

**Development of an Antigen Detection Based
Point-of-Care Test for the Diagnosis of
Primary Syphilis**

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Submitted in partial fulfillment of the requirements for the degree of

MASTER OF MEDICAL SCIENCE

In the Discipline of Medical Microbiology,

Department of Infection Prevention and Control

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University of KwaZulu-Natal, Durban

2011

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DECLARATION

I, **Meleshni Naicker**, declare that:

- (i) The research reported in this dissertation, except where otherwise indicated, is my original work.
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"As the candidate's supervisor I agree/~~do not agree~~ to the submission of this dissertation"

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Date

ACKNOWLEDGEMENTS

I wish to extend my eternal gratitude and appreciation to the following:

My supervisor, Professor AW Sturm, Department of Infection Prevention and Control, for providing the opportunity for me to work on this project, for your guidance, motivation and helpful suggestions during the course of this study. Thank you for your confidence in my ability as a student and for helping me to develop as a scientist in this chosen field.

National Research Foundation, for providing financial assistance.

Dr Siva Danaviah and Staff at Africa Centre, Shymala Padayachee and Staff at the HIV Pathogenesis Programme, Karnishree Govender, Mona Pillay, Devan Moodley, Dr Bronwyn Joubert, Dr Kamini, Gounden, Dr Manormoney Pillay, Fazana Karim, Kavitha Govender, Melisha Sukkhu, Logan Pillay, Jay Murugan, Mantha Makume, Anitha Pillay, Professor Preshnie Moodley, Simbarashe Mabwe, Dr Farouk Amod, Thank you for your kind assistance, helpful advice and generous support.

Linda Bester and Staff at Biomedical Resource Unit Westville campus, for providing experimental rabbits and animal ethical training

Shiran Singh and Rajiv Singh, UKZN, Medical School student LANs, for providing technical support and for your willingness to assist me at all times.

My Family and Friends, thank you for your constant prayers, support and encouragement throughout my years of study.

My Grandfather, Mr Johnny Naidoo, thank you for your continuous love, support, prayers, encouragement and regular phonecalls throughout my years of study.

My late Grandparents, for always being there for me in spirit and guiding me throughout my studies.

My Parents, Roy and Pinky Naicker, my Sister, Yegeshni and my Brother, Vinolin, for your continuous love, support, guidance and patience throughout my studies as well as all my other endeavours. For the tremendous amount of faith that you'll have in me, for cheering me up when I was down and encouraging me to do my best and to even extend beyond boundaries I never knew I could. For your constant prayers and presence. Thank you.

Yegeshni and Vinolin Naicker, thank you both for your technical assistance and your willingness to assist me at all times.

GOD, Thank You for providing me with wonderful opportunities, for the support that I have of family and friends, for strength and determination to undertake this project and courage to overcome all odds. Thank You for your constant love, guidance and protection. Thank You for the completion of this dissertation.

DEDICATION

**This dissertation is dedicated to my parents, Roy and Pinky Naicker,
sister, Yegeshni and brother, Vinolin**

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LIST OF ABBREVIATIONS

%	percent
µg	microgram
µL	microlitre
µM	micromolar
6xHis	hexahistidine fusion tag
bp	base pair
CSF	cerebrospinal fluid
ddNTPs	dideoxyribonucleoside triphosphates
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
dsDNA	double-stranded DNA
<i>E. coli</i>	<i>Escherichia coli</i>
eg.	example
EK	enterokinase
ELISA	Enzyme Linked Immunosorbent Assay
FCA	Freund's complete adjuvant
FTA	Fluorescent treponemal antibody
FTA-ABS	Fluorescent treponemal antibody – absorption
GUD	genital ulcer disease
HATTS	Hemagglutination treponemal test for syphilis
HCl	hydrochloric acid
HIV	Human Immunodeficiency Virus
i.e.	that is
IC	immunochromatographic

IPTG	isopropylthio- β -galactoside
kb	kilobase
kbp	kilobase pair
KCl	potassium chloride
kDa	kilodalton
LB	Luria Bertani
M	molar
mg	milligram
MgCl₂	magnesium chloride
MHA-TP	Microhaemagglutination assay for antibodies to <i>T.pallidum</i>
mL	millilitre
mm	millimetre
mM	millimolar
N- terminal	amino-terminal
ng	nanogram
°C	degrees celcius
OD	optical density
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween 20
PBSTM	phosphate buffered saline + Tween 20 + non fat dry milk
PCR	Polymerase chain reaction
pmol	picamolar
POC	point-of-care
RIT	Rabbit infectivity testing
rpm	revolutions per minute

RPR	Rapid Plasma Reagin
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
STD	sexually transmitted disease
STI	sexually transmitted infection
subsp	subspecies
<i>T. pallidum</i>	<i>Treponema pallidum</i> subsp <i>pallidum</i>
<i>T. carateum</i>	<i>Treponema carateum</i>
Taq	<i>Thermus aquaticus</i> DNA polymerase
TEMED	N ² , N ² , N ² , N ² - tetramethylenediamine
TPHA	<i>Treponema pallidum</i> haemagglutination assay
TPI	<i>T. pallidum</i> immobilization test
Tromp	<i>T. pallidum</i> rare outer membrane protein
Tromp1	<i>T. pallidum</i> rare outer membrane protein (31 kDa)
TRUST	Toluidine red unheated syphilis test
U	unit
US	United States
USR	Unheated serum regain test
UV	ultraviolet
V	volts
VDRL	Venereal Disease Research Laboratory
viz.	namely
WHO	World Health Organization
x g	gravitational force
α	alpha
β	beta

ABSTRACT

Aim: To develop an antigen detection based, point-of-care test that will rapidly exclude syphilitic infection in patients presenting with genital ulcers.

Materials and Method: *T. pallidum* subsp *pallidum*, Nichols strain, was propagated by intra-testicular inoculation of rabbits. *T. pallidum* DNA was obtained by suspending the testicular extract in ProbeTec lysis buffer followed by heating. Crude DNA was purified and concentrated. Specific primers were used for the amplification of the gene encoding the 31 kDa *T. pallidum* rare outer membrane porin protein (termed “Tromp1”). The amplified gene was cloned in frame with the pET100/D-TOPO vector that carries the N-terminal Xpress epitope and polyhistidine fusion tags. A screening PCR, restriction digest and DNA sequencing were used to confirm the presence of the *tromp1* insert. Isolated plasmid DNA, pET100/D/*tromp1* and the pET100/D/*lacZ* (positive control) were transformed into BL21 (DE3) pLysS *E. coli* cells for expression of recombinant Tromp1 and β -galactosidase as fusion proteins. SDS-PAGE and Western blot analysis were applied for detection of the recombinant proteins.

Results: The gene encoding the 31 kDa Tromp protein was successfully cloned and sequenced. Multiple sequence alignment showed 100% homology between the cloned *tromp1* gene sequence and its reference sequence. In addition, a screening PCR for transformation products and restriction digest of isolated plasmid DNA confirmed the presence of the *tromp1* insert. Following gene expression, SDS-PAGE gel analysis showed no difference in the banding pattern between IPTG induced and uninduced lysates. The positive control however, showed a bright and distinct band at its

expected size range of ~121 kDa. A Western blot and ELISA using specific antibodies to the N-terminal Xpress epitope fusion tag confirmed the absence of recombinant Tromp1 protein.

Discussion and Conclusion: The results show that the *tromp1* insert was successfully cloned and maintained up until the expression level. However expression of recombinant Tromp1 in BL21 (DE3) pLysS *E. coli* cells, for use as antigen in the serodiagnosis of primary syphilis was not achieved, despite several attempts to optimize gene expression. Expression of the positive control gene confirmed that growth and induction were properly performed.

CHAPTER 1

INTRODUCTION

Syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum* subsp *pallidum*. This species belongs to the order *Spirochaetales*, the family *Spirochaetaceae* and the genus *Treponema* (Tramont EC, 2005). The genus *Treponema* comprises pathogenic and non-pathogenic species indigenous to human and animals (Radolf JD, 1991). Among the human pathogens, there are four, namely; *Treponema pallidum* subsp *pallidum* the syphilis-causing agent; *Treponema pallidum* subsp *endemicum* causes endemic syphilis (bejel); *Treponema pallidum* subsp *pertenue* causes yaws and *Treponema carateum* causes pinta (Singh and Romanowski, 1999). *T. pallidum* subsp *pallidum* is the “most invasive of the pathogenic treponemes” as it “produces highly destructive lesions in almost any tissue of the body” (Radolf JD, 1991).

Syphilis begins as an ulcerated lesion termed “chancre” at the site of entry. If untreated, the organism disseminates from its initial site to produce a maculopapular rash characteristic of secondary stage syphilis. The disease may then enter an asymptomatic latent stage which may be cleared by the immune system or reappear as tertiary stage syphilis with the potential to infect any tissue in the body.

Syphilis was first reported in Europe in the late 15th century. By the 16th century the disease had progressed to become a major public health issue earning its name, “the great pox” (Chiu and Radolf, 1994). Syphilis continued to be an important global health problem throughout the 20th and into the 21st century. In the year 1999, the

World Health Organization estimated the occurrence of 12 million new cases of syphilis, worldwide, most of which occurred in the developing countries (World Health Organization, 2001). It has been reported that syphilis occurs mainly among the poor, those who lack access to health care as well as those who have multiple sexual partners (Peeling and Hook, 2006).

The introduction of penicillin therapy in the late 1940s along with improved preventive measures and the implementation of STI control programs resulted in reduction of the levels of syphilis over the years. In addition, due to the scarce laboratory facilities for diagnosing GUD in resource-poor settings, a syndrome-based approach to the management of STIs had been introduced and implemented across several developing countries. The syndromic management approach is based on the identification of a syndrome i.e. a group of symptoms a patient complains of along with easily recognizable signs upon clinical examination. If an STI syndrome is identified, combination treatment is provided that covers the majority of organisms responsible for that syndrome in a particular geographical area (Altini and Coetzee, 2005).

Till the development of PCR based tests, the inability to cultivate *T. pallidum in vitro*, or to sustain the organism in cell culture through multiple generations, has made it difficult to obtain a definitive diagnosis of syphilis. Furthermore, a specimen source from the latent and late stages cannot be obtained, since lesions are absent (Karp *et al.*, 2009). Clinical symptoms, the identification of treponemes in lesion exudate and antibody detection tests form the basis for diagnosing syphilis. Darkfield microscopy can detect motile treponemes within exudate obtained from lesions of patients with

primary, secondary or early congenital syphilitic infection. It is at these stages of a syphilitic infection that treponemes in the lesions are in abundance and darkfield examination is most successful. However, *T. pallidum* subsp *pallidum* cannot be distinguished from the other pathogenic subspecies by means of microscopy (Ceighton ET, 1990; Larsen *et al.*, 1995). Furthermore, darkfield microscopy is not suitable for examining specimens obtained from oral and anal lesions due to the difficulty in distinguishing *T. pallidum* from commensal treponemes present in these cavities (Chiu and Radolf, 1994; Ryan KJ, 2004). In addition, darkfield microscopy requires laboratory skill and experience to perform. Therefore, in parts of the world where this is feasible, PCR has replaced darkfield microscopy.

Serological tests provide a more practical method for diagnosing syphilis and are the only means for identifying asymptotically infected persons (Radolf JD, 1991). Serological tests for syphilis fall into two categories: nontreponemal and treponemal tests.

Nontreponemal tests (VDRL and RPR) detect antibodies to a combination of cardiolipin, cholesterol and lecithin. These antibodies are however not exclusive to patients with *T. pallidum* infection as these may test positive for other treponemal diseases and nontreponemal diseases involving tissue damage and a variety of autoimmune diseases (Ryan KJ, 2004). Hence, a reactive nontreponemal test itself cannot confirm a *T. pallidum* infection.

Treponemal tests (TPHA and FTA-ABS) are more specific to *T. pallidum* since they detect antibodies to specific *T. pallidum* antigens (Ryan KJ, 2004). They are therefore

used to confirm reactive nontreponemal tests of presumed syphilitic patients. Unlike the nontreponemal tests, they cannot be used for assessment of response to treatment because antibodies to treponemal antigens can persist for years. Hence, test results remain positive for years, if not for life (World Health Organization, SDI, 2006; Ryan KJ, 2004). However they can be used as screening tests since treponemal tests are only falsely reactive in approximately 1% of the general population (Goldman and Lantz, 1971). Treponemal tests are costly and technically more difficult to perform than nontreponemal tests. Furthermore treponemal tests take at least 1-2 hours or more to obtain results, which is longer than with nontreponemal tests.

In this study we aimed to develop a rapid point-of-care test that uses immobilized specific antibody to detect whole treponemes in primary ulcer secretions. The test was planned to be an immunochromatographic (IC) test strip incorporating specific antibodies raised against the 31 kDa *T. pallidum* rare outer membrane porin protein. Performing such a test, requires only a minimum of technical training, can be performed within minutes at room temperature and does not require the use of any equipment.

While *T. pallidum* once used to be among the leading bacterial causes of genital ulcer disease (GUD) in African and other developing countries, it has now been superseded by herpes simplex virus type 2 (HSV-2) infection, as recent studies in Africa have shown (Nilsen *et al.*, 2007; Mwansasu *et al.*, 2002; Ahmed *et al.*, 2003; Moodley *et al.*, to be published). With decreasing levels of syphilis, it is not surprising that patients presenting with genital ulcer syndrome, most often suffer from another sexually transmitted infection. Despite this, patients are treated for syphilis as well,

since it is included in the syndromic management guidelines for genital ulcer syndrome. The “unnecessary” treatment with penicillin for syphilis does result in hypersensitivity reactions and may hypothetically result in the development of resistance in *T. pallidum* and other bacterial species. For this reason, the proposed rapid point-of care test is aimed to exclude the possibility of a syphilitic infection when used to diagnose individuals presenting with genital ulcers. Overall, this will reduce the number of patients unnecessarily treated for syphilis.

CHAPTER 2

LITERATURE REVIEW

(2.1) The Genus *Treponema*

Treponema pallidum subspecies *pallidum* is the causative agent of venereal syphilis. This species belongs to the order *Spirochaetales*, the family *Spirochaetaceae* and the genus *Treponema* (Tramont EC, 2005). The genus *Treponema* comprises pathogenic and non-pathogenic species indigenous to human and animals (Radolf JD, 1991). Among the human pathogens, there are four, namely; *Treponema pallidum* subsp *pallidum*, the syphilis-causing agent; *Treponema pallidum* subsp *endemicum* causes endemic syphilis (bejel); *Treponema pallidum* subsp *pertenue* causes yaws and *Treponema carateum* causes pinta (Radolf JD, 1991; Norris and Larsen, 1995).

T. pallidum subsp *pallidum* is the “most invasive of the pathogenic treponemes” as it “produces highly destructive lesions in almost any tissue of the body” (Radolf JD, 1991). “*T. pallidum* subsp *pertenue* and *endemicum* are intermediate in invasiveness” causing destructive lesions in only bones and soft tissue. *T. carateum* is the least invasive species causing only cutaneous disease (Radolf JD, 1991). These pathogenic treponemes are closely related in their morphology, their antigenic structure, by DNA homology and by their ability to adhere to mammalian cells. Furthermore, no metabolic, structural or virulence marker differences among the pathogenic treponemes have been found and their antigenic relatedness results in similar seropositive outcomes among patients infected with all pathogenic treponemes (Tramont EC, 2005). Despite the fact that all of the species are closely related in many ways, they do express immunological differences as shown in rabbit models in

which experimental treponematoses infection by one species induced complete protection against reinfection by the homologous species, but only partial protection against heterologous species. In addition, although there is no established reason for this, they cause distinctly different diseases (Tramont EC, 2005). Also differences are noted in the host range, modes of transmission and geographic distribution which assists in speciation and subspeciation (Chiu and Radolf, 1994).

Several non-pathogenic species have also been identified in humans. These are found in particular as part of the normal flora of the intestinal tract, the oral cavity and genitalia of male and female (Radolf JD, 1991). *Treponema paraluiscliviculi* is the only known animal pathogen among the treponema species (Chiu and Radolf, 1994).

(2.2) Historical Background of Syphilis

The origin of syphilis has been debated over the centuries, following its emergence in Europe in the late 15th century, coinciding with the return of Christopher Columbus from the New World. Three main theories have been proposed: the Columbian theory; the pre-Columbian theory and the mutation/virulence theory. The Columbian theory suggests that syphilis was imported from the New World by Christopher Columbus and his crew and then spread by mercenaries in the army of Charles VIII of France during his siege of Naples (Chiu and Radolf, 1994). The pre-Columbian theory on the other hand states that syphilis was well-established in Europe before the return of Columbus but had been confused with other diseases of similar symptomatology, such as leprosy (Baskan and Tunali, 2002). According to the mutation/virulence theory, syphilis has evolved out of yaws, a disease originating in Africa. Yaws is caused by *Treponema pertenue*. The earliest cases of yaws have been

described nearly 4400 years earlier than the first syphilis cases (Baskan and Tunali, 2002). The mutation/virulence theory postulates that *T. pertenue* became more virulent due to mutational events and became *T. pallidum* causing syphilis. These mutational events could perhaps have taken place in the New World. Columbus and his crew may have possibly brought this virulent strain from the New World, and due to the lack of resistance in the Europeans, it resulted in a rapid spread of the disease (Baskan and Tunali, 2002).

However, the disease had been established and became recognized as a distinct STD by the end of the 15th century. By the 16th century, syphilis had reached epidemic proportions in Europe and eventually spread across the world, earning its name “the great pox”. It then became highly endemic in all levels of European society (Chiu and Radolf, 1994). By the mid-19th century, “the cause, epidemiology and clinical manifestations of syphilis” had been established (Richard PH, 1842; Tramont EC, 2005). By the end of the 19th century, syphilis was perceived as an “imitator” since it could mimic the effects of other diseases (Baskan and Tunali, 2002). Syphilis continued to be an important global health problem throughout the 20th and into the 21st century. By the 20th century important milestones in syphilis research had been achieved. In 1905, Schaudinn and Hoffman discovered the *Treponema pallidum* spirochete and its association with syphilis (Schaudinn and Hoffman, 1905; Singh and Romanowski, 1999; Chiu and Radolf, 1994). They demonstrated the presence of “spirochetes in Giemsa-stained smears of fluid from secondary syphilitic lesions” (Schaudinn and Hoffman, 1905; Singh and Romanowski, 1999). The year 1906 saw the development of the first serodiagnostic test for syphilis by August von Wasserman. This paved the way for the development of other serologic tests for

diagnosing syphilis (Singh and Romanowski, 1999; Wassermann *et al.*, 1906). During the same year, Paul Ehrlich made his discovery of the first treatment for syphilis in the form of an arsenic containing compound (Tramont EC, 2005). In the 1940s, the efficacy of penicillin was demonstrated when Mahoney and colleagues (1943) had successfully treated the first four patients with primary syphilis using penicillin (Singh and Romanowski, 1999; Mahoney *et al.*, 1943). Today penicillin continues to remain the drug of choice for therapy of all stages of syphilis and no resistance to the drug appears to have developed (Chiu and Radolf, 1994).

(2.3) Epidemiology of Syphilis

In 1947, in the United States, the incidence of primary and secondary syphilis was 66.4 cases per 100 000 population. By 1956, these rates had declined to 3.9 cases per 100 000 (Nakashima *et al.*, 1996; Kilmarx and St. Louis, 1995). This was attributed to the availability of penicillin therapy, changes in sexual behavior and improved preventive measures. However since the 1950's the United States has had successive epidemics with peaks at approximately every 10 years. There has been an overall increase in the maximum rates during these epidemics. In the last major epidemic, the rates peaked in 1990 at 20.3 per 100 000 population, the highest rate since 1949 (Kilmarx and St. Louis, 1995). This was largely related to crack cocaine usage and the exchange of illegal drugs for sex. During the 1990's a national elimination program was initiated which had reduced the syphilis incidence rates to the lowest level recorded i.e. a rate of 2.5 cases per 100 000 population in the year 1999. (MMWR, 2001-a). However, this was not long lasting. Since the year 2000, the

number of syphilis cases, in the United States increased once again, mainly among men who have sex with men (MMWR, 2001-b, 2002 and 2003).

The World Health Organization estimated that 12 million new cases of syphilis had occurred worldwide in 1999 (Figure 1), most of which occurred in the developing countries (World Health Organization, 2001). It has been reported that syphilis occurs mainly among the poor and those who lack access to health care as well as those who have many sexual partners (Peeling and Hook, 2006).



Figure 1: Global distribution of syphilis cases, among adults, in 1999
(World Health Organization, 2001)

In South Africa, syphilis prevalence over the period 1997 to 2008, has shown a downward trend as was reported in the 2008 National Antenatal Sentinel HIV and Syphilis Prevalence Survey. The survey investigated syphilis prevalence among antenatal clinic attendees in South Africa (Figure 2). The results showed a decrease

among pregnant women presenting at public antenatal care clinics from more than 10% in 1997 to 1.9% in 2008.

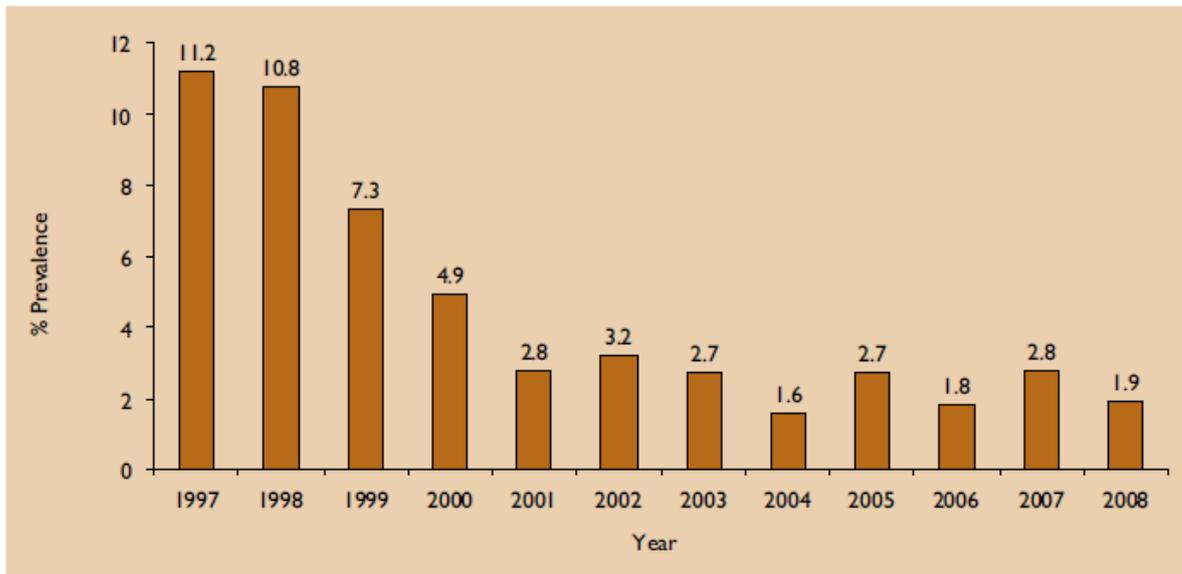


Figure 2: National syphilis prevalence trends among antenatal women, South Africa, 1997 to 2008 (National Department of Health, South Africa Report, 2008)

The syphilis prevalence among antenatal women by province in South Africa was also determined in the survey, for the period 2006 to 2008 (Table 1). In 2008, the Northern Cape showed with 6.8% the highest prevalence. This province has had high prevalences for the past three years: 6.9% in 2006, 5.4% in 2007 and 6.8% in 2008. Western Cape showed a significant increase in syphilis prevalence from 1.9% in 2006 to 5.6% in 2007 (the highest prevalence by province in South Africa recorded in that year). However, it decreased considerably to 3.8% in 2008. Similarly, Gauteng, Mpumalunga, Eastern Cape, North West and Limpopo all showed an increase in prevalence from 2006 to 2007, followed by a significant reduction by 2008. Only Kwazulu-Natal showed a continuous reduction in syphilis amongst first time antenatal clinic attendees from 2006 through to 2008 (1.0 in 2006, 0.8 in 2007 and 0.6 in 2008). In Free State the prevalence remained at 2.5% from 2006 to 2007 but declined slightly

to 2.3% in 2008. Northern Cape was the only province which showed an increase in prevalence in 2008 (National Department of Health, South Africa Report, 2008).

Province	RPR prev. 95% CI		RPR prev. 95% CI	
	2006	2007	2006	2008
National	1.8 (1.7–2.0)	2.8 (2.6–3.0)	1.9 (1.7–2.0)	1.9 (1.7–2.0)
Northern Cape	6.9 (5.0–8.9)	5.4 (4.2–6.9)	6.8 (5.2–8.7)	6.8 (5.2–8.7)
Western Cape	1.9 (1.4–2.4)	5.6 (4.9–6.3)	3.8 (3.1–4.6)	3.8 (3.1–4.6)
Gauteng	2.3 (1.9–2.7)	3.8 (3.3–4.3)	2.7 (2.3–3.1)	2.7 (2.3–3.1)
Free State	2.5 (1.8–3.1)	2.5 (1.9–3.0)	2.3 (1.7–3.1)	2.3 (1.7–3.1)
Mpumalanga	1.1 (0.7–1.5)	1.8 (1.3–2.4)	0.7 (0.4–1.2)	0.7 (0.4–1.2)
Eastern Cape	2.6 (2.0–3.1)	3.0 (2.5–3.6)	1.9 (1.5–2.4)	1.9 (1.5–2.4)
North West	1.8 (1.2–2.4)	2.7 (2.1–3.5)	1.5 (1.1–2.2)	1.5 (1.1–2.2)
KwaZulu-Natal	1.0 (0.8–1.4)	0.8 (0.6–1.1)	0.6 (0.4–0.8)	0.6 (0.4–0.8)
Limpopo	0.6 (0.4–0.9)	1.4 (1.1–1.9)	0.4 (0.3–0.7)	0.4 (0.3–0.7)

Table 1: Syphilis prevalence by province among first time antenatal attendees in South Africa from 2006 to 2008 (National Department of Health, South Africa Report, 2008)

The survey also looked at syphilis prevalence trends among first time antenatal attendees by age group for the years 2006 to 2008 (Figure 3). The results showed a decrease in prevalence from 2007 to 2008 in all age groups.

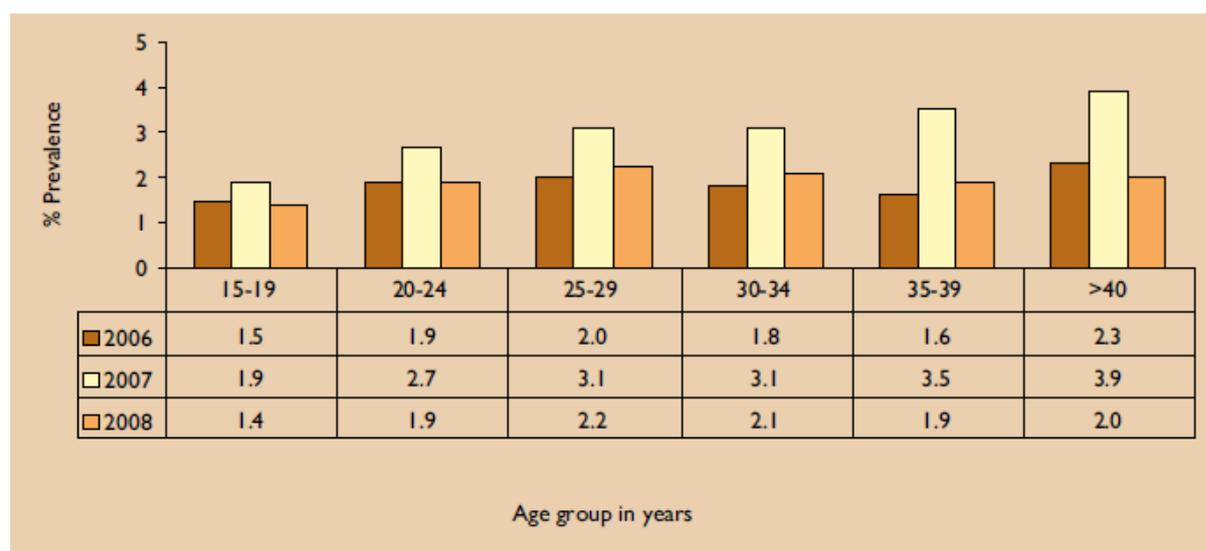


Figure 3: Syphilis prevalence trends among antenatal women by age group, South Africa, 2006 to 2008 (National Department of Health, South Africa Report, 2008).

One of the manifestations of STIs is genital ulcer disease (GUD). Genital ulcer disease can be the result of either bacterial or viral causative agents. Among the bacterial causes of GUD is *T. pallidum* which along with *H. ducreyi* (the cause of chancroid), used to be among the leading etiological agents of GUD, representing 70% - 90% of cases (Pham-Kanter *et al.*, 1996). However since the late 1980s the situation has markedly changed so that the relative importance of *T. pallidum* and *H. ducreyi* decreased significantly, whereas that of herpes simplex virus type 2 (HSV-2) increased.

Several researchers reported on the etiology of GUD in Africa. Most studies applied PCR techniques on secretions obtained from genital ulcers. These studies confirmed the decreasing prevalence of *T. pallidum* and *H. ducreyi* with a significant increase in HSV-2 prevalence.

A cohort study conducted by Moodley *et al.*, reported on the trends in the etiology of GUD in Durban, South Africa from the period, 1984 until 2004 (to be published). This study recruited GUD patients attending the largest clinic in Durban. “This 20-year period saw the introduction of syndromic management, the unfolding of the HIV epidemic and the introduction of improved diagnostics test systems” (Moodley *et al.*). The results showed that syphilis, chancroid and granuloma inguinale dominated during the first decade but decreased over time to be superceded by increasing prevalence in genital herpes, LGV and ulcers without diagnosis, in the later years. Similar trends were observed in HIV-infected and HIV-uninfected patients (Figure 4), with the exception of genital herpes. Genital herpes was the only disease that showed

a consistently higher prevalence in HIV-infected patients, indicating an association between this increase and HIV-1 infection (to be published).

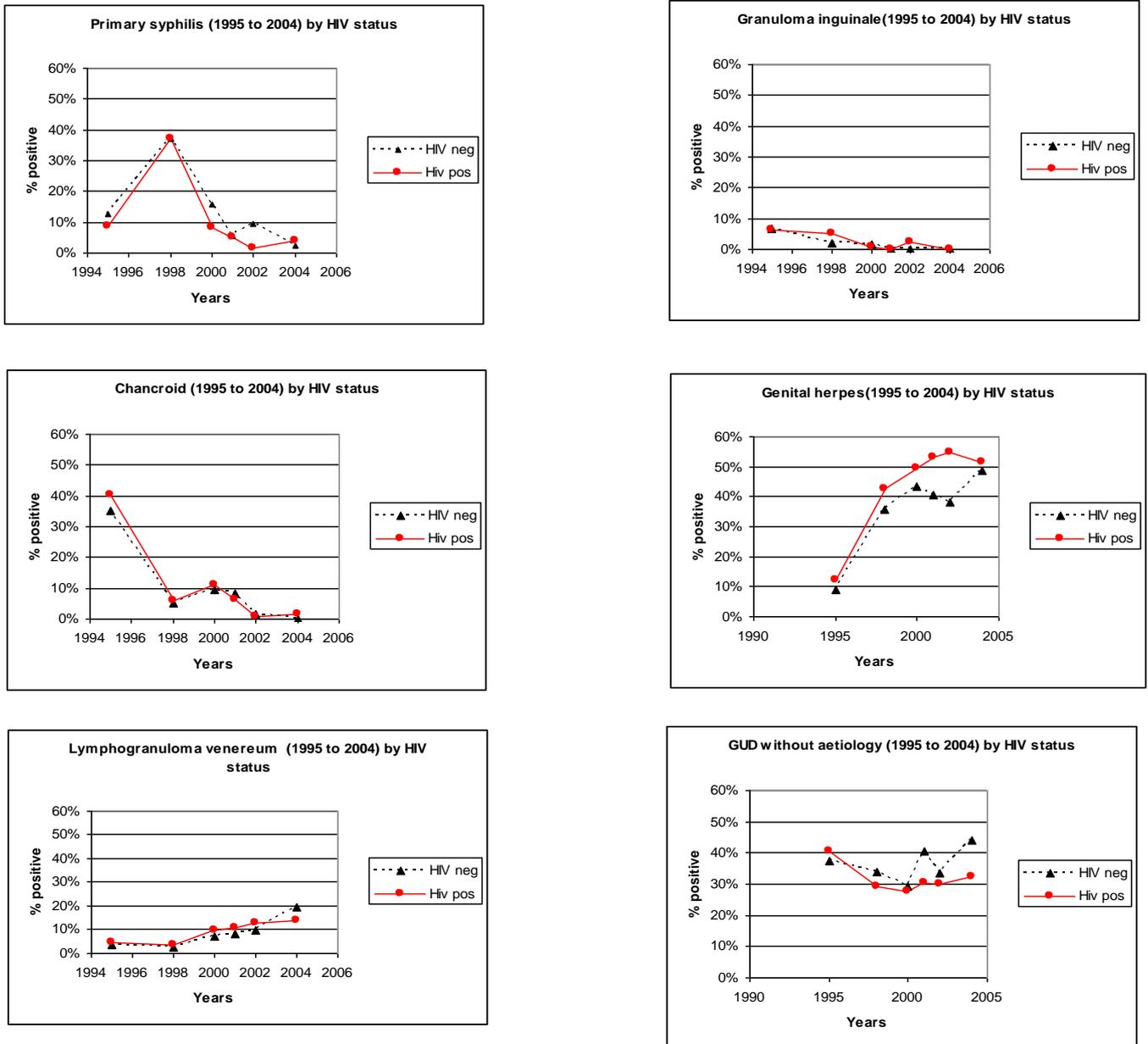


Figure 4: Trends in the aetiology of genital ulcer disease in HIV-1 infected and uninfected patients in KwaZulu-Natal for the period, 1995 to 2004 (Moodley *et al.*, to be published)

In a more recent study conducted on 301 patients with GUD in Dar es Salaam, Tanzania (2006), the authors reported the following figures: HSV-2 represented 83% of all identified pathogens, HSV-1 8%, *T. pallidum* 4% and *H. ducreyi* 5% (Nilsen *et al.*, 2007). This study among others (Chen *et al.*, 2000, Lai *et al.*, 2003), confirmed the decreasing levels of the bacterial causes of GUD with *T. pallidum* displaying the lowest prevalence recorded for that year.

The downward trend in bacterial causes of GUD has been attributed to effective implementation of syndromic management in public health facilities. Syndromic management for GUD does not include treatment for genital herpes. In addition, the awareness of HIV infection and hence the adoption of safer sexual practices may have further contributed to the decline.

(2.4) Structure and Morphology of *T. pallidum*

T. pallidum is a slim (0.15 μm), helical spirochete measuring 5 to 15 μm in length with regular spirals of a wavelength of 1 μm and an amplitude of about 0.3 μm (Sherris JC, 2004 and Ryan KJ, 2004). *T. pallidum* stains poorly with aniline dyes. However it can be readily seen by immunofluorescence darkfield microscopy or silver impregnation histologic techniques (Sherris JC, 2004). Viable *T. pallidum* “exhibits characteristic motility that consists of rapid rotation about its longitudinal axis and bending, flexing, and snapping about its full length” (Radolf JD, 1991).

Similarly with all spirochetes, *T. pallidum* consists of an outer membrane surrounding the periplasmic space, a peptidoglycan-cytoplasmic membrane complex, a protoplasm

cylinder and endoflagella (Radolf *et al.*, 1995). Because of this double-membrane ultrastructure, *T. pallidum* has been classified as a gram negative bacterium like *E. coli* (Radolf *et al.*, 1995). However several studies conducted by Radolf and colleagues have shown substantial differences between the outer membrane of *T. pallidum* and that of *E. coli*. They demonstrated that the outer membrane of *T. pallidum* lacks lipopolysaccharide (LPS), is far more fragile and appears to have a lower protein/lipid ratio when compared to the outer membrane of conventional gram negative bacteria. Furthermore its flagella (organelles of motility) are located within the periplasmic space and not extracellular as with conventional gram negative bacteria (Chiu and Radolf, 1994). Hence, these are named endoflagella.

The low protein/lipid content provides a possible explanation as to why specific antibodies present in sera from syphilis patients bind poorly to viable *T. pallidum*. This was initially attributed to a protective capsule or outer coat of proteins and mucopolysaccharides obtained from the host and located external to the organism's outer membrane (Chiu and Radolf, 1994). However freeze-fracture and deep-etch electron microscopy studies by Radolf and colleagues proved that the poor antibody binding of *T. pallidum* was a result of "a paucity of integral membrane proteins" (Radolf *et al.*, 1995) with only some of these proteins extending through the outer membrane to become surface exposed. Freeze-fracture electron microscopy has been instrumental in understanding the molecular architecture of the *T. pallidum* outer membrane. This technique visualizes proteins within cleaved lipid bilayers as being intramembranous particles (IMPs). Upon examination of the *T. pallidum* outer membrane, it revealed 100-fold less intramembranous particles as compared to conventional gram negative bacteria and that these particles are of uniform size

suggesting only a few different protein species present in the outer membrane (Radolf *et al.*, 1995). In contrast, fracture faces through the cytoplasmic membrane showed a greater particle density than its corresponding face of the outer membrane. It was estimated that the cytoplasmic membrane of *T. pallidum* has a ten fold greater density than the outer membrane. In addition, cytoplasmic membrane particles are randomly distributed and heterogenous in size thus suggesting a diversity of proteins. These studies reported that the bacterium's most immunogenic proteins are lipoproteins that are anchored to the cytoplasmic membrane thus rendering them inaccessible to antibodies directed against these molecules.

Because of this low density of membrane spanning proteins, these have been termed "Tromps", treponemal rare outer membrane proteins (Blanco *et al.*, 1990). It has been suggested that the low density of surface exposed protein antigens may permit *T. pallidum* to evade host immune defenses, thereby contributing to the pathogenesis of the organism (Radolf *et al.*, 1995).

(2.5) Transmission of *T. pallidum*

Transmission of syphilis occurs mainly by sexual intercourse with an individual who has primary or secondary syphilitic lesions. Late disease is however not infectious (Sherris JC, 1984). Other forms of sexual activity such as kissing, oral-genital and oral-anal contact may also transmit infection. Moreover, body fluids such as urine and semen may also harbor treponemes. Nonsexual modes of transmission such as blood transfusions, accidental direct inoculation and the sharing of needles among

intravenous drug users may also occur but are thought to be rare (Chiu and Radolf, 1994).

Transplacental transmission to the developing fetus may occur at any time during pregnancy, giving rise to “congenital syphilis”, which is analogous to secondary syphilis in the adult (Ryan KJ, 2004). Perinatal infection may also occur when the newborn comes into contact with an infectious lesion or through inoculation with maternal blood at the time of delivery (Chiu and Radolf, 1994).

(2.6) Pathogenesis of *T. pallidum*

Due to the inability to grow *T. pallidum in vitro*, it is difficult to obtain sufficient numbers of organisms to perform studies. Therefore, the mechanisms involved in the pathogenesis of syphilis are not clearly understood. Current information into the pathogenesis of the organism is based on observations of human disease and experiments conducted in animal (rabbit) models.

From these observations, it is presumed that *T. pallidum* enters its human host through small breaks in the skin or through penetration of intact mucous membranes where it slowly multiplies with little initial tissue reaction. It produces a lesion at the point of entry known as the chancre. In women, the lesion may occur “on the labia, the walls of the vagina or the cervix” (Radolf JD, 1991). In men, it occurs “on the shaft or glans of the penis” (Radolf JD, 1991). A chancre may also occur on the lips, tongue, tonsil, anus or other areas of the skin depending on the mode of transmission (Radolf JD, 1991).

Microscopically, syphilis is characterised by endarteritis. Endothelial cells within small arterioles, swell and proliferate, causing a reduction in local blood supply which further results in necrosis. The ulcerated primary lesion is termed the “chancre” which contains large amounts of spirochetes. Endarteritis is also accompanied by the infiltration of lymphocytes, monocytes and plasma cells at the site of infection (Ryan KJ, 2004).

Experiments conducted both on humans and rabbits using standard inocula, have shown that fewer than ten spirochetes are capable of causing infection (Chiu and Radolf, 1994). Animal studies have further demonstrated the potential of the organisms to appear within minutes in the lymph nodes and their ability to disseminate widely within hours (Cumberland and Turner, 1949; Raiziss and Severac, 1937).

Mechanisms describing the entry of *T. pallidum* into cells is still unclear. However, *in vitro* studies have demonstrated the organism’s ability to attach to mammalian cells (Fitzgerald *et al.*, 1975; Hayes *et al.*, 1977). In addition, these studies showed rapid invasion of cells, following minutes after cell inoculation. The authors suggest that the organism’s desire to enter cells may be an attempt to escape from an unfavourable environment. Alternatively, there may be a specific stage in treponemal growth that requires an intracellular residence (Fitzgerald *et al.*, 1975). Further *in vitro* studies have revealed that *T. pallidum* may contain receptors on its surface specific for fibronectin and other extracellular matrix components thus mediating treponemal attachment (Chiu and Radolf, 1994). It has also been suggested that attachment may occur via specific attachment ligands which may serve as potential vaccine candidates

(Baseman and Hayes, 1980; Thomas *et al.*, 1985). It has also been demonstrated that *T. pallidum* attaches to various mammalian tissue culture cell lines *in vitro* including lines derived from genitourinary epithelia, a property not shared by nonpathogenic treponemes (Chiu and Radolf, 1994).

An important feature of *T. pallidum* infection is its ability to disseminate away from its initial site of entry. To accomplish this, the organisms must be able to move through the viscous extracellular connective tissue matrix (ECM) that occurs between tissue cells (Radolf JD, 1991). According to Radolf, *T. pallidum* produces an enzyme that can degrade hyaluronic acid which is a major component of ECM (Radolf JD, 1991). Hematogenous dissemination occurs as well. This results in the organisms invasion of various organ systems, presumably by penetrating the endothelial cells lining small blood vessels (Chiu and Radolf, 1994). *In vitro* studies using cultured human endothelial cells have shown that *T. pallidum* rapidly traverses “between tight endothelial cell junctions”, indicative of its invasive properties (Chiu and Radolf, 1994). It suggests that tissue invasion may be a critical virulence factor for the organism.

There is a general belief that the organisms motility greatly contributes towards its invasiveness. The invasiveness of *T. pallidum* is best observed in congenital syphilis, where *T. pallidum* is one of few bacterial pathogens of humans capable of traversing the tissue barriers separating mother and fetus. Congenital infection occurs mainly by transplacental invasion, however other routes of fetal infection such as passage through the chorioamnion may also occur (Chiu and Radolf, 1994).

(2.7) Physiology - Growth and Metabolism

“Most of our knowledge of the physiology, metabolism and antigenic structure of *T. pallidum* is derived from the Nichols strain which has been maintained in rabbits since 1912” (Singh and Romanowski, 1999; Nichols and Hough, 1913). *T. pallidum* cannot survive and multiply outside the mammalian host (Lafond and Lukehart, 2006). Its infectious capability is lost within a few hours. Limited replication has been obtained with careful control of oxygen tension and pH in primary tissue culture systems, where Fieldsteel and colleagues were able to achieve 100-fold multiplication through several generations (Lafond and Lukehart, 2006; Fieldsteel *et al.*, 1981; Fieldsteel *et al.*, 1982; Ryan KJ, 2004). However, *T. pallidum* subsp *pallidum* cannot be cultured continuously *in vitro*. Instead it has to be propagated by intra-testicular inoculation of rabbits to obtain a sufficient number of organisms for research and diagnostic purposes.

T. pallidum multiplies by longitudinal, binary fission with a generation time of about 30 to 33 hours *in vivo* (Chiu and Radolf, 1994) and 30 to 50 hours *in vitro* (Lafond and Lukehart, 2006; Fraser *et al.*, 1998). There are several factors that may contribute to the organism’s slow replication rate. Firstly, *T. pallidum* has limited metabolic capacity. It lacks the tricarboxylic acid cycle and the electron transport chain related to that. Hence it depends solely on glycolysis for the synthesis of ATP (Lafond and Lukehart, 2006; Fraser *et al.*, 1998). Glycolysis produces a net yield of 2 ATP molecules as compared to an organism that undergoes aerobic respiration (eg. *E. coli*) and produces 38 ATP molecules resulting in a 19 times greater energy production per substrate equivalent as compared to that of *T. pallidum*. *T. pallidum*’s limited

metabolic capacity is explained by the bacterium's small genome size of 900 kilobase pairs, making it one of the smallest prokaryotic genomes (Chiu and Radolf, 1994). On analysis of its genome, it indicates that there is an absence of pathways for the utilization of alternative carbon sources for energy and *de novo* synthesis of enzymes and nucleotides (Fraser *et al.*, 1998). Furthermore, it lacks pathways for the synthesis of amino- and fatty acid, but carries the necessary enzymes required for interconversion of amino acid and fatty acids. Hence, it has been suggested that with the aid of specific transporters, the organism derives essential macromolecules from its host and then uses interconversion pathways to generate others (Fraser *et al.*, 1998).

Secondly, *T. pallidum* is microaerophilic and requires low levels of free oxygen (2 to 10%) (Sherris JC, 1984). Its sensitivity to oxygen is explained by the fact that *T. pallidum* lacks the enzymes catalase and superoxide dismutase, that detoxify reactive oxygen species (Fraser *et al.*, 1998). It is suggested that the organism derives protection against oxygen radicals from its host cells (Cox CD, 1983; Cox *et al.*, 1990).

Thirdly, *T. pallidum* is unstable at high temperatures as demonstrated by Julius Wagner von Jauregg in 1918, whereby he used heat therapy to treat for late neurosyphilis. Patients were inoculated with malaria-infected blood and later treated with quinine. This regimen resulted in elevated body temperature which appeared to have destroyed *T. pallidum* in the central nervous system. Similar effects were observed when alternative methods for raising body temperature were used (Wagner von Jauregg J, 1918; Lafond and Lukehart, 2006). Other conditions such as cold,

dessication as well as a wide range of detergents and disinfectants also inactivates the *T. pallidum* organism (Ryan KJ, 2004).

(2.8) Syphilis in Humans – Clinical Manifestations

The clinical manifestations of syphilis in an untreated individual is divided into several stages:

(2.8.1) Primary Syphilis

The first clinical manifestation of syphilis is a lesion that develops at the site of entry. It begins as an indurated, painless papule that progresses over one to several weeks to form an ulcer. The ulcer when fully developed with a clean, firm base and round discrete borders is termed the chancre (Chiu and Radolf, 1994) and can vary in size from a few millimetres to more than 2 cm (Ryan KJ, 2004). Following exposure, the primary chancre may develop on average after 3 weeks, with an incubation period ranging between 10 to 90 days (U.S. Department of Health, Education, and Welfare, 1968). The size of the inoculum determines the development of the lesion. The larger the inoculum size, the more rapid the lesion will develop. The primary chancre is highly infectious and full of spirochetes (Ryan KJ, 2004) thus making the primary stage a highly infectious stage.

In heterosexual males, chancres commonly occur on the penis or in the oral cavity. In homosexual males lesions can be localized in the rectum and anal canal. In women, chancres usually occur on the vulva, labia or cervix (Ryan KJ, 2004). Due to these inconspicuous locations in both women and homosexual men, primary chancres are not always apparent. Furthermore they are painless. Hence the diagnosis of syphilis

may be delayed until further disease manifestations are identified (Lafond and Lukehart, 2006). Diagnosis of syphilis can also prove complicated when chancres are not of its classic morphology. Non-indurated chancres with “irregular borders” have been observed (Chapel TA, 1978). If untreated, the lesion heals spontaneously after 4 to 6 weeks. However, in approximately 15% of patients, the lesion may remain present at the onset of secondary syphilis.

Some clinical manifestations of Primary Stage Syphilis

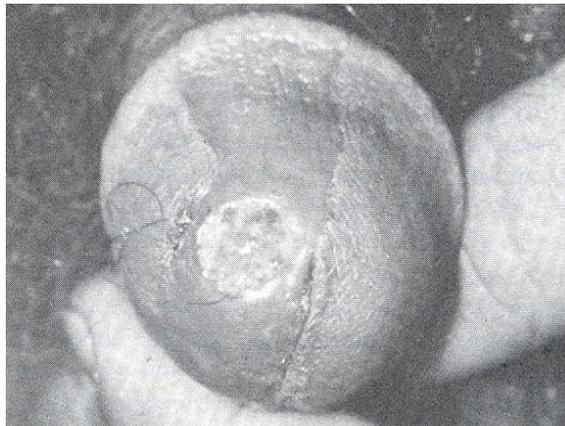


Figure 5: A syphilitic chancre on the glans penis. Corey, L. (1984) Sexually transmitted diseases p. 637-643. *In* Sherris, J.C. (ed.) Medical microbiology, An introduction to infectious diseases, Elsevier Science Publishing Co., Inc, New York

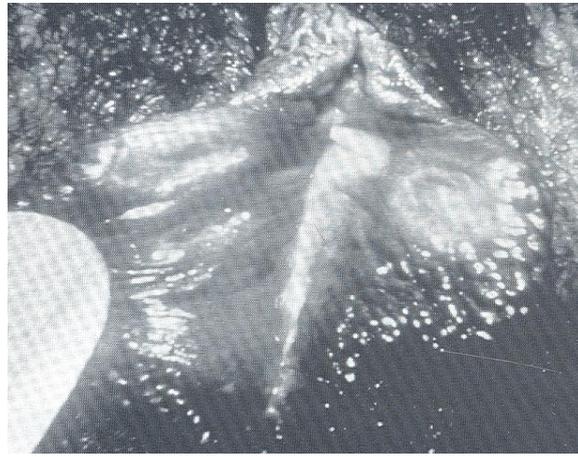


Figure 6: “Kissing” syphilitic chancres on the labia minora. Chiu and Radolf (1994) Syphilis p. 694-714. *In* Hoeprich *et al.*, (ed.) Infectious diseases, A treatise of infectious processes, 5th ed. J.B. Lippincott Company, Philadelphia

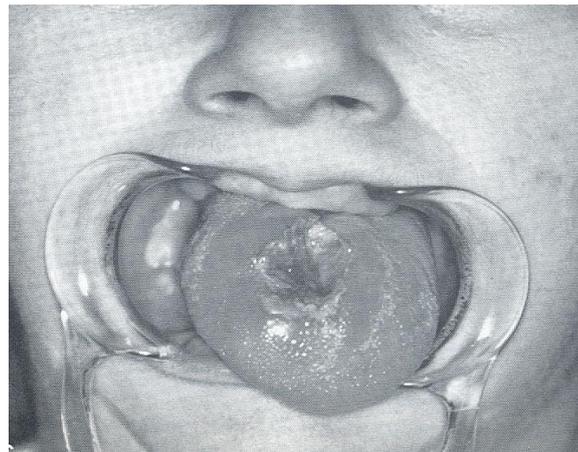


Figure 7: Chancre on the tongue. Chiu and Radolf (1994) Syphilis p. 694-714. *In* Hoeprich *et al.*, (ed.) Infectious diseases, A treatise of infectious processes, 5th ed. J.B. Lippincott Company, Philadelphia

(2.8.2) Secondary Syphilis

Two to eight weeks following the appearance of the chancre, clinical manifestations of secondary stage syphilis begin to appear. This stage results from the multiplication

and dissemination of *T. pallidum* from its initial site of infection to other host tissues (Ryan KJ, 2004). The most characteristic symptom of secondary syphilis is a widespread mucocutaneous rash. The rash is characterized by pale, distinct lesions that are distributed on the trunk and extremities including the palms, soles and face (Lafond and Lukehart, 2006). The lesions can be described as macular, maculopapular, papular, papulosquamous and pustular, with macular and maculopapular lesions predominating (Chiu and Radolf, 1994; Lafond and Lukehart, 2006). The lesions are symmetric, varying from several millimetres to several centimetres in diameter (Chiu and Radolf, 1994). Other manifestations of secondary syphilis include generalized nontender lymph node enlargement accompanied by fever, malaise and other manifestations of systemic infection (Ryan KJ, 2004).

Other mucocutaneous manifestations of secondary syphilis include patchy alopecia and thinning of the eyebrows and beard, diffuse redness of the tonsils and pharynx as well as superficial ulcers of the oral and genital mucosa. In addition, 10% of patients develop painless, mucosal warty erosions called condylomata lata in warm, moist areas such as the genitalia and perineum (Chapel TA, 1980; Mindel *et al.*, 1989).

Secondary syphilitic lesions are full of spirochetes and thus highly infectious (Ryan KJ, 2004). If untreated, the lesions heal over several weeks but may be accompanied by scarring or hyper- or hypopigmentation. In the majority of cases however, healing occurs without scarring (Singh and Romanowski, 1999). Secondary infection gets resolved in only one third of untreated patients via host immune responses. In the remaining two thirds of patients, the disease enters a latent state (Ryan KJ, 2004).

Some Clinical Manifestations of Secondary Stage Syphilis

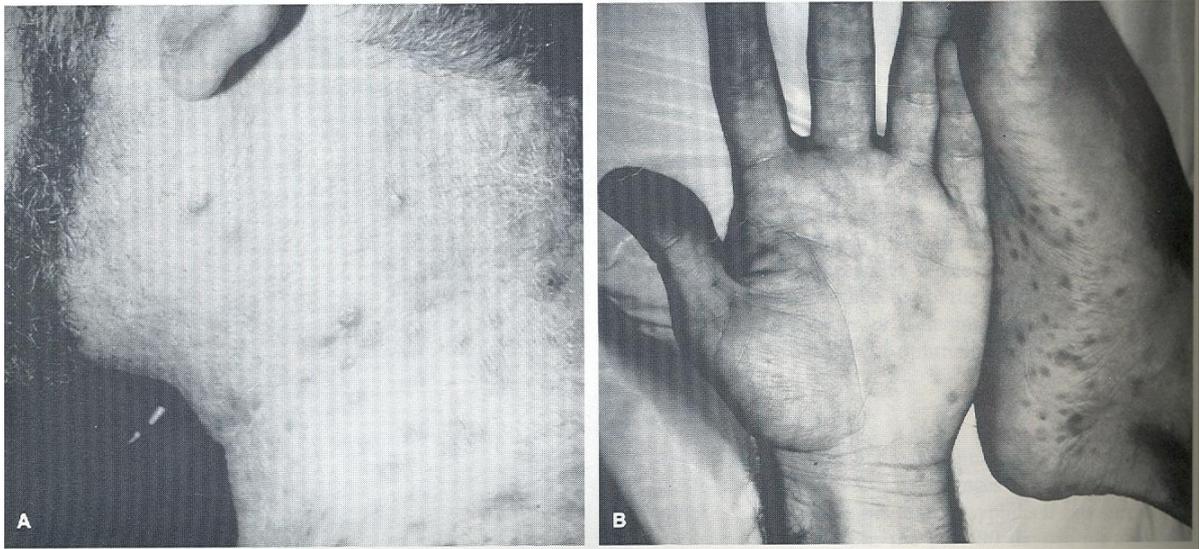


Figure 8: (A) Papulosquamous rash of secondary syphilis; (B) Papulosquamous lesions of palms and soles characteristic of secondary syphilis. Chiu and Radolf (1994) Syphilis p. 694-714, *In* Hoepfich *et al.*, (ed.) Infectious diseases, A treatise of infectious processes, 5th ed. J.B. Lippincott Company, Philadelphia

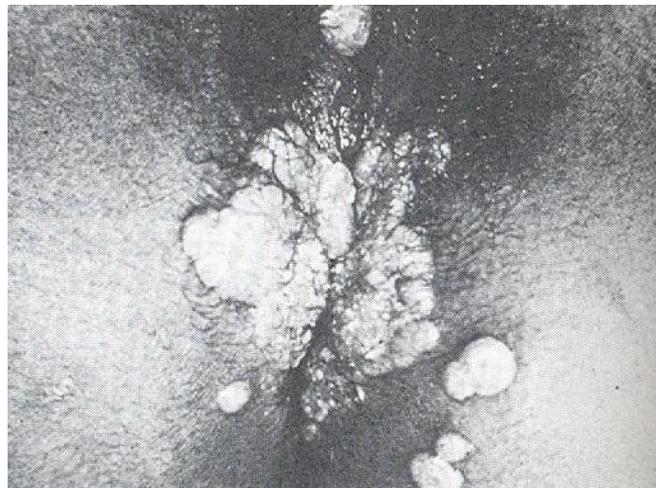


Figure 9: Condylomata lata, characteristic of secondary syphilis. Corey, L. (1984) Sexually transmitted diseases p. 637-643, *In* Sherris, J.C. (ed.) Medical microbiology, An introduction to infectious diseases, Elsevier Science Publishing Co., Inc, New York

(2.8.3) Latent Syphilis

During latent syphilis there are no clinical signs or symptoms or abnormalities in the CSF, however serologic test results are positive. It comprises two stages: early latent syphilis and late latent syphilis, based upon the approximation of the time of infection.

Early latent syphilis generally occurs within one year of infection. During early latency, approximately 25% of untreated patients may experience relapses of secondary syphilis as demonstrated by the Oslo study (Gjestland T, 1955).

Late latent syphilis occurs after one year of infection. During this stage, serologic testing is positive but sexual transmission is unlikely. In late latent syphilis, the patient harbours the infectious organisms in their spleen and lymph nodes (Radolf JD, 1991). Here relapses cease as the patient develops immunity to relapses and resistance to reinfection (Ryan KJ, 2004).

Transmission of *T. pallidum* during latency is possible from relapsing secondary lesions and through blood transfusions and mother-to-child transmissions. Latent syphilis ends when the patient has been treated appropriately or when symptoms of tertiary stage syphilis become apparent.

(2.8.4) Tertiary Syphilis

Approximately one third of untreated patients develop tertiary stage syphilis some 5 to 20 years after initial infection (Ryan KJ, 2004). Tertiary syphilis is a slow progressing, inflammatory stage with the potential to affect any tissue or organ in the body. Today with appropriate antibiotic treatment for syphilis, the late manifestations of tertiary syphilis are rarely seen. Tertiary syphilis is subdivided into neurosyphilis, cardiovascular syphilis and late benign gummatous syphilis. Cardiovascular and gummatous syphilis used to be more prevalent manifestations of tertiary syphilis before penicillin became available, however neurosyphilis is currently the dominant form of tertiary syphilis.

Neurosyphilis may manifest as meningovascular syphilis, general paresis or tabes dorsalis. Meningovascular syphilis involves gummatous and obliterative vascular changes of the meninges. This is associated with an increased number of cells and an increased protein concentration in the CSF as well as with focal neurologic changes (Corey L, 1984). In general paresis, there is extensive cortical degeneration of the brain with vast amounts of treponemes found in affected areas (Corey L, 1984). Other changes include reduced memory, hallucinations or psychosis (Corey L, 1984). In tabes dorsalis there is “demyelination of the posterior columns and dorsal roots and damage to dorsal root ganglia” (Corey L, 1984). The latter causes “ataxia, wide-based gait, foot slap and loss of the sensations of position, pain and temperature” (Corey L, 1984).

Cardiovascular syphilis is mainly the result of “local inflammation induced by the multiplication of treponemes within the wall of the thoracic aorta” (Radolf JD, 1991). The most common manifestation of cardiovascular syphilis is aortitis. The aortitis may be asymptomatic and is “detected on chest radiograph by linear calcification or widening of the aorta” (Chiu and Radolf, 1994). There are three major symptomatic forms of cardiovascular syphilis. These include aortic insufficiency, coronary ostial stenosis and aneurysm. Aortic insufficiency is the result of dilation of the aortic root, whilst angina is the most common manifestation of coronary ostial stenosis (Chiu and Radolf, 1994). Aneurysms are associated with a variety of syndromes including “aortic valve incompetence, pressure necrosis of structures adjacent to the aorta or rupture of the aorta” thus resulting in sudden death (Corey L, 1984; meddean.luc.edu/lumen/Meded/mech/cases/case21/syphilis.htm)

A localized, granulomatous reaction to *T. pallidum* infection is referred to as a gumma. Gummas are highly destructive and commonly occur in skin, bones and joints but can potentially affect any organ. It manifests similarly to that of other mass-producing tissue lesions such as tumours. Diagnosis of a gumma involves serologic evidence of the disease along with its characteristic histologic features (Corey L, 1984).

(2.8.5) Congenital Syphilis

Congenital syphilis refers to the transplacental transmission of *T. pallidum* from an infected mother to her developing fetus. Symptoms of congenital syphilis are analogous to that of secondary syphilis in an adult (Ryan KJ, 2004). Congenital

syphilis may occur at any stage in pregnancy with the risk of transmission to the unborn being greater during early maternal syphilis than in the later stages (Sheffield *et al.*, 2002). If infected pregnant women are treated appropriately during their first two trimesters of pregnancy, the possibility of negative outcomes will be reduced. On the other hand, late or inadequate treatment could result in spontaneous abortion, fetal damage, fetal death, stillbirth or disease in the infant (Ryan KJ, 2004; Lukehart and Lafond, 2006).

(2.9) Treatment for Syphilis

Since its introduction in the late 1940s, till today, penicillin remains the preferred antibiotic for treating all stages of syphilis including congenital syphilis (Chiu and Radolf, 1994). *In vitro* and *in vivo* animal studies have demonstrated the exquisite susceptibility of *T. pallidum* to this agent. The bacterium is killed by low levels of penicillin G. A concentration of 0.03 IU/mL (0.018 µg/mL) of penicillin is regarded therapeutic (Chiu and Radolf, 1994). However, the preparations used, the dosage and duration of treatment depend on the clinical stage and manifestations of the disease (Kinghorn GR, 1999). No resistance of *T. pallidum* to penicillin appears to have developed.

Health care facilities in developing countries often lack the necessary equipment and trained laboratory personnel to make a diagnosis of syphilis on-site. To overcome this, a syndrome-based approach to the management of STIs has been introduced and implemented across several developing countries. The syndromic management approach is based on the identification of a syndrome i.e. a group of symptoms a

patient complains of along with easily recognizable signs upon clinical examination. If an STI syndrome is identified combination treatment is provided that covers the majority of organisms responsible for that syndrome in a particular geographical area (Altini and Coetzee, 2005, Vuylsteke B, 2004). For example, a patient whom clinically presents with a genital ulcer will be treated according to syndromic management guidelines for genital ulcers. This includes treatment for primary syphilis. Later stages of syphilis do not present as part of an STI syndrome and are therefore not covered by the syndromic management approach.

(2.10) Laboratory Diagnosis of Syphilis

An accurate diagnosis of syphilis has proven complicated due to the inability to culture *T. pallidum in vitro* or stain it with simple laboratory stains. Furthermore, specimen source from latent and late stages are not available since lesions are absent (Karp *et al.*, 2009). Over the years, scientists, worldwide, have sought for alternative laboratory methods to identify syphilitic infection in the various stages of disease. Various methods have been established up to date. Clinical symptoms, the identification of *T. pallidum* in lesion exudate and antibody detection tests form the basis for diagnosing syphilis (World Health Organization, 1985).

(2.10.1) Darkfield Microscopy

“In many cases, clinical manifestations are highly characteristic” (Radolf JD, 1991). If one or more lesions are apparent, then darkfield microscopy can be used to visualize viable treponemes within lesion exudate (Radolf JD, 1991). Since its emergence in the 1920s, darkfield microscopy has proven to be the most rapid and

direct means of identifying treponemes within exudate obtained from lesions of patients with primary, secondary or early congenital syphilitic infection (Chiu and Radolf, 1994; Tramont EC, 2005). It is at these stages of a syphilitic infection that treponemes in lesions are in abundance and darkfield examination is most successful. *T. pallidum* subsp *pallidum* has characteristic morphology and motion that distinguishes it from other spiral organisms. It has a corkscrew appearance and moves in a spiralling motion with a 90° undulation about its midpoint (Tramont EC, 2005). Prepared slides should be viewed immediately after specimen collection for the presence of motile treponemes where the identification of a single treponeme is sufficient for diagnosis (Chiu and Radolf, 1994). The organisms lose motility if viewing of the slides is delayed. *T. pallidum* subsp *pallidum* cannot be distinguished from the other pathogenic subspecies by means of microscopy (Creighton ET, 1990; Larsen *et al.*, 1995). Furthermore, darkfield microscopy is not suitable for examining specimens obtained from mucosal surfaces (from oral and anal lesions) due to the difficulty in distinguishing *T. pallidum* from commensal treponemes present in these cavities (Chiu and Radolf, 1994).

Preparations should be viewed for at least ten minutes before being considered negative (Chiu and Radolf, 1994). Positive identification of treponemes upon darkfield examination provides a “specific and immediate diagnosis of syphilis” (Larsen *et al.*, 1995). However, a negative examination does not exclude the diagnosis of syphilis. It is possible that too few organisms are present in the specimen to be detected due to the lesion being in the healing stage of infection or the treponeme may have altered by systemic or topical disease treatment (Larsen *et al.*, 1995). In addition, the presence of numerous erythrocytes, air bubbles or tissue

fragments may obscure visualization of treponemes and the darkfield specimen will have to be reported as unsatisfactory (Larsen *et al.*, 1995). Furthermore, an inexperienced observer can be easily misled by the presence of artifacts such as cotton fibres and Brownian motion (Larsen *et al.*, 1995). Hence, darkfield microscopy requires considerable skill and experience to make an accurate diagnosis. It has been reported that the sensitivity of darkfield microscopy approaches 80% (Daniels and Ferneyhough, 1977; Larsen *et al.*, 1995).



Figure 10: The presence of treponemes as viewed by darkfield microscopy. (CDC guidelines depts.washington.edu/nnpts/online_training/std_handbook)

(2.10.2) Animal Infectivity Testing

Animal infectivity testing is the oldest and most sensitive method for the detection of *T. pallidum* infection in clinical specimens. It is considered the gold standard for determining the sensitivity of other detection methods including PCR (Grimprel *et al.*, 1991; Sanchez *et al.*, 1993; Lukehart *et al.*, 1988). Numerous animals from hamsters

to chimpanzees were used for maintaining treponemes or to determine infectivity. However not all these animals developed noticeable signs of infection or reactive serologic tests (Larsen *et al.*, 1995; Wilcox and Guthe, 1966) to proceed further. Among these, the rabbit proved to be most practical animal for maintaining treponemes and to determine its development of infection. In addition, a syphilitic lesion is produced at the site of inoculation in the rabbit.

(2.10.2.1) Rabbit Infectivity Testing (RIT)

Rabbit infectivity testing (RIT) became the gold standard for detecting *T. pallidum* from clinical specimens, to which other methods are compared. The sample, either CSF or whole blood is inoculated (within 30 minutes of collection) into rabbits intratesticularly and then monitored for the development of orchitis with treponemes visible in testicular extract (Chiu and Radolf, 1994). In rabbits that have not developed orchitis yet, serum samples are subjected to serologic testing for syphilis at monthly intervals. If the rabbit fails to seroconvert after 90 days, the RIT is negative. Rabbits that seroconvert are sacrificed and its testes extracted and minced. The presence of treponemes in this extract is indicative of a positive RIT (Chiu and Radolf, 1994). If there are no treponemes seen, a second rabbit should be inoculated with the testicular extract of the first one (Chiu and Radolf, 1994).

RIT is highly sensitive, and can approach 100% if the number of treponemes exceeds 23 (Magnuson *et al.*, 1956). However, RIT is cumbersome, expensive and requires 3 to 6 months to complete (Larsen *et al.*, 1995; Singh and Romanowski, 1999). Hence, this method proves impractical for routine clinical use.

(2.10.3) Serologic Tests for the Diagnosis of Syphilis

Serological tests provide a more practical method for diagnosing syphilis and provide the only means for identifying asymptotically infected persons (Radolf JD, 1991). Over the years, numerous serological tests have been developed that aim to detect one of two different types of antibodies. Therefore two categories of serological tests are recognised: **nontreponemal tests**, that detect antibodies to a combination of cardiolipin with cholesterol and lecithin and **treponemal tests** that detect antibodies directed to specific *T. pallidum* antigens. Both types of tests should be performed when diagnosing syphilis as the treponemal tests serve as the confirmatory tests to positive nontreponemal test reactions.

(2.10.3.1) History of the Development of Serologic Tests for Syphilis

The year 1906 saw a major breakthrough in treponemal research with the development of the first serologic test for syphilis called the Wasserman test, an adaptation of the complement fixation test that was discovered by Bordet and Gengou in 1901 (Wasserman *et al.*, 1906). Liver extract obtained from newborns whom had died from congenital syphilis, was used as the antigen in this test. The Wassermann antigen was initially considered to be specific but it was later revealed that other tissues such as beef heart extract could also serve as antigen (Eagle H, 1937; Larsen *et al.*, 1995). The addition of cholesterol and lecithin increased the sensitivity of the antigens (Eagle H, 1937; Larsen *et al.*, 1995). The complement fixation tests had contributed significantly towards the diagnosis of syphilis, but was accompanied with

some drawbacks as well. The test was difficult to perform, required the use of several reagents and a period of 24 hours to complete.

This test was later followed by the precipitation test as developed by Michaelis in 1907 (Michaelis L, 1907) and Meinicke in 1917 (Meinicke E, 1917). These tests used watery and sodium chloride extracts of syphilitic livers, respectively. These tests did not rely on complement.

Kahn, in 1922, introduced a flocculation test that did not need complement and could be viewed within a few hours macroscopically (Kahn RL, 1922). This test underwent several modifications each time improving its sensitivity and specificity. However, this test used crude extract of tissue which varied in quality, hence making standardization difficult.

In 1941, Pangborn isolated the active antigenic component from beef heart, a phospholipid called cardiolipin (Pangborn MC, 1941). The combination of cardiolipin with lecithin and cholesterol, resulted in the formation of a serologically active antigen for detecting syphilis antibodies. Furthermore, the antigen could be standardized both chemically and serologically thus ensuring greater reproducibility of test results (Rudolph and Larsen, 1993).

(2.10.3.2) Nontreponemal Tests

The earliest cardiolipin antigens used to measure syphilis antibodies were crude extracts prepared from beef livers or beef hearts, as mentioned earlier. However this

often produced false positive reactions. Today with the use of the pure preparation of the cardiolipin-cholesterol-lecithin complex, fewer false positive reactions are obtained (Tramont EC, 2005). It is believed that these lipids are derived from the host and become integrated with the *T. pallidum* membrane during their interaction in infection. Antibodies directed to this lipid complex are termed, “reagin” (Ryan KJ, 2004). But note, that the cardiolipin complex is not exclusive to *T. pallidum* infection. Reagin antibodies may be produced in response to other treponemal diseases as well as nontreponemal diseases resulting in tissue damage (Catterall RD, 1972; Ryan KJ, 2004). Hence, due to the non-specific nature of these antibodies, a reactive nontreponemal test itself cannot be used to provide a definite diagnosis of *T. pallidum* infection. Further serological evidence is required.

Nontreponemal tests serve two purposes: Firstly, they are the primary screening tests for patients with suspected syphilis. Secondly, serial measurement of nontreponemal antibody titres is done to assess disease activity and response to treatment (Chiu and Radolf, 1994). This is because the height of the antibody titre is directly related to activity of disease. Reactivity to nontreponemal tests develops only 1 to 4 weeks following the appearance of the primary syphilitic chancre (Morton RS, 2004; Kinghorn GR, 1999). Secondary syphilis is accompanied by the highest titres (Morton RS, 2004; Kinghorn GR, 1999).

The most commonly used nontreponemal tests are the Venereal Disease Research Laboratory (VDRL) test, the Rapid Plasma Reagin (RPR) test and the Reagin Screen Test (RST).

The VDRL slide test is the standard nontreponemal test, in which heated serum (56°C) is tested for its ability to flocculate cardiolipin-cholesterol-lecithin antigen suspension (Tramont EC, 2005). This test can be rapidly performed, is reproducible and provides acceptable levels of sensitivity and specificity (Harris *et al.*, 1946). It is now most often used to monitor a patient's response to treatment (Tramont EC, 2005). Modifications of the VDRL for routine screening of syphilis include the unheated serum reagin (USR) test, the rapid plasma reagin (RPR, 18mm-circle) card test, its automated version, the automated reagin test (ART), the reagin screen test (RST) and the toluidine red unheated syphilis test (TRUST).

In each of these tests, reagin antibody is detected by measuring the flocculation or agglutination of the antigen suspension. In the VDRL and USR tests, antigen-antibody flocculation is observed with a microscope whilst other tests view agglutination macroscopically. The RPR and TRUST further use charcoal and red paint pigment respectively, added to the USR reagent to enhance visualization of the antigen-antibody agglutination. These reactions can therefore be viewed macroscopically (Kinghorn GR, 1999; Creighton ET, 1990; Larsen SA, 1990; and Larsen *et al.*, 1984).

Of these macroscopic tests, the RPR is most widely used. It has several advantages as compared to the VDRL. These are "antigen stability, the ability to use plasma instead of serum, and macroscopic observation" (McGrew *et al.*, 1968; Lafond and Lukehart, 1995). Nevertheless, the VDRL is still in use within certain laboratories. The sensitivities of the RPR and VDRL vary among the different stages of disease. The tests are negative upon the appearance of the primary chancre, i.e. disease of short

duration. Following several weeks of infection, the tests provide positive readings. The reported mean sensitivities of RPR and VDRL during primary syphilis are 86% and 78% respectively, whilst the sensitivities of both tests during secondary syphilis reaches 100% (Larsen *et al.*, 1995).

Nontreponemal test results are positive early in infection but revert back to normal following successful treatment. Hence, it allows one to monitor the duration of the infection and response to treatment thereafter.

In summary, nontreponemal tests are cost-effective, simple and easy to perform and sensitive. They can be used to screen large amounts of sera in areas of high prevalence as well as to monitor duration of infection and treatment status.

However, since they detect antibodies to the cardiolipin complex they are non-specific and may result in false positives due to cross-reacting antibodies in patients with viral infections (Epstein–Barr, hepatitis, varicella, measles), leprosy, lymphoma, tuberculosis, malaria, endocarditis, connective tissue disease and liver disease. False positive results can occasionally occur in pregnant women, HIV infected patients, intravenous drug use or contamination (Ryan KJ, 2004; Chiu and Radolph, 1994). Hence, if positive, they must be confirmed by one or more specific treponemal tests. Also, false negatives may occur with undiluted sera due to an excess of antibody or the presence of blocking antibodies, known as the “prozone effect” (Chiu and Radolph, 1994; Kinghorn GR, 1999; Jurado *et al.*, 1993).

(2.10.3.3) Treponemal Tests

Treponemal tests are specific to *T. pallidum* since they detect antibodies directed to *T. pallidum* antigens (Ryan KJ, 2004). They are used to confirm positive nontreponemal test results of presumed syphilitic patients. The most commonly used treponemal tests are haemagglutination tests and immunofluorescence tests.

(a) *T. pallidum* Immobilization (TPI) test

The year 1949, saw the development of the first treponemal antibody test, by Nelson and Mayer, which they called, the *T. pallidum* immobilization (TPI) test (Nelson and Mayer, 1949). This test uses testicular tissue extract of rabbits, containing viable *T. pallidum* (Nichols strain) as its antigen. It “is based on the ability of patient’s antibody and complement to immobilize living treponemes, as observed by darkfield microscopy” (Larsen *et al.*, 1995). The TPI test was initially considered the standard treponemal test in the United States (Larsen *et al.*, 1984; Larsen *et al.*, 1995) and it was specific. However, the test proved complicated, technically difficult, expensive and time-consuming (Larsen *et al.*, 1995). By the 1970s, the test was found to be less-sensitive and specific (Hederstedt B, 1976; Rein *et al.*, 1980) when compared to other treponemal tests. Nevertheless, the TPI test continues to be used in the United States but in research laboratories only (Larsen *et al.*, 1995).

(b) Fluorescent-Antibody Tests

The fluorescent treponemal antibody (FTA) test, introduced in 1957 was a major breakthrough in the quest for developing more efficient treponemal antigen tests (Deacon *et al.*, 1957). This test required that the patient's serum sample be diluted 1:5 in saline solution and then reacted with a suspension of killed treponemes that were fixed onto a slide. This was followed with a fluorescein labelled anti-human immunoglobulin. The slides are examined under a microscope with UV light whereby fluorescence of the treponemes indicates the presence of anti-treponemal antibodies in patients' sera. Non-specific reactions were encountered in this FTA test due to the occurrence of cross reaction with common antigens shared by both *T. pallidum* and non-pathogenic treponemes that form part of the normal flora in humans (Deacon and Hunter, 1962). Hence, the FTA-test was superceded by the more specific and sensitive FTA absorption (FTA-ABS) test whereby the serum sample is first diluted in a sorbent made from the nonpathogenic Reiter treponeme, for the removal of nonspecific anti-spirochetal antibodies often found in normal serum (Hunter *et al.*, 1964; Deacon and Hunter, 1962; Ryan KJ, 2004).

Today, the FTA-ABS and the FTA-ABS double-staining test that requires the use of incident illumination microscopes (Hunter *et al.*, 1979) continue to remain standard treponemal tests for syphilis (Larsen *et al.*, 1995). However, since the FTA-ABS requires fluorescence microscopy, it requires this expensive piece of equipment as well as trained personnel. A further disadvantage to this test is that it is standardized at one serum dilution (1:5) and as with most immunofluorescence tests, its interpretation can be subjective. It therefore requires great attention to detail, is

difficult to standardize from one laboratory to another and is difficult to quantify (Tramont EC, 2005). Hence, the search for reliable, efficient yet inexpensive treponemal tests continued.

(c) Haemagglutination Tests

In 1965, Rathlev reported the first of the haemagglutination techniques to the serologic diagnosis of syphilis (Rathlev T, 1965). In her study, she used formalinized, tannine treated sheep erythrocytes that were sensitized with ultra-sonicated Nichols strain derived antigen. If treponemal antibodies are present in the patient's sera, it resulted in agglutination of the sensitized erythrocytes.

Modifications to Rathlev's method later led to the development of other haemagglutination tests such as the microhemaagglutination assay for antibodies to *T. pallidum* (MHA-TP), HATTS and the *Treponema pallidum* haemagglutination assay (TPHA). These tests also measure the ability of sera to agglutinate erythrocytes that were previously sensitized with *T. pallidum* sonicates. Sheep erythrocytes are used in the MHA-TP test (Tomizawa and Kasamatsu, 1966), turkey erythrocytes in the HATTS test (Wentworth *et al.*, 1978) and fowl erythrocytes in the TPHA test (ImmuTrep TPHA, Omega Diagnostics). These tests are performed in a microtitre plate.

The FTA-ABS test is still more sensitive, however the haemagglutination tests are cheaper and easier to perform and as result, most laboratories continue to perform haemagglutination tests for routine work.

In summary, treponemal tests are generally more specific and sensitive than the nontreponemal tests, with the FTA-ABS test being highly sensitive in all stages of syphilis (Jaffe HW, 1984). The haemagglutination tests are however easier and cheaper to perform than the fluorescent-antibody tests and therefore most laboratories use these tests routinely. None of these tests provide information regarding disease activity.

Treponemal tests do have some disadvantages. Firstly, they are costly and technically more difficult to perform than nontreponemal tests. Secondly treponemal tests cannot distinguish between an active and a past treated infection. This is because antibodies produced in response to specific treponemal antigens persist for years in the individual (World Health Organization, SDI, 2006), hence causing test results to remain positive for years, if not for life (Ryan KJ, 2004). Therefore it cannot be used to monitor response to treatment. Thirdly, most treponemal tests take 1-2 hours or more to obtain results. Lastly, although specific for syphilis, it has been reported that treponemal tests are falsely reactive in approximately 1% of healthy people who have no history or clinical findings of syphilis (Goldman and Lantz, 1971). The inability to completely remove antibodies against commensal spirochetes is suggested to be the major cause for these falsely reactive treponemal tests (Chiu and Radolf, 1994).

(2.10.4) Molecular-based Methods for the Diagnosis of Syphilis

(2.10.4.1) Polymerase Chain Reaction (PCR)

Up until now, PCR has not yet been standardized for use in the routine clinical diagnosis of syphilis. However, several research groups have designed primer

sequences against genes coding for membrane lipoproteins of *T. pallidum*. These genes are conserved in *T. pallidum* and therefore provide specific PCR targets (Larsen *et al.*, 1995).

In 1991, they amplified the 658 bp gene encoding the 47 kDa surface protein, a lipoprotein that is genetically dominant in the human immune response to *T. pallidum* infection (Jones *et al.*, 1984). They performed the test on clinical specimens such as “amniotic fluid, neonatal sera and neonatal CSF” (Jones *et al.*, 1984; Larsen *et al.*, 1995) for diagnosis of congenital syphilis in neonates. When these results were verified with that of RIT, it produced an overall sensitivity of 78% (Grimprel *et al.*, 1991; Sanchez *et al.*, 1993). It has been suggested that this lack of sensitivity may be attributed to nonspecific inhibitors of the PCR reaction. Attempts were then made to eliminate PCR inhibition encountered in clinical specimens, by investigating alternative methods for isolating DNA from clinical samples (Grimprel *et al.*, 1991).

Noordhoek *et al* reported on the use of their PCR to diagnose neurosyphilis, by detecting the presence of treponemes in small volumes of CSF. They initially amplified the gene encoding a 39 kDa membrane protein, then continued with second nested PCR primers since the initial primers from this gene showed a lack of specificity (Noordhoek *et al.*, 1991).

A further difficulty was encountered analogous to that seen with serodiagnostic testing of neurosyphilis. It could not differentiate between patients with an active or past infection. Patients who were previously infected with *T. pallidum* and had sought treatment still came up positive on PCR. At present, PCR cannot differentiate

between the presence of a small number of viable treponemes and that of dead treponemes whose DNA can yet still be amplified (Larsen *et al.*, 1995).

The amplification of other genes used in the detection of *T. pallidum* in specimens include segments of the TmpA (a 42 kDa membrane protein) and the 4 D antigen (an oligomeric protein that has multiple forms) (Hay *et al.*, 1990). However, the PCR had not been evaluated against known serologic tests hence “the reported sensitivity could not be compared with that of standard clinical methods” (Larsen *et al.*, 1995).

(2.10.4.2) The Use of Recombinant Antigens in Serologic Tests

More recently, researchers have attempted to clone and express specific *T. pallidum* genes coding for antigens for direct use in serologic tests for syphilis. These tests are mainly in the form of an ELISA or Western blot. The majority of the cloned antigens to date appear to be membrane-associated lipoproteins that are highly antigenic. The lipid component serves as a hapten that increases antigenicity of that protein (Schouls LM, 1992).

Prior to recombinancy, two major drawbacks were encountered in the development of serologic tests using specific *T. pallidum* antigens. The first was the limited number of treponemes available following cultivation in rabbits, resulting in insufficient quantities of recovered treponemal protein for use in the test (Larsen *et al.*, 1995).

With recombinant DNA technology, large quantities of recombinant treponemal proteins can be expressed in *E. coli* but a major problem encountered here was

purification. The recombinant proteins had to be purified before use in order to eliminate background response due to antibodies directed against *E. coli* found in normal human sera (Larsen *et al.*, 1995). This however, was costly. An alternative was that the sera be absorbed with *E. coli* to remove anti-*E. coli* antibodies before use. However, this was not consistently reproducible thus leading to variations in background (Larsen *et al.*, 1995).

The second drawback encountered in the development of specific *T. pallidum* antigen tests was cross-reactivity with commensal spirochetes. Cross-reactivity was also observed with some “specific treponemal proteins because of reactivity to the lipid component of *T. pallidum* membrane lipoproteins” (Communicable Disease Centre, 1964). This problem could be overcome by the use of nonacylated recombinant fusion proteins that could eliminate any nonspecific reactivity (Centres for Disease Control, 1988; Sanchez *et al.*, 1993). This may however result in some loss in sensitivity as well.

The TmpA membrane lipoprotein was the first antigen to be cloned for use in a serologic test for the diagnosis of syphilis (Schouls *et al.*, 1989). The TmpA gene encoding a 42 kDa protein was used in the development of an ELISA for syphilis and was shown to be both sensitive and specific (Schouls *et al.*, 1989). Its sensitivity was compared to the TPHA and FTA-ABS serological tests for syphilis, to which the results were almost equal (Schouls *et al.*, 1989; Ijesslmuiden *et al.*, 1989). The authors further reported of a significant correlation between the anti-TmpA antibody titer and the VDRL titre for sera obtained from treated syphilitic patients. Based on

these findings, the authors suggested that this test could prove significant in monitoring treatment status (Schouls *et al.*, 1989; Ijesslmuiden *et al.*, 1989).

Since then, several other recombinant proteins have been cloned and used towards the development of ELISA serodiagnostic tests for syphilis. Among these are the 34 kDa membrane protein TmpB (Schouls *et al.*, 1989) and the 35 kDa membrane protein TmpC (Schouls *et al.*, 1991) as well as proteins secreted from the cytoplasm (Norris *et al.*, 1993; Strugnell *et al.*, 1990).

Extensive research has been done on the 47 kDa penicillin-binding protein, which is abundantly produced by *T. pallidum* (Jones *et al.*, 1984). This protein is immunodominant and does not cross-react with antibodies against commensal spirochetes. An ELISA system incorporating this protein has been investigated by several laboratories but the product has not been made available to date (Larsen *et al.*, 1995).

(2.11) The Use of Serologic Tests for Syphilis in Point-of-Care Situations

As already discussed, there are numerous serologic tests for the diagnosis of syphilis currently available on the market. There are still some in the process of being developed and others that are under evaluation. Each of these tests has its own advantages and disadvantages as outlined earlier. Traditionally in the diagnosis of syphilis, nontreponemal tests are used for screening of sera of at risk patients and these results are confirmed by the more specific treponemal tests. The confirmatory tests, however advantageous, takes several hours to complete and most of these don't

allow single serum testing. One must also take into consideration the time taken for the nontreponemal test to be performed, followed by its confirmatory test and the time taken to analyze the results of both tests. In point-of-care situations such as the STD clinics, prenatal clinics or drug treatment centres this would not be feasible. Firstly, the clinics do not have the trained laboratory personnel to perform and interpret the necessary confirmatory tests nor the time to perform these, since clinics are usually busy environments. In these situations treatment should begin immediately and not be delayed waiting for the results of the confirmatory test to be made available.

This point is especially important in cases where patients are unable to return to the clinic in a timely manner eg. in rural areas where clinics are usually far out. In such situations, a diagnosis of primary syphilis is often based on clinical symptoms and clinical history upon which treatment is then administered syndromically.

Clinics may send the samples off-site for testing. But by the time the results are received, the patient has already been treated syndromically and has left the clinic. On the other hand, the patient may instead be told to return to the clinic once the test results are available. Failure of the patient to do so would result in him/her not receiving appropriate treatment and on time, if the test result is positive. Further unfortunate consequences of this would be long term complications in that patient and onward transmission to their sexual partners.

There is also the risk of HIV transmission. It has been reported that syphilis may be a predisposing factor for the transmission of HIV. According to Chiu and Radolf, syphilitic chancres disrupt normal epithelium or mucosal barriers, thus providing

portals of entry for HIV into the systemic circulation. Furthermore, the base of a syphilitic chancre contains numerous activated lymphocytes and macrophages, the cells that are potential targets and reservoirs for HIV (Chiu and Radolf, 1994).

Hence, the use of a rapid point-of-care test for the early detection and treatment of syphilis is critical. Such a test would also prove extremely beneficial in reducing the prevalence of congenital syphilis. The World Health Organization has reported that the mortality rate of infants to congenital syphilis is greater than one million infants a year worldwide (World Health Organisation, SDI, 2006). Congenital syphilis can be prevented if infected mothers are identified early and treated accordingly. For several years, rapid POC tests for the detection of treponemal antibodies have been widely used for the screening of pregnant women.

According to the WHO guidelines for syndromic management of STIs, it states that antimicrobial regimens should cover 95% of STI cases. In 2004, Moodley and colleagues had established that in KwaZulu-Natal syphilis, chancroid and granuloma inguinale together made up just 5% of the etiology (Figure 4). Other published studies investigating the etiology of GUD in South Africa, confirmed the decreasing prevalence of these agents. Considering that these guidelines were to be applied towards the management of GUD, would mean the withdrawal of the antimicrobials used to treat these less prevalent infections, from the local guidelines. However, South African policy makers are reluctant to withdraw such treatment in fear of a rapid re-emergence of the diseases. This implies that patients currently presenting with GUD continue to receive the full treatment regimen for all causes of GUD. To avoid treatment of a large number of patients for infections that they do not have, the

development of rapid point-of-care tests for the exclusion of *T. pallidum* and *H. ducreyi* in ulcers is critical.

In summary, a rapid point-of-care test is one that can be used in all health care settings (including resource limited settings) to allow for immediate informed treatment choices (World Health Organization, 2006). Such a test needs to be cost-effective, simple and easy to perform, not require sophisticated equipment or special storage and transport conditions, can be performed on-site without trained laboratory personnel and its results need to be available in a matter of minutes and be interpreted with ease.

(2.12) Aim and Motivation for Current Study

In the above context, this study is aimed towards the development of a rapid point-of-care test to show that patients are rather not infected with syphilis (i.e. an on-site test that effectively demonstrates the exclusion of a syphilitic infection when used to diagnose individuals presenting with genital ulcers). This requires an antigen detection test with a high sensitivity (and preferably also high specificity) which allows for definite, reliable negative test results. This will prevent administering of “unnecessary” treatment with penicillin for syphilis thus further preventing a painful injection, hypersensitivity reactions in patients and the possible development of resistance in *T. pallidum* and other bacterial species.

At the moment, darkfield microscopy provides the only POC diagnosis for primary syphilis (Section 2.10.1) and such technique is not available in resource limited

settings or general/routine clinic levels. Also trained laboratory personnel are required to perform this technique since great attention to detail is necessary for viewing treponemes. Serologic tests show positive findings only weeks following the appearance of the primary syphilitic chancre. This study aims at the development of a rapid test to detect primary syphilis using primary lesion material.

This POC test also aims to be specific to *T. pallidum* subsp *pallidum* by using its rare outer membrane protein as the target antigen for detection. In 1995, Blanco and colleagues reported their findings of a treponemal outer membrane porin protein, a 31 kDa protein which they termed “Tromp1” (Blanco *et al.*, 1995). Other proteins belonging to the Tromp family of proteins include the 65- and 28 kDa proteins. It has been suggested that the Tromps play a pivotal role in the pathogenesis of syphilis and immunity (Blanco *et al.*, 1995).

The 31 kDa protein was chosen for the purposes of this study because it is significantly enriched in the *T. pallidum* outer membrane preparation as was demonstrated by Blanco and colleagues in 1994 (Blanco *et al.*, 1994). In addition, this protein was reported to have “significant amino acid sequence homology to a family of streptococcal surface adhesins” (Blanco *et al.*, 1996) thus suggesting a similar function of Tromp1, in *T. pallidum*, and hence its potential role in syphilis pathogenesis (Blanco *et al.*, 1996). In another study conducted in 1990, freeze-fracture electron microscopy of the *T. pallidum* outer membrane was used to demonstrate IRS-antibody mediated aggregation of Tromp. This further suggested that Tromp1 could be a key surface-exposed target for specific antibody-mediated killing or opsonisation (Blanco *et al.*, 1996).

For this study, antiserum was to be raised against the 31 kDa Tromp protein and be immobilized onto the immunochromatographic test strip for detection of whole treponemes. Hence, whilst other available serologic tests detect treponemal antibodies in syphilitic sera, which can result in cross reactivity, this POC test plans to use immobilized specific antibody to detect whole treponemes in ulcer secretions.

Isolating the protein from the organism itself would be extremely difficult for several reasons. Firstly, the *T. pallidum* outer membrane is highly fragile and there is a lack of suitable procedures to isolate and purify the outer membrane. Secondly, the amount of recoverable protein will be less and some protein would have been lost during the purification procedure. Thirdly, only a limited number of treponemes are obtained following its cultivation in rabbits. This would mean that several rabbits would have to be sacrificed in order to obtain sufficient number of organisms for the isolation of the desired, rare protein for study. To avoid this, it was decided, for this study, to use recombinant DNA technology to clone and express the gene encoding the 31 kDa Tromp protein. The recombinant protein would then be used to raise anti-treponemal antibodies in rabbits. Specific antibodies would then be immobilized onto an immunochromatographic test strip for the detection of whole treponemes in primary ulcer secretions.

CHAPTER 3

MATERIALS AND METHODS

(3.1) Propagation of *T. pallidum*

Treponema pallidum subsp *pallidum* (Nichols strain) was maintained by passaging the organisms by intra-testicular injection in a New Zealand white male rabbit free of treponemal infection, as previously described by Fieldsteel *et al* (1981) with slight alterations. Ethical approval for use of animals was granted on March 5th, 2008, under the reference number 028/08/Animal.

On day 1, *T. pallidum* (Nichols strain) was inoculated intra-testicularly into a rabbit. The rabbit was first anaesthetized using a combination of 12 mg xylazine and 6 mg ketamine injected intramuscularly. Following sedation, the scrotal area was prepared for inoculation as follows: The area was depilated and disinfected sequentially with 2% chlorohexidine in alcohol (Habitane) and povidone iodine (Betadine; Woudine-Kyron). Each testis was inoculated with approximately 0.5 mL of 1:5 diluted suspension of testicular tissue from a former rabbit containing *T. pallidum*. On days 4 and 9 postinfection, the rabbit was inoculated intramuscularly with 4 mg/mL of hydrocortisone acetate. On day 12, at the time of peak orchitis the rabbit was euthanized. The following procedure was followed. Preparation of the rabbit was executed by trained staff at the Biomedical Resource Unit, Westville campus, UKZN. The rabbit was anaesthetized with a combination of ketamine and xylazine. The rabbit was then euthanized by injecting pento-sodium barbitone directly into the heart.

The testes were removed aseptically. Each testis was placed into a sterile beaker containing 10 mL of phosphate buffered saline with 20% fetal calf serum, for transport from the animal facility to the laboratory.

(3.1.2) Harvesting of *T. pallidum*

After removal of extraneous tissue, the testes were chopped into fine pieces while submerged in 10 mL of PBS as described in Section 3.1. An additional 5 mL of PBS containing 20% fetal calf serum was added to the suspension. The suspension was then placed on a shaker for 30 minutes at 37°C. To concentrate the treponemes and remove redundant tissue, the suspension was then centrifuged in a clinical centrifuge at 845 x g for 10 minutes. The supernatant was aspirated and transferred to sterile 1.5 mL Eppendorf tubes in 1 mL volumes each. The presence and viability of treponemes was confirmed using darkfield microscopy.

(3.2) Crude DNA Extraction

The treponemal suspension was aliquoted in 1 mL volumes in 1.5 mL Eppendorf tubes as described in Section 3.1.2. Each of these was centrifuged at 46,620 x g for 30 minutes and the supernatant discarded. The first pellet was reconstituted in 200 µL ProbeTec lysis buffer (BD ProbeTec ®, Becton Dickinson). The suspension was transferred to the second tube and the pellet in that tube was resuspended in the same volume of buffer. This was repeated till all pellets were concentrated into 200 µL in a single Eppendorf tube. This suspension was placed in a heating block at 113°C for 30 minutes to aid lysis. The resulting crude DNA was then further purified.

(3.3) DNA Purification

Ultra-pure DNA was obtained using the DNA Clean and Concentrator-5 kit from ZymoResearch (ZymoResearch Corp). This kit uses the principle of fast-spin column chromatography to rapidly yield high-quality, purified DNA. The procedure followed was according to the manufacturer's instructions:

Two volumes of specially formulated DNA Binding buffer was added to each volume of crude DNA preparation (100 μ L aliquots) and mixed by vortexing. The mixture was then transferred to the provided Zymo-spin column within a microcentrifuge collection tube (provided with the kit) and centrifuged at 9 391 x g for 30 seconds. The flow-through fluid was discarded whilst the DNA remained in the column matrix. This was followed by adding 200 μ L of provided Wash buffer to the column and centrifugation at 9 391 x g for 30 seconds. This wash step was repeated once and the collection tube discarded. The column was inserted into a sterile 1.5 mL microcentrifuge tube and 30 μ L of nuclease-free water (Fermentas/Inqaba) was applied directly to the centre of the column matrix without touching the matrix. This was followed by centrifugation at 9 391 x g for 30 seconds to elute pure *T. pallidum* DNA. The concentration and purity of recovered DNA was then determined.

(3.4) Measurement of DNA Concentration and Purity

Concentration and purity of recovered DNA was determined using the Nanodrop 2000, which is based on the principle of spectrophotometry, whereby the amount of ultraviolet radiation absorbed by a solution of DNA (or protein) is directly

proportional to the amount present in that sample. Nucleic acid solutions are measured at 260 nm whilst proteins are measured at 280 nm. Pure DNA in solution should have an A_{260}/A_{280} ratio of purity ranging from 1.8 to 2.0. A ratio that is less than 1.8 indicates the presence of contaminants such as proteins and phenols. These samples should be subjected to further purification.

For measuring DNA, the nucleic acid icon was selected. The instrument was allowed a few seconds to initialize and then zeroed. Spectrophotometers are usually zeroed against reagent blanks which contain everything but the compound to be measured. For the purpose of this study, the Nanodrop was zeroed against a distilled water blank, since the *T. pallidum* DNA was eluted in water. A volume of 1.5 μL of distilled water was placed onto the Nanodrop applicator and the arm placed down into the water. The blank icon was selected and the instrument zeroed. The arm was lifted and the water was wiped off with soft tissue gauze. Following this, 1.5 μL of sample was applied and the measure icon selected. Absorbance readings at 260 nm and 280 nm along with the A_{260}/A_{280} purity ratio were obtained. An A_{260}/A_{230} ratio was also obtained indicating any possible chemical contaminants. Nucleic acid concentration measurements were obtained in nanogram per microlitre ($\text{ng}/\mu\text{L}$). The DNA sample was stored at -20°C until use.

(3.5) Polymerase Chain Reaction (PCR) – Amplification of the *tromp1* Porin Gene

(3.5.1) PCR Setup

The PCR master mix was prepared in a room separate from where DNA extraction and DNA addition was carried out. Electrophoresis and analysis of PCR products (amplicons) was performed in the separate amplicon laboratory to prevent contamination. In preparation for PCR, the benchtop, pipettes, tip boxes and tube racks were swabbed with 10% bleach followed by 70% ethanol.

(3.5.2) PCR

PCR was used to amplify the gene encoding the 31 kDa Tromp porin protein. The article titled “Recombinant *Treponema pallidum* rare outer membrane protein 1 (Tromp1) expressed in *Escherichia coli* has porin activity and surface antigenic exposure” by Blanco and colleagues in 1996, was used as a reference article for the amplification of the *tromp1* porin gene.

The aim of the PCR was to generate a PCR product lacking the region encoding the Tromp1 signal peptide, hence resulting in the expressed protein being secreted rather than the membrane bound product. This ensures that the protein be produced in abundance in the soluble fraction thus allowing for easier purification. The primers published by Blanco *et al* were used to generate PCR products lacking the region encoding the signal peptide. For the purpose of this study, a CACC nucleotide overhang was added onto the N-terminal region of the existing forward primer to facilitate directional cloning at a later stage (5'-**CACCCGCGGATCCATTCGGTAGCAAGGATGCCGCA**-3'). No changes were made to the reverse primer (5'-CCGGAATTCCTAGCGAGCCAACGCAGCAAC-3'). Both primers were purchased from Roche Diagnostics. The primers were

reconstituted according to the manufacturer's instructions and diluted to a concentration of 0.5 μM for the PCR reactions.

These primers were used in a standard mastermix to generate *tromp1* PCR products. However the initial results showed nonspecific bands. Therefore PCR optimization was performed to determine optimal concentrations of MgCl_2 and dNTPs to be used in the reaction.

(3.5.3) Optimization of the Concentration of dNTPs and MgCl_2 for PCR

Optimization was performed by varying the concentration of MgCl_2 and dNTPs in the mastermix. The concentration of MgCl_2 used in PCR reactions varies from 1 mM to 5 mM, whilst the dNTP concentration should be between 50 μM to 500 μM . To optimize the performance of the PCR, varying concentrations of MgCl_2 (Roche Diagnostics) and dNTPs (Roche Diagnostics) were used (Table 1), in a mastermix containing 5 μl of 10x Taq buffer (100 mM, Roche Diagnostics) with KCl (500 mM, Fermentas/Inqaba Biotec), 0.05 μM of each primer (Roche Diagnostics), 0.001% gelatin, 0.25 μL of Taq (0.05 U/ μL , Roche Diagnostics) and 10 μL of DNA template (55.4 ng/ μL) in a final volume of 50 μL . Each reaction mixture was prepared independently in a 0.2 mL sterile Eppendorf tube (Lasec). Preparation of the mastermix and DNA addition was done on ice at all times.

dNTPs (μM)	MgCl_2 (mM)				
	1	2	3	4	5
A 50 μM	0	0.5 mM	1 mM	1.5 mM	2 mM
B 100 μM	0	0.5 mM	1 mM	1.5 mM	2 mM
C 200 μM	0	0.5 mM	1 mM	1.5 mM	2 mM
D 250 μM	0	0.5 mM	1 mM	1.5 mM	2 mM

Table 1: Concentration of dNTPs and MgCl_2 used in PCR optimization

PCR amplification was performed in the GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The PCR reaction mix was denatured by heating to 94°C for 2 minutes. The following PCR cycling conditions were used for 30 cycles: denaturation at 94°C for 30 s; followed by annealing at 45°C for 30 s and extension at 72°C for 30 s. A final extension consisted of one cycle at 72°C for 10 minutes. The thermocycler was programmed to maintain the amplified PCR products, at 4°C , until removal. The amplified products were analysed under a UV trans-illuminator following agarose gel electrophoresis.

(3.5.4) Agarose Gel Electrophoresis

To determine the integrity and yield of the amplified products as well as to establish whether these were of the desired size, the products were subjected to electrophoresis in an ethidium bromide containing 2% agarose gel (Appendix A-5) in TBE buffer. Standard markers of known molecular weight were run alongside the product to estimate its molecular weight.

To achieve this, 1.4 g of molecular grade agarose (Whitehead Scientific) was weighed out into a beaker to which 70 mL of 0.5x TBE buffer (Appendix A-3) was added. The solution was warmed in a microwave oven until the agarose was completely dissolved. Then, 70 μ L of a 0.05% ethidium bromide solution (Appendix A-4) was added under gentle swirling. The mixture was then allowed to cool to approximately 45°C. Extra caution was taken when handling ethidium bromide as it is known to be a potent carcinogen. Any ethidium bromide wastes and tips were discarded in the provided ethidium bromide disposal buckets.

In the meanwhile, the gel cartridge was set up. The tray and combs were washed and then swabbed with 70% ethanol. Two combs producing 15 wells each were placed along the length of the agarose gel to create two rows of wells for the loading of samples. The gel solution was poured into the gel cartridge and allowed to solidify at room temperature for about 30 minutes. Thereafter the combs were removed resulting in neatly formed wells. The gel cartridge was placed into an electrophoresis tank filled with 0.5x TBE buffer.

The amplification products were removed from the thermocycler, gently tapped and spun down for a few seconds. Then 5 μ L of each sample was mixed with 1 μ L of sample loading buffer (Appendix A-6) on a parafilm sheet and these mixtures were then loaded into the wells in the agarose gel. Samples (which are negatively charged) were loaded in a manner such that they would run from the negative anode (black) towards the positive cathode (red). DNA molecular weight marker XIV (100 to 1500 bp ladder) from Roche Diagnostics was used to determine the size of the DNA fragments generated by PCR. The lid of the electrophoresis tank was closed and

electrophoresis was performed for 60 minutes at 100 V. Remaining amplified products were stored at -20°C until further use.

After electrophoresis, the powerpack was switched off and the lid of the tank was lifted. The gel cartridge was removed from the tank and the gel was placed into the Gene Genius gel dock (Bio Imaging Systems) to obtain an image. The GeneSnap Software from Syngene was applied to analyze the images.

It was established that a final concentration of 1 mM MgCl₂ and 200 µM dNTPs was efficient for this PCR reaction as indicated by the brightest and most clearly defined band at the desired region. However, nonspecific secondary bands also appeared in the lower molecular weight region.

A second PCR was then performed using the optimized mastermix but with 2 µL of template DNA instead of 10 µL. This provided the same results and also prevented wastage of template DNA. However, the nonspecific secondary bands remained present. Therefore, the annealing temperature and primer concentration was optimized using the mastermix with optimal MgCl₂ and dNTP concentrations.

(3.5.5) Gradient PCR

Annealing temperature and primer concentration were simultaneously optimized using the MiniOpticon Real-Time PCR system from BioRad. This optimization can often be achieved in a single experiment. Commonly known as Gradient PCR, this technique allows the empirical determination of an optimal annealing temperature

using the least number of steps. Other components of the PCR reaction can be optimized simultaneously as was done here, by varying the primer concentration against an annealing temperature gradient.

Primer solutions containing 0.5 μM , 0.1 μM , 0.05 μM and 0.025 μM were prepared for both the forward and reverse primers. Four sets of mastermixes were prepared as per primer dilution with primer concentration being the only changing factor in the mastermixes. The constitution of these different mastermixes in a volume of 24 μL is shown in table 2. Phusion High-Fidelity DNA polymerase (Finnzymes Reagents) was used to create blunt ended PCR products to facilitate directional cloning as required by the chosen cloning kit.

Reagent	Final Concentration	Volume per reaction (μL)
(1) Nuclease-free Water	-	13.9
(2) Phusion Buffer	1x	1
(3) MgCl_2	1 mM	1
(4) dNTPs	200 μM	0.5
(5) Forward primer	0.05 μM ; 0.01 μM ; 0.005 μM or 0.0025 μM	2.5
(6) Reverse primer	0.05 μM ; 0.01 μM ; 0.005 μM or 0.0025 μM	2.5
(7) Gelatin	0.001%	2.5
(8) Phusion DNA polymerase	0.008 U/ μL	0.1
(9) Template DNA	55.4 ng/ μL	1
Total		<u>25 μL</u>

Table 2: Components of reaction mix for primer concentration/temperature gradient

PCR

Volumes of 24 μL of the different mastermixes were added to each well of a MiniOpticon plate, resulting in six rows with decreasing primer concentration per well. This was followed by the addition of 1 μL of *T. pallidum* DNA extract to each of the wells. The plate was tapped gently and spun down for a few seconds.

Using the gradient function of the system, an annealing temperature gradient of 56 to 71°C [56; 59; 61; 65; 68 and 71°C] was set to run in decreasing manner as per column of primer dilution. The MiniOpticon system was programmed accordingly using the BioRad CFX Manager software. The plate outlay for the gradient PCR is shown in table 3.

	P ₁ - 0.05 μM	P ₂ - 0.01 μM	P ₃ - 0.005 μM	P ₄ - 0.0025 μM
T ₁ – 71°C	•	•	•	•
T ₂ – 68°C	•	•	•	•
T ₃ – 65°C	•	•	•	•
T ₄ – 61°C	•	•	•	•
T ₅ – 59°C	•	•	•	•
T ₆ – 56°C	•	•	•	•

Table 3: Plate outlay for the gradient PCR (P: final primer concentration; T: temperature)

The plate was inserted into the detector of the MiniOpticon and its door was closed. The instrument was set to amplify for 35 cycles. Each cycle consisted of a denaturation step at 98°C for 30 s and 98°C for 10 s; annealing temperature gradient [56; 59; 61; 65; 68 and 71°C] for 30 s; an initial extension at 72°C for 30 s; followed by a final extension at 72°C for 10 min. The thermocycler was programmed to maintain the amplified PCR products, at 4°C, until removal.

Amplified products were analysed by means of agarose gel electrophoresis as described in Section 3.5.4. Upon analysis, bands corresponding with the desired size of ~884 base pairs were found in all the lanes where 0.05 μ M of each of the primers was used. No bands were identified in the lanes where lower concentrations of primers were used. The best results were obtained at annealing temperatures of 68°C and 56°C where the bands were most defined.

An annealing temperature of 68°C was used for the remaining work. A second PCR now using the optimized conditions was carried out to confirm the presence of the *tromp1* gene. Nuclease-free water was used as a negative control. Upon confirmation of the presence of the gene, the amplification product was removed from -20°C storage and purified using the PureLink PCR Purification Kit from Invitrogen.

(3.5.6) Purification of Amplified (*tromp1*) Product

The PureLink PCR Purification Kit from Invitrogen is designed to rapidly and efficiently remove primers, dNTPs, enzymes and salts from PCR products. The kit applies selective binding of dsDNA to its silica-based membrane in the presence of chaotropic salts. All centrifugation steps were carried out in a microcentrifuge.

Four volumes of a high cut-off PureLink Binding buffer with isopropanol, was added to one volume of PCR product. The sample was then added to a provided PureLink spin column in a collection tube and centrifuged at 10 000 x g for 1 minute. The flow-through fluid was discarded. The addition of binding buffer serves to adjust the conditions for the binding of dsDNA to the spin column. The dsDNA binds to the

silica-based membrane in the column and impurities are removed by thorough washing with provided Wash buffer as follows: 650 μ L of Wash buffer with ethanol is added to the column which is then centrifuged at 10 000 x g for 1 minute and the flow-through fluid discarded. The column was then centrifuged at maximum speed (18 407 x g) for 2-3 minutes to remove any residual wash buffer. The spin column was then placed into a clean 1.7 mL elution tube supplied with the kit and dsDNA was finally eluted in water. A 50 μ L volume of nuclease-free water (Fermentas/Inqaba Biotec) was added to the centre of the spin column with much care taken not to touch the membrane. The column was incubated at room temperature for 1 minute and then centrifuged at maximum speed (18 407 x g) for 2 minutes. The column was discarded. The elution tube now contains the purified PCR product which was analyzed on agarose gel electrophoresis to confirm its purity. The product was stored at -20°C until further use.

(3.6) Cloning of the *tromp1* Amplification Product

The purified amplification products from Section 3.5.6 were cloned into the pET100/D-TOPO vector according to the manufacturer's instructions. This vector is included in the Champion pET Directional TOPO Expression Kit purchased from Invitrogen.

The Champion pET Directional TOPO Expression Kit from Invitrogen was chosen for cloning and expressing of the *tromp1* gene. This kit directionally clones the blunt-ended amplified DNA into the pET100/D TOPO vector for high-level, T7-regulated expression in *E. coli*. The amplified DNA is directionally cloned by adding four

bases (CACC) to the 5' end of the existing forward primer. The vector contains a complementary GTGG overhang that recognizes the CACC overhang on the PCR product. It invades the 5' end of the PCR product, anneals to the added bases and stabilizes the PCR product in the correct orientation (Invitrogen user manual, 2006).

(a) pET100/D-TOPO Vector

The amplified *tromp1* gene was cloned into the pET100/D-TOPO vector. “The pET TOPO vectors are designed to facilitate rapid, directional cloning of blunt-ended PCR products for regulated expression in *E. coli*” (Invitrogen user manual, 2006).

Important Features of the pET100/D-TOPO Vector:

- The vector contains the ampicillin resistance gene for the selection of transformants. This ampicillin resistance gene encodes the protein β -lactamase, which is secreted into the medium where it hydrolyzes ampicillin, thus inactivating the antibiotic.
- pET100 vector allows the expression of recombinant protein with an N-terminal tag containing the Xpress epitope and a 6x-His fusion tag. This allows the production of recombinant fusion proteins that may be easily detected and purified.
- The N-terminal tag also includes an enterokinase (EK) recognition site for cleavage of the tag from the protein after purification, using enterokinase (EKMax).

- A ribosomal binding site (RBS) is situated upstream of the start codon (ATG) in the N-terminal tag to ensure optimal spacing for proper translation.
- It contains the TOPO cloning site for the directional cloning of blunt-ended PCR products.
- The T7 *lac* promoter allows for the high-level, IPTG-induced expression of the recombinant protein in *E. coli* strains that express T7 RNA polymerase.
- It contains the *lacI* open reading frame that encodes the Lac repressor to reduce basal transcription of the gene of interest.
- The *lac* operator serves as the binding site for the Lac repressor to reduce basal transcription of the gene of interest.
- The T7 transcription termination region permits efficient transcription termination.
- It contains the pBR322 origin of replication for the low-replication and maintenance in *E. coli*.

(b) T7 Regulated Expression

“The Champion pET Expression System uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*” (Invitrogen user manual, 2006).

The BL21 (DE3) *E. coli* strain “carries the DE3 bacteriophage lambda lysogen” which further contains a *lac* construct. This construct comprises the following:

- *lacI* gene which encodes the lac repressor
- the T7 RNA polymerase gene under control of the *lacUV5* promoter in the host chromosome

- a portion of the *lacZ* gene.

The *lac* construct is inserted into the *int* gene thus inactivating this gene. Meanwhile the *lacI* gene encoding the Lac repressor goes on to repress the expression of T7 RNA polymerase from the *lacUV5* promoter. The addition of an inducer, isopropylthio- β -galactoside (IPTG), “allows expression of T7 RNA polymerase from the *lacUV5* promoter” (Invitrogen user manual, 2006). When sufficient T7 RNA polymerase is produced, it binds to the T7 *lac* promoter on the pET TOPO vector, thus driving the expression of the recombinant protein (Invitrogen user manual, 2006).

Previous studies have demonstrated that there is constantly some basal level expression “of T7 RNA polymerase from the *lacUV5* promoter in the λ DE3 lysogens” even in the absence of the inducer IPTG (Studier and Moffatt, 1986; Invitrogen user manual, 2006). This does not pose a problem as long as the product coded for by the gene of interest is not toxic to the *E. coli* host. This is because basal level expression of a toxic gene product would lead to plasmid instability and cell death. To accommodate for this, the pET vector is designed with a T7 *lac* promoter (as mentioned above) which further contains a *lac* operator sequence placed downstream from the T7 promoter. “The *lac* operator serves as the binding site for the Lac repressor, encoded by a *lacI* gene” (Invitrogen user manual, 2006). The Lac repressor binds to the T7 *lac* promoter via its *lac* operator sequence thus blocking T7 RNA polymerase and basal transcription of the gene of interest.

There are cases however, that the gene product of interest is too toxic to BL21 Star (DE3) cells that other *E. coli* host strains are required for the expression studies. The toxicity of the *tromp1* gene to certain *E. coli* host cells was observed by Blanco *et al.*

They reported that the lethality for *E. coli* transformants harbouring the intact *tromp1* gene is similar to that observed for many recombinant gram negative porin proteins expressed in *E. coli* (Blanco *et al.*, 1995).

The instructions for users of the Champion pET Expression Kit from Invitrogen suggest that for known toxic genes, it is best to perform the expression experiment in the BL21 Star (DE3) **pLysS** strain which they also supply. This strain differs in that it contains the pLysS plasmid which produces T7 lysozyme. “T7 lysozyme binds to T7 RNA polymerase and inhibits transcription. This activity results in reduced basal levels of T7 RNA polymerase, leading to reduced basal expression of T7-driven heterologous genes.” (Invitrogen user manual, 2006). Hence, the overall yield of recombinant protein obtained from BL21 Star (DE3) cells is greater than the yield obtained from the BL21 Star (DE3) pLysS cells (Invitrogen user manual, 2006).

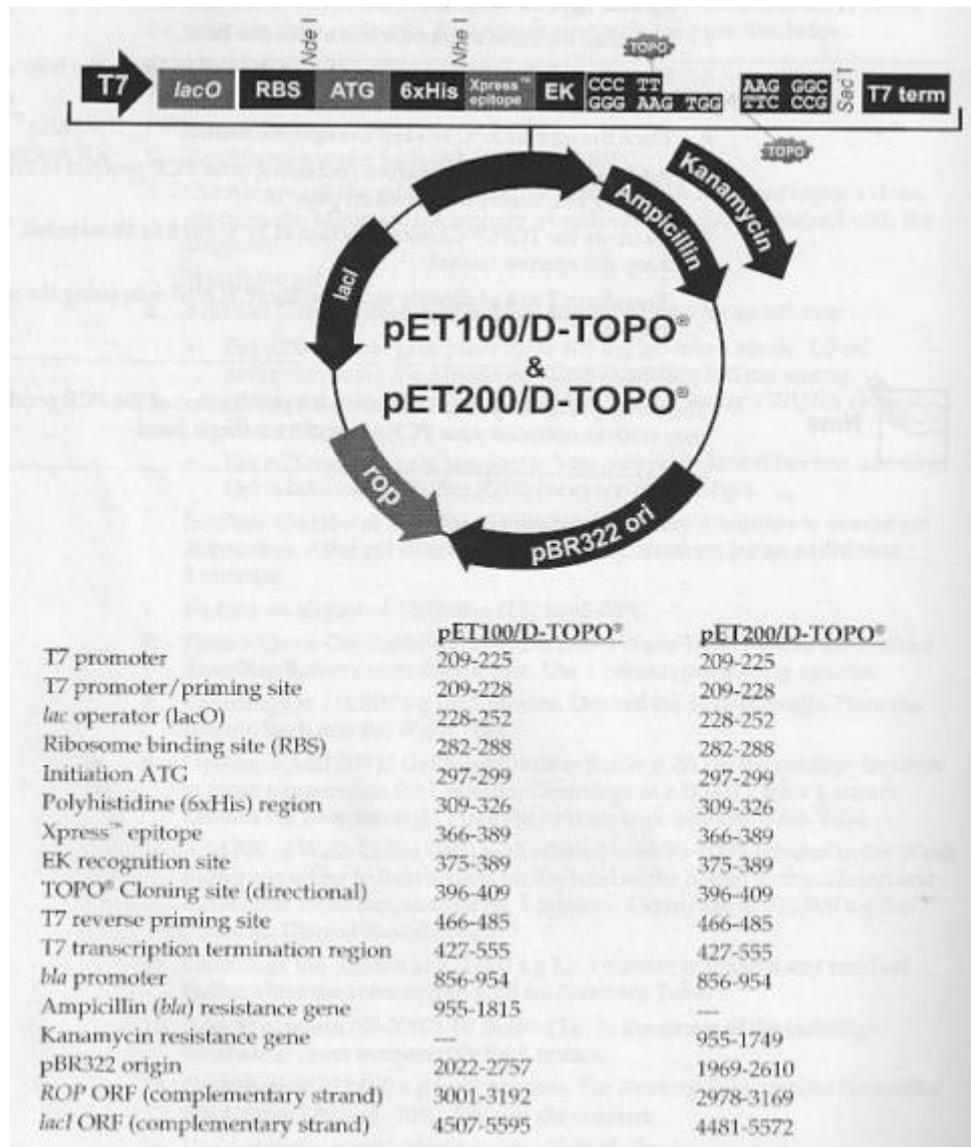
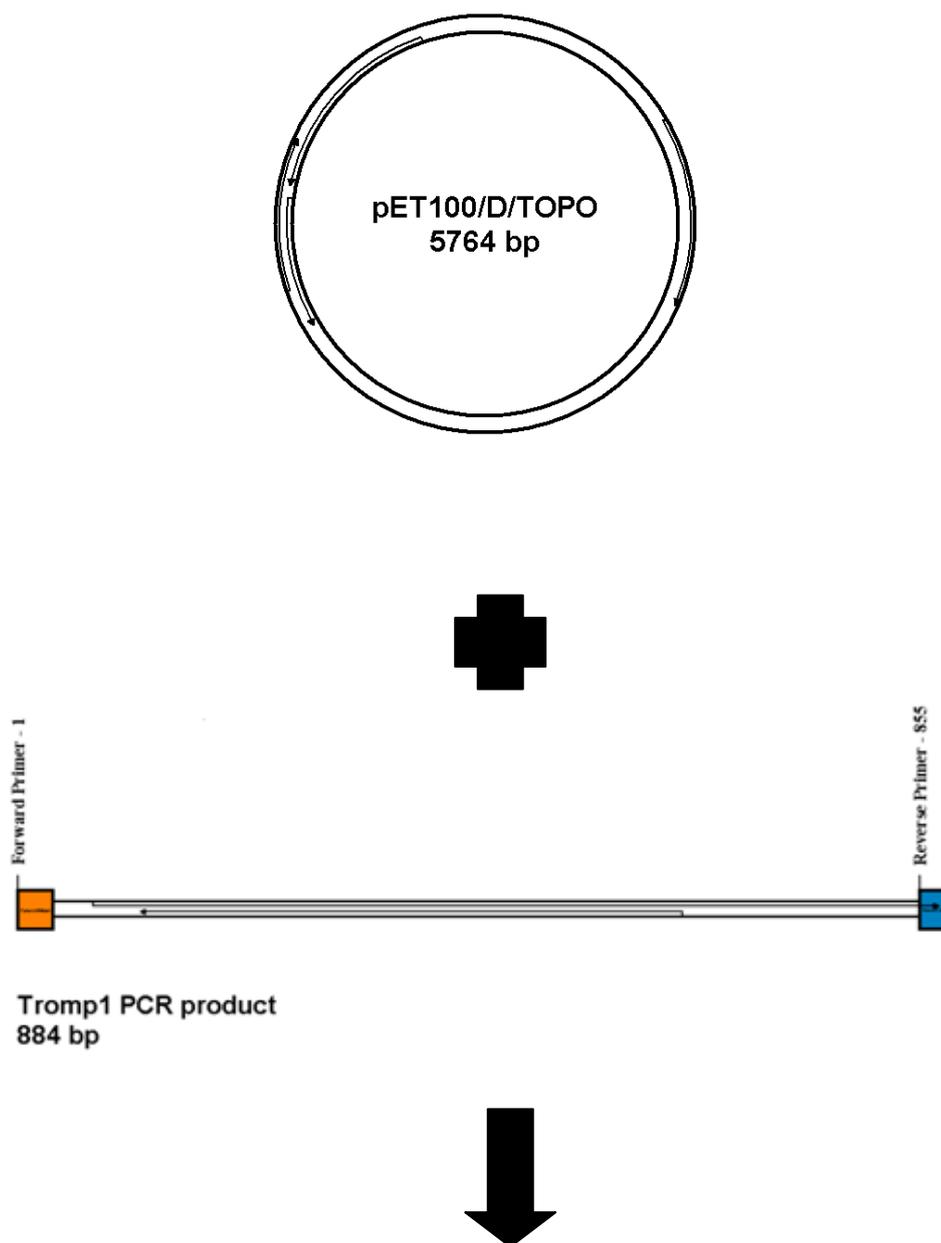


Figure 1: Map and features of pET100/D-TOPO (Invitrogen user manual, 2006)

(3.6.1) Evaluation of pET constructs using pDRAW32 1.1.109

pET constructs can be quickly evaluated for expression of the desired target protein using pDRAW32 1.1.109. Figure 2 shows the computer generated pET100/D/*tromp1*, plasmid DNA construct.



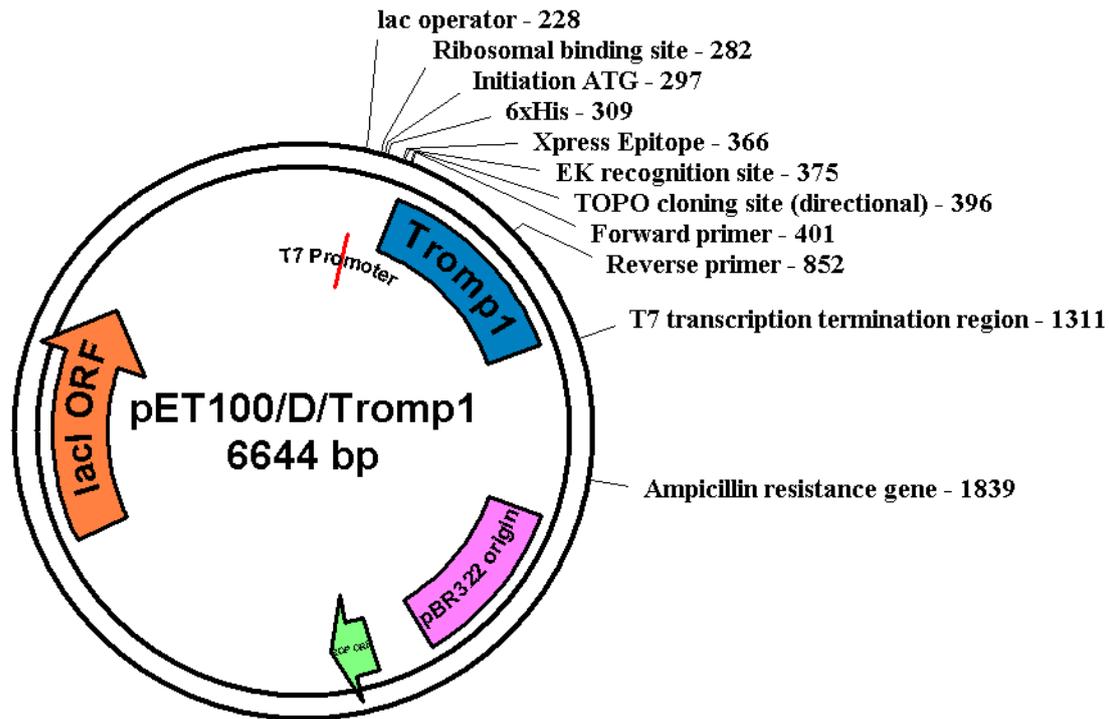


Figure 2: Evaluation of the pET100/D/*tromp1* using pDRAW32 1.1.109

(3.6.2) Ligation of the *tromp1* Amplification Product into pET 100/D-TOPO Vector

It had been advised in the instruction manual that in order to obtain the highest cloning efficiency, a 0.5:1 to 2:1 molar ratio of PCR product:TOPO vector should be used. This is because the efficiency decreases significantly if the ratio of PCR product:TOPO vector is <0.1:1 or >5:1. It had also been suggested that for the pET TOPO vector, a 1 to 5 ng of a 1 kb PCR product be used in the cloning reaction that will result in a suitable number of colonies. Since the size of the PCR product in this study is <1 kb, it was decided that the product be diluted to 5 ng/ μ L for the cloning ligation reaction. A range of 0.5 to 1 μ L of this dilution was used in the reaction.

(3.6.2.1) TOPO Cloning Reaction

The PCR product along with the provided salt solution and water was thawed on ice and the cloning reaction was carried out on ice. The vector was removed from -20°C storage only when ready to use. Two 0.5 mL reaction tubes were labelled according to the varying concentrations of product insert being tested. The final ligation reaction mix therefore consisted of water, salt solution, DNA insert and vector in a final volume of 6 μL (Table 4).

	Reaction 1	Reaction 2
Water	3.5 μL	3 μL
Salt solution	1 μL	1 μL
DNA insert	0.5 μL	1 μL
Vector	1 μL	1 μL
Total	<u>6 μL</u>	<u>6 μL</u>

Table 4: Components of ligation reaction

The ligation reaction ingredients were mixed gently and incubated at room temperature for 30 minutes. The One Shot TOP 10 chemically competent *E. coli* cells (Invitrogen) were placed on ice and 3 μL of each cloning/ligation reaction was added to one of two vials (50 μL each) of One Shot TOP 10 chemically competent *E. coli*, mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds and immediately transferred back onto ice for a further 2 minutes following which 250 μL of SOC medium, at room temperature, (Invitrogen) was

added to each vial without mixing. The tubes were capped tightly, taped onto a clean rack and then placed horizontally in a shaking incubator, 230 rpm at 37°C for 1 hour. Turbidity of the culture after 1 hour incubation indicated growth. For plating out the *E. coli* cells involved in the transformation reaction, a hockey glass stick was dipped into 100% ethanol and flamed, cooled slightly, flamed and cooled once again for approximately 40 seconds. Then, using the heat sterilized glass stick, 100 µL and 50 µL from each transformation mix was spread on prewarmed selective LB agar plates (Appendix B-2), containing 100 µg/mL of ampicillin (Capital Lab Supplies) for selection (Appendix B-3). The plates were incubated overnight at 37°C. It was necessary to plate out two different volumes of transformation mix to ensure that at least one plate will have well-spaced colonies for further processing.

After approximately 16 hours of incubation, the plates were removed from the incubator and analysed for transformants. Growth was best observed on the plates spread with transformation reaction mix 2, containing 1µL (5 ng/µL) of DNA insert. Well-spaced colonies were best observed on the plates inoculated with 50 µL of each transformation reaction mix whilst too dense growth occurred on the plates inoculated with a 100 µL reaction mix volume.

(3.6.3) Analysing for Positive Transformants

(3.6.3.1) Subcultures of TOP10 *E. coli* Transformants

This cloning kit does not accommodate for typical blue-white screening (in the presence of X-gal medium) that is commonly used for confirming the presence of

inserts. Instead a screening PCR was performed to analyze for positive transformants. Firstly, spot inoculated subcultures of transformed TOP10 *E. coli* colonies were prepared. This entailed grids being drawn on the surface of the covers of prewarmed LB agar plates (containing 100 µg/mL ampicillin) using a black marker and each grid was numbered. Using sterile loops, randomly selected colonies were picked off the original LB agar plates and spot inoculated onto the agar within each grid. This therefore provided numbers to each colony that enabled us to identify those that were positive according to the results of the screening PCR. The subculture plates were incubated, inverted at 37°C overnight for approximately 16 hours. The next morning, transformant colonies were screened for the presence of the *tromp1* insert.

(3.6.3.2) Screening Transformant Colonies for Recombinant DNA

A varying number of randomly selected colonies were picked off the grids of the subcultured LB agar plates and suspended in 100 µL of prewarmed LB media (Appendix B-1) containing 100 µg/mL of ampicillin. The tubes were taped to a clean rack and incubated horizontally at 37°C for 90 minutes with gentle shaking. In the meanwhile the plates were sealed with parafilm and stored in a plastic bag at 4°C until further use.

For the screening PCR reaction, the same primers used in the previous PCR reaction (Section 3.5.2) were used as they hybridise at the ends of the insert. However, since this was just a screening PCR that did not require the use of expensive Phusion products, a different cheaper polymerase was used. Furthermore since there is no blue-white screening, it would mean that several colonies will have to be picked for

analyzing positive clones and therefore several reactions would have to be conducted. Therefore Taq polymerase along with its 10x PCR buffer from Roche Diagnostics were used to minimize cost. The reaction mix was made to a final volume of 20 μ L as shown in table 5.

Reagent	Final Concentration	Volume per reaction (μL)
(1) Nuclease-free water	-	11.6
(2) 10x PCR buffer	1x	2
(3) MgCl ₂ (25 mM)	1 mM	0.8
(4) dNTPs (10 mM)	0.2 mM	0.4
(5) Forward primer (CACC)	0.05 μ M	2
(6) Reverse primer (BE2)	0.05 μ M	2
(7) Taq polymerase	0.05 U/ μ L	0.2
(8) Clone – <i>E. coli</i> broth culture	-	1
Total		<u>20</u>

Table 5: Components of reaction mix for screening PCR

Following incubation, 1 μ L broth culture of each clone was added to the mastermix that had already been prepared and aliquoted at 19 μ L volumes into 0.2 mL Eppendorf tubes. The remaining broths were incubated for an additional 4 hours before being stored at 4°C until further use.

The PCR reaction mix was denatured by heating to 94°C for 5 minutes in a Gene Amp PCR system 9700 thermocycler. The following PCR cycling conditions were used for 35 cycles: denaturation at 94°C for 30 s; followed by annealing at 55°C for 30 s and an extension at 68°C for 4 minutes. A final extension consisted of one cycle at 68°C

for 10 minutes. The thermocycler was programmed to maintain the amplified PCR products, at 4°C, until removal.

The amplified products were then analyzed on agarose gel electrophoresis as described in Section 3.5.4. When positive clones were identified, the remaining of the cultures were transferred to 3 mL each of prewarmed LB broth media containing 100 µg/mL of ampicillin and incubated horizontally, overnight at 37°C with shaking (230 rpm).

The next day, the cultures were removed from the incubator and 150 µL of each culture was mixed with 850 µL of autoclaved pure glycerol aliquots and stored at -80°C should the need arise to revive these cultures for future use. A 1.5 mL culture volume was used for the preparation of pure plasmid DNA (minipreps) from these recombinant *E. coli* cultures.

(3.6.3.3) Isolation of Pure Plasmid DNA

Plasmid DNA was prepared using the PureYield Plasmid Miniprep System from Promega. This system provides a rapid method to purify plasmid DNA using a silica-membrane column. The purified plasmid DNA can be used without further manipulation for DNA sequencing or other standard molecular biology techniques. The method outlined below was used following the manufacturer's instructions.

In preparation for plasmid DNA isolation, 1.5 mL of bacterial culture was centrifuged at maximum speed (18 407 x g) for 1 minute in a microcentrifuge. After discarding of the supernatant, the pellet was resuspended in 600 µL of nuclease-free water

following which 100 μL of Cell Lysis Buffer (supplied) was added and mixed by inverting the tubes six times. This was followed by adding 350 μL of cold Neutralization solution (supplied) and mixed thoroughly by inverting. The suspension was centrifuged at maximum speed (18 407 x g) for 3 minutes in a microcentrifuge and the supernatant was transferred to a PureYield Minicolumn (supplied) without disturbing the cell debris pellet. The minicolumn was placed into a supplied collection tube and centrifuged at maximum speed (18 407 x g) for 15 seconds. Its flow-through fluid was discarded and the minicolumn was placed back into the same collection tube.

For the washing process, 200 μL of supplied Endotoxin Removal Wash was added to the minicolumn followed by centrifugation at maximum speed (18 407 x g) for 15 seconds. Endotoxin Removal Wash aims to remove protein, RNA and endotoxin contaminants from purified plasmid DNA. A 400 μL volume of supplied Column Wash Solution was then added to the minicolumn which was centrifuged at maximum speed (18 407 x g) for 30 seconds.

Finally, for the elution of plasmid DNA, the minicolumn was transferred to a clean 1.5 mL microcentrifuge tube and 30 μL of nuclease-water was added directly to the centre of minicolumn matrix, without touching the matrix. The column was allowed to stand at room temperature for 1 minute and then centrifuged at maximum speed (18 407 x g) for 1 second to elute the plasmid DNA. The minicolumn was discarded and the microcentrifuge tube now contained pure plasmid DNA.

The concentration of the plasmid DNA was determined using the Nanodrop 2000 and stored at -20°C until use. The DNA was later diluted in preparation for restriction digestion.

(3.6.3.4) Plasmid DNA Restriction Digest

Plasmid DNA was digested with both restriction enzymes, EcoRI and BamHI since the primers used for the amplification of the *tromp1* gene contains restriction sites for these enzymes at their 5' ends as shown in table 6.

Primer	Restriction Enzyme	Restriction Site	Primer Sequence
Forward primer	BamHI (5' end)	ggatcc	5'-CACCCGCGGATCCATTCGGTAGCAAGGATGCCGCA-3'
Reverse primer	EcoRI (5' end)	gaatc	5'-CCGGAATTCCTAGCGAGCCAACGCAGCAAC-3'

Table 6: Restriction enzymes used to digest plasmid DNA

Digestion was carried out by mixing 100 ng of plasmid DNA, 1 µL each of EcoRI (Fermentas/Inqaba Biotec) and BamHI (Fermentas/Inqaba Biotec), 2 µL of 10x Tango buffer (Fermentas/Inqaba Biotec), made up to a final volume of 20 µL with nuclease-free water. Tango buffer was provided along with the enzymes to simplify buffer selection for double digests. The samples were mixed gently and quickspun for a few seconds. Restriction digest was performed in a Gene Amp PCR system 9700 thermocycler at 37°C (temperature at which most restriction enzymes function best) for 120 minutes. This was followed by a final incubation at 65°C for a further 20 minutes to inactivate the enzymes. Digested products were then analysed on agarose

gel electrophoresis (Section 3.5.4) to confirm the presence of the *tromp1* gene following cloning. The products were electrophoresed alongside two molecular weight markers. Molecular weight marker XIV (100 to 1500 bp) was used to show the *tromp1* insert (884 bp) whilst Marker X (0.07 to 12.2 kbp) was used to show the vector (5764 bp) and uncut plasmid DNA (6644 bp) of higher molecular weights.

(3.7) DNA Sequencing

Sequencing was performed to confirm that the *tromp1* insert was cloned into the correct reading frame of the pET100/D-TOPO expression vector. The isolated plasmid DNA (recombinant DNA) served as the template for the fluorescent-based cycle sequencing reaction. Varying dilutions of template DNA were used in the sequencing reaction. Results were only achieved when the DNA was used neat.

After much optimization (Appendix C – Sequencing Optimization), the existing PCR primers had to be redesigned for the sequencing reaction as follows: In one set of primers, the restriction sites and the CACC nucleotide overhang (forward primer) of the PCR primers were removed. This resulted in a primer set with the following nucleotide sequences:

Forward Sequencing primer: 5'-ATTCGGTAGCAAGGATGCCGCA-3'

Reverse Sequencing primer: 5'-CTAGCGAGCCAACGCAGCAAC-3'

A second set of primers were designed to a sequence internal to the primers on either end of the insert. This resulted in a primer set with the following nucleotide sequences:

Forward Sequencing primer: 5'-CATGATAGCGGATGCTGTC-3'

Reverse Sequencing primer: 5'-AACAACTCGCCTCCAAT-3'

The new sequencing primers, purchased from Roche Diagnostics, were reconstituted according to the manufacturer's instructions and diluted to 3.2 pmol/ μ L for the cycle sequencing reaction.

(3.7.1) Cycle Sequencing Reaction

During the cycle sequencing reaction, the DNA fragment to be sequenced is denatured and the resulting template is extended by the incorporation of deoxynucleotides (dNTPs) by DNA polymerase. At some stage during the reaction a fluorescent-labelled dideoxynucleotide (ddNTP) that lacks a hydroxyl group attached to the 3' carbon gets incorporated into the growing chain thus replacing the normal dNTP. This causes premature halting of DNA synthesis since the lack of the 3' hydroxyl group means that it cannot form a bond with the next nucleotide. Hence, this results in a series of incomplete DNA fragments whose sizes provide information concerning the linear sequence of bases in the DNA.

The ABI Prism BigDye Terminator v3.1 cycle sequencing kit provides the required reagent components for the sequencing reaction premixed in a single tube of ready reaction mix. These include deoxynucleotide triphosphates, fluorescent-labelled dideoxynucleotides (dye terminators), AmpliTaq DNA polymerase, FS, rTth pyrophosphatase, magnesium chloride and buffer. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or

double-stranded DNA templates, PCR fragments and on large templates such as BAC clones. The sequencing enzyme, AmpliTaq DNA polymerase, FS, is a variant of *Thermus aquaticus* DNA polymerase and contains a point mutation in the active site resulting in less discrimination against dideoxynucleoside triphosphates (ddNTPs). This mutation is one in which “the phenylalanine is substituted by a tyrosine at position 667” (Parker *et al.*, 1996). This mutation allows for improved incorporation of chain-terminating ddNTPs as opposed to the wild-type Taq DNA polymerase. This leads to a more even peak intensity pattern (Parker *et al.*, 1996). The enzyme also contains a second mutation in the amino terminal domain that eliminates the 5'-3' nuclease activity of AmpliTaq DNA polymerase (ABI Prism BigDye Terminator v3.1 cycle sequencing kit, user manual).

The mastermix was prepared for both the forward and reverse primers independently as shown in table 7.

Reagent	Volume (μL) per well	Volume (μL) per 96-well plate
(1) Nuclease-free water	5.25	504
(2) Ready reaction mix	2	192
(3) Primer	0.25	24

Table 7: Components of mastermix for cycle sequencing reaction

A volume of 7.5 μL of each mastermix was aliquoted into each well of a MicroAmp 96-well reaction plate (purchased from ABI). This was followed by a volume of 2.5 μL of plasmid DNA (neat) to each well. The plate was then sealed with an ABI

Prism optical adhesive cover and spun down for a few seconds. The adhesive cover was removed and replaced with a rubber mat. The plate was put onto a Gene Amp PCR system 9700 thermocycler. The reaction mix was denatured at 95°C for 10 minutes. The following cycling conditions were used for 30 cycles: denaturation at 96°C for 10 seconds; followed by annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. The thermocycler was programmed to maintain the amplified PCR products, at 4°C, until removal.

(3.7.2) Sequencing Product Clean-up

After the sequencing reaction, the extension products were subjected to a clean-up to remove excess reagents and to precipitate the DNA.

Fresh stocks of sodium acetate and ethanol were prepared prior to the clean-up. 70% ethanol was prepared in the morning and kept at refrigerated temperature until use since cold ethanol increases the precipitation of DNA. The sodium acetate/ethanol solution was prepared as shown in table 8.

Reagent	x1 (µL)	x120 (one full plate) µL
3 M sodium acetate (pH 5.2)	5	600
100% ethanol	50	6000
Total	55	6600

Table 8: Preparation of sodium acetate/ethanol solution

A volume of 55 µL of sodium acetate solution was added to each well without mixing. The plate was sealed with an adhesive cover. The plate was vortexed and

centrifuged in a clinical centrifuge at 3000 x g for 20 minutes. The cover was then removed and the plate was gently inverted onto a folded paper towel and centrifuged in this position at 150 x g for 5 minutes. The plate was removed from the centrifuge and 150 μ L of 70% cold ethanol was immediately added to each well. This step had to be performed quickly because a delay in the addition of ethanol will result in the formation of dye blobs on the sequencing data. The plate was sealed with an adhesive cover and centrifuged at 3000 x g for 5 minutes. Once again, the adhesive cover was removed and the plate was gently inverted onto a folded paper towel and centrifuged in this position at 150 x g for 1.5 minutes. The plate was dried at 50°C for 5 minutes in a thermocycler. The plate was sealed with an adhesive cover, then wrapped in foil and stored at -20°C until ready for use.

When ready to sequence, the dried DNA pellets in each well was reconstituted with 10 μ L of formamide, vortexed and then spun down for a few seconds at 150 x g. The DNA was then denatured at 95°C for 2 minutes before being put onto the sequencer.

(3.7.3) Sequencing Analysis

The sequencing data of recombinant clones were analyzed by means of the Geneious Pro 5.0.2. software. The consensus sequence of the recombinant clones was blasted against the *tromp1* reference sequence (obtained from GenBank under the accession number U16363.1) available on NCBI (blast.ncbi.nlm.nih.gov). This sequence was also translated using the Translate tool from ExPASy AU.

(3.8) Transformation of the *E. coli* Expression Host with Recombinant DNA

The BL21 Star (DE3) and BL21 Star (DE3) pLysS *E. coli* strains were included in the Champion pET Directional TOPO Expression Kit, from Invitrogen, for use as hosts for expression studies. Due to the toxicity of the Tromp1 protein (Blanco *et al.*, 1996) the BL21 (DE3) **pLysS** *E. coli* cells was used as the expression host in this study. pLysS confers resistance to chloramphenicol. Therefore when preparing LB agar plates, 34 µg/mL chloramphenicol (Capital Lab Supplies, Appendix B-4) was added in addition to 100 µg/mL of ampicillin. In addition, the BL21 (DE3) *E. coli* lacking pLysS was also transformed to test for growth. The kit also provided a pET TOPO vector containing the *lacZ* gene (encoding β-galactosidase) for use as a positive expression control. The pET100/D/*lacZ* positive control plasmid was also used to transform host cells. A vial of the BL21 (DE3) pLysS one shot cells was thawed on ice for about 5 minutes prior to use. The plasmid DNA was diluted to 5 ng/µL of which 1 µL of this was used to transform the cells. Cells and DNA were mixed by gentle tapping. The vials were incubated on ice for 30 minutes and then incubated for 30 seconds in a water bath at 42°C. The vials were removed from the water bath and immediately placed on ice for 2 minutes after which 250 µL of SOC medium (at room temperature) was added to each vial without mixing. The vials were taped onto a microcentrifuge rack and placed on its side in a shaking incubator (230 rpm) at 37°C for 1 hour.

Following incubation, 30 µL and 100 µL of each transformation reaction mix was plated out onto two LB agar plates containing 100 µg/mL ampicillin and 34 µg/mL

chloramphenicol. The plates were inverted and incubated at 37°C overnight for approximately 16 hours.

The next day, following colony growth, spot inoculated subcultures for both the positive control and the *tromp1* transformant colonies were prepared using LB agar plates containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol (Section 3.6.2.1). The plates were incubated at 37°C overnight for approximately 16 hours. These plates are referred to as the masterplates.

This was followed with a screening PCR, as described in Section 3.6.3.2 to ensure that the insert was still present and maintained in the clones at this stage. Plasmid DNA was then isolated from positive clones (Section 3.6.3.3) and sequenced (Section 3.7). Sequencing was performed to confirm the results of the cloning PCR and to ensure correct orientation of the insert. Confirmation of this allowed proceeding with the expression study.

(3.9) Expression Study

(3.9.1) Expression of Recombinant Tromp1 protein

Single positive transformants, from the masterplates (control and *tromp1* transformants) were picked and cultured in 10 mL of LB media containing 100 µg/mL ampicillin and 34 µg/mL of chloramphenicol for selection of *E. coli* cells containing the expression plasmid. The cultures were incubated overnight at 37°C with shaking (230 rpm).

Immediately after this incubation period, the optical density at 600 nm (OD_{600}) of each culture was obtained using the Cary MinUV software. With each OD_{600} reading, a blank containing uninoculated LB media was used. Readings were done in triplicate. When the expected OD_{600} of 0.6 to 1.0 was reached (usually after 15 hours), the cultures were removed from the incubator. A 1:20 dilution of each culture was prepared using fresh LB media containing the appropriate antibiotics. This allowed the cells to quickly return to logarithmic growth (OD_{600} of 0.05 to 0.1) to reach the appropriate cell density. The cultures were incubated until they reached mid-log growth ($OD_{600} = 0.6$).

The mid-log cell density for expression of soluble recombinant Tromp1 at OD_{600} had to be optimized. The expression study was reproduced several times with OD_{600} readings of 0.3, 0.4, 0.6 and 0.8.

When the appropriate OD_{600} was reached, the cultures were induced by adding isopropylthio- β -galactoside, IPTG (Invitrogen, Appendix B-5) to a final concentration of 0.5 to 1 mM. A range in the concentrations of IPTG was used to optimize the correct concentration for the purposes of this expression study. The cultures were split into two 5 mL volumes each and IPTG was added to one of these. The uninduced half served as a control for each induced culture.

(3.9.2) Pilot Expression Study

Immediately after the addition of IPTG to each culture, 500 μ L from the induced and uninduced cultures were removed and transferred to clean 1.5 mL Eppendorf tubes.

The aliquots were centrifuged at maximum speed in a microcentrifuge for 30 seconds and the supernatant was aspirated. The cell pellets were stored at -20°C until use. These are the zero time point samples. Similarly, samples were taken from each culture every hour for 7 hours.

(3.10) Lysis of *E. coli* cells

The cell pellets were thawed at room temperature, resuspended in 500 μL of cold lysis buffer (Appendix D) and incubated on ice for 30 minutes. The bacterial suspensions were sonicated on ice 3 times for 20 seconds until the samples were no longer viscous. After each round of sonication the samples were placed on ice for 20 seconds. The sonicates were then centrifuged in a microcentrifuge at maximum speed for 10 minutes and the supernatant (soluble fraction) was transferred to a sterile Eppendorf tube and stored at -20°C . The pellets (insoluble fraction) were also stored at -20°C . Both, the supernatant and pellet protein preparations were subjected to Sodium dodecyl sulphate – polyacrylamide gel electrophoresis for analysis of its protein content.

(3.11) Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

(SDS-PAGE)

(3.11.1) SDS-PAGE Setup

The supernatant and pellet protein preparations were analysed on SDS-PAGE according to the procedure by Laemmli (1970). Both mini and large gels were prepared using the SDS-PAGE systems from BioRad.

The glass plates (11 cm x 11 cm) were sandwiched together with 0.35 mm spacers in between to create a mould for the gel preparation. The assembled plates were then clamped together and fixed onto a SDS-PAGE preparation stand. The bottom and sides of the plates were sealed with warmed 3% agarose (Appendix E-1). A 12% acrylamide separation gel (Appendix E-14) was prepared by mixing together 6.7 ml of sterile deionized water, 8 mL of 30% acrylamide mix, 5 mL of 1.5 M Tris-HCl (pH 8.8), 0.2 mL of 10% SDS, 20 μ l of N', N', N', N'- tetramethylethylenediamine (TEMED) and 0.1 mL of 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ in a glass beaker (Appendix E). The solution was gently swirled and immediately transferred into the gel slab using a 1 mL pipette. Sufficient space was allowed for the addition of a 1 cm stacking gel. Before the gel began to polymerize, its surface was overlaid with 20% ethanol (Appendix E-7). This step aligns the gel and also prevents the diffusion of oxygen into the gel which would otherwise inhibit polymerization. The gel was allowed to polymerize for 45 minutes at room temperature. The solution remaining in the beaker serves as a reference to indicate that the separating gel has polymerized. After polymerization, the ethanol overlay was removed with blotting paper.

A 5% stacking gel (Appendix E-14) was prepared by mixing together 6.1 mL of sterile deionized water, 1.3 mL of 30% acrylamide mix, 5 mL of 0.5 M Tris-HCl (pH 6.8), 0.1 mL of 10% SDS, 10 μ L of TEMED and 0.05 mL of 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ in a glass beaker (Appendix E). The stacking gel solution was poured directly onto the

surface of the polymerized separation gel. A comb was inserted into the stacking gel solution, taking care not to trap any air bubbles underneath the teeth of the comb. The gel was allowed to polymerize for 30 minutes at room temperature. After polymerization, the comb was carefully removed, creating neatly formed wells for sample loading. The wells were washed with triple distilled water to remove any unpolymerized acrylamide.

Two gels could be prepared and electrophoresed simultaneously. Furthermore, they serve to balance one another during the electrophoretic run. The gel preparations were securely mounted onto the electrophoresis apparatus and the electrophoresis tank was filled with 10x electrode buffer [0.25 M Tris-HCl (pH 8.3); 1.92 M glycine and 1% SDS] (Appendix E-8).

(3.11.2) Sample Preparation for SDS-PAGE

The supernatant and pellet protein preparations from Section 3.10 were brought to room temperature. Equivalent volumes of supernatant and 2x SDS-PAGE sample loading buffer (Appendix E-12) were mixed together and heated at 95°C for 5 minutes. The pellets were reconstituted in 100 μ L of 1x SDS-PAGE sample loading buffer (Appendix E-13) each, and then heated at 95°C for 5 minutes.

A 10 μ L volume of supernatant sample and 5 to 7 μ L of pellet sample was loaded into the wells of the SDS-PAGE gel. The protein samples were electrophoresed along with a standard molecular weight marker. The ready-to-use PageRuler Unstained

Protein Ladder, from Fermentas/Inqaba Biotec, is a mixture of 14 recombinant, highly purified proteins with molecular weights ranging from 10 to 200 kDa.

The protein samples were electrophoresed for 4 hours at 100 V for large gels and 2 hours at 100 V for minigels. After electrophoresis, the gels were carefully removed from the system and then immersed for 1 hour in isopropanol/acetic acid solution (Appendix E-9) to fix the polypeptides onto the gel. The gels were stained with Coomassie Brilliant Blue R250 staining solution (Appendix E-10) overnight on a rocker at room temperature. The next day, the gels were destained for 2 hours in 7% methanol/5% acetic acid solution (Appendix E-11), with a change in solution at least three times. The gels were stored in triple distilled water, until photographs were taken.

(3.12) Western Blotting

Western blotting was further used to identify and locate the recombinant Tromp1 fusion protein based on its ability to bind to specific antibodies.

(3.12.1) Setup of the Mini-Trans Blot Electrophoretic Transfer Cell Apparatus

To achieve equilibration, the gel was immersed in 100% methanol whilst setting up the Western blot apparatus. A large container was filled with ice cold Towbin transfer buffer (Appendix F-1). A gel holder cassette containing black and white holder ends were opened and immersed in transfer buffer. A filter pad was firmly placed on the black holder end whilst still being immersed in buffer. Similarly two

sheets of pre-cut filter paper were placed on the filter pad. A nitrocellulose membrane cut to the dimension of the acrylamide gel was placed on the filter paper. The gel was then placed onto the nitrocellulose membrane. This was followed by two more sheets of filter paper and a final closing filter pad. The gel cassette holder was then closed and inserted into the tank filled with Towbin transfer buffer. A Bio-ice cooling unit (kept frozen at -20°C) was inserted into the tank to prevent the system from overheating during the electrophoretic transfer, otherwise the gel will stick to the membrane. A magnetic stirrer was placed at the bottom of the tank to obtain a consistent equilibrium of buffer. The gel was electrophoresed at 90 V, 400 mA for 3 hours. At the end of the run, the system was dismantled. The gel was stained with Coomassie staining solution (Appendix E-10) and destained with 7% acetic acid, 5% methanol (Appendix E-11) as performed in Section 3.11. This was to ensure that all protein bands had been successfully transferred onto the nitrocellulose membrane blot. Hence, the gel should be clear when the transfer is complete. The membrane blot was marked with a pencil at its right hand corner to indicate its correct side and was handled with a pair of sterile tweezers at all times. The blot was washed briefly with triple distilled water and then stained with Ponceau S solution (Appendix F-2) for 1 minute, to stain the protein. The solution was then drained and the blot was rinsed with triple distilled water until its background was reduced and dried for approximately 1.5 hours between two Whattman filter papers.

(3.12.2) Immunodetection for the Presence of N-terminal Xpress Fusion Proteins

The membrane blot was blocked for 1 hour in 20 mL 1x PBS containing Tween-20 and 5% non-fat dry milk (PBSTM, Appendix F-3), at room temperature on a shaker. The membrane was then washed three times with 1x PBS containing 0.1% Tween-20 (PBST, Appendix F-4) at 5 minutes per wash.

Anti-Xpress antibodies (purchased from Invitrogen) directed against recombinant proteins containing the N-terminal leader peptide, Xpress epitope (fusion tag), was used as the primary antibody. The Anti-Xpress antibody is a mouse monoclonal IgG antibody that recognizes the Xpress epitope sequence, -Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys found in this leader peptide. The primary antibody was diluted 1:5000 in 1x PBS containing 0.05% Tween-20 and 5% non-fat dry milk (PBSTM, Appendix F-3) and incubated for one hour at room temperature on a shaker. The membrane was then washed three times with 1x PBS containing 0.1% Tween-20 (PBST) at 5 minutes per wash, as above.

Goat Anti-Mouse polyclonal IgG conjugated to horseradish peroxidase (HRP), from AbD Serotec, was used as the secondary antibody. The antibody was reconstituted according to the manufacturer's instructions. The reconstituted secondary antibody was diluted 1:5000 in 1x PBS containing 0.05% Tween-20 and 5% non-fat dry milk (PBSTM, Appendix F-3) and incubated for one hour at room temperature on a shaker.

A 5 mL volume of BM Blue POD precipitating chromogenic substrate (Roche Diagnostics) was added to the membrane and incubated in the dark, at room

temperature until the appearance of bands. The membrane was washed with triple distilled water and dried between two Whatmann filter papers. BM Blue POD is a chromogenic substrate for the detection of peroxidase. It produces a permanent dark blue band or spot at the peroxidase binding site on the membrane.

(3.13) Enzyme Linked Immunosorbent Assay (ELISA)

An indirect ELISA was performed to confirm the results of SDS-PAGE and Western Blot. It is possible that the protein may be present, but in far too low a concentration to be detected by these two assays mentioned above. ELISA produces a colour change should the protein be present at low concentrations.

The supernatant protein preparations (taken at all time points at every hour during expression, following IPTG induction) were used as the antigen for the ELISA. The protein antigen was coated on an ELISA microtitre plate. The antigen was diluted 1:2 with 1x Coating buffer (Appendix G-1). A 100 μ L volume of antigen-coating solution was added to all wells. The plate was covered with a lid and placed into a sealed humidified container and incubated overnight at room temperature. The next morning, the well contents were decanted to remove any residual antigen-coating solution. The wells were then washed three times with 1x PBST Wash buffer (Appendix G-2). Remaining protein binding sites were blocked by the addition of 100 μ L of Blocking buffer (Appendix G-3) to each well. The plate was covered with a lid and incubated for one hour at room temperature. After incubation the well contents were decanted and washed three times with 1x PBST Wash buffer.

Anti-Xpress antibodies (Invitrogen) directed against recombinant proteins containing the N-terminal leader peptide, Xpress epitope (fusion tag), was used as the primary antibody for this ELISA. The primary antibody was diluted 1:2000, 1:5000 and 1:10000 with PBS containing 0.01% Tween-20 and 3% non-fat dry milk (Appendix G-4). A 50 μ L volume of each dilution was added to appropriate wells and the plate was incubated in a sealed, humidified container for one hour at room temperature. After incubation the well contents were decanted and washed three times with 1x PBST Wash buffer.

Goat Anti-Mouse polyclonal IgG conjugated to horseradish peroxidase (HRP), purchased from AbD Serotec, was used as the secondary antibody for this ELISA. The reconstituted antibody was diluted 1:15000 with PBS containing 0.01% Tween-20 and 3% non-fat dry milk (Appendix G-4). A 50 μ L volume of secondary antibody was added to each well and the plate was incubated in a sealed, humidified container for one hour at room temperature. After incubation, the well contents were decanted and washed three times with 1x PBST Wash buffer.

SIGMAFAST OPD (o-phenylenediamine dihydrochloride) (Sigma-Aldrich) tablets were used as substrate for the detection of peroxidase activity in the ELISA. The substrate solution was prepared according to the manufacturer's instructions. A 200 μ L volume of substrate solution was added to each well and the plate was incubated in the dark at room temperature for 30 minutes or until a colour developed. After the incubation period the reaction was stopped by adding 50 μ L of 3 M HCl. Stopped reactions were then read at 492 nm in a MicroELISA Autoreader.

CHAPTER 4

RESULTS

(4.1) Propagation of *T. pallidum*

The rabbit testicular tissue extract showed a high concentration of motile spirochetes when viewed by darkfield microscopy (Figure 1) at a magnification of x450. Just a single rabbit was required for this work. This extract was used throughout the study.

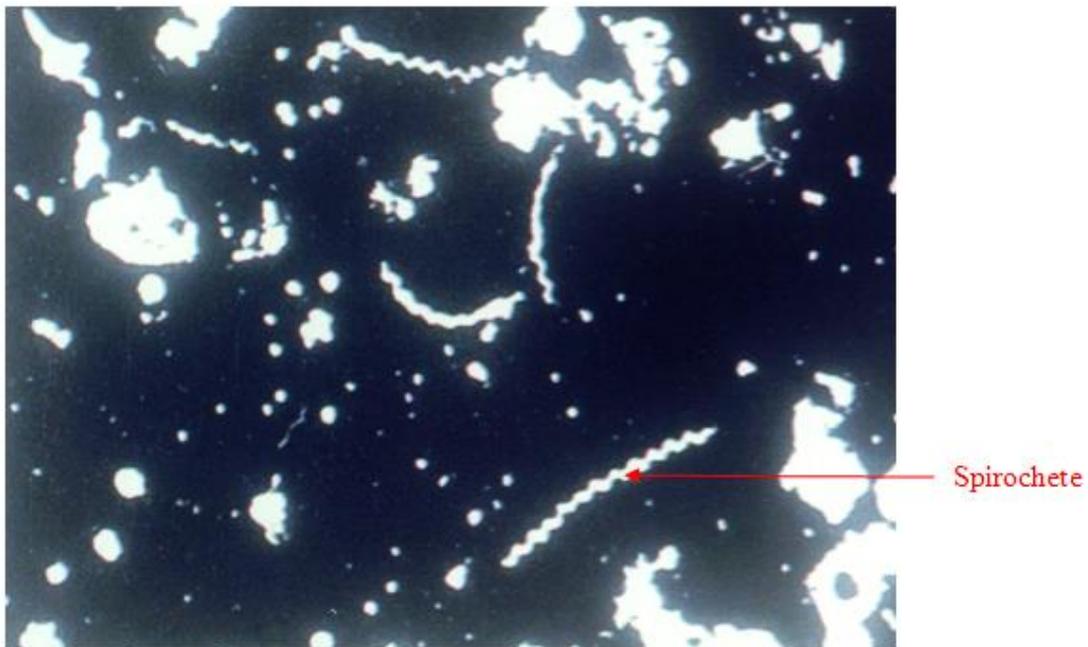


Figure 1: The presence of viable treponemes as viewed by darkfield microscopy,
(magnification, x450)

(4.2) *T. pallidum* DNA Extraction and Purification

T. pallidum DNA was obtained by suspending the testicular extract in ProbeTec lysis buffer (BD ProbeTec ®, Becton Dickinson) followed by heating. The obtained crude DNA was purified as described in Section 3.2. The concentration of recovered DNA as determined by spectrophotometry (Nanodrop 2000) was estimated to be 55.4 ng/μL with a purity (A_{260}/A_{280} ratio) of 1.96 (Figure 2).

Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
10/12/2009 3:05:34 PM	55.4	ng/μl	1.109	0.566	1.96	2.16	DNA	50.00

Figure 2: Results of quantification of purified *T. pallidum* DNA as determined using the Nanodrop 2000

(4.3) Optimization of the Concentration of dNTPs and MgCl₂ for PCR

Figures 3 a and b show the results of optimization of the concentration of dNTPs and MgCl₂ in the mastermix to be used for amplification of the *tromp1* gene by conventional PCR.

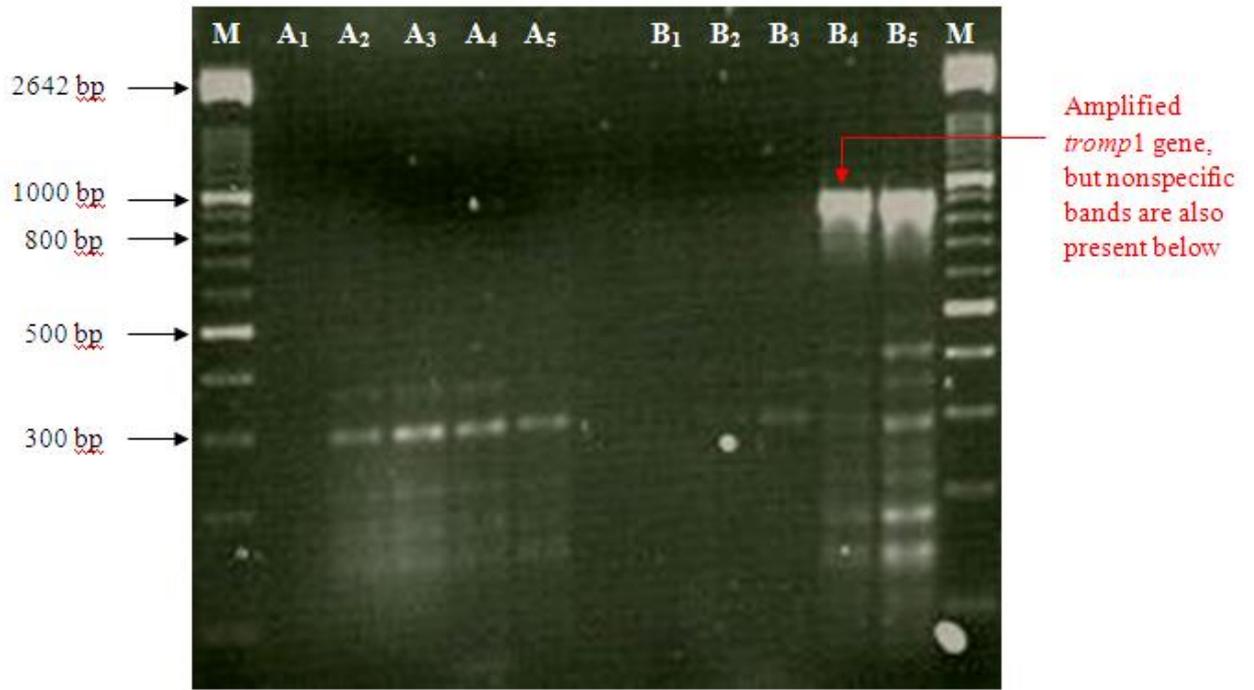


Figure 3 a

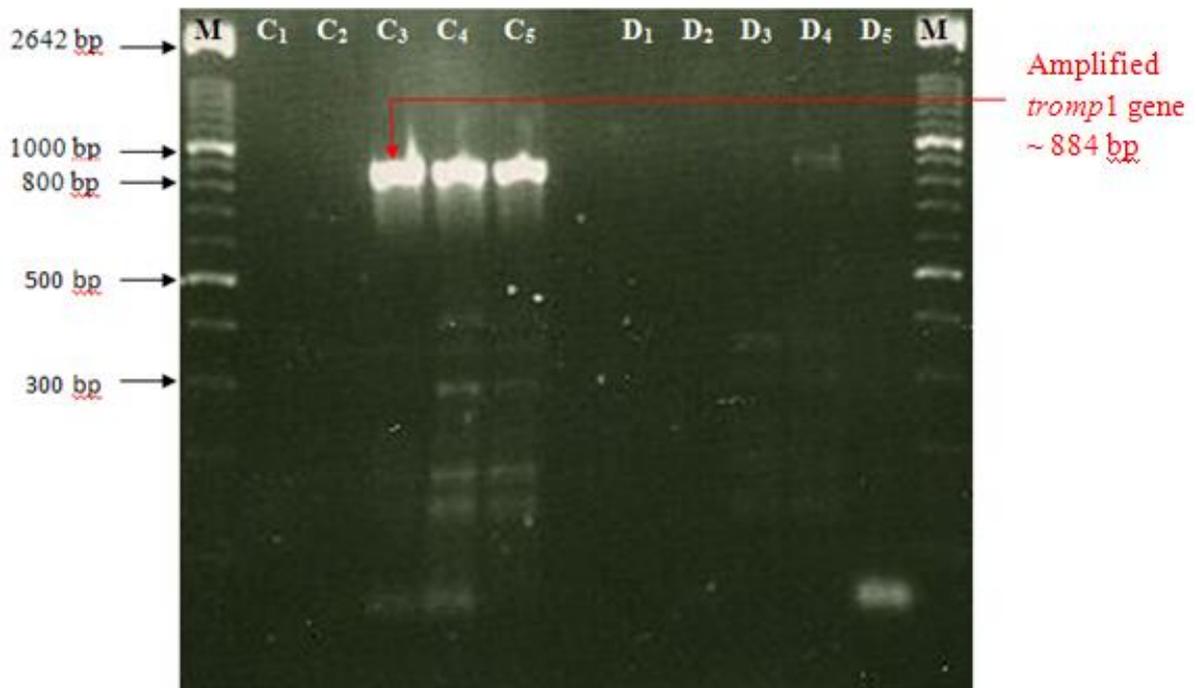


Figure 3 b

Figures 3 a and b: Optimization of the concentration of dNTPs and MgCl₂ for the amplification of the *tromp1* gene

It was established that a final concentration of 1 mM MgCl₂ and 200 μM dNTPs (lane C₃) was efficient for this PCR reaction as indicated by the brightest and most defined band at the desired region of ~884 bp. In addition, lane C₃ contained fewer nonspecific bands as opposed to C₄₋₅ and B₄₋₅. DNA molecular weight marker XIV (100 to 1500 bp) was used to determine the size of the amplified products (lane M).

(4.4) Primer Concentration/Temperature-Gradient PCR

Figure 4 a and b shows the result of optimization of primer concentration and annealing temperature for the PCR reaction.

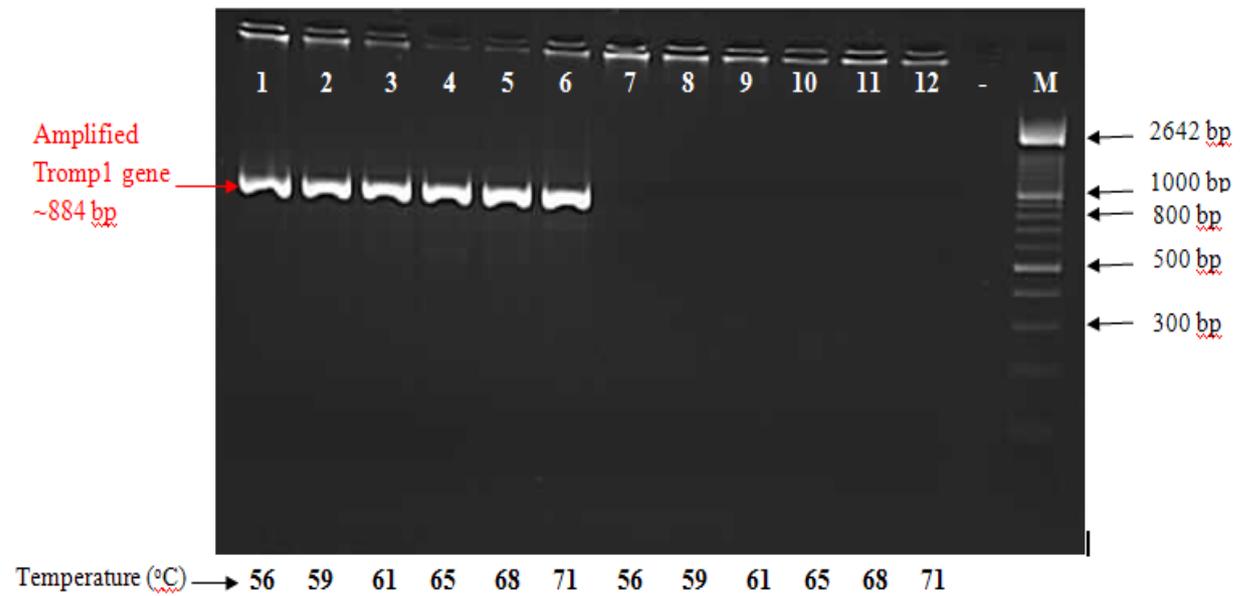


Figure 4 a

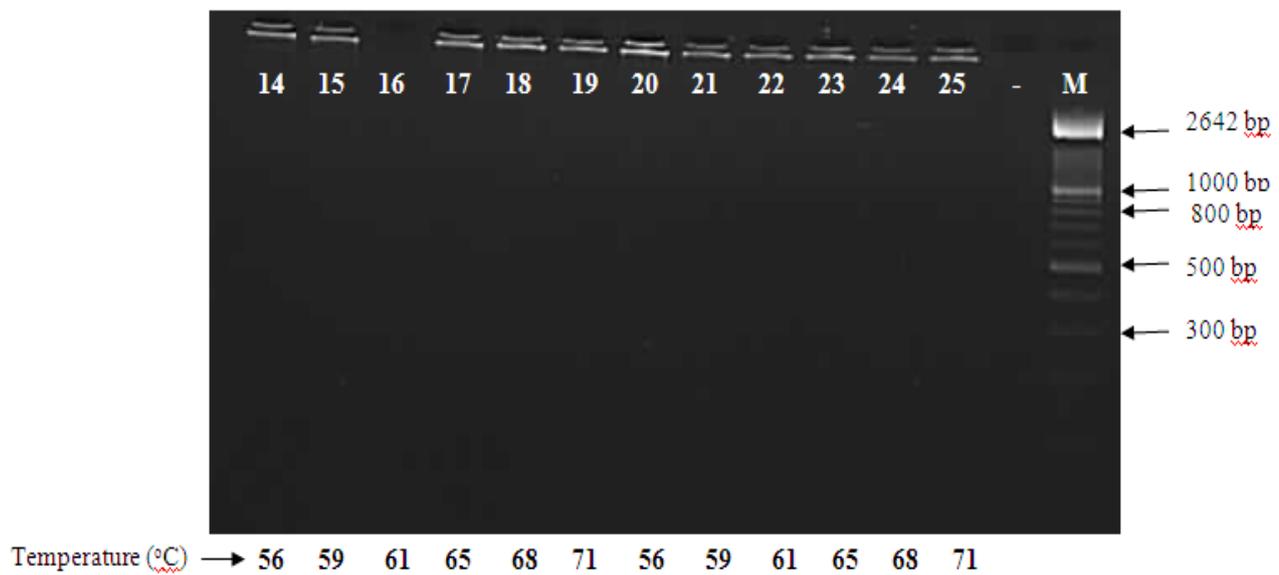


Figure 4 b

Figure 4 a and b. Agarose gel electrophoresis showing the amplification products obtained at different annealing temperatures and different primer concentrations.

Lanes 1-6 shows the *tromp1* amplification product (lacking the signal peptide) obtained with a final concentration of primers of 0.05 μM measured at varying annealing temperatures (56°C to 71°C). Lanes 7 to 12 show the results obtained over the same temperature range with a final primer concentration of 0.01 μM . Lanes 14 to 19 and lanes 20 to 25 show the results obtained with a final primer concentration of 0.005 μM and 0.0025 μM respectively. However, no bands were identified in these lanes, indicating that the optimal conditions for the *tromp1* PCR was a final primer concentration of 0.05 μM for each of the forward and reverse primers and an annealing temperature of 56 to 71°C. An annealing temperature of 68°C was used throughout the study.

Figure 5 shows the results following the amplification of the *tromp1* gene under optimized PCR conditions, prior to the purification of the amplified product.

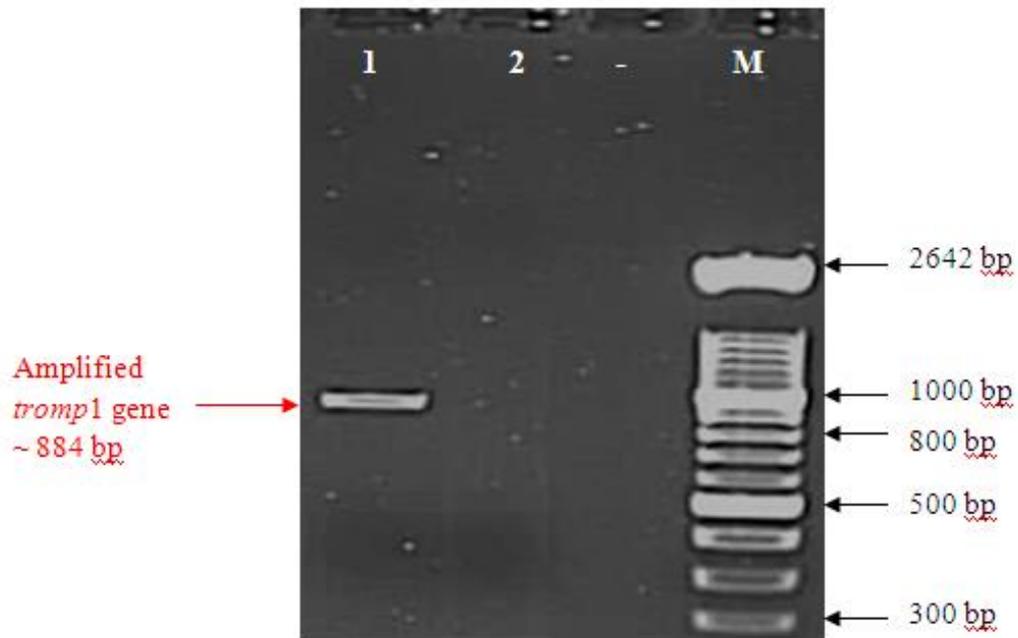


Figure 5: Amplification of the *tromp1* gene using the optimized mastermix and reaction conditions. Lane 1: *tromp1* gene; lane 2: negative control; M: molecular weight marker

(4.5) Purification of the *tromp1* Amplification Product

Figure 6 shows the electrophoresis results of the amplified *tromp1* gene product following purification based on the selective binding of double stranded DNA to silica-based membrane in the presence of chaotropic salts. The presence of a single band of the expected molecular weight confirms the purity.

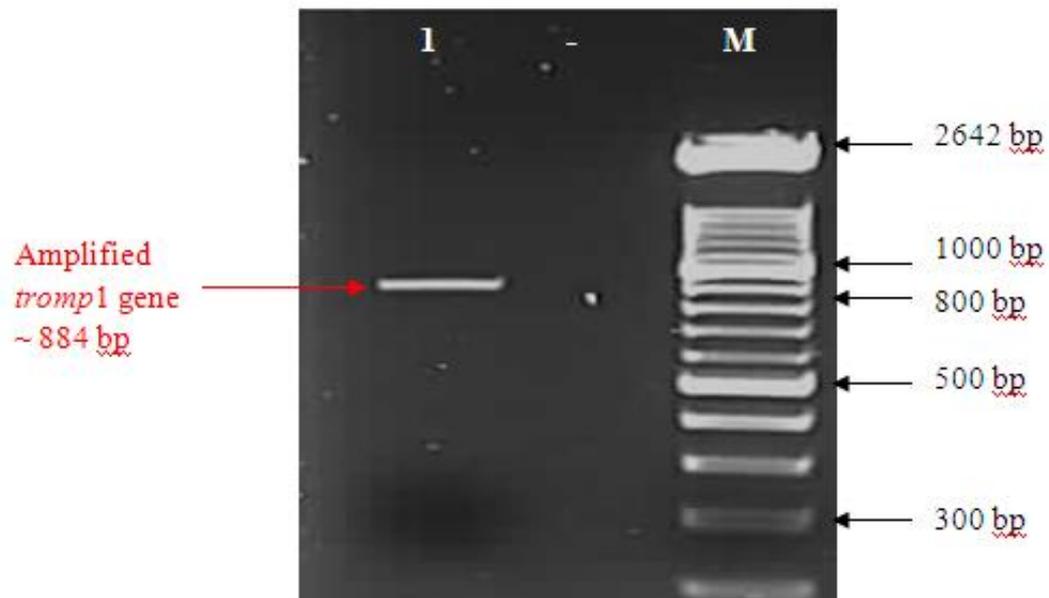


Figure 6: Agarose gel electrophoresis results of purified PCR product (lane 1);
lane 2: Marker XIV

(4.6) Cloning of the *tromp1* Amplification Product

TOP10 *E. coli* cells were transformed with pET100/D/*tromp1*. Figure 7 shows transformed colonies following an overnight incubation.

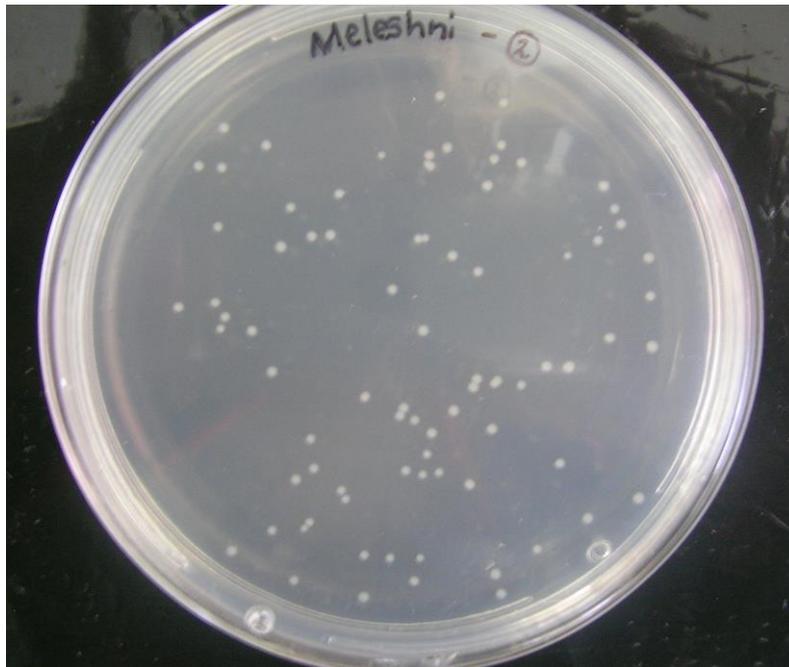


Figure 7: Selective LB agar plate containing ampicillin, showing single colonies of competent TOP10 *E. coli* cells transformed with pET100/D/*tromp1*

(4.7) Screening Transformant Colonies for Recombinant DNA

A varying number of randomly chosen colonies were screened for the presence of *tromp1* inserts by PCR. Colonies were picked off from the subcultures on the LB agar plates and suspended in 100 μ L LB broth followed by incubation at 37°C for 90 minutes. A PCR targeting the *tromp1* insert was performed using the mastermix and conditions as discussed in Section 3.6.3.2. The results are shown in figure 8. All colonies screened contained the *tromp1* insert as indicated by the presence of bands of the required molecular weight.

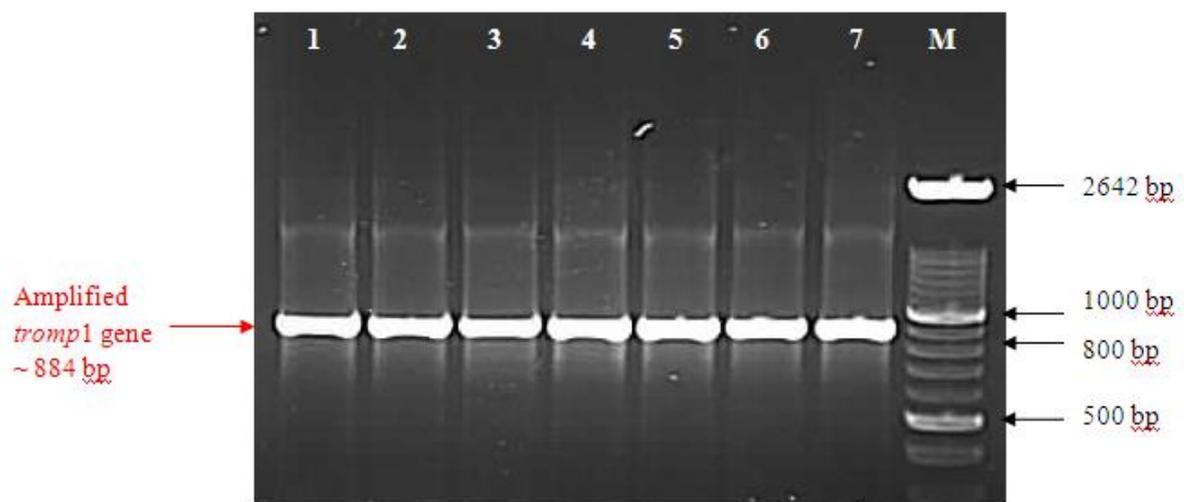


Figure 8: Amplification products of the 884 bp inserts (lanes 1 to 7) from seven *E. coli* colonies.

(4.8) Restriction Digest of Pure Plasmid DNA

Figure 9 shows a restriction digest of the pET100/D/*tromp1* plasmid DNA using BamHI and EcoRI restriction enzymes. These endonucleases were selected for the digest since the primers used for the amplification of the *tromp1* gene contain their restriction endonuclease sites at the 5' ends. The forward primer contains the BamHI restriction site whilst the reverse primer contains the EcoRI restriction site. The *tromp1* gene band is clearly visible as well as the bands representing the plasmid and the vector.

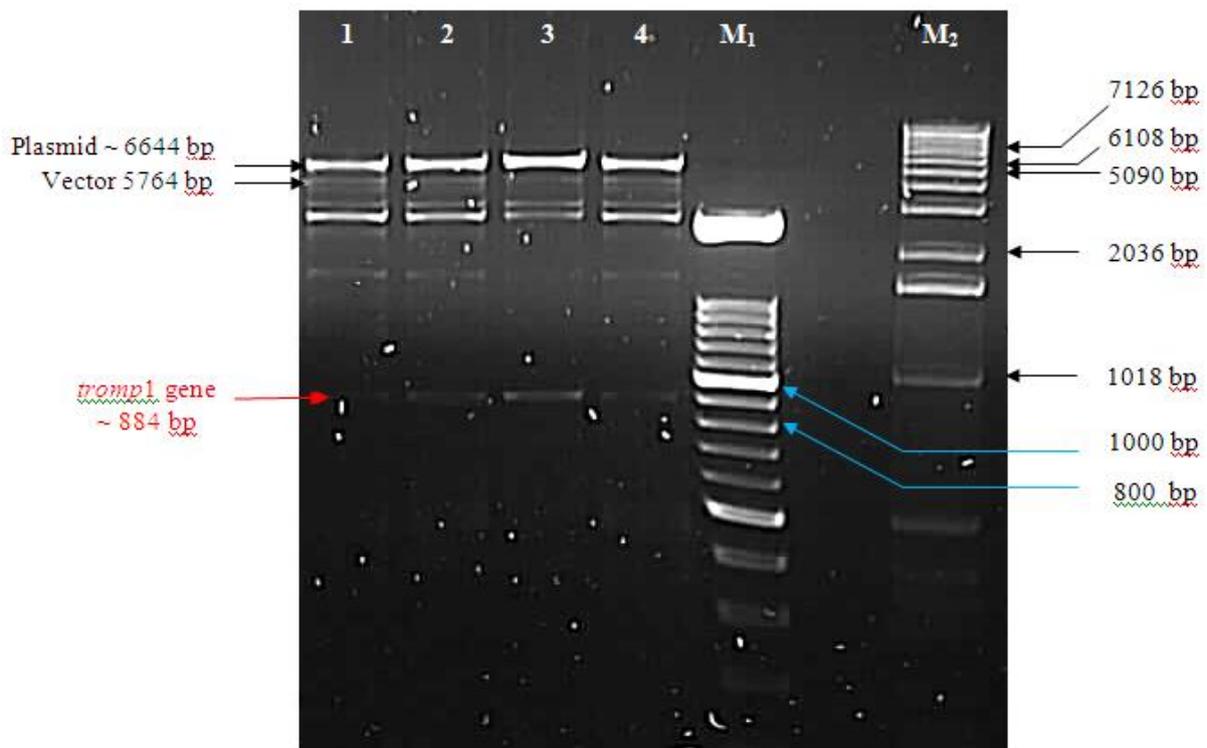


Figure 9: Agarose gel electrophoresis of plasmid DNA digested with BamHI and EcoRI restriction enzymes (Lanes 1 to 4). Bands of ~6644 bp region represent undigested plasmid DNA whilst the bands at ~5764 bp represent the vector only. M₁: molecular weight marker XIV; M₂: molecular weight marker X (0.07 to 12.2 kbp).

(4.9) DNA Sequencing - Optimization

Sequencing was performed to confirm that the *tromp1* insert was cloned into the correct reading frame of the pET100/D-TOPO expression vector. The isolated plasmid DNA (recombinant DNA) served as the template for the fluorescence-based cycle sequencing reaction. After using varying dilutions of the template DNA for the reaction that proved unsuccessful, it was decided that the DNA be used neat for all sequencing reactions.

The primers used for the PCR reaction (Section 3.5.2) were initially used for the cycle sequencing reaction as well (Table 1). The primers were diluted to a final concentration of 3.2 pmol/ μ L for the reaction. However, after several attempts these primers failed to produce a sequencing signal for analyses (Appendix C).

<u>Initial PCR primer set</u>	
Forward primer	5'-CACCCGCGGATCCATTCGGTAGCAAGGATGCCGCA-3'
Reverse primer	5'-CCGGAATTCCTAGCGAGCCAACGCAGCAAC-3'

Table 1: Nucleotide sequences of the initial PCR primer pair

It was then decided that the PCR primers would be redesigned for use in the sequencing reaction in an attempt to decrease the high GC content of both primers and shorten their overall length (Table 2). In one set of primers, the restriction sites and the CACC nucleotide overhang (forward primer) of the PCR primers were removed. A second set of primers was designed to a sequence internal to the primers on either end of the insert. This resulted in primer sets with the following nucleotide sequences:

<u>Primer set 1</u>	
Forward primer 1	5'-ATTCGGTAGCAAGGATGCCGCA-3'
Reverse primer 1	5'-CTAGCGAGCCAACGCAGCAAC-3'
<u>Primer set 2</u>	
Forward primer 2	5'-CATGATAGCGGATGCTGTC-3'
Reverse primer 2	5'-AACAACTCGCCTCCAAT-3'

Table 2: Nucleotide sequences of the primer sets used for the sequencing reaction

The new sequencing primers were reconstituted according to the manufacturer's instructions and diluted to a final concentration of 3.2 pmol/ μ L. Both newly designed primer sets successfully produced sequencing data and were used for all sequencing reactions henceforth.

(4.10) DNA Sequencing of Inserts Following Transformation of *E. coli* TOP10**Cells with pET100/D/*tromp1***

Figure 4.10 shows the DNA sequence of the *tromp1* gene as inserted into the pET100/D-TOPO expression vector, i.e. the plasmid DNA obtained from a positive clone. This sequence was compared to the *tromp1* reference sequence (Figure 4.11) obtained from GenBank (www.ncbi.nlm.nih.gov).

```

ggccacgatgctccggcgtagaggatcgagatctcgatcccgcgaaattaatacgactcactataggggaattgtgagc
ggataacaattcccctctagaataatTTTgtttaactttaagaaggagatatacatatgCGGGgttctcatcatcatcatcat
ggtatggctagcatgactggtggacagcaaatgggtcgggatctgtacgacgatgacgataaggatcatcccttcaaccgc
ggatccattcggtagcaaggatgccgcagcggacgggaaaccctggtgtcaccaccattggcatgatagcggat
gctgtcaaaaacatcgctcaaggtgatgtgcatctaaaggggtgatgggtcctggtgtgaccgcacctgtacac
ggctactgcgggggatgtggaatggctcgggaatcggatctcctctgtacaacgggtgcacctggaaaccaag
atgggcgaggtgtttcaaaactgcgcgggagccgttggtagttgcagtttctgagactattccggtgtctcagcgt
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acgaaagctgtgcaagctgtgccgggaaaaactcgcgaattactcaacgttatcaggcgtaccagcagcagtt
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agtgcgcatgatatgcaggaactggcagcgttattgcgcagcgtaaactccctgctatcttattgagagttctattc
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tttctgatgcgatgggggatcgggtacgagcgagggtacctacgtagggatggtaacacacaatatcgatacga
tcgttgctcgttggctcgttaggaattccggaagggcgagctcaacgatccggctgctaacaagcccgaaggaag
ctgagttggctgctgccaccgctgagcaataactagcataacccttggggcctctaaacgggtcttgaggagttttgctg
aaaggaggaactatatccggatcccgaagaggccggcagtagccggcataccaagcctatgctacagcatccag
ggtgacggt

```

KEY:

	pET100/D-TOPO
	Forward and Reverse primers
	<i>T. pallidum</i> (Tromp1) gene sequence
	Vector start codon
	Stop codon
	Ribosomal binding site
	T7 promoter/priming site

Figure 10: DNA sequence of the *tromp1* gene inserted into the pET100/D-TOPO expression vector. Highlighted in black bold print is the entire *tromp1* DNA sequence lacking the signal peptide. Shown in red, is the expression vector with the underlined regions indicating the N-terminal polyhistidine and Xpress fusion tags respectively. The forward and reverse primers are highlighted in blue.

The *tromp1* amino acid sequence begins with a 40-residue hydrophobic region presumed to be the signal peptide (Blanco *et al.*, 1995). However since the signal peptide was not required for this study and had been deliberately excluded so as to express non-exported, soluble protein, the sequence (Figure 10) lacks a start codon (atg). Instead it uses the start codon on the expression vector, located downstream from its ribosomal binding site. The T7 promoter/priming site is situated upstream from the ribosomal binding site. The stop codon of the *tromp1* gene occurs within the reverse primer. This sequence showed 100% homology when blasted against the *tromp1* reference sequence (Figure 11) obtained from GenBank (www.ncbi.nlm.nih.gov) under the accession number U16363.1.

atgcatcaaaattcacccaagcagtgctcacttgatacgtgaaagaatatgtgcctgcgtgctcgcgcttgcatgctgaccg
gttttacgcacgcattcggtagcaaggatgccgcagcggacgggaaaccctggttgcaccaccattggcatgatagcg
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gaggtgtttccaaactgcgcgggagccgcttgtagttgcagtttctgagactattccggtgtctcagcgttttcttggagg
aagcagagttc gatccgcatgtgtggttgatgtaaagctgtggtcttattcgggaaggcagtgtagaaagcttgcaag
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ggcgcaaggcgcagtcgctgcctgctgaaaggcgtgtgttggtgaccgctcatgatgcgttcggctattttagccgtgcgta
tggtttgaggtgaaggggtgcaaggggtgagcaccgctcgggaagccagtcgcatgatatgcaggaactggcagcgt
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aggcaagagggcacgtagtgagattggaggcagttgtttctgatgcgatgggggatgcgggtacgagcagggtac
ctacgtagggatggtaacacacaatatcgatacgtcgttctgcgttggtcgcctagcgtggaatgtagagttgagtggt
agaagagccgttggtggtcggctgtatggcagc

Figure 11: *tromp1* reference gene sequence obtained from GenBank (www.ncbi.nlm.nih.gov) under accession number U16363.1

(4.11) Multiple Sequence Alignment

A nucleotide blast between the cloned *tromp1* gene and its reference sequence (accession number U16363.1) was performed on NCBI (blast.ncbi.nlm.nih.gov). Figures 4.12 a and b show the nucleotide blast and multiple sequence alignment of the cloned *tromp1* gene sequence against its reference sequence, respectively. This shows a 100% identity of the cloned gene.

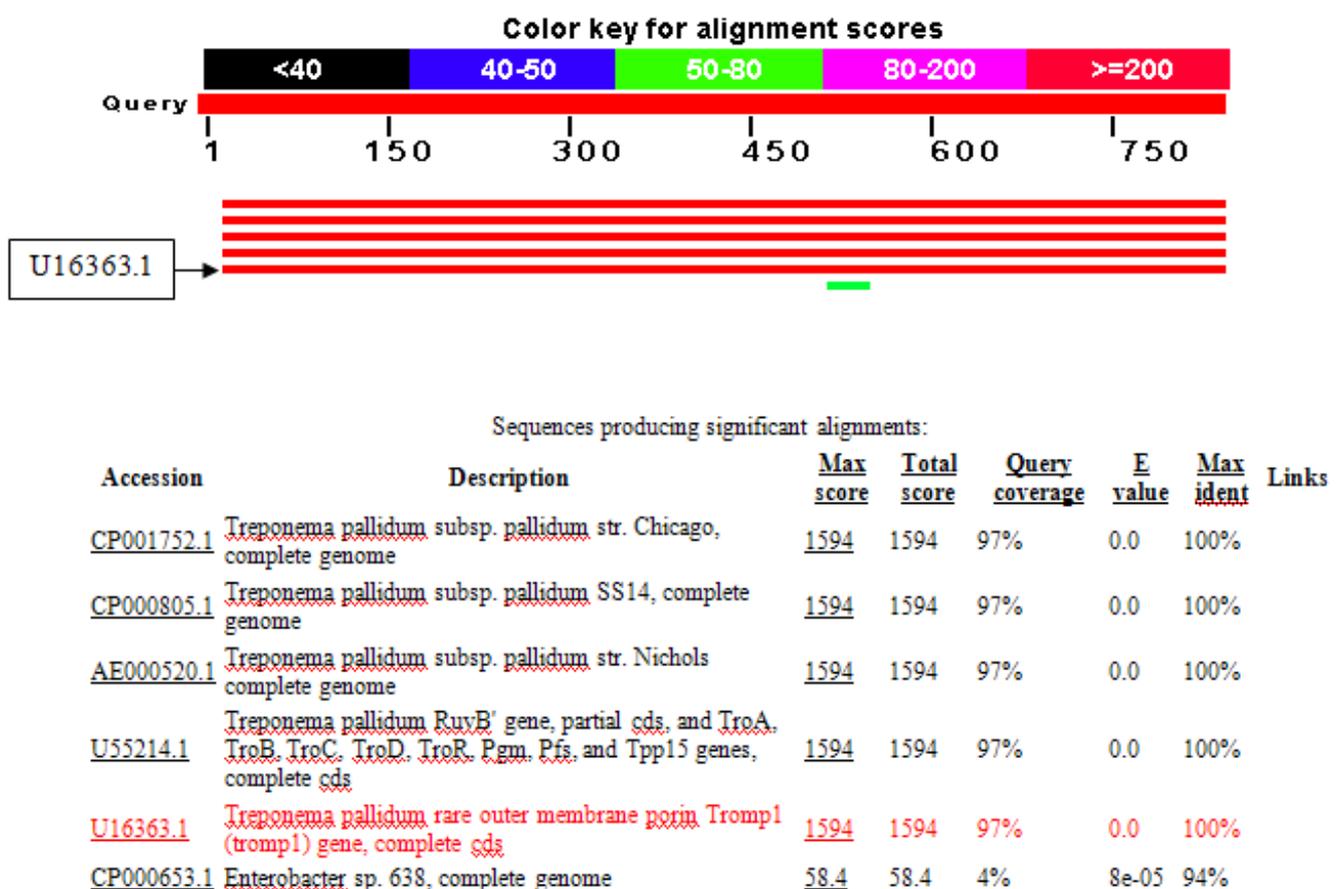


Figure 12 a: Results of a nucleotide blast of the cloned *tromp1* insert against the nucleotide collection available on NCBI (blast.ncbi.nlm.nih.gov). The results show 100% homology to the *tromp1* reference sequence, U16363.1.

[gb|U16363.1|TPU16363](#) Treponema pallidum rare outer membrane porin Tromp1 (tromp1)
gene, complete cds
Length=1014

Score = 1594 bits (863), Expect = 0.0
Identities = 863/863 (100%), Gaps = 0/863 (0%)
Strand=Plus/Plus

```

Query 13  CATTTCGGTAGCAAGGATGCCGCAGCGGACGGGAAACCCCTGGTTGTCACCACCATTGGCA 72
          |||
Sbjct 95  CATTTCGGTAGCAAGGATGCCGCAGCGGACGGGAAACCCCTGGTTGTCACCACCATTGGCA 154

Query 73  TGATAGCGGATGCTGTCAAAAACATCGCTCAAGGTGATGTGCATCTAAAGGGGTTGATGG 132
          |||
Sbjct 155 TGATAGCGGATGCTGTCAAAAACATCGCTCAAGGTGATGTGCATCTAAAGGGGTTGATGG 214

Query 133 GTCCTGGTGTGACCCGCACCTGTACACGGCTACTGCGGGGGATGTGGAATGGCTCGGGA 192
          |||
Sbjct 215 GTCCTGGTGTGACCCGCACCTGTACACGGCTACTGCGGGGGATGTGGAATGGCTCGGGA 274

Query 193 ATGCGGATCTCATCCTGTACAACGGGTTGCACCTGGAACAAGATGGGCGAGGTGTTTT 252
          |||
Sbjct 275 ATGCGGATCTCATCCTGTACAACGGGTTGCACCTGGAACAAGATGGGCGAGGTGTTTT 334

Query 253 CCAAAGTGCAGCGGAGCCGCTTGGTAGTTGCAGTTTCTGAGACTATTCCGGTGTCTCAGC 312
          |||
Sbjct 335 CCAAAGTGCAGCGGAGCCGCTTGGTAGTTGCAGTTTCTGAGACTATTCCGGTGTCTCAGC 394

Query 313 GTCTTTCTCTTGAGGAAGCAGAGTTCGATCCGCATGTGTGGTTTGTATGTAAGCTGTGGT 372
          |||
Sbjct 395 GTCTTTCTCTTGAGGAAGCAGAGTTCGATCCGCATGTGTGGTTTGTATGTAAGCTGTGGT 454

Query 373 CTTATTCGGTGAAGGCAGTGTACGAAAGCTTGTGCAAGCTGTTGCCGGGAAAAACTCGCG 432
          |||
Sbjct 455 CTTATTCGGTGAAGGCAGTGTACGAAAGCTTGTGCAAGCTGTTGCCGGGAAAAACTCGCG 514

Query 433 AATTTACTCAACGTTATCAGGCGTACCAGCAGCAGTTGGATAAGCTTGACGCGTACGTT 492
          |||
Sbjct 515 AATTTACTCAACGTTATCAGGCGTACCAGCAGCAGTTGGATAAGCTTGACGCGTACGTT 574

Query 493 GCGCAAGGCGCAGTCGCTGCCTGCTGAAAGGCGTGTGTTGGTGACCGCTCATGATGCGT 552
          |||
Sbjct 575 GCGCAAGGCGCAGTCGCTGCCTGCTGAAAGGCGTGTGTTGGTGACCGCTCATGATGCGT 634

Query 553 TCGGCTATTTTAGCCGTGCGTATGGTTTTGAGGTGAAGGGGTTGCAAGGGGTGAGCACCG 612
          |||
Sbjct 635 TCGGCTATTTTAGCCGTGCGTATGGTTTTGAGGTGAAGGGGTTGCAAGGGGTGAGCACCG 694

Query 613 CTTCGGAAGCCAGTGCATGATATGCAGGAAGTGGCAGCGTTTATTGCGCAGCGTAAAC 672
          |||
Sbjct 695 CTTCGGAAGCCAGTGCATGATATGCAGGAAGTGGCAGCGTTTATTGCGCAGCGTAAAC 754

Query 673 TCCCTGCTATCTTTATTGAGAGTTCTATTCCGCACAAAAACGTTGAAGCGTTAAGGGATG 732
          |||
Sbjct 755 TCCCTGCTATCTTTATTGAGAGTTCTATTCCGCACAAAAACGTTGAAGCGTTAAGGGATG 814

Query 733 CGGTGCAGGCAAGAGGGCACGTAGTGCAGATTGGAGGCGAGTTGTTTTCTGATGCGATGG 792
          |||
Sbjct 815 CGGTGCAGGCAAGAGGGCACGTAGTGCAGATTGGAGGCGAGTTGTTTTCTGATGCGATGG 874

Query 793 GGGATGCGGGTACGAGCGAGGGTACCTACGTAGGGATGGTAACACACAATATCGATACGA 852
          |||
Sbjct 875 GGGATGCGGGTACGAGCGAGGGTACCTACGTAGGGATGGTAACACACAATATCGATACGA 934

Query 853 TCGTTGCTGCGTTGGCTCGCTAG 875
          |||
Sbjct 935 TCGTTGCTGCGTTGGCTCGCTAG 957

```

Figure 12 b: Multiple sequence alignment (blast.ncbi.nlm.nih.gov) of the cloned *tromp1* insert against the reference sequence

(4.12) Transformation of BL21 (DE3) pLysS *E. coli* cells with pET100/D/*tromp1*

Figure 13 shows single colonies following overnight growth of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/*tromp1* (plasmid DNA), in preparation for the expression study. The transformed *E. coli* cells were spread on an LB agar plate containing ampicillin and chloramphenicol as selective agents for the plasmid and pLysS respectively.



Figure 13: Selective LB agar plate containing ampicillin and chloramphenicol, showing single colonies of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/*tromp1*

Figure 14 shows single colonies following overnight growth of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/*lacZ*, which serves as a positive control in the expression study. The control vector contains the *lacZ* gene (encoding for β -galactosidase) that has been directionally cloned into it.



Figure 14: Selective LB agar plate containing ampicillin and choramphenicol, showing single colonies of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/*lacZ* (positive control)

(4.13) Subcultures of BL21 (DE3) pLysS *E. coli* Transformants

Figures 15 and 16 show spot inoculated subcultures of the pET100/D/*tromp1* and pET100/D/*lacZ* transformed *E. coli* colonies. This method provides a clone of desired colonies and allocates numbers to each of these for referral purposes.

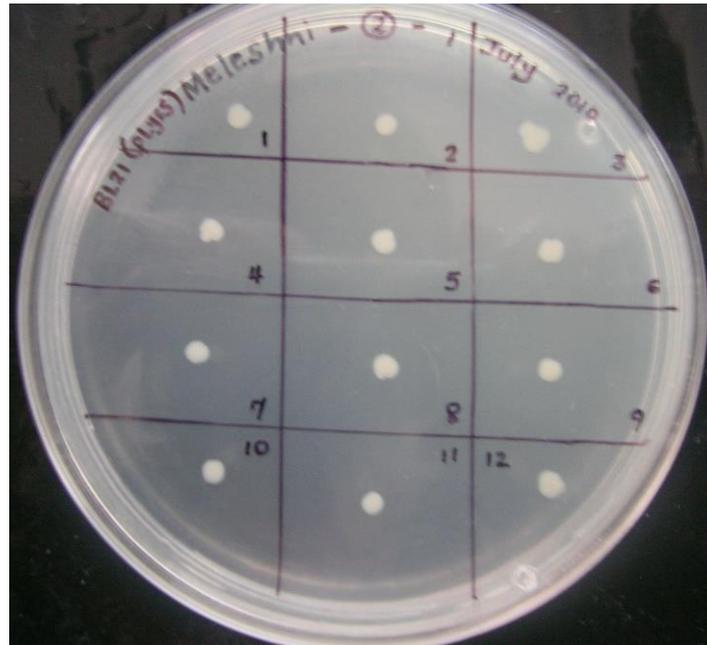


Figure 15: Subcultures of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/*tromp1*

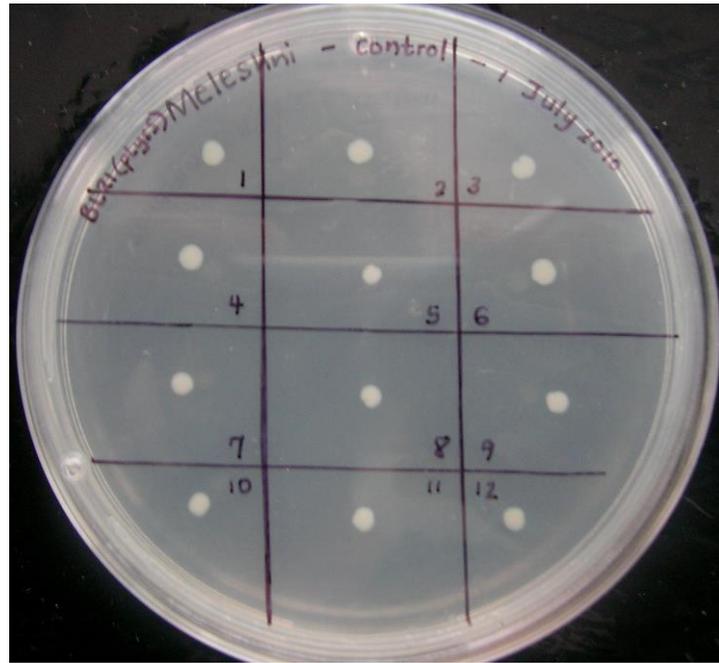


Figure 16: Subcultures of BL21 (DE3) pLysS *E. coli* cells transformed with pET100/D/lacZ (positive control)

(4.14) Screening Transformant Colonies for Recombinant DNA

Figure 17 shows the results of the PCR reaction on progeny of individual BL21 (DE3) pLysS *E. coli* colonies transformed with pET100/D/*tromp1*. These results show that the *tromp1* insert is present at this stage and has been successfully transformed into the *E. coli* expression host.

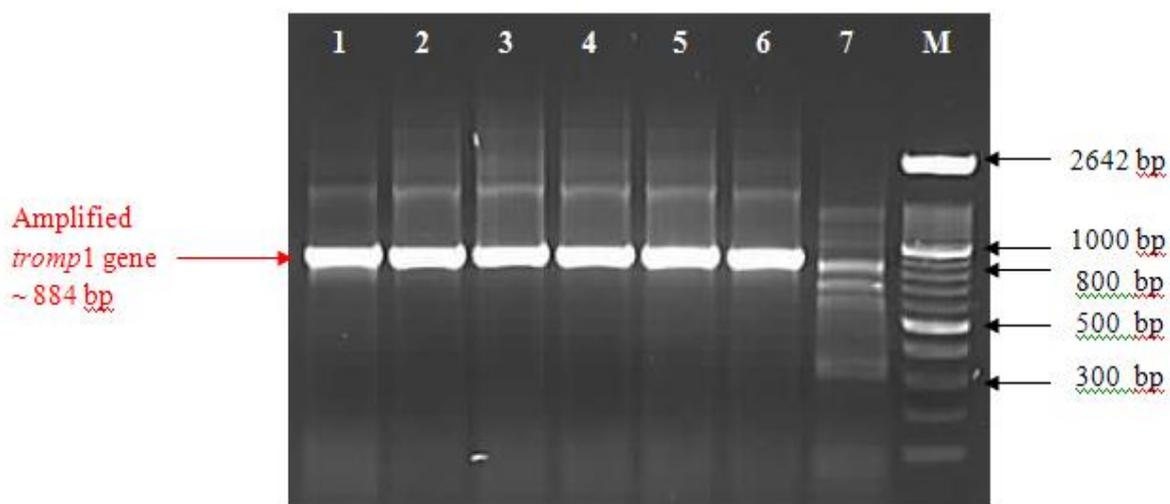


Figure 17: Agarose gel electrophoresis results showing the 884 bp amplification products (lanes 1 to 6) for the selection of recombinant clones. Lane 7 shows the amplification product resulting from the transformed expression control.

(4.15) DNA Sequencing of the *tromp1* Insert Isolated from Transformed BL21

(DE3) pLysS *E. coli*

Figure 18 shows the DNA sequence of the *tromp1* insert following isolation of the plasmid DNA from transformed BL21 (DE3) pLysS *E. coli* cells.

```

aaaaacatcgctcaaggtgatgtgcatctaaaggggtgatgggtcctgggtgtgacccgcacctgtacacggctactgcg
ggggatgtggaatggctcgggaatgcggatctcatcctgtacaacgggtgcacctggaaccaagatgggcgaggtgtt
ttccaaactgcgcgggagccgcttggtagttgcagttctgagactattccggtgtctcagcgtctttctcttgaggaagcaga
gttcgatccgatgtgtggttgatgtaaagctgtggtcttattcggggaaggcagtgacgaaagcttgcaagctgttcc
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ggcgcagtcgctgcctgctgaaaggcgtgtgttggtgaccgctcatgatgcgttcggctatatttagccgtgcgtatggtttga
ggtgaaggggttcaaggggtgagcaccgcttcggaagccagtgcgcatgatatgcaggaactggcagcgttattgcgc
agcgtaaactccctgctatctttattgagagttctattccgcacaaaaacgttgaagcgttaagggatgcgggtgcaggcaaga
gggcacgtagtgag

```

Figure 18: DNA sequence of the overlapping region of the *tromp1* insert isolated from the transformed BL21 (DE3) pLysS *E. coli* host, against the *tromp1* reference sequence obtained from GenBank (www.ncbi.nlm.nih.gov), accession number U16363.1.

(4.16) Multiple Sequence Alignment

A nucleotide blast of the cloned *tromp1* insert and its reference sequence (accession number U16363.1) was performed on NCBI (blast.ncbi.nlm.nih.gov). Figures 19 a and b shows the results of a nucleotide blast and multiple sequence alignment of the cloned *tromp1* insert against its reference sequence, respectively. This nucleotide blast shows a 100% homology between the two sequences.

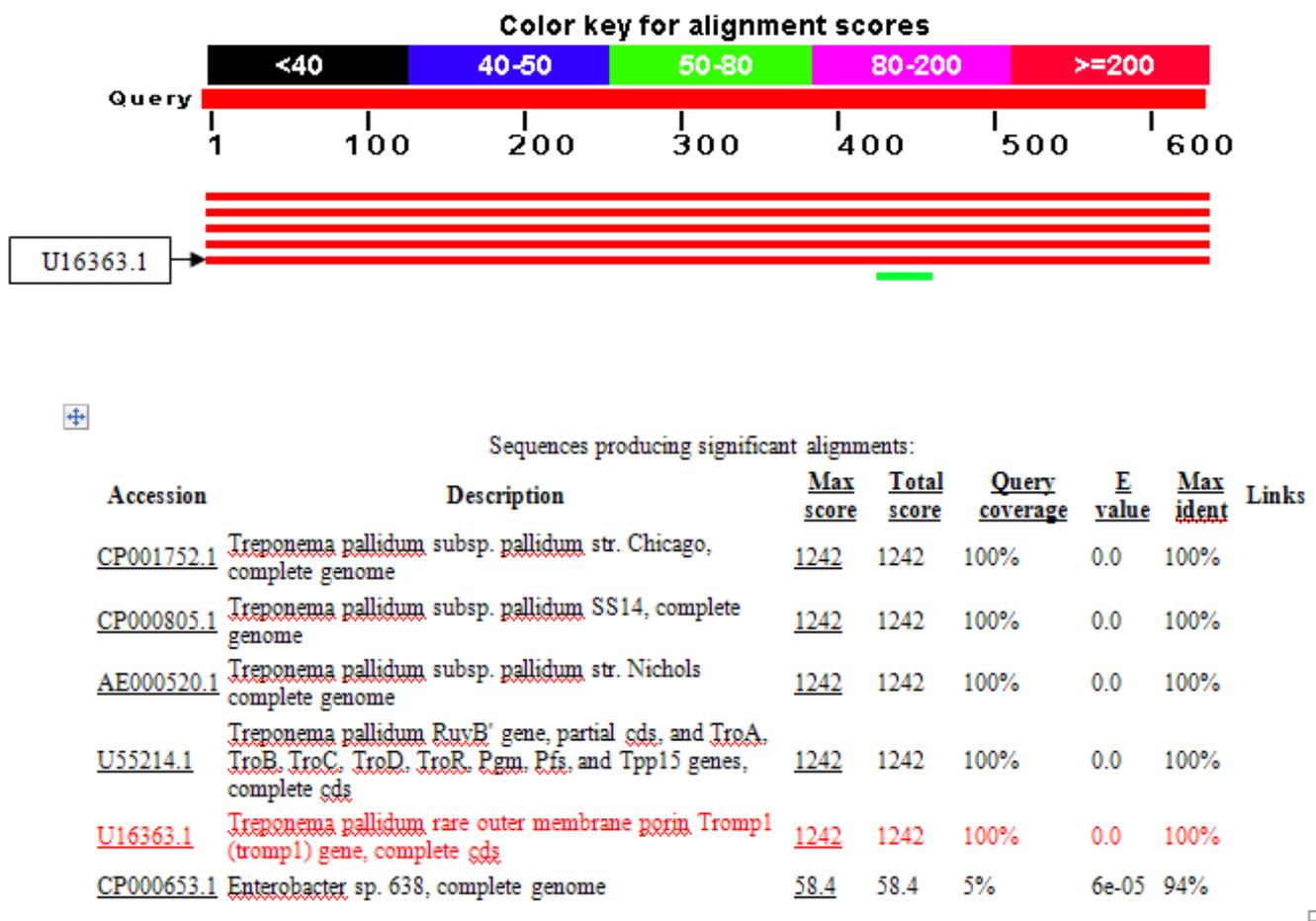


Figure 19 a: Nucleotide blast of the cloned *tromp1* insert against the nucleotide collection available on NCBI (blast.ncbi.nlm.nih.gov). The results show 100% homology to the *tromp1* reference sequence, U16363.1.

[gb|U16363.1|TPU16363](#) Treponema pallidum rare outer membrane porin Tromp1 (tromp1) gene, complete cds
 Length=1014

Score = 1242 bits (672), Expect = 0.0
 Identities = 672/672 (100%), Gaps = 0/672 (0%)
 Strand=Plus/Plus

```

Query 1      AAAAACATCGCTCAAGGTGATGTGCATCTAAAGGGGTTGATGGGTCCCTGGTGTGACCCG 60
            |
Sbjct 172     AAAAACATCGCTCAAGGTGATGTGCATCTAAAGGGGTTGATGGGTCCCTGGTGTGACCCG 231

Query 61     CACCTGTACACGGCTACTGCGGGGATGTGGAATGGCTCGGGAATGCGGATCTCATCCTG 120
            |
Sbjct 232     CACCTGTACACGGCTACTGCGGGGATGTGGAATGGCTCGGGAATGCGGATCTCATCCTG 291

Query 121    TACAACGGGTTGCACCTGGAAACCAAGATGGGCGAGGTGTTTTCCAAACTGCGCGGGAGC 180
            |
Sbjct 292    TACAACGGGTTGCACCTGGAAACCAAGATGGGCGAGGTGTTTTCCAAACTGCGCGGGAGC 351

Query 181    CGCTTGGTAGTTGCAGTTTCTGAGACTATTCCGGTGTCTCAGCGTCTTCTCTTGAGGAA 240
            |
Sbjct 352    CGCTTGGTAGTTGCAGTTTCTGAGACTATTCCGGTGTCTCAGCGTCTTCTCTTGAGGAA 411

Query 241    GCAGAGTTCGATCCGCATGTGTGGTTTATGTAAGCTGTGGTCTTATTCGGTGAAGGCA 300
            |
Sbjct 412    GCAGAGTTCGATCCGCATGTGTGGTTTATGTAAGCTGTGGTCTTATTCGGTGAAGGCA 471

Query 301    GTGTACGAAAGCTTGTGCAAGCTGTTGCCGGGAAAAACTCGCGAATTTACTCAACGTTAT 360
            |
Sbjct 472    GTGTACGAAAGCTTGTGCAAGCTGTTGCCGGGAAAAACTCGCGAATTTACTCAACGTTAT 531

Query 361    CAGGCGTACCAGCAGCAGTTGGATAAGCTTGACGCGTACGTTTCGGCGCAAGGCGCAGTCG 420
            |
Sbjct 532    CAGGCGTACCAGCAGCAGTTGGATAAGCTTGACGCGTACGTTTCGGCGCAAGGCGCAGTCG 591

Query 421    CTGCCTGCTGAAAGGCGTGTGTGGTGACCGCTCATGATGCGTTCGGCTATTTTAGCCGT 480
            |
Sbjct 592    CTGCCTGCTGAAAGGCGTGTGTGGTGACCGCTCATGATGCGTTCGGCTATTTTAGCCGT 651

Query 481    GCGTATGGTTTTGAGGTGAAGGGGTTGCAAGGGGTGAGCACCGCTTCGGAAGCCAGTGCG 540
            |
Sbjct 652    GCGTATGGTTTTGAGGTGAAGGGGTTGCAAGGGGTGAGCACCGCTTCGGAAGCCAGTGCG 711

Query 541    CATGATATGCAGGAACGGCAGCGTTTATTGCGCAGCGTAAACTCCCTGCTATCTTTATT 600
            |
Sbjct 712    CATGATATGCAGGAACGGCAGCGTTTATTGCGCAGCGTAAACTCCCTGCTATCTTTATT 771

Query 601    GAGAGTTCTATTCGACAAAAACGTTGAAGCGTTAAGGGATGCGGTGCAGGCAAGAGGG 660
            |
Sbjct 772    GAGAGTTCTATTCGACAAAAACGTTGAAGCGTTAAGGGATGCGGTGCAGGCAAGAGGG 831

Query 661    CACGTAGTGCAG 672
            |
Sbjct 832    CACGTAGTGCAG 843
  
```

Figure 19 b: Multiple sequence alignment (blast.ncbi.nlm.nih.gov) of the cloned *tromp1* insert against the *tromp1* reference sequence, following transformation of BL21 (DE3) pLysS *E. coli*.

(4.17) Gene Expression

Gene expression is studied in the log phase of the *E. coli* growth curve. This is determined by measuring increasing turbidity in a spectrophotometer at OD₆₀₀. The *E. coli* cultures were incubated until their growth reached OD₆₀₀ readings of 0.3, 0.4, 0.6 and 0.8. These values were chosen based on recommendations for mid-log growth in the Invitrogen user manual (2006) and publications in the literature (Blanco *et al.*, 1996). SDS-PAGE analysis was applied for detection of the gene product, viz Tromp1 protein.

Figures 20, 21 and 22 show the SDS-PAGE analysis of the supernatant and pellet protein preparations obtained following lysis of the *E. coli* cells. This was done at different time intervals, following induction with IPTG. In addition, induced *E. coli* lysates were analyzed against their corresponding uninduced lysates for observation of the desired protein of a molecular weight of 34 to 35 kDa.

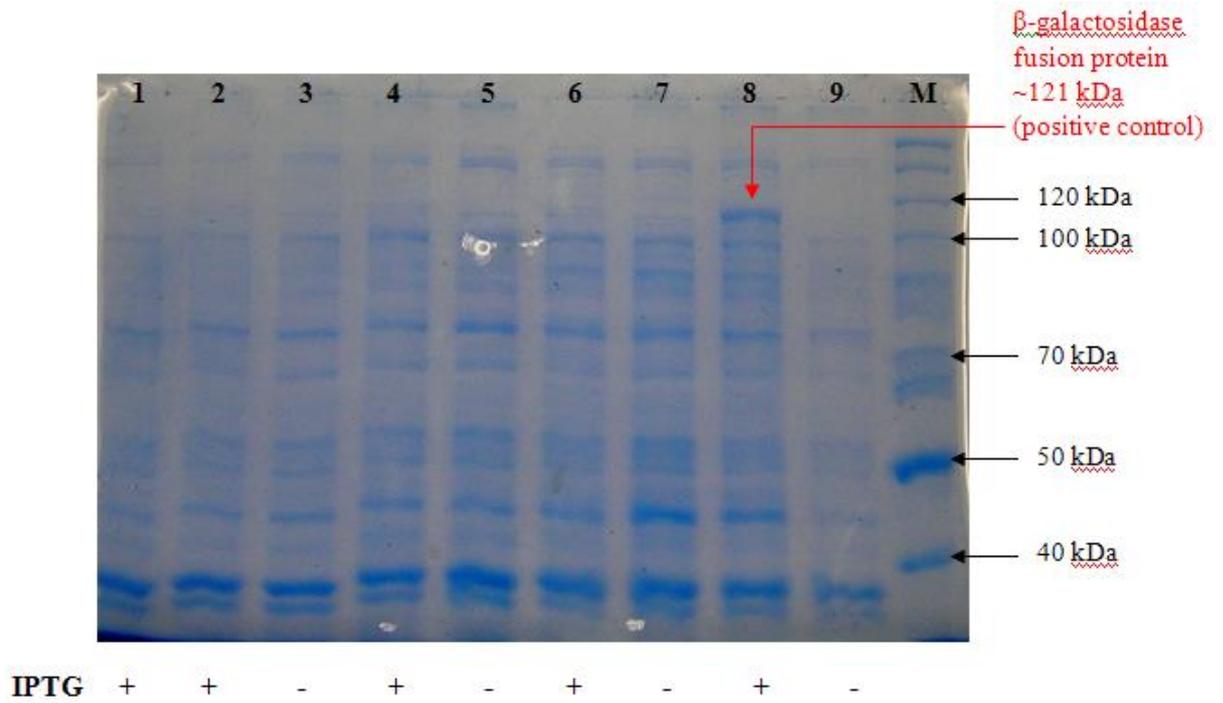


Figure 20: Coomassie blue – stained SDS-PAGE gel analysis of protein supernatant samples obtained after 4 hours of IPTG induction

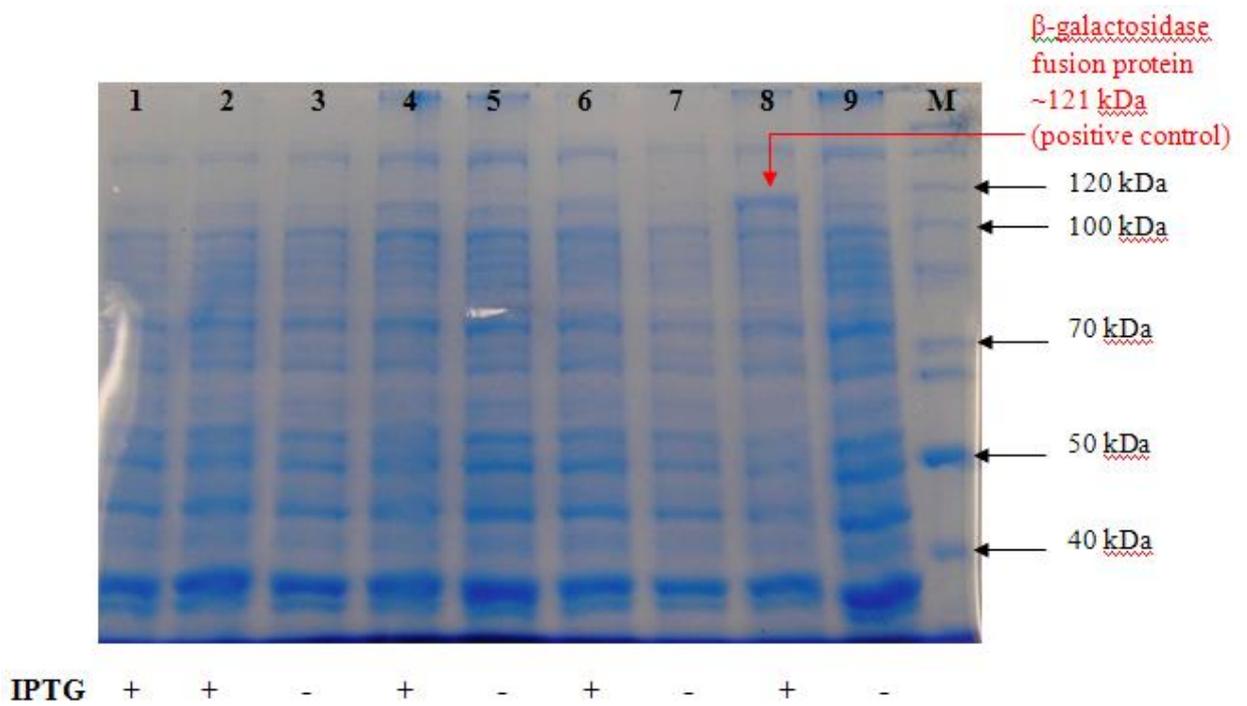


Figure 21: Coomassie blue – stained SDS-PAGE gel analysis of protein supernatant samples obtained after 6 hours of IPTG induction

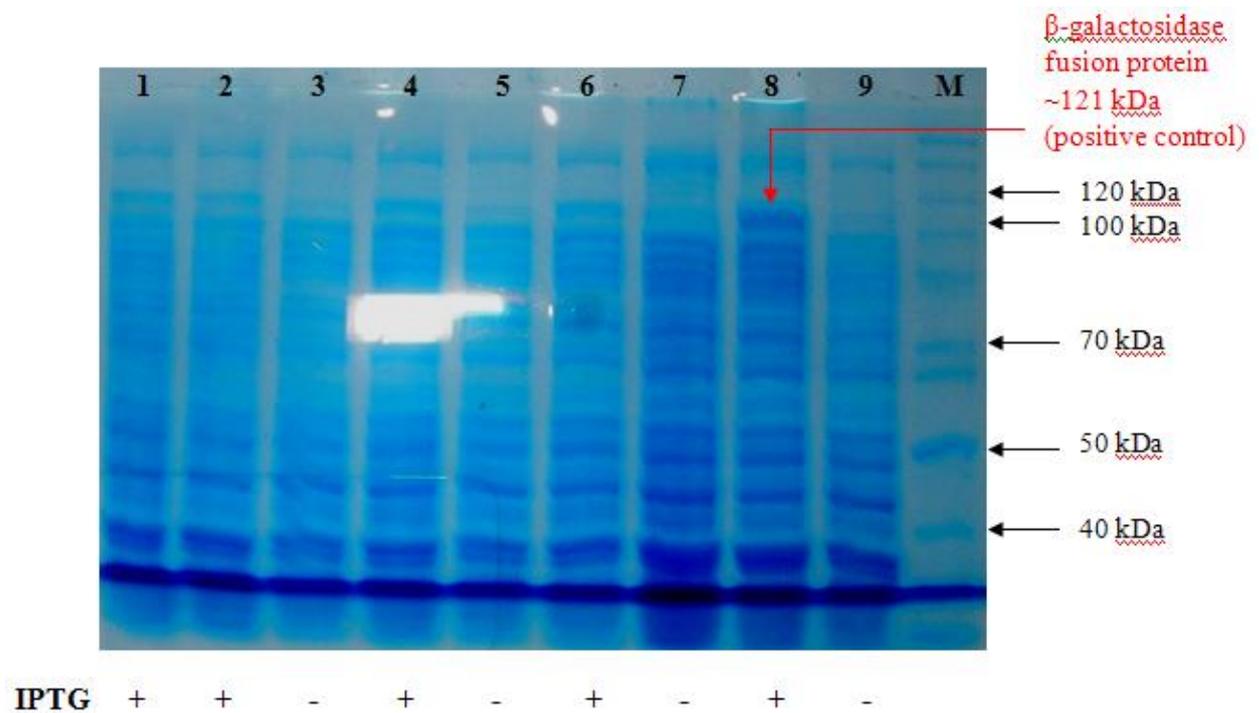


Figure 22: Coomassie blue – stained SDS-PAGE gel analysis of protein pellet samples obtained after 6 hours of IPTG induction

Lanes:

1. Sample 2 induced with IPTG to a final concentration of 0.5 mM
2. Sample 2 induced with IPTG to a final concentration of 1 mM
3. Uninduced sample 2
4. Sample 3 induced with IPTG to a final concentration of 1 mM
5. Uninduced sample 2
6. Sample 4 induced with IPTG to a final concentration of 1 mM
7. Uninduced sample 4
8. Positive expression control induced with IPTG to a final concentration of 1 mM
9. Uninduced positive expression control
10. PageRuler protein molecular mass marker (10 to 200 kDa)

Protein preparations obtained at various time points at one hour intervals following IPTG induction, were analysed by SDS-PAGE and stained with Coomassie blue staining solution. Each induced *E. coli* lysate was run along its corresponding uninduced lysate which served as a negative control. The recombinant protein is visible as a band of increasing intensity over time in the expected size range.

However, SDS-PAGE gel analysis showed no difference in the banding pattern between the IPTG induced and uninduced lysates, for both the supernatant (Figures 20 and 21) and pellet protein preparations (Figure 22). Furthermore, no difference was noted between lysates that were induced with IPTG to final concentrations of 0.5 mM and 1 mM.

The positive control however, shows a bright and distinct band at its expected size range of ~121 kDa. This confirms that growth and induction were properly performed. The recombinant protein of interest is 31 kDa. However, the addition of the N-terminal fusion tag increases the size of the recombinant protein by 3 kDa. One should also account for the additional amino acids between the fusion tag and the start of the protein. Five vector-encoded amino acids are present at the N-terminus of this recombinant protein. Therefore, the product to look for has a molecular weight of 34 to 35 kDa.

(4.18) Western Blot Analysis

A Western blot analysis was performed following SDS-PAGE. Western Blot takes SDS-PAGE analysis further in that it detects the protein of interest from within a mixture of proteins based on its ability to bind specific antibodies. In this study anti-Xpress antibodies specific to the N-terminal Xpress epitope fusion tag were used to detect the recombinant protein.

One should expect to see a permanent dark blue band or spot at the peroxidase binding site on the nitrocellulose membrane indicating specific antigen-antibody binding and hence the presence of the recombinant protein. However no band was seen. Instead, the membrane remained clear (results not shown).

(4.19) Enzyme Linked Immunosorbent Assay (ELISA)→ **Increasing Primary Antibody dilution**↓ **Increasing Incubation Time**

	1	2	3	4	5	6	7	8	9
A	0.088	0.092	0.094	0.096	0.084	0.091	0.093	0.082	0.089
B	0.094	0.099	0.099	0.097	0.094	0.094	0.096	0.091	0.092
C	0.096	0.100	0.097	0.097	0.098	0.094	0.095	0.091	0.096
D	0.104	0.093	0.096	0.099	0.098	0.097	0.096	0.073	0.099
E	0.095	0.097	0.095	0.099	0.093	0.095	0.095	0.103	0.099
F	0.093	0.095	0.097	0.097	0.093	0.093	0.095	0.095	0.095
G	0.094	0.096	0.101	0.096	0.095	0.094	0.092	0.092	0.092
H	0.095	0.096	0.098	0.095	0.099	0.090	0.096	0.094	0.095

Table 3: ELISA using anti-Xpress antibodies to demonstrate any possible levels of detectable protein in lysed *E. coli* cell supernatant extract

KEY:

Well number	Primary Antibody dilution
1-3	1:2000
4-6	1:5000
7-9	1:10 000

Well number	Increasing incubation time (hours) following IPTG induction
A	0 hour
B	1 hour
C	2 hours
D	3 hours
E	4 hours
F	5 hours
G	6 hours
H	7 hours

An indirect ELISA was performed to confirm the results of SDS-PAGE and Western Blot. As with Western Blot, ELISA too uses specific antibody to detect the recombinant protein of interest.

Rows A to H contain the samples obtained at one hour intervals following IPTG induction during the 7 hour expression study. Row A contains the zero time point samples from the moment of induction and thereafter the samples are arranged according to increasing hours of incubation following IPTG induction.

Theoretically, protein expression should occur in increasing amounts from time point 0 onwards until it reaches a peak. This should be followed by a temporary plateau phase. Finally a downward trend in protein expression is expected to follow. However the results in Table 3 show no such pattern in protein expression. Furthermore the values obtained are far too low to indicate the presence of even a little amount of protein. This therefore confirms the results established from SDS-PAGE and Western Blot, suggesting the absence of the production of the recombinant Tromp1 protein.

CHAPTER 5

DISCUSSION

The aetiology of genital ulcer disease in South Africa has changed over time from predominantly primary syphilis and chancroid to genital herpes and lymphogranuloma venereum. Despite this, the guidelines for syndromic management of GUD still include penicillin for primary syphilis. The aim of this project was to develop a rapid point-of-care test for the exclusion of primary syphilis. This would prevent administering of unnecessary treatment for syphilis, preventing a painful injection, hypersensitivity reactions in patients and the possible development of resistance in *T. pallidum* and other bacterial species.

This test would be an immunochromatographic (IC) test strip incorporating specific antibodies raised against the 31 kDa *T. pallidum* rare outer membrane porin protein (Tromp1), in its test region on the test strip. The 31 kDa protein was the first *T. pallidum* membrane-spanning outer membrane protein identified (Blanco *et al.*, 1994). The protein was chosen for this study due to its high concentration in the *T. pallidum* outer membrane and the uniqueness of this protein to *T. pallidum* subsp *pallidum*.

To be able to produce the required capture antibodies, an amount of soluble Tromp1 protein was needed. In order to obtain reasonable quantities of pure Tromp1 in soluble form, the protein was to be cloned and expressed in *E. coli* cells as a fusion protein, lacking its signal peptide and purified.

PCR conditions for the amplification of the *tromp1* gene were optimized until a single distinct band at the desired region of 884 bp was obtained. Following cloning and ligation of amplified *tromp1* in the pET100/D-TOPO vector, transformants were screened for the presence of inserts by performance of a PCR. Random colonies selected for the PCR all contained the *tromp1* insert, indicative of “positive clones”. These results were further confirmed by DNA sequencing and digestion of plasmid DNA by means of restriction enzymes. Similar steps were carried out to ensure that the *tromp1* insert was still maintained following transformation of the BL21 (DE3) pLysS *E. coli* host cells to be used for expression of the gene. Confirmation of this allowed continuation of the experiments to attempt T7-regulated expression of soluble Tromp1 protein.

Despite variation in incubation time and IPTG expression inducer concentrations, SDS-PAGE analysis showed that no recombinant protein was expressed by the BL21 (DE3) pLysS *E. coli* host cells. The protein profiles of both the IPTG-induced and uninduced lysates appeared to be exactly the same. However, the positive expression control gene encoding the β -galactosidase fusion protein was expressed as indicated by the bright distinct band at its expected size range of ~121 kDa. This band was only identified after IPTG induction. No band of similar intensity was identified in the uninduced control lane. The uninduced lysate served as a negative control. Expression of β -galactosidase fusion protein from *E. coli* indicates that growth and induction were properly performed during the expression study. The absence of expressed recombinant Tromp1 fusion protein was later confirmed by Western blot and ELISA using antibodies directed against the Xpress fusion tag.

It was initially suspected that the *tromp1* gene was perhaps cloned in the incorrect reading frame for transcription and translation to occur. The Geneious Pro 5.0.2. software was used to confirm that the gene was correctly cloned in frame with the N-terminal peptide containing the Xpress epitope and the 6xHis fusion tag. The start codon, ribosomal binding site and T7 promoter/priming site were all identified upstream from the polyhistidine fusion tag as expected.

Secondly, the presence of rare (untranscribed) codons was ruled out. This was based on a codon code usage calculator (Graphical Codon Usage Analyzer) to determine the compatibility between the expression *E. coli* and the *tromp1* gene. This was considered because differences in codon usage preference among organisms can lead to various problems that concern heterologous gene expression (<http://gcua.schoedl.de>).

The presence of unexpected stop codons generated by mutation was also ruled out. This can especially occur when cloning PCR products. However sequencing revealed the absence of such mutations.

Optimal induction conditions of the host *E. coli* cultures is essential in the expression of heterologous genes and this needs further optimization. The expression of the Tromp1 fusion protein in the BL21 (DE3) pLysS *E. coli* cells should be examined at different growth/induction temperatures, preferably below 37°C, the temperature that was used throughout this study. Yin *et al* (2007) reported on the expression of the GFP-heparinaseI fusion protein in BL21 (DE3) *E. coli* cells examined at various induction temperatures of 15°C, 20°C and 32°C. A soluble form of this protein was

observed at 32°C with an even greater amount of soluble protein observed at 15°C, the induction temperature that was used for all remaining experiments henceforth. An induction temperature of 34°C could also be examined as was suggested in several protocols. It has been suggested that by decreasing the growth or induction temperature, the rate at which the target protein is produced slows down and therefore chances of improper folding and aggregation of polypeptides decreases. Such improper folding is often seen with protein overexpression in *E. coli* (Kataeva *et al.*, 2005). However, no incubator was available of which the temperature could be altered. Therefore an induction temperature of 37°C was used throughout this study.

In addition, the IPTG-inducing concentration could be further optimized by using a wider range. IPTG to a final concentration varying from 0.1 mM to 2 mM should be investigated.

The level of protease expression in the BL21 (DE3) pLysS *E. coli* cells in comparison to the levels of expressed recombinant Tromp1 protein should be considered. Proteases are enzymes that catalyze the hydrolytic breakdown of proteins into peptides or amino acids. To account for this, the expression experiment could in future be repeated in the presence of protease inhibitors to protect against different degrading enzymes.

The BL21 (DE3) pLysS strain contains the pLysS plasmid which further carries the T7 lysozyme gene. Co-expression of this T7 lysozyme gene was shown to reduce background expression since this lysozyme binds to the T7 RNA polymerase and by doing so, inhibits this enzyme before induction with IPTG occurs (Pan and Malcolm,

2000). Therefore, the pLysS strain is the host of choice for expressing toxic proteins that could kill the *E. coli* cells before induction. Unfortunately, the same lysozyme molecules can cause bacterial lysis during growth and continue inhibiting T7 RNA polymerase following induction, resulting in overall reduced protein expression levels (Pan and Malcolm, 2000). Since Tromp1 is a known toxic protein (Blanco *et al.*, 1996), the BL21 (DE3) pLysS strain was used as the expression host for recombinant Tromp1 in this study. This may have resulted in inhibition of the T7 RNA polymerase even during induction, with as a result, undetectable levels of recombinant Tromp1 protein. An attempt was made to transform the *tromp1* gene into BL21 (DE3) lacking pLysS along with the strain that contains the pLysS plasmid. However, no growth was observed on the plates containing transformants lacking pLysS. This confirms the toxicity of Tromp1 for *E. coli* cells. Following this observation, expression of recombinant Tromp1 protein was performed in the pLysS strain during the remainder of the study.

In future, BL21 (DE3) *E. coli* cells lacking the pLysS plasmid could be grown in medium supplemented with 1% glucose. This would be followed by culture in glucose free medium for the final growth cycles. By changing to a medium with a poor carbon source for these final growth cycles, higher expression levels that are typical of the BL21 (DE3) cells can be readily obtained following induction (Pan and Malcolm, 2000). Culturing in the presence of glucose has previously shown decreased background expression of the T7 polymerase gene and partial improvement of plasmid stability in BL21 (DE3) cells (Pan and Malcolm, 2000). This is considered to be the effect of catabolite repression on the *lacUV5* promoter, in the λ DE3 lysogens, which drives basal expression of the T7 polymerase gene.

An alternative approach would be to clone the *tromp1* gene as a fusion to a “carrier” protein for the production of soluble heterologous proteins in *E. coli*. Several carrier proteins are available including maltose-binding protein (MBP), thioredoxin (Trx), glutathione S-transferase (GST), intein, calmodulin-binding protein (CBP), NusA and cellulose-associated protein (CAP) (Shih *et al.*, 2002). The choice of carrier protein depends on the gene to be expressed, since they do not function equally well with all heterologous proteins (Esposito and Chatterjee, 2006). It has been reported that when used as a fusion partner to the gene of interest, the gene coding for the carrier protein can increase translation efficiency and promote proper folding of their partners that in turn leads to the enhanced solubility of the desired protein (Esposito and Chatterjee, 2006). Some of these fusion carrier proteins play a dual role in serving as affinity tags as well, thus increasing the efficiency of soluble protein purification (Esposito and Chatterjee, 2006).

Kataeva *et al* (2005) reported on the soluble protein expression of selected *Shewanella oneidensis* MR-1 and *Clostridium thermocellum* JW-20 proteins in *E. coli* (Kataeva *et al.*, 2005). MBP was selected as the carrier protein in their study based on its efficiency at improving the stability and solubility of its passenger proteins. This study further showed that MBP significantly increased the solubility of its passenger proteins following a decrease in induction temperature when compared to unfused variants. This is because a decrease in induction temperature slows down production of the desired protein thereby allowing greater time for proper folding of target polypeptides (Kataeva *et al.*, 2005).

In another study, MacDonald *et al* (2003) investigated the effect of N-terminal fusion partners on protozoan α and β tubulin gene expression levels in *E. coli* BL21 (DE3) pLysS strain. They demonstrated that high-solubility fusion partners MBP and GST produce a five-fold increase in soluble protein yields when compared with the lower solubility fusion partner polyhistidine, the fusion partner used in the study presented in this dissertation (MacDonald *et al.*, 2003).

If expression of recombinant Tromp1 protein continues to fail, after having taken the above suggestions into consideration, an alternative method would be to obtain monoclonal antibodies to the various surface proteins previously identified in *T. pallidum*. The antibody preparation would then be analysed on SDS-PAGE. The most abundant antibody produced in response to treponemal infection could then be isolated and purified for use as capture antibodies in the immunochromatographic test strip for the direct detection of whole treponemes in primary lesion material.

Several studies have already identified and characterized the *T. pallidum* outer membrane proteins, suggesting that they may “represent potential surface-exposed virulence determinants and targets of host immunity” (Blanco *et al.*, 1997). Norris and Sell have previously reported a 39 kDa polypeptide that appears to be a major surface constituent of *T. pallidum*. They suggest that this protein may play a pivotal role in induction of immunity to syphilis (Norris and Sell, 1984). In 1994, Blanco and colleagues had established a novel method to isolate the *T. pallidum* outer membrane and characterize its protein constituents. Among their findings, they reported the identification of the 65, 31 and 28 kDa proteins. These proteins belong to the Tromp family of proteins as they are exclusively associated with the outer membrane. When

tested against sera obtained from rabbits inoculated with *T. pallidum* and from humans with syphilis, all three proteins proved to be antigenic. Their significant enrichment in the outer membrane preparation made the Tromp proteins “leading candidates for membrane-spanning outer membrane proteins of *T. pallidum*” (Blanco *et al.*, 1994). Hence, in addition to the 31 kDa Tromp protein chosen for the purposes of the study presented here, these other rare outer membrane proteins could also be used.

A second alternative method would be to harvest enough treponemes following intra-testicular cultivation in rabbits. This would be followed by the isolation of the *T. pallidum* outer membrane. Outer membrane isolation could be achieved using the method described by Blanco and colleagues whereby they harvested outer membranes by immersing the treponemes in 50 mM cold citrate buffer at pH 3.2 (Blanco *et al.*, 1994). A more recent and gentle method for *T. pallidum* outer membrane isolation was described by Radolf and colleagues (Radolf *et al.*, 1995). This method is based upon plasmolysis of treponemes in hypertonic sucrose followed by isopycnic sucrose density gradient ultracentrifugation. Isolated outer membrane fractions will then be sonicated on ice to release protein antigens. After centrifugation, the supernatant containing soluble protein will be aspirated and subjected to SDS-PAGE and Coomassie blue staining and destaining as described by Laemmli (Laemmli UK, 1970). Protein bands of interest will be excised and eluted from the gel followed by purification and quantification of desired protein. A major drawback to this approach is the need for several rabbits for this procedure, since only limited numbers of treponemes can be obtained following intra-testicular cultivation. Secondly, the outer membrane of *T. pallidum* is extremely fragile. Therefore conventional methods used

for isolating outer membranes from other gram negative bacteria do not apply. More gentle methods as those explained above are suggested for *T. pallidum* outer membrane isolation. Thirdly, treponemes obtained from rabbit infection may be contaminated with host tissue, which can complicate the separation and purification of *T. pallidum* molecules (Blanco *et al.*, 1994; Blanco *et al.*, 1997).

This work is part of a project that aims at designing a point-of-care test that is able to establish whether a patient with genital ulcer disease is infected with *Treponema pallidum* and/or *Haemophilus ducreyi*. Both these pathogens are nowadays rare in such patients and an immunochromatography test that detects both would prevent many patients from receiving antibiotics that are not indicated. Such a project is ambitious and it was obvious from the onset that this likely needed multiple approaches of which several would fail. This study attempted to produce sufficient recombinant Tromp1 protein of *T. pallidum* to raise antibodies to be used as capture antibodies in the immunochromatography strip. The method used turned out to be unsuccessful. Future studies need to take this further. Several suggestions have been made to increase the chances for successful production of recombinant Tromp1 protein. In addition, alternative methods have been discussed as well.

CHAPTER 6

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CHAPTER 7

APPENDICES

(7.1) Appendix A : Preparation of PCR Reagents:

1. 0.5 mM EDTA (1 L)

EDTA 2H₂O – 186.1 g

Water – 800 mL

EDTA was dissolved in triple distilled water with the aid of a magnetic stirrer.

Thereafter, the pH of the solution was adjusted to 8.0 with 20 g of sodium hydroxide (NaOH) pellets and the volume was brought up to 1 L.

2. 5x TBE Buffer (1 L)

Trizma base – 54 g

Boric acid crystals – 27.5 g

0.5 mM EDTA – 20 mL

All the reagents were dissolved in 800 mL of triple distilled water. The pH of the solution was adjusted to 8.0 and the volume was brought up to 1 L.

3. 0.5x TBE Buffer (1 L)

5x TBE Buffer	– 100 mL
Water	– 900 mL

Stored at room temperature.

4. 0.05% Ethidium Bromide Solution (100 mL)

Ethidium Bromide	– 0.05 g
Water	– 100 mL

Ethidium bromide was dissolved in triple distilled water and stored at 4°C.

5. 2% PCR agarose gels

Reagent	Large gel	Medium gel	Small gel
Agarose (molecular grade)	3 g	1.4 g	0.45 g
0.5x TBE buffer	150 mL	70 mL	30 mL
Ethidium bromide	150 µl	70 µL	30 µL

Table 1: Preparation of 2% PCR agarose gels

6. Sample Loading Buffer

Bromophenol blue (without xylene)	– 0.05 g
Sucrose	– 5 g
Water	– 20 mL

The loading buffer was aliquoted in volumes of 1 mL into sterile 1.5 mL Eppendorfs and stored at 4°C.

(7.2) Appendix B: Preparation of Cloning Reagents

1. LB (Luria-Bertani) Medium

(1.0% Tryptone; 0.5% Yeast extract; 1.0%NaCl, pH7)

Bacto Tryptone	– 10 g
Yeast extract	– 5 g
Sodium chloride (NaCl)	– 10 g

All reagents were dissolved in 800 mL of triple distilled water. The pH of the solution was adjusted to 7.0 with sodium hydroxide (NaOH) and the volume was brought up to 1 L. The solution was autoclaved and stored for 1 month at 4°C.

Antibiotics were added to the medium as required as follows:

10 µL of ampicillin (100 µg/mL in PBS) to every 10 mL volume of LB medium

10 µL of chloramphenicol (34 µg/mL in pure ethanol) to every 10 mL volume of LB medium

2. LB agar plates

LB medium was prepared as above but 15 g/L Bacto agar was added before autoclaving. Immediately after autoclaving, the solution was cooled to 55°C in a waterbath. Antibiotics were added as above and the agar solution was poured into 10 cm plates at 20 mL volumes each. The agar plates were allowed to harden at room temperature, then inverted, wrapped in foil and stored at 4°C for up to 1 month.

3. Ampicillin Stock (100 µg/mL)

1 g of ampicillin was dissolved in 10 mL of Phosphate buffer (pH 8.0). The solution was aliquoted in volumes of 1 mL into sterile 1.5 mL Eppendorfs, covered with foil and stored at -20°C. It was used at 100 µg/mL.

4. Chloramphenicol Stock (34 µg/mL)

0.34 g of chloramphenicol was dissolved in 10 mL of ethanol. The solution was aliquoted in volumes of 1 mL into sterile 1.5mL eppendorfs, covered with foil and stored at -20°C. It was used at 34 µg/mL.

5. IPTG Stock (238.31 mg/mL)

0.23831 g of IPTG was dissolved in 1 mL of triple distilled water. The solution was aliquoted in volumes of 200 µL into sterile 1.5 mL Eppendorfs, covered with foil and stored at -20°C.

(7.3) Appendix C: Optimization of Sequencing Reaction

The primers used for the PCR reaction were initially used for the cycle sequencing reaction as well. The primers were diluted to a final concentration of 3.2 pmol/ μ L. However, the use of these primers failed to provide any sequencing signal for analysis after several attempts.

In a first attempt to optimize the sequencing reaction, the primers were further diluted to a final concentration of 2.5 pmol/ μ L, to facilitate primer binding as suggested by a colleague. It has been suggested that an increased primer concentration such as 3.2 pmol/ μ L may require that the annealing temperature (cycle sequencing reaction) be increased as well. It was therefore decided to lower the primer concentration to accommodate for the average annealing temperature. However, this also failed to provide sequencing signal.

A second attempt at optimization investigated the ABI Prism Big Dye Kit to ensure that it was in working order. The same kit was used to sequence a colleague's samples other than that being investigated and this proved to be successful.

The conditions of the sequencer were then optimized. Its ramp rate was decreased to allow the primer enough time to bind to the template DNA. The annealing temperature was also increased but neither of these attempts proved successful.

Finally, the primers used in the reaction were investigated. It had been suggested that the primers were initially too long (30 to 35 base pairs). In addition, both primers

contained a high GC content with the forward primer having an even greater GC content after the addition of the CACC nucleotide overhang (the requirements for directional cloning). Taking the above into consideration, it was therefore decided to redesign these primers for the sequencing reaction.

In one set of primers, the restriction sites and the CACC nucleotide overhang (forward primer) on the initial PCR primers were simply removed:

Forward Sequencing primer: 5'-ATTCGGTAGCAAGGATGCCGCA-3'

Reverse Sequencing primer: 5'-CTAGCGAGCCAACGCAGCAAC-3'

A second set of primers were designed to a sequence internal to the primers on either end of the insert:

Forward Sequencing primer: 5'-CATGATAGCGGATGCTGTC-3'

Reverse Sequencing primer: 5'-AACAACTCGCCTCCAAT-3'

The new sequencing primers were reconstituted according to the manufacturer's instructions and diluted to a final concentration of 3.2 pmol/ μ L. Both newly designed primer sets successfully produced sequencing data and were used for all sequencing reactions henceforth.

(7.4) Appendix D: Preparation of Lysis Buffer**(50 mM Tris-HCl; 10% glycerol; 0.1% Triton X-100)****For 100 mL:**

500 mM Tris-HCl, pH 8.0 (autoclaved)	– 10 mL
Glycerol	– 10 mL
Triton X-100	– 0.1 mL

Made up to 100 mL with triple distilled water and stored at 4°C.

Lysis Buffer additives:

The following additives were included in the lysis buffer only when ready to use.

To every 10 mL of lysis buffer the following were added:

10 mg/mL Lysozyme	– 100 µL
10 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor	– 100 µL
Protease cocktail	– 1 µL

Lysozyme Stock (10 mg/mL)

0.01 g of lysozyme was dissolved in 1 mL of triple distilled water and kept on ice at all times.

(7.5) Appendix E: Preparation of SDS-PAGE Solutions**1. 3% Agarose**

Agarose	- 3 g
Water	- 100 mL

The agarose was added to triple distilled water and allowed to dissolve by heating in a microwave.

2. 30% Acrylamide mix

Acrylamide	- 60 g
Bisacrylamide	- 1.6 g
Water	- 200 mL

The acrylamide and bisacrylamide was dissolved in triple distilled water. The solution was filtered using filter paper. The bottle was covered with foil and stored at 4°C.

3. 4x Separating gel buffer (1.5 M Tris-HCl, pH 8.8)

Tris	- 36.3 g
Water	- 150 mL

Tris was dissolved in triple distilled water and its pH was adjusted to 8.8 with HCl.

The solution was autoclaved and stored at 4°C.

4. 4x Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Tris	- 3 g
Water	- 50 mL

Tris was dissolved in triple distilled water and its pH was adjusted to 6.8 with HCl.

The solution was autoclaved and stored at 4°C.

5. 10% SDS

SDS	- 10 g
Water	- 100 mL

SDS was dissolved in triple distilled water and stored at room temperature. A mask was worn when handling SDS.

6. 10% Ammonium Persulfate (NH₄)₂S₂O₈

Ammonium Persulfate	- 0.1 g
Water	- 1 mL

Ammonium persulfate was dissolved in triple distilled water. Ammonium persulfate solution had to be made up fresh before use.

7. 20% Ethanol overlay

Ethanol	- 20 mL
Water	- 80 mL

The ethanol was dissolved in triple distilled water.

8. 10x Electrode Buffer (0.25 M Tris-HCl; 1% SDS; 1.92 M Glycine)

Tris	– 3.028 g
Glycine	– 14.413 g
SDS	– 1 g
Water	– 1000 mL

Tris and glycine were dissolved in 500 mL of triple distilled water. SDS was then added to the solution without swirling since SDS is extremely foamy. Instead the solution was allowed to heat in a waterbath for a few minutes until the SDS was dissolved. The pH of the solution was adjusted to 8.3 and the solution was stored at room temperature.

9. Rapid Staining Fixing Solution (25% isopropanol; 10% acetic acid)

Isopropanol	– 250 mL
Acetic acid	– 100 mL
Water	– 750 ml

The isopropanol and acetic acid were dissolved in triple distilled water and stored at room temperature.

10. Coomassie Blue Staining Solution (0.06% Coomassie Blue; 10% acetic acid)

Coomassie Blue R250 dye	- 0.6 g
Acetic acid	- 100 mL
Water	- 900 mL

The Coomassie blue dye was dissolved in acetic acid and the solution was brought up to 1 L with triple distilled water. The staining solution was stored at room temperature.

11. Destaining Solution (7% acetic acid; 5% methanol)

Acetic acid	- 70 mL
Methanol	- 50 mL
Water	- 880 mL

The acetic acid and methanol were dissolved in triple distilled water and stored at room temperature.

12. 2x SDS-PAGE Sample Loading Buffer

0.5 M Tris-HCl, pH 6.8	– 2.5 mL
Glycerol (100%)	– 2 mL
β -mercaptoethanol	– 0.4 mL
Bromophenol blue	– 0.02 g
SDS	– 0.4 g

All reagents were combined and brought to a final volume of 10 mL with triple distilled water. The solution was aliquoted and stored at -20°C until needed.

13. 1x SDS-PAGE Sample Loading Buffer

0.5 M Tris-HCl, pH 6.8	– 1.25 mL
Glycerol (100%)	– 1 mL
β -mercaptoethanol	– 0.2 mL
Bromophenol blue	– 0.01 g
SDS	– 0.2g

All reagents were combined and brought to a final volume of 10 mL with triple distilled water. The solution was aliquoted and stored at -20°C until needed.

14. Gel preparation for SDS-PAGE

<u>Reagents</u>	<u>Large gel</u>	<u>Small gel</u>
Distilled water	13.4 mL	4 mL
30% Acrylamide mix	16 mL	3.33 mL
1.5 M Tris-HCl, pH 8.8	10 ml	2.5 mL
10% SDS	400 μ L	100 μ L
10% Ammonium persulfate	200 μ L	50 μ L
TEMED	40 μ L	5 μ L
Total volume	40 mL	10 mL

Table 2: Preparation of a 12% Separating gel

<u>Reagents</u>	<u>Large gel</u>	<u>Small gel</u>
Distilled water	12.2 mL	3 mL
30% Acrylamide mix	2.6 mL	670 μ L
0.5 M Tris-HCl, pH 6.8	5 mL	1.25 mL
10% SDS	200 μ L	50 μ L
10% Ammonium persulfate	100 μ L	25 μ L
TEMED	20 μ L	2 μ L
Total volume	20 mL	5 mL

Table 3: Preparation of a 5% Stacking gel

(7.6) Appendix F: Preparation of Western Blot Reagents**1. Towbin Transfer buffer (1 L)****(20 mM Tris; 192 mM glycine; 20% methanol; pH 8.3)**

Water	– 800 mL
Tris	– 2.4 g
Glycine	– 14.4 g
Methanol	– 200 mL

The reagents were dissolved in 800 mL of triple distilled water and the pH was adjusted to 8.3. The buffer was stored at 4°C.

2. Ponceau S Solution (0.1% Ponceau; 1% Acetic acid)

Ponceau S stain powder	- 0.1 g
Acetic acid	- 1 mL
Water	- 50 mL

The Ponceau S stain powder and acetic acid were dissolved in 50 mL of triple distilled water whilst placed on a stirrer bath. Thereafter the volume of the solution was brought up to 100 mL with triple distilled water. The solution was stored at room temperature.

3. Blocking buffer (PBSTM)

1x PBST – 100 mL

Non-fat dry milk powder – 5 g

Stored at 4°C.

4. Wash buffer (PBST)

(1xPBS containing 0.1% Tween-20)

1x PBS – 100 mL

Tween-20 – 100 µL

Stored at 4°C.

(7.7) Appendix G: Preparation of ELISA Reagents**1. Coating Buffer (Bicarbonate Buffer) 1 L**

Water	– 900 mL
Sodium carbonate (Na ₂ CO ₃)	– 5.3 g
Sodium Bicarbonate (NaHCO ₃)	– 4.2 g

The Na₂CO₃ and NaHCO₃ were dissolved in 900 mL of triple distilled water. The pH of the solution was adjusted to 9.6 and brought up to a final volume of 1 L with triple distilled water. The buffer was stored at 4°C.

2. 1x Phosphate buffered saline/Tween-20 (PBST) Wash buffer

Ten phosphate buffered saline tablets were dissolved in 1 L of triple distilled water for the preparation of 10x PBS. The solution was sterilized by autoclaving. A 10 mL volume of 10x PBS was diluted with triple distilled water to make up 100 mL of 1x PBS. For every 100 mL of 1x PBS, 10 µL (0.01%) of Tween-20 was added. The solution was stored at 4°C. Tween-20 was added only as required.

3. Blocking buffer

Water	– 900 mL
Sodium carbonate (Na ₂ CO ₃)	– 5.3 g
Sodium Bicarbonate (NaHCO ₃)	– 4.2 g
Non-fat dry milk powder	– 50 g

All reagents were dissolved in 900 mL of triple distilled water. The pH of the solution was adjusted to 9.6 and the volume of the solution was brought up to 1 L with triple distilled water and stored at 4°C.

4. Primary and Secondary Antibody diluent

PBS/Tween-20	– 100 mL
Non-fat dry milk powder	– 3 g

Stored at 4°C.

(7.8) Appendix H: Conversion of Centrifuge “rpm” values to “g” values

R = radius (in millimeters)

N = speed (in r.p.m.) divided by 1000

RCF = gravitational acceleration “g”

Primary Calculations:

$$\text{RCF (x g)} = 1.118 \text{ R} \cdot \text{N}^2$$

$$\text{Speed (r.p.m.)} = 946 \sqrt{\text{RCF}/\text{R}}$$

$$\text{Radius (mm)} = \text{RCF}/1.118 \text{ N}^2$$

$$\text{RCF} = 1.12 \times 185 \text{ mm} \times (17000\text{rpm divided by } 1000) \times (17000\text{rpm divided by } 1000)$$

$$= 1.12 \times 185 \text{ mm} \times 17 \times 17$$

$$= \underline{59,881 \text{ x g}}$$

