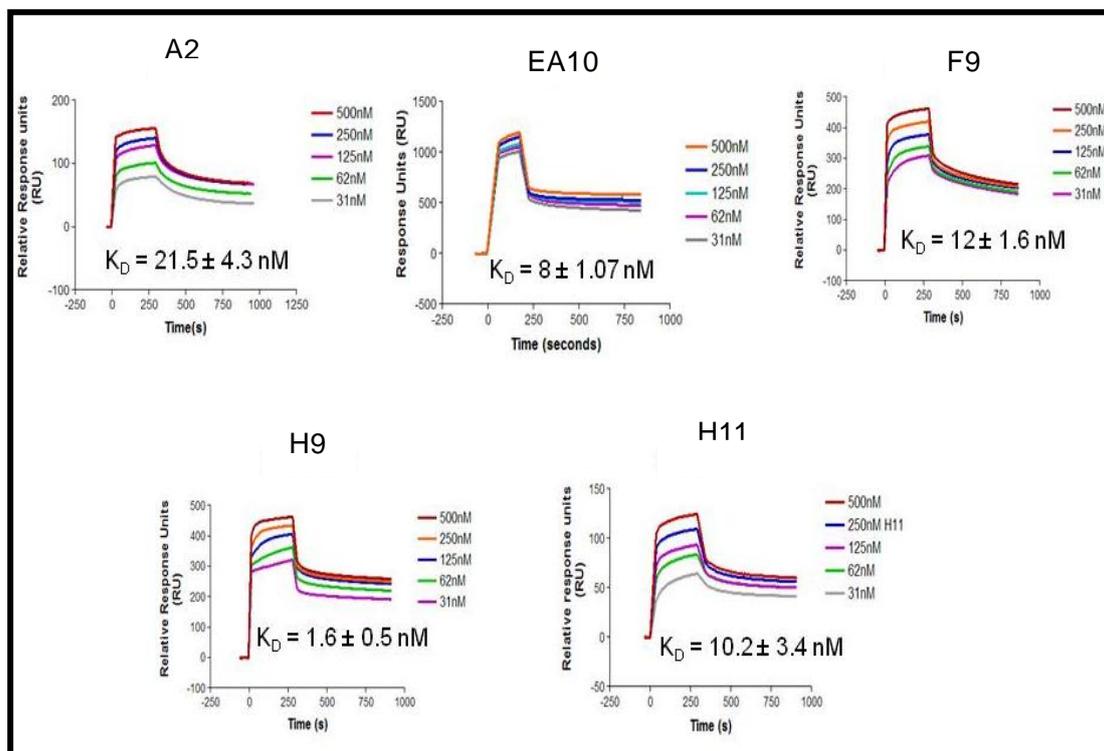
Figure 3-12: Kinetic study of anti CFP-10/ESAT-6 aptamers.
CFP-10 attached to a CM5 chip was used to capture the anti-CFP-10/ESAT-6 aptamers. The aptamers were injected seperately and at different concentrationsto determine the dissociation constant for each aptamer.

Table 3-2: Kinetic parameters and standard deviations of the six selected aptamers.

Aptamer	Kd (1/s)	Ka (1/Ms)	kD (M)	SD (M)
A2	1.05 E-03	5.12 E+04	2.15 E-08	4.3 E-09
EA10	5.01 E-04	5.63 E+04	8.98 E-09	1.07 E-09
F9	6.17 E-04	4.86 E+04	1.28 E-08	1.6 E-09
H9	1.96 E-04	1.37 E+05	1.60 E-09	0.5 E-09
H11	6.00 E-04	6.16 E+04	1.02 E-08	3.4 E-09

3.3.7 - Determination of whether aptamer folding affects binding to the target

For the aptamers to be used a possible diagnostic, it was important to determine if the aptamers needed a refolding step to be able to detect the CFP-10 protein. One batch of EA10 was folded as described (Section 3.2.1.2) and another batch was used directly after thawing to determine if the aptamer could be used without the refolding step. The results showed that the aptamer can be used without the refolding step (Figure 3-13), which bodes well for the future use of these aptamers as diagnostic reagents.
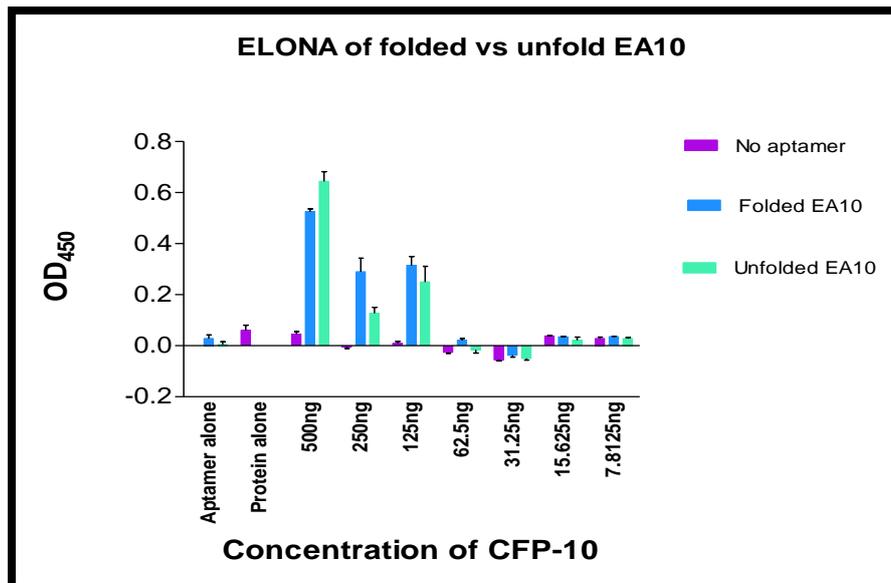
Figure 3-13: ELONA of folded versus unfolded aptamer binding to CFP-10.
One batch of EA10 was refolded and the other was used directly after thawing. The refolding step is not necessary for the binding of this specific aptamer, as no significant difference was seen between the folded and the unfolded aptamer.

3.4 – DISCUSSION

Many partitioning methods are used for SELEX, for example affinity columns containing immobilised ligands (Ellington and Szostak, 1990) and BIASelection (Khati, 2002). However, the nitrocellulose membrane filter used in this study, is a common partitioning matrix used in SELEX, as proteins have a high affinity for nitrocellulose (Tuerk and Gold, 1990). One problem with this method is the selection of sequences possessing a high affinity for the nitrocellulose membrane. The problem is more pronounced when using ssDNA libraries. DNA is more hydrophobic than RNA due to the absence of the 2'-OH group on the sugar moiety, and thus has a higher tendency to bind to the filters, producing high backgrounds. To avoid the selection and the carry-over of such sequences, two precautions can be adopted. The first is to pre-soak the nitrocellulose membranes in a 0.5 M potassium hydroxide (KOH) solution for 20 minutes (Toulme unpublished data). The second is to pass the library pool over the membrane before starting the selection so as to discard the nitrocellulose membrane-binders (Toulme unpublished data). Both methods were used to decrease the probability of selecting nitrocellulose-binding sequences. Even though the precautions were taken, a further negative selection against the nitrocellulose membrane was performed during selection to remove membrane-binders amplified during the selection.

Another important consideration in the SELEX process is the method of generating ssDNA. Although a variety of methods have been shown to generate ssDNA (Avci-Adali

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et al., 2010, Blank et al., 2001, Espelund et al., 1990, Fitzwater and Polisky, 1996, Gyllensten and Erlich, 1988, Hamula et al., 2008, Higuchi and Ochman, 1989, Hultman et al., 1989, Jones et al., 2006, Pagratis, 1996, Paul et al., 2009, Stoltenburg et al., 2005, Wu and Curran, 1999, Zelada-Guillen et al., 2009), these studies report the selections using two common methods of generating ssDNA, namely streptavidin-coated magnetic beads and exonuclease. The T7-based selection protocol to generate ssDNA was also used. The T7-based method resulted in the majority of the aptamers isolated in this study. The T7-based method does not pose the problem of eluting the ssDNA from a polyacrylamide gel, nor the added problem faced by streptavidin-coated magnetic beads where there is a carry-over of dsDNA and streptavidin. The asymmetric PCR typically has the disadvantage of low-yield, antisense strand interference and the common occurrence of smeared bands (Cao et al., 2009). This is not the case with the T7-based method, which has high yields of ssDNA and clean single bands of ssDNA. The strand separation using streptavidin-coated magnetic beads had to be abandoned after just one round of selection due to no ssDNA recovery. Usually with streptavidin-coated magnetic bead strand separations the problem is that the streptavidin elutes and can result in the re-hybridisation of both strands (Avci-Adali et al., 2010). However, in this study, we had a unique case of no ssDNA or streptavidin elution when using NaOH or heat elution which was suggested by the manufacturers. Even when heat and NaOH where combined as an elution method the ssDNA was not eluted. Due to the lack of ssDNA elution we had to abandon the biotin-based method after the first round of selection.

In this study, the CFP-10/ESAT-6 heterodimer was used as a target protein, because it is a potent T-cell antigen and no studies have reported the use of this antigen for TB diagnostics. A total of 24 aptamers were obtained in the study. Only six of the selected aptamers have been further characterised based on competition ELONA and specificity studies. Five of the selected six aptamers showed high affinity for the target protein with dissociation constants in the lower nanomolar range. Moreover, this study, as well as previous studies have shown that aptamers can be chemically synthesised, which allows their rapid production in large quantities with excellent reproducibility of results (Yang et al., 2005, Liu and Lu, 2005, Laurenson et al., 2011). These aptamers were further tested in an ELONA-based TB diagnostic.

Chapter 4. EVALUATION OF LIMIT OF DETECTION AND DETECTION OF MTB ANTIGENS IN CLINICAL SPUTUM SAMPLES USING APTAMERS

SUMMARY

Despite the enormous global burden of tuberculosis (TB), conventional approaches to diagnosis continue to rely on tests that have major drawbacks. The improvement of TB diagnostics relies, not only on good biomarkers, but also upon accurate detection molecules. This chapter provides a proof-of-concept for the aptamer-based TB diagnostic study. One suitable aptamer, called EA10, was evaluated by ELONA using sputum samples obtained from 20 TB patients and 48 control patients (those with latent TB infection, symptomatic non-TB patients, and healthy laboratory volunteers). Culture positivity for *Mycobacterium tuberculosis* (Mtb) served as the reference standard. Accuracy and cut-points were evaluated using ROC curve analysis. The EA10 aptamer had a sensitivity and specificity of 100% and 68.75% using Youden's index. This preliminary proof-of-concept study suggests that a diagnosis of active TB using anti-CFP-10/ESAT-6 aptamers applied to human sputum samples is feasible.

4.1 – INTRODUCTION

TB is a major fatal infectious disease and has become a major public health problem in developing countries. The most recent WHO figures estimate a worldwide TB incidence of 8.8 million per year and 1.45 million deaths annually (Who, 2011). For decades the gold standard for diagnosing TB is a combination of rapid identification of bacilli by direct microscopy and culture for subtype and antibiotic sensitivity. Diagnosing active TB has become more problematic with the increase in HIV infection. Patients with HIV have difficulty producing sputum, as they often have extra-pulmonary TB. Timely diagnosis of active pulmonary TB cases is important for the control of the disease. Despite the enormous burden of TB, conventional approaches to diagnosis used today rely on tests that have major drawbacks. Many of these tests are slow and lack both specificity and sensitivity.

Approximately 40–50% of patients with pulmonary TB are smear-positive. Sputum must contain at least 5000 bacilli/ml for them to be detectable by microscopy. It is estimated that 10% of smear-negative patients are also culture-negative. Patients with smear-negative, culture-positive TB appear to be responsible for about 17% of TB transmission (Behr et al., 1999). Although microscopy is not very accurate, it remains the most rapid technique and in developing countries it is often the only test available for diagnosis of pulmonary TB (Dinnes et al., 2007). The major drawback of sputum smear microscopy is its poor sensitivity, which is estimated to be between 35% - 70% (Steingart et al., 2006).

The culture method is the most sensitive of currently available tests, with sensitivity rates of up to 98%. However, the method is more expensive than microscopy and requires a high standard of technical competence. Culture is a good means to identify bacilli and drug resistance. However, it requires up to eight weeks for the isolation of Mtb from a clinical specimen. In 10–20% of cases the bacillus is not successfully cultured (Andersen et al., 2000).

Other means of diagnosing TB include nucleic acid amplification tests (NAATs), mycobacteriophage tests, interferon gamma release assays (IGRAs), the lipoarabinomannan (LAM) antigen-detection assay (Dheda et al., 2010, Dheda et al., 2009) and recently, the fully automated GeneXpert™. There are NAATs that can detect small amounts of DNA or RNA from Mtb. These tests are thought to be 90-100% accurate on smear-positive patients and 60-70% accurate on smear-negative cases (Drobniewski et al., 2003). The LAM antigen-detection assay is attractive because it uses urine, which is a sterile and easily obtainable biological fluid. Furthermore, this assay can be applied to other body fluids. However, LAM has little clinical utility in a high burden

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setting as it has poor sensitivity of between 13% and 21% (Dheda et al., 2010). The GeneXpert™ has advantages in that it is rapid and simple to use, with very little training required. However, the drawback is that the test is currently expensive and not a point-of-care tool. Furthermore, the sensitivity of the GeneXpert™ in the South African setting was found to be suboptimal in smear-negative, HIV-infected patients (Boehme et al., 2010).

Aptamers, by virtue of their high specificity and high sensitivity, could serve as tools for the early and specific detection of active TB and meet the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to the end user) diagnostic guidelines recommended by WHO for developing countries (Mabey et al., 2004). Since aptamers can be produced using chemical synthesis or by PCR, the cost of producing aptamers is 10-50 times less than those for producing antibodies (Low et al., 2009). Recently, Qin and colleagues developed aptamers against the MPT64 proteins of Mtb (Qin et al., 2009). They suggested that the aptamers can be used as a new diagnostic for Mtb. A sandwich assay was developed based on aptamer-protein complexes and reported a sensitivity of 86.3% and a specificity of 88.5% (Qin et al., 2009).

The purpose of this chapter is to report on the limit of detection of a ssDNA aptamer, namely EA10, that was isolated against the CFP-10/ESAT-6 heterodimer. The aptamer was also used as a proof-of-principle in a TB diagnostic test using sputum samples from patients with active TB.

4.2 – MATERIALS AND METHODS

Ethics statement
Approval for the use of sputum samples for this study was obtained from the University of Cape Town, Health Science Faculty Research Ethics Committee (Ethics number: REC REF: 421/2006). Written consent was received from patients whose sputum is used in the study.

4.2.1 - Specificity testing
Aptamer specificity was determined in an ELONA against lysates of different bacteria found in the oral cavity, namely: *Pseudomonas aeruginosa* (ATCC 29212, Dr. Lyndy McGaw, University of Pretoria); *Streptococcus pyogenes* (ATCC 12344, Dr. Gerda Fouche, CSIR Biosciences*), Staphylococcus aureus* (ATCC 29213, Dr. Lyndy McGaw, University of Pretoria)*; Mycobacterium smegmatis, Mycobacterium bovis* (Prof. Eric

Rubin, Harvard Medical School)*; Mycobacterium tuberculosis* (H37Rv, Dr. Kheertan Dheda, UCT); and *Corynebacterium xerosis* (Dr. Gerda Fouche, CSIR Biosciences). Each bacterial culture was grown in 200 ml of their respective growth media and pelleted by centrifugation at 4000 rpm (Allegra X-22, Beckman Coulter, Inc.). The pellets were then resuspended in 3 ml of PBS and lysates were obtained by bead beating the resuspended pellets. The lysates were then used in a Bicinchoninic Acid Protein assay (BCA) to determine total protein concentrations. Equal concentrations of total protein from each bacterial lysate was coated onto 96-well micro-titre plates in a $NaHCO_3$ buffer, and then incubated overnight at 4°C. The plates were washed with 1 x PBS-T (pH 7) and blocked with a 5% fat-free milk solution for 1 hour at 4°C. The plates were then washed three times with 1 x PBS-T, after which 150 nM biotinylated EA10 aptamer was added and incubated for 2 hours at room temperature. This was followed by three washes with 1 x PBS-T and the addition of HRP-conjugated streptavidin (diluted 1:10000 in 1 x PBS-T), and incubation for 2 hours at 37°C. The plates were then washed four times with 1 x PBS-T, after which TMB detection substrate was added. A change in colour to blue, which could be observed with a naked eye, indicated that the aptamers bound to the CFP-10 protein. The reaction was stopped with a 2 M sulphuric acid solution, at which point the blue colour observed changed to yellow. The plates were then read on the MultiSkan Go plate reader at a wavelength of 450 nm. Each plate had CFP-10 and an aptamer-alone control respectively, which were averaged and then subtracted from each well to eliminate background noise.

4.2.2 - Limit of detection

Serial dilutions of CFP-10 was bound to a 96-well micro-titre plate in a $NaHCO_3$ buffer and incubated overnight at 4°C. The plates were then washed with 1 x PBS-T (pH 7) and blocked with a 5% fat-free milk solution for 1 hour at 4°C. The plates were then washed 3 times with a 1 x PBS-T after which 150 nM biotinylated EA10 aptamer was added and incubated for 2 hours at room temperature. The plates were processed and read as described above in section 4.2.1.

4.2.3 – Sputum sample analysis

To determine the use of the ssDNA aptamers as detection reagents, one suitable ssDNA aptamer, denoted EA10, was tested in a small case controlled study. Forty sputum samples that have been well characterised were obtained from the Lung Infection and Immunity Unit, Groote Schuur Hospital and the University of Cape Town (South Africa), were used for the clinical study. A further 15 laboratory volunteers working at the CSIR donated one to two sputum samples for testing. Samples were grouped into two definite categories as shown in the study flow diagram (Figure 4-1). Each laboratory volunteer

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was assigned a random number for anonymity and the samples were labeled with the random number as well as 1 or 2 (depending on the day the sample was taken). All sputum samples obtained were liquefied in a 0.1% dithiothreitol (DTT) solution. The sputum samples were bound to a 96-well micro-titre plate in a $NaHCO_3$ buffer and incubated overnight at $4°C$. The plates were processed and read as described above in section 4.2.1. Aptamers selected from the same parental library against an unrelated target (human CD7) were used as negative controls (LM-B12 and LM-H9). Subsequently, a specificity and sensitivity comparison, by means of an ELONA, between EA10 and H11 was performed using a small subset of samples (28 sputum samples). The results were averaged and the standard deviation was calculated for each sputum sample. Each experiment was repeated twice to determine repeatability of the assay by calculating the coefficient of variance. The diagnostic performance of the assay was evaluated using ROC curve analysis (SigmaPlot 12.0, Statsoft, Inc.; GraphPad Prism 5.04, GraphPad Software, Inc.) and cut-points were determined based on Youden's index.
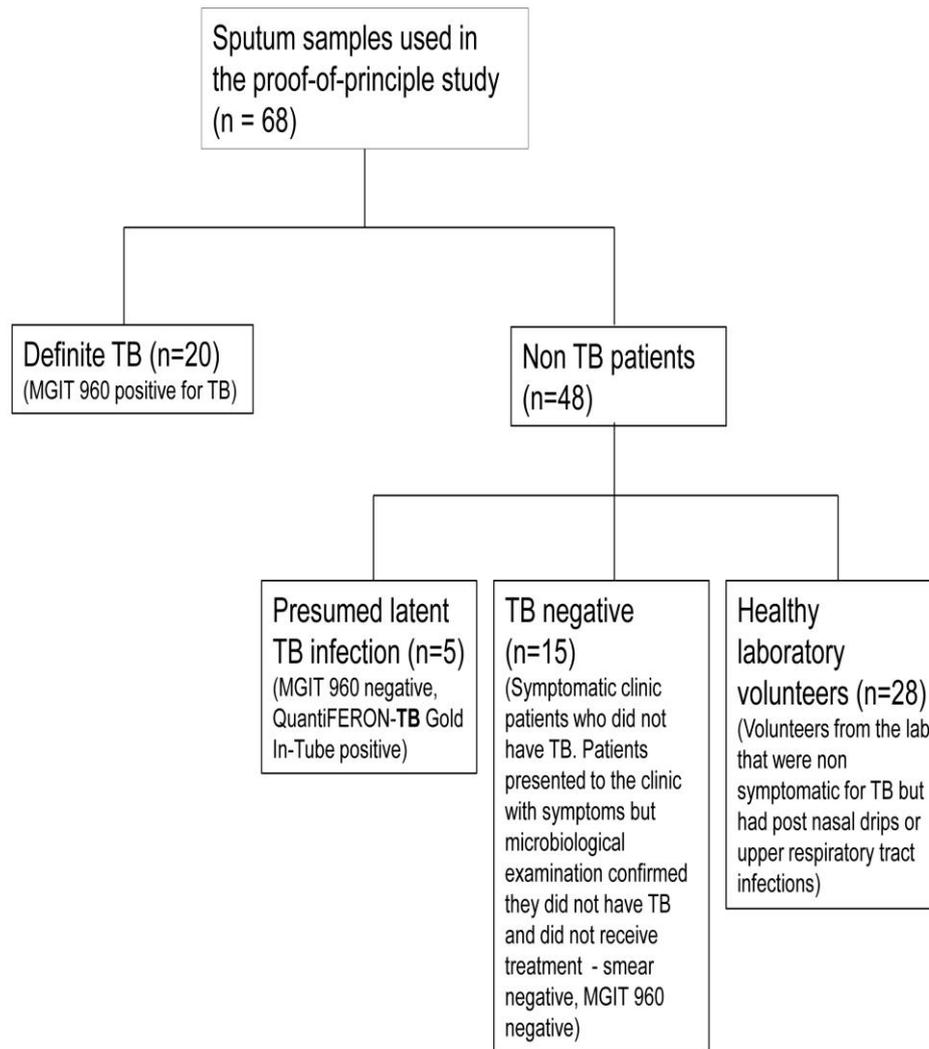
Figure 4-1: Study flow diagram.
Study plan and patient categorisation of the 68 participants in active (definite) TB, latent TB, TB negative and healthy donors that were evaluated in the proof-of-principle study.

4.3 – RESULTS

4.3.1 - Specificity

To determine the specificity of the ssDNA aptamers, lysates were prepared from bacteria inhabiting the oral cavity and evaluated for binding to the aptamers in an ELONA. This was important if the aptamers were going to be used in an initial diagnostic screening on sputum samples. The six tested aptamers had variable binding, although none of the aptamers were able to bind to the lysates of *Streptococcus pyogenes* or *Corynebacterium xerosis* (Figure 4-2). Interestingly, only E1 and H11 seemed highly specific to Mtb. It was decided that one aptamer, namely EA10, would be tested in clinical samples. EA10 was chosen to assess clinical sputum samples to determine if the

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aptamers had potential as a diagnostic because it had cross-reactivity with *Staphylococcus aureus*.
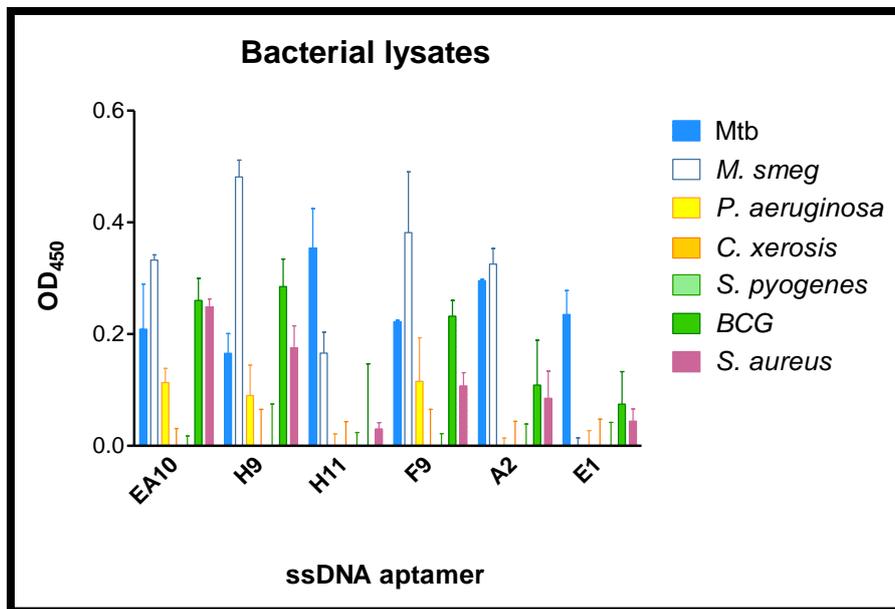
Figure 4-2: Bacterial lysate specificity ELONA for ssDNA aptamer EA10.
The specificity was done using lysates from bacterial cultures of Mtb, *M. smeg*, *M. bovis BCG, P. aeruginosa*, *S. aureus* and *S. pyogenes*.

4.3.2 – Limit of detection

To determine the limit of detection of CFP-10 by EA10 in the ELONA, a serial dilution of CFP-10 was preformed. The serial dilutions of CFP-10 coated to a 96-well micro-titre plate revealed that 150 nM of EA10 is able to detect as little as 31 ng of CFP-10 (Figure 4-3). The best fit curve had an R^2 value of 0.85, indicating a goodness of fit of the dilutions to the linear regression line.
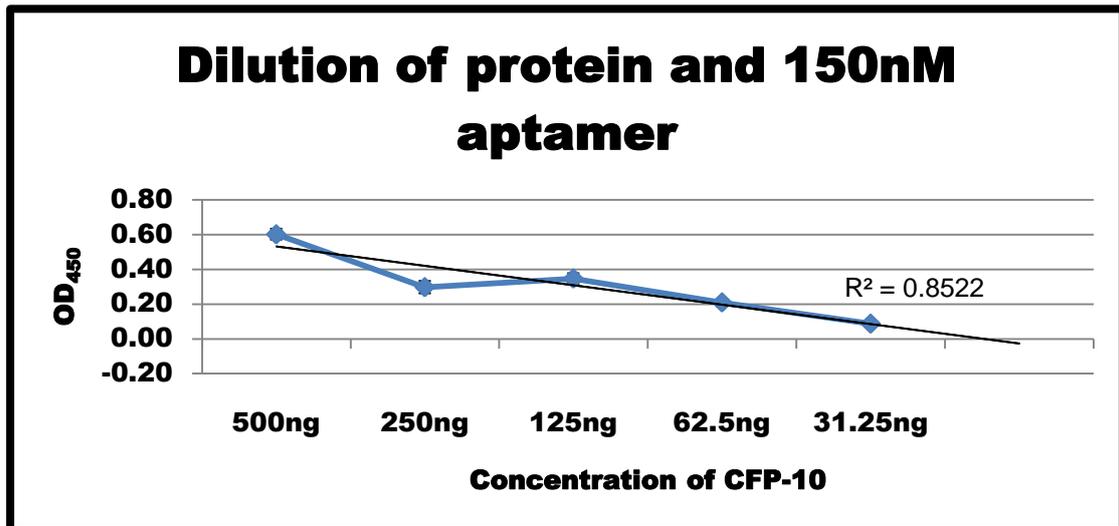
Figure 4-3: Binding of aptamer EA10 to serial dilutions of CFP-10.
Serial dilutions of CFP-10 were coated onto a 96-well micro-titre ELISA plate and binding of EA10 to these dilutions were tested for a limit of detection. The results reveal that the limit of detection for 150 nM of EA10 is 31 ng, with an R^2 of 0.85.

4.3.3 – Sputum sample evaluation

To evaluate the aptamers for detecting TB infection using clinical sputum samples, the utility of EA10 was tested in 68 sputum samples in an ELONA. The samples fall in the following categories (a) smear-positive, culture-positive; (b) negative-smear, culture-positive; (c) smear-negative, culture-negative, Quantiferon in-tube gold positive; and (d) smear-negative, culture-negative, Quantiferon in-tube gold negative (Table 4-1). The initial twenty samples were screened to determine if the assay would work. Once it had been established that the assay had potential, sputum from either smear-positive, culture-positive; smear-negative culture-negative individuals or laboratory volunteers (Table 4-2) were used to test the aptamer in a larger sample size.

Table 4-1: Characterisation of the sputum samples obtained from Groote Schuur Hospital/UCT
Samples were obtained from Groote Schuur Hospital/UCT. The numbers indicate the anonymised patient identity.

| Smear-negative, culture-negative, QuantiFERON in-tube gold negative and TSPOT negative (No TB)	Smear-negative, culture-negative, QuantiFERON in-tube gold positive and TSPOT positive (latent TB)	Smear-negative and culture-positive	Smear-positive and culture-positive
35	54	11	5
52	66	36	17
139	67	65	39
143	76	85	59
161	101	88	94

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Table 4-2: Characterisation of the sputum samples for second round of testing
Samples were obtained from Groote Schuur Hospital/UCT. The numbers indicate the anonymised patient identity.

Smear-positive and culture-positive	Smear-negative, culture-negative	Laboratory volunteers
S2045	589	350917
S2044	434	443723
S2063	539	447612
S2062	428	481114
S2048	489	318906
S2060	377	3495
S2042	487	796741
S2041	409	40703
S2065	386	54885
S2052	488	125636
		163729
		514002
		188683
		762206
		926945

Based on the ROC curve analysis with a 99% confidence interval, the study showed that in 86% (p<0.0001) of randomly selected cases, a TB positive individual had a test value (OD450) higher than a TB-negative individual. In this study, the cut-point for positive readings based on the ROC curve analysis using the Youden's index was OD450 = 0.2. Using this cut-off, the assay has a specificity of 68.75% and sensitivity of 100%. The ssDNA aptamer, EA10, was able to detect 20 of the 20 positive samples as positive, although three of 20 samples were borderline cases, i.e. just on the cut-off point. For the purpose of this study, they were classed as a positive (Figure 4-4, Table 4-3). The ssDNA aptamer correctly detected 33 of the 48 negative samples (Figure 4-4, Table 4-3). Eight of the laboratory volunteers had one sputum sample that gave a false positive reading; two of these eight laboratory volunteers had false positive results for both of the sputum samples obtained (Figure 4-4, Table 4-3). As expected, LM-B12 and LM-H9, which were used as a negative control, did not detect TB in the sputum samples (Figure 4-4). The coefficient of variance ranged between 2 and 10%, indicating the repeatability of the test. A more specific aptamer (H11) was compared to EA10 using 28 samples. The data shows that the two aptamers were comparable with a specificity of 60% and sensitivity of 65% using the Youden's index (Figure 4-5).
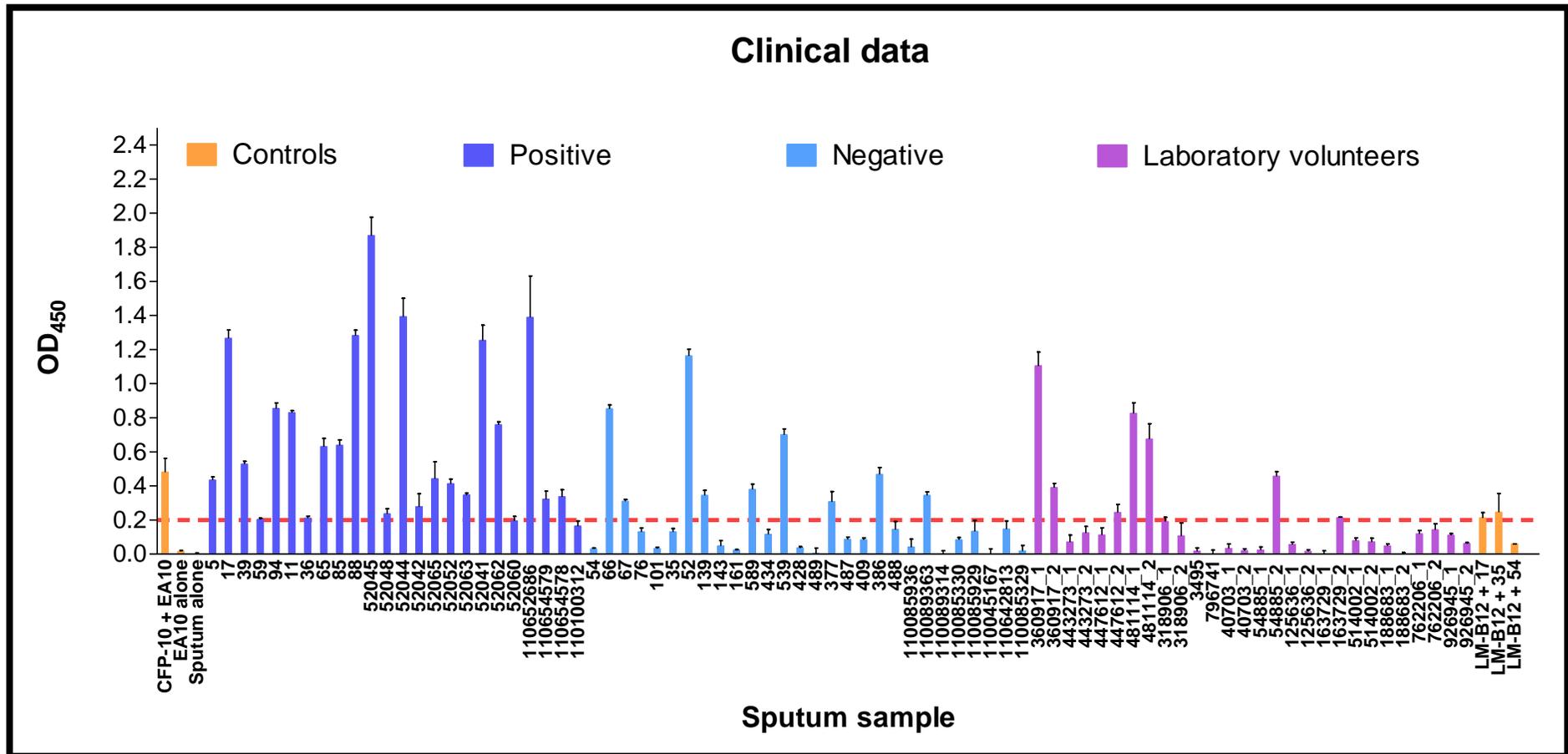
Figure 4-4: ELONA of sputum samples using ssDNA aptamer EA10.
The cut-off for positive was set at an OD_{450} of 0.2, as illustrated by the dotted red line and was determined by a 99% confidence interval of a known negative sample. An aptamer selected using the same library but a different target was used as a control (LM_B12). Error bars denote standard deviations, the experiment was done in triplicate and each sample was repeated in two independent studies. The coefficient of variance between the two studies was less than 10% for all samples.

Table 4-3: Characterisation of sputum samples as determined by detection of CFP-10 by EA10
The numbers indicate the anonymised patient identity.

Positive TB samples	Comment	Negative TB samples	Comment
5		35	
11		54	Latent TB
17		76	Latent TB
36	Border line of cut-off	101	Latent TB
39		143	
59	Border line of cut-off	161	
65		434	
85		428	
88		489	
94		487	
S2045		409	
S2048		488	
S2044		443723_1	
S2042		443723_2	
S2063		447612_1	
S2041		318906_1	
S2062		318906_2	
S2060	Border line of cut-off	3495	
S2065		796741	
S2052		40703_1	
66	False positive (Latent TB)	40703_2	
67	False positive (Latent TB)	54885_1	
52	False positive reading	125636_1	
139	False positive reading	125636_2	
589	False positive reading	163729_1	
539	False positive reading	514002_1	
377	False positive reading	514002_2	
386	False positive reading	188683_1	
360917_1	False positive reading	188683_2	
360917_2	False positive reading	762206_1	
447612_2	False positive reading	762206_2	
481114_1	False positive reading	926945_1	
481114_2	False positive reading	926945_2	
54885_2	False positive reading		
163729_2	False positive reading		
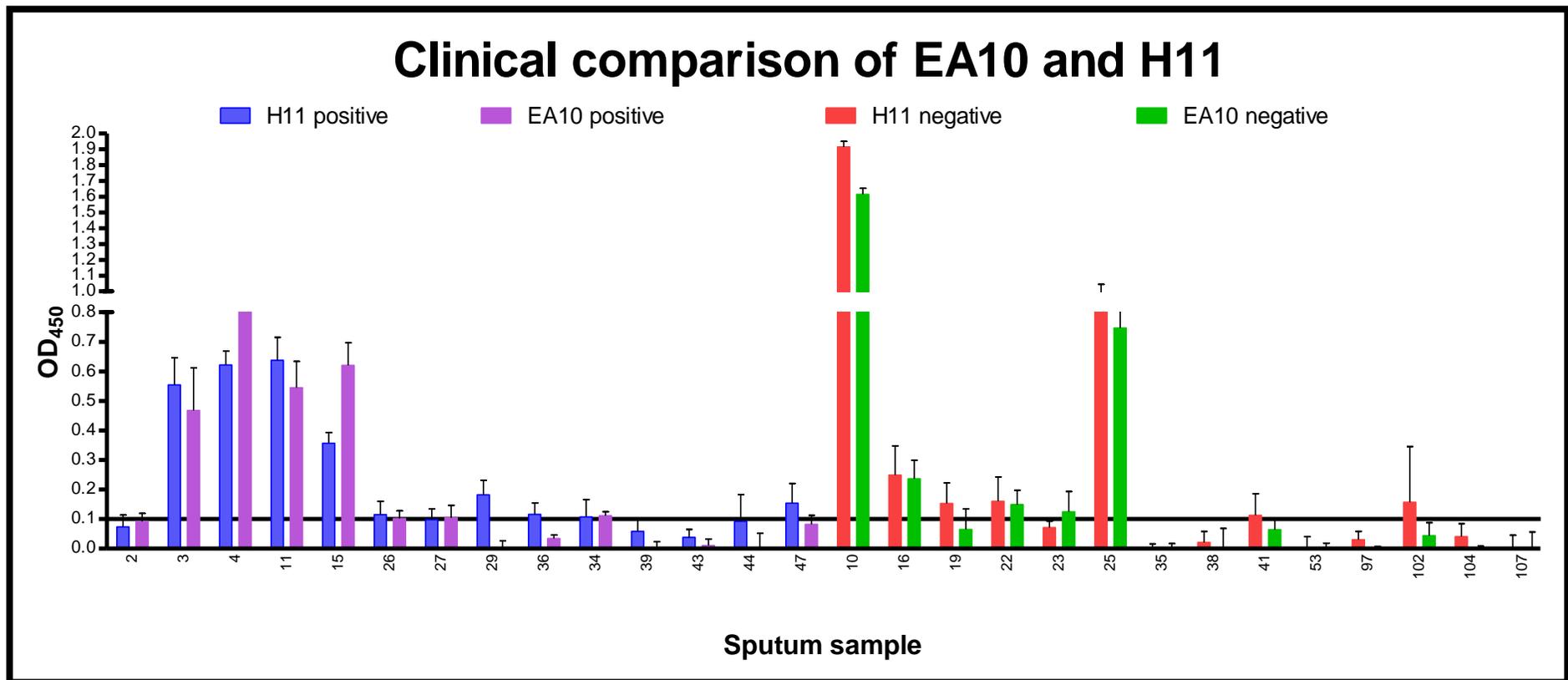
Figure 4-5: Comparison of EA10 and H11 using sputum samples.
The sensitivity and specificity of EA10 and a more specific aptamer H11 were compared using 28 sputum samples. Using Youden's index, the cut-point for positive samples was set at an OD_{450} of 0.1 and is indicated by the solid line. Data are presented as means ± standard deviation of the mean.

4.4 – DISCUSSION

We isolated aptamers against the CFP-10/ESAT-6 heterodimer to be used as potential TB detection reagents. In a small case controlled study, the aptamer-based assay had a sensitivity of 80 - 100% and a specificity of 68.75% using Youden's index, which is encouraging for development of the aptamers as detection reagents for active TB.

Oral cavity bacteria was chosen for this study due to the wide diversity of bacteria present that includes gram-positive bacteria, gram-negative bacteria, anaerobic bacteria, mycobacteria and fungal species. Lower respiratory tract bacteria are present at much lower concentrations than oral cavity bacteria and require the use of specialised techniques such bronchoscopes to obtain. The bacterial lysate study revealed that the aptamers were able to recognise *S. aureus, M. smegmatis, M. bovis* BCG and, in some cases*, P. aeruginosa*. This may be due to the fact that *M. smegmatis* secretes the CFP-10 protein (Converse et al., 2005), and although the RD1 region has been removed from *M. bovis BCG*, the mycobacterium genome has 23 ESAT-6 homologues (such as CFP-10, Esx G and Esx H). Thus, the aptamers might recognise one of the other ESAT-6-like proteins that are encoded by one of the other 10 genomic loci (Maciag et al., 2009). ESAT-6-like homologues have also been described in the genomes of other gram-positive bacteria like *Bacillus subtilis, B. anthracis, Clostridium acetobutylicum, Listeria monocytogenes*, and *S. aureus* (Gey Van Pittius et al., 2001, Tan et al., 2006). The presence of these ESAT-6-like homologues in *S. aureus* may explain why aptamers raised against the CFP-10/ESAT-6 heterodimer also bind to lysates of *S. aureus*.

Despite binding of the aptamer to *M. smegmatis*, *M. bovis* BC*G* and *S. aureus*, EA10 was selected and tested for its ability to detect TB using sputum samples. EA10 had a specificity of 68.75% using Youden's index, which is lower than that observed for antibodies raised to detect the CFP-10 monomer (Feng et al., 2011). Sensitivity and specificity of the superior specific binding aptamer, H11, was not significantly different from EA10. Possible reasons for some of the false positive readings in this study might be as a result of *S. aureus* or other bacterial co-infections (Gey Van Pittius et al., 2001) or from misclassification bias due to subclinical TB; however, the patients did not return to the hospital and thus no further follow up could be done to clarify this point. Additionally, the lowered specificity may be attributed to antigen persistence in patients with previous TB infection (Gomez et al., 2004). Alternatively, long-term persister organisms could be secreting the culture filtrate proteins at very low levels, detectable by the aptamers but not by antibodies.

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An encouraging aspect of the aptamer-based diagnostic assay, in this preliminary study, is that it performed reasonably well in both HIV-infected and HIV-uninfected patients. Further studies, after appropriate optimisation, will be required in HIV-infected individuals. Diagnostic tests may vary in their performance in HIV-infected individuals. GeneXpert™ has sub-optimal negative predictive values in HIV-infected individuals (Boehme et al., 2010), whilst the urine LAM assay performs optimally in HIV-infected individuals with low CD4 counts (Peter et al., 2010, Peter et al., 2012). The sensitivity of the aptamer-based test is comparable to that of an antibody-based ELISA in a recent study by Feng *et al.* (Feng et al., 2011), with both studies having sensitivities of over 80% for the detection of CFP-10 in clinical specimens. Compared to the antibody-based assays, aptamer-based assays have greater stability under a wide range of conditions, and can be repeatedly used without losing their binding ability. Aptamers are, therefore, a promising class of detection reagents for the development of improved diagnostics (Yang et al., 2005).

Limitations of the current aptamer-based assay are the lack of a rapid readout, as ELONA takes several hours to complete, as well as antigenic cross-reactivity. The cross-reactivity of the aptamer-based assay may be improved through optimisation of the aptamers, which may include truncation of the current 90-mer parent aptamers to minimum sequences essential for binding to the target, which in some cases improves the kinetics of the aptamers (Shangguan et al., 2007). By truncating the aptamers not only will the cost of production be reduced, but it may also prevent non-specific binding. Furthermore, a sandwich assay can be developed that makes use of two aptamers; one as a capture and the other as a detector molecule. By making use of such a sandwich assay the specificity of either the capture or the detector aptamer can be lower, provided that the combination of the two is highly specific to the antigen in question.

This chapter illustrates that it is feasible to develop aptamers to relatively TB-specific antigens and thereby detect these antigens in human biological samples obtained from patients with and without active TB. However, further optimisation is required to improve performance outcomes, followed by validation in larger appropriately designed studies using biological samples from different body compartments. This will lay the foundation for their inclusion and evaluation in point-of-care detection platforms.

Chapter 5.   GENERAL DISCUSSION AND CONCLUSION

5.1 - SUMMARY OF FINDINGS

This study reported a rapid protocol for the purification and refolding of recombinant hexa-histidine tagged CFP-10 and ESAT-6 expressed in *E. coli*. The protocol described within this thesis is a rapid one-step purification protocol that eliminates the need for multiple chromatography and anion exchange steps of previous protocols. An *E. coli* contaminating protein was removed, resulting in 99% pure and biologically active recombinant CFP-10 and ESAT-6 proteins.

The purified proteins obtained were used as targets for the selection of ssDNA aptamers against the CFP-10/ESAT-6 heterodimer. The aptamers selected showed strong binding kinetics and good specificity. One aptamer, namely EA10, was used in a proof-of-principle study showing that the aptamers can be used as a TB diagnostic for clinical sputum samples from patients with active TB.

The clinical sample evaluation revealed that the EA10 aptamer had a specificity of 68.75% and a sensitivity of 80-100%. However the sample size of the clinical study was small and needs to be increased.

5.2 - IMPLICATIONS OF THIS STUDY IN RELATION TO CURRENT TB DIAGNOSTICS

It has been shown that aptamers could successfully replace antibodies based on their specificity and sensitivity in ELISA, *in situ* fluorescent hybridization, and Western blotting thereby creating a niche for aptamers in diagnostic applications (Kulbachinskiy, 2007). To date, aptamers have been used for measuring concentrations of various metabolites and protein factors, for detection of toxins, identifying specific types of cells and tissues, and pathogenic microorganisms (Bruno and Kiel, 1999, Charlton et al., 1997, Hesselberth et al., 2000, Homann and Goringer, 1999, Proske et al., 2005). The use of aptamers in a diagnostic application has been demonstrated by the use of the adenosine, cocaine and thrombin aptamers. These aptamers were modified for attachment to gold nanoparticles and are used in a colorimetric readout system for their specific target (Xia et al., 2010).

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Aptamers have an advantage over other conventional diagnostic tools as they can have modified bases allowing their use to span a wide range of diagnostic applications (Gold et al., 2010). They are more stable to heat, pH and organic solvents. They are cost-effective as they can be obtained in large amounts through chemical synthesis resulting in little batch-to-batch variation (Lee et al., 2007), and with the patent on the SELEX coming to an end in 2013 licenses will not be required for their commercial exploitation.

There are currently two studies that reported aptamers against an Mtb antigen (Qin et al., 2009) and the whole bacterium (Chen et al., 2007). However, no aptamers have ever been raised against ESAT-6, CFP-10 or the CFP-10/ESAT-6 heterodimer. These antigens have been used to elicit an immune response in a number of TB diagnostic kits (Brock et al., 2001, Chapman et al., 2002, Pai et al., 2008, Van Pinxteren et al., 2000, Perkins, 2000, Shah et al., 2006, Wedlock et al., 2002). However, to date there is no diagnostic that measures the antigen directly. Thus, the purpose of this study was to isolate aptamers against the CFP-10/ESAT-6 heterodimer that could be a used as a potential diagnostic.

The sensitivity of the ELONA using aptamer EA10 falls between the ranges for smear microscopy (Perkins, 2000, Shah et al., 2006, Wedlock et al., 2002) and PCR (Dinnes et al., 2007, Pai et al., 2008). This shows that the ELONA is comparable with current diagnostics on the market. However, what favours the aptamers as a useful tool for diagnostics is the low cost to produce them and the high sensitivity and specificity observed.

5.3 - CHALLENGES AHEAD

The challenges that lie ahead include improving both the sensitivity and specificity of the aptamers, e.g. by using an antigen capture and detection aptamers and by developing an aptamer-based point-of-care diagnostic assay.

5.4 - FUTURE WORK

The other 23 aptamers that still need to be tested to determine if there are aptamers that can distinguish between different mycobacterial species, yet retain high specificity and sensitivity in clinical samples. The aptamers need to be tested in a larger clinical case study to determine if they are indeed able to compete with current diagnostic tests. The aptamers will still need to be truncated to determine the shortest sequence possible that

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will yield similar if not better results. This will aid in the reduction of manufacturing cost as well as increase the specificity of these aptamers. This work is in progress and forms part of a larger project outside the scope of this thesis.

To determine the best diagnostic platform for the aptamers, the aptamers will be tested in a lateral flow device and in a tube-based colorimetric read-out system where they are covalently bound to nanoparticles. This was the case with the cocaine aptamers (Liu et al., 2006a) and thrombin aptamers (Xia et al., 2010), respectively. The most promising application of these aptamers as a diagnostic would be in designing a microchip that would incorporate a large number of different aptamers that could simultaneously detect a variety of target molecules, as has been illustrated for human proteins in previous studies (Gold et al., 2010, Kulbachinskiy, 2007).

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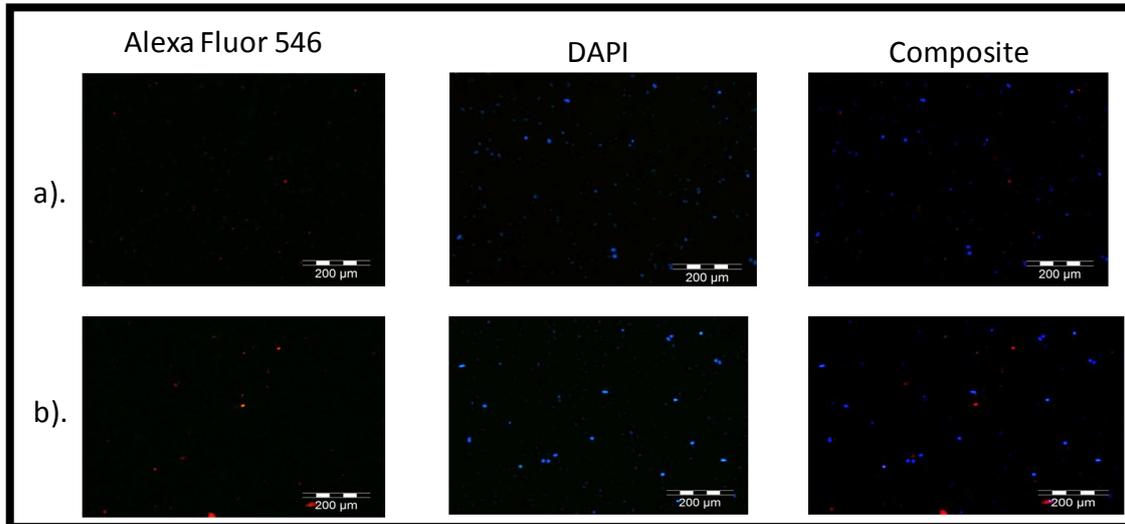
Appendix I Figure 1: Fluorescently labelled ESAT-6 and CFP-10 binding to macrophages.
Macrophages were incubated in the presence of Alexa Fluor 546-labeled ESAT-6 (a) and CFP-10 (b)
The right hand panels show microscope composite images of the same cells viewed separately for DAPI
(blue) and Alexa Fluor 546 (red) fluorescence.

**Appendix II Table 1: *M-fold* structures of all selected aptamers.**

A1	
A2	
A5	

A10	
A11	
B3	

B7	
D12	

E1	
E9	

EA10	
EC12	
F1	

F3	
F9	
F11	

G6	
G7	

H9	
H10	
H11	

I1	
J1	