The development of novel antigens for improved syphilis diagnosis

by

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BSc, University of Victoria, 2008

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

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Syphilis is a disease caused by the bacterium *Treponema pallidum* subsp. *pallidum*, which is generally transmitted through sexual contact, or vertically from a mother to her fetus. Syphilis is effectively treated with penicillin yet remains prevalent worldwide, due in part to the shortfalls of current diagnostic tests. Traditional serological testing algorithms screen with diagnostic tests specific for non-treponemal antibodies followed by subsequent screening of reactive samples for treponeme-specific antibodies. Limitations exist with both the sensitivity and specificity of non-treponemal and treponemal tests. Specific enzyme immunoassays, chemiluminescence assays and rapid point-of-care tests have been developed that contain the *T. pallidum* proteins TpN15 (Tp0171), TpN17 (Tp0435), TpN47 (Tp0574), and/or TpN44 (Tp0768; TmpA). These tests have also been shown to have suboptimal sensitivities, highlighting the need for identification of novel syphilis diagnostic candidates. In this study, soluble recombinant versions of two previously identified diagnostic candidates, Tp0326 and Tp0453, as well as a novel Tp0453-Tp0326 chimera were produced. The sensitivity of these recombinant proteins in enzyme-linked immunosorbant assays (ELISA) for diagnosis of syphilis was determined by screening characterized serum samples from primary, secondary, and latent stages of infection (n=169). The specificity was determined by screening

uninfected individuals (n=13), false positives identified via the standard testing algorithm (n=19), and potentially cross-reactive infections caused by *Leptospira*, *B. burgdorferi, H. pylori,* Epstein-Barr virus, hepatitis B virus, hepatitis C virus, and cytomegalovirus (n=38). The sensitivities for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera were found to be 86%, 98% and 98%, respectively. The specificities for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera were found to be 99%, 100% and 99%, respectively. These findings suggest that Tp0453 and the Tp0453-Tp0326 chimera show promise as novel syphilis-specific diagnostic candidates for accurate detection of all stages of infection and for future development into numerous diagnostic test formats including enzyme immunoassays, chemiluminescence assays, and rapid point-of-care tests.

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Abbreviations

BLAST	Basic Logical Alignment Search Tool
BamA	β-barrel assembly machinery protein A
BCA	Bicinchoninic acid
CSF	Cerebrospinal fluid
CIA	Chemiluminescence immunoassays
DFM	Dark field microscopy
DC	Dendritic cell
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ECM	Extracellular matrix
FTA	Fluorescent Treponemal antibody
FTA-ABS	FTA absorption
Gly	Glycine
ICS	Immunochromatographic strips
IgM	Immunoglobulin M
IgG	Immunoglobulin G
LA	Latex agglutination
LPS	Lipopolysaccharide
MHA-TP	Microhemagglutination assay for antibodies to T. *pallidum*
PAMP	Pathogen-associated molecular patterns
PRR	Pattern-recognition receptors
PG	Peptidoglycan
PBS	Phosphate buffered saline
POC	Point-of-care

PCR	Polymerase chain reaction
PMNs	Polymorphonuclear lymphocytes
POTRA	Polypeptide transport-associated
RIT	Rabbit infectivity test
RPR	Rapid plasma reagin
Ser	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TRUST	Toluidine red unheated serum test
TPI	*Treponema pallidum* immobilization
TP-PA	*Treponema pallidum* particle agglutination
VDRL	Venereal Disease Research Laboratory

Acknowledgments

I would like to thank most of all my supervisor, Dr. Caroline E. Cameron, for her continuing support through the duration of this project – she has provided me with unparalleled guidance, not only the scientific aspects of this research, but also in my future goals and directions through numerous discussions and exposure to the many facets of scientific research and medicine. I would also like to thank my committee members Drs. Terry Pearson and Brad Nelson for their council throughout this study, and the insight they have contributed to both my project and personal aspirations. My coworkers in Dr. Cameron's laboratory have also contributed greatly through both their vast intellect and camaraderie, especially Rebecca Hof, Dr. Simon Houston, Charmaine Wetherell, and former lab members Teresa Brooks, and Julia Hassler. Furthermore, I would like to thank my fellow graduate students (past and present), Dr. Azad Eshghi, Mike Cummings, Morteza Razavi, Dr. Barry Duplantis, and Ralph McWhinnie for their support, scientific discussions, and even moderate commiseration and mutual debauchery. By the same token I would like to thank Matthew Pope in Dr. Pearson's laboratory for his technical savvy and ginger intuition. I would also like to thank Dr. Laura L.E. Cowen for her guidance in the statistics section of this project and Dr. Chris Upton for his open-door support throughout my studies. Finally, I would like to thank Dr. Muhammad G. Morshed and Yvonne Simpson at the British Columbia Centre for Disease Control, who contributed greatly to the development of my immunoassay and in doing so, the positive results of this study.

Dedication

Science is like the ocean. At times it seems like the squall will never end as you paddle in a futile attempt against the current, only to be bashed day and night by the crashing waves of frustration and despair. At other times it seems like you have floated into the doldrums and have to cope with what seems to be unending boredom as you are forced to learn the value of patience. However, at times you experience the elation of success, as you ride the wave of knowledge and realize why you have thrown yourself into the unbridled power of science – to pay tribute to those that came before you by contributing a small piece to the knowledge of the world we live in, and in doing so inevitably bask in the beautiful complexity of nature.

This thesis is dedicated to the people who have supported me through the trying times and revelled with me in my successes. Most of all I would like to dedicate this to my parents, Harry and Kathleen, and my sister Kaitlyn. Your unconditional support in every facet of my life is the pillar from which I draw my strength. None of this could have been done without you. I would also like to thank the rest of my family who are the solid foundation that this pillar was built on, especially my grandma Mary Croxton who's strength and determination created the family that I am lucky to have today. Finally I would like to dedicate this to my amazing friends who understand the lengthy times I recede to my studies, yet are there to celebrate when I come out to play.

Chapter 1: Introduction

1.1 Syphilis – a brief history

Approximately 15,000 BC, in some warm climate, spirochetes passed from the external environment and took up residence in a human being (Hayden, 2003). As these spirochetes evolved they became more dependent on their host, relying on host-to-host transmission in order to maintain their survival (Canale-Parola, 1977). Eventually they evolved to elicit effective transmission through skin-lesions and/or oral contact; modes characteristic of the related diseases pinta, bejel, and yaws (de Melo *et al.*). At some point some spirochetes found that invasion of the epithelium during sexual contact was a highly successful mode of transmission and this was the beginning of what evolved to be *Treponema pallidum* susp. *pallidum;* the causative agent of syphilis.

In 1495, King Charles VIII of France laid siege to Naples, the aftermath of which included the first recorded syphilis epidemic. Scholars believe that during the war syphilis was effectively spread by hundreds of prostitutes who had accompanied the French army into battle. During this period Nicolas Squillacio, a Sicilian doctor, described the manifestations of the new, highly prevalent disease:

"The purulent pustules spread in a circle, and there is an abundance of the most virulent lupus. The signs of the sickness are these: there are itching sensations and an unpleasant pain in the joints; there is a rapidly increasing fever; the skin is inflamed with revolting scabs, and is completely covered with swellings and tubercules which are initially of a livid red colour, and then become blacker. After a few days a sanguine humour oozes out; this is followed by excrescences which look like tiny sponges which have been squeezed dry; the sickness does not last more than a year, although the skin remains covered in scars which parts… I exhort you to provide some new remedy to remove this

plague from the Italian people. Nothing could be more serious than this curse, this barbarian poison" (Hayden, 2003).

Due to the recent return of Columbus from the new world in 1493, and the presence of sick Spaniards fighting for both France and Naples, syphilis was thought to be a disease brought back from the new world (Hayden, 2003). This theory has stimulated heated debates among scholars, who have utilized numerous scientific methods including gene sequencing and paleopathology in an attempt to elucidate whether syphilis is a new world or old world disease (10, 69). Regardless of its origins, after the siege of Naples the mercenaries who fought for King Charles disbanded to their respective homes throughout Europe, spreading the disease (Hayden, 2003). Over the years, this affliction has been known by many names, the majority of which involve blaming one's neighbours or enemies for the illness. Syphilis has been called the Neapolitan sickness, the *Morbus Gallicus* (French sickness), the great pox, the Canton rash (after the first Chinese port to open to European trade), and the Chinese ulcer (when it spread from China to Japan). Syphilis has been known as the 'great imitator' due to its ability to mimic numerous other conditions and diseases. In 1530, the Italian physician and poet Girolamo Fracastoro wrote the poem *Syphilus sive morbus gallicus,* which told the story of a shepherd named Syphilus who had defied the god Apollo, and in doing so was cursed with the disease. Fracastoro thus coined the name "Syphilis," and it has been used ever since to describe the disease caused by *Treponema pallidum* susp. *pallidum* (Hayden, 2003).

By the end of the nineteenth century, sypholologist Alfred Fournier estimated that 15 percent of the population of Paris had contracted syphilis. In Vienna, novelist Stefan

Zweig wrote that one or two out of ten young men had the disease, which was usually contracted from a prostitute. It was even said that some young men believed that receiving their first chancre (open sore characteristic of syphilis) was indicative of their transition into manhood (Hayden, 2003). In 1767 the causative agent of syphilis had still not been elucidated, so in order to test if syphilis and gonorrhoea were the same disease, the Scottish physician and venerologist John Hunter infected himself with gonorrhoeal puss. Unfortunately, Hunter seems to have infected himself with the etiologic agents of both diseases, leading him to conclude that syphilis and gonorrhoea were the same disease and setting syphilis research back decades (Singh *et al.*, 1999, LaFond *et al.*, 2006). In 1838, Philippe Ricord finally proved that syphilis and gonorrhoea were two separate diseases in an experiment that involved the inoculation of 2,500 people, including Paris prostitutes, with gonorrhoeal pus (Hayden, 2003, Singh *et al.*, 1999, LaFond *et al.*, 2006).

For over 500 years physicians and scientists have attempted to understand syphilis in order to treat the disease and eradicate it from society. Many people are suspected to have contracted syphilis, including Vincent van Gogh, Friedrich Nietchez, Ludwig van Beethoven, Oscar Wilde, Al Capone and Adolf Hitler. At the end of the 19th century it was believed that in rare instances syphilis could even produce genius (Hayden, 2003). Considering the length of time our greatest minds have been battling this disease and that it is not only present today but increasing in incidence, it is safe to say that not only is syphilis is one of the most intriguing afflictions, but that *Treponema pallidum* subspecies *pallidum* is a highly successful pathogen.

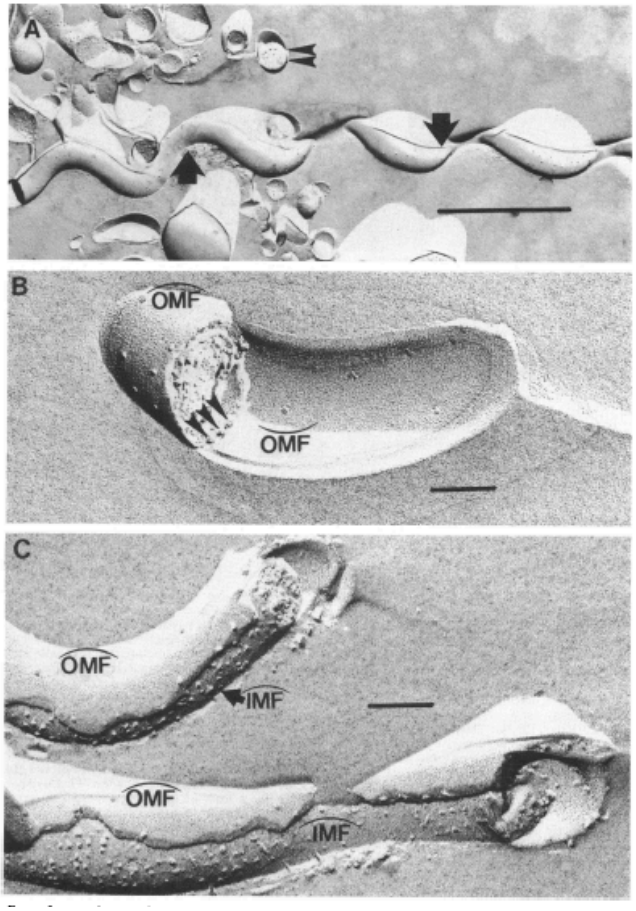

Figure 1: Freeze-fracture electron micrographs of *T. pallidum.*

(A) Low-magnification view showing a treponeme fractured over a 4.7 μm length of the OM convex face (indicated by arrows); a transverse fracture is indicated by arrowheads. Bar – 1 μm. (B) High-magnification view of the convex OMF and the concave OMF of *T. pallidum*; endoflagella are visible in the transverse fracture (indicated by arrowheads). Bar = 0.1 μm. (C) High-magnification view of the convex OMF and IMF of *T. pallidum*. Bar = 0.1 μm (Walker *et al*., 1989). This research was originally published in J Bacteriol. Walker, E. M., G. A. Zampighi, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1989. Demonstration of rare protein in the outer membrane of *Treponema pallidum* subsp. *pallidum* by freeze-fracture analysis. J Bacteriol **171:**5005-11© the American Society for Biochemistry and Molecular Biology.

1.2 *Treponema pallidum* subspecies *pallidum*

The causative agent of syphilis is the bacterium *Treponema pallidum* subsp. *pallidum.* This bacterium is a member of the phylum Spirochaetes, which include other spiral shaped human pathogens including *Leptospira, Borrelia burgdorferi and Borrelia*

recurrentis. The species *Treponema pallidum* has four known subspecies: *pallidum, pertenue, carateum*, and *endemicum,* which cause the human diseases syphilis, yaws, pinta, and endemic syphilis, respectively. These related diseases can be distinguished from each other by their clinical manifestations, epidemiological characteristics, and genetic markers (Cejkova *et al.,* LaFond *et al.*, 2006). Genetically these subspecies are extremely similar, with recent studies indicating that the subspecies *pallidum* and *pertenue* have an overall sequence identity of 99.8% (Centurion-Lara *et al.*, 2006, Cejkova *et al.*).

Treponema pallidum subsp. *pallidum* (hereafter referred to as *T. pallidum)* is spiral in shape, 6-15 μm in length, and 0.2 μm in diameter (LaFond *et al.*, 2006). Due to its narrow stature, conventional microscopes and gram staining are not sufficient to visualize the bacterium. Schaudinn and Hoffman first visualized *T. pallidum* spirochetes using Giemsa-stained smears, but today in most laboratory and clinical settings dark-field microscopy is employed (LaFond *et al.*, 2006, Ratnam, 2005, Schaudinn, 1905). The outer membrane is an extremely fragile, fluid lipid bilayer devoid of lipopolysaccharide (LPS) (Fraser *et al.*, 1998). Freeze-fracture analysis of whole treponemes shows a paucity of outer membrane proteins (Figure 1), with numbers predicted to be less than 1% of those found in bacteria such as *E. coli* (Walker *et al.*, 1989). Most gram-negative bacteria possess a peptidoglycan (PG) layer bound to the underside of their outer membrane, which increases its rigidity. The PG of *T. pallidum* is found midway within the periplasm, loosely attached to the inner membrane (Liu *et al.*, 2010, Izard *et al.*, 2009). Recent research suggests that the PG layer of *T. pallidum* adds structural stability to the

bacterium and stabilizes the flagellar motor, which enables its characteristic corkscrew motility (Jepsen *et al.*, 1968, Liu *et al.*, 2010, Izard *et al.*, 2009).

Four *T. pallidum* genomes (Nichols, SS14, DAL-1, and Chicago) have been fully sequenced, and found to have 99.9% sequence homology between strains. These genomes, have lengths ranging from 1,138,011- 1,139,971 base pairs, which is short in comparison to other bacteria such as the model organisms *Escherichia coli* (K-12 is 4.6 Mb) and *Bacillus subtilis* (4.2 Mb) (21, 36, 62, 89, 111). The *T. pallidum* genomes encode only 1041 open reading frames (ORFs), also small in relation to *Escherichia coli* and *Bacillus subtilis*, which have 4288 and 4100 ORFs respectively. *T. pallidum* is an obligate parasite, and the limited number of protein coding regions adds support to previous research showing limited metabolic capabilities in relation to carbon source utilization, the lack of a functioning Krebs cycle, and an inability to perform beta-oxidation of fatty acids (Nichols *et al.*, 1975, Schiller *et al.*, 1977). Genome sequence analysis confirmed the lack of enzymes related to alternative carbon source utilization, the electron transport chain, the Krebs cycle, and fatty acid metabolism (Fraser *et al.*, 1998). This deficiency in metabolic capabilities helps to explain why *T. pallidum* has not been successfully cultivated *in vitro,* with tissue culture cultivation yielding maximal organism increases of only 100-fold (Fieldsteel *et al.*, 1981). In 1912 Nichols *et al.* successfully inoculated rabbits with cerebral spinal fluid containing *T. pallidum*, and since then rabbits have been the optimal model for studying syphilis due to multi-generation propagation, maintenance of virulence and the presence of disease manifestations similar histologically and clinically to primary and secondary disease (Nichols *et al.*, 1913, Baker-Zander *et al.*, 1980). The Nichols strain of *T. pallidum* has

been maintained in rabbits since its isolation in 1912, and its preserved virulence for humans was confirmed in 1976 when a technician was accidentally infected while inoculating rabbits (Nichols *et al.*, 1913, Fitzgerald *et al.*, 1976). The generation time of *T. pallidum* is quite slow, doubling only every 30-33 hours (Magnuson *et al.*, 1948, Cumberland *et al.*, 1949)

With such a dependence on its host for nutrients, it is expected that *T. pallidum* would have an extensive collection of surface proteins for nutrient transport. Genome analysis identified 57 ORFs encoding 18 potential transporters with specificity for carbohydrates, amino acids, and positively charged ions, predicted to be associated with the inner membrane (Fraser *et al.*, 1998). No known homologues for porins or transporters involved in nutrient transport through the outer membrane have yet been identified. The protein Tp0453 is believed to reside on the outer membrane, and current research suggests that it may be involved in nutrient transport through its insertion and subsequent disruption of the outer membrane (Hazlett *et al.*, 2005, Luthra *et al.*, 2011).

1.3 *T. pallidum* pathogenesis

T. pallidum is highly infectious. One study analyzing infectivity, using human volunteers, found that the dose required to produce a positive infection in 50% of test subjects was only 57 organisms (Magnuson *et al.*, 1956). The fragility of the outer membrane and paucity of outer membrane proteins makes standard biological experimentation difficult with *T. pallidum* – treponemes begin to die once they are removed from the host, and infectiousness is lost within hours or days of harvest (LaFond *et al.*, 2006). Due to these limitations, genetic manipulation of *T. pallidum* has not yet been achieved, making the elucidation of virulence factors extremely difficult. Genome

analysis of *T. pallidum* fails to identify traditional virulence factors such as exotoxins, or the presence of a type III secretion system (Fraser *et al.*, 1998). A number of putative hemolysin orthologs, similar to those found in *B. burgdorferi,* were identified, yet researchers have failed to produce these proteins with any hemolytic activity (Fraser *et al.*, 1998, Fraser *et al.*, 1997). Key pathogenic mechanisms used by *T. pallidum* are the invasion of host tissues, rapid dissemination throughout the body, and immune system evasion. Although still poorly understood, progress has been made in elucidating the processes behind these mechanisms.

1.3.1 Adhesion

Adhesion is the first critical step in the pathogenesis of most organisms since this process effectively facilitates the colonization of host tissues and therefore the initiation of infection. Previous studies have shown that *T. pallidum* attaches to eukaryotic cells from humans, rabbits, and rats, and that this adherence could be reversed in the presence of serum from previously infected rabbits (Fitzgerald *et al.*, 1977, Hayes *et al.*, 1977). Adherence to HEp-2 cells can also be reduced by treating eukaryotic cells with trypsin prior to binding by *T. pallidum*, suggesting that treponemes interact with membrane proteins on host cells (Baseman *et al.*, 1980). Research has shown that *T. pallidum* binds to the extracellular matrix (ECM) structural components of human kidney tissue fibronectin, laminin, collagen, and hyaluronic acid (Fitzgerald *et al.*, 1984). Investigations by Cameron *et al.* identified a putative outer membrane protein, Tp0751, which binds in a dose-dependent manner to multiple isoforms of the extracellular matrix component laminin, a glycoprotein found in the basement membrane that underlies endothelial cell layers (Cameron, 2003, Cameron *et al.*, 2005). Further studies indicated

that when Tp0751 was heterologously expressed in the non-adherent spirochete *Treponema phagedenis*, Tp0751 was found to reside on the surface of the bacteria, and imparted the ability to bind laminin (Cameron *et al.*, 2008).

1.3.2 Dissemination

T. pallidum rapidly gains access to the blood and lymphatic systems and disseminates widely throughout its host within the first several hours (Lukehart, 1992). Investigations using the rabbit model showed that *T. pallidum* enters the bloodstream within minutes to hours after intratesticular or mucous membrane infection (Cumberland *et al.*, 1949). Treponemes are believed to access and leave the circulatory system through the invasion of intracellular junctions between epithelial cells (Thomas *et al.*, 1988). Research investigating humans infected with syphilis discovered that *T. pallidum* could be found in almost every tissue and major organ system including the heart, liver, kidneys, and even the central nervous system (Singh *et al.*, 1999). Even in the presence of a rapid humoral and cellular immune response, *T. pallidum* persists, which is believed to be due in part to its ability to spread throughout the body and "hide" from the immune system (Lukehart, 1992). Rapid, widespread dissemination seems to be a major pathogenic mechanism of *T. pallidum*.

In order to disseminate, treponemes need to be able to pass through numerous tissues. One way that *T. pallidum* may penetrate tissues is by stimulating host cells to synthesize interstitial collagenase MMP-1, which is involved in the breakdown of type I collagen – a major component of skin and tendons, as well as the scaffolding for internal organs. Chung *et al* found that when treponemes were added to human dermal fibroblast cultures, the amount of MMP-1 secreted and its respective mRNA levels were increased,

thus providing evidence that in order to traverse host tissues, *T. pallidum* stimulates host cells to produce enzymes capable of degrading surrounding connective tissue (Chung *et al.*, 2002). The process by which *T. pallidum* induces the production of MMP-1 is unknown. Tp0751, described above for its function as an adhesion protein, has been shown to also be involved in *T. pallidum's* rapid dissemination. Investigations by Houston *et al.* revealed that not only could Tp0751 bind human fibrinogen, but that it may represent a novel protease capable of degrading the host components fibrinogen and laminin (Houston *et al.*, 2011). Fibrinogen is upregulated during infection and subsequently degraded through thrombin-catalysis to produce fibrin – a process which forms clots acting to localize and contain pathogens thereby preventing dissemination and successful invasion (Levi *et al.*, 2004). The capacity to degrade both fibrinogen and laminin suggests that Tp0751 enables *T. pallidum* to migrate through the host ECM and avoid containment by fibrin clots. This suggests that Tp0751 is responsible, at least in part, for the characteristic rapid dissemination of *T. pallidum* – a crucial virulence factor for pathogenesis.

1.4 Immune system response to *T. pallidum*

One method by which the immune system signals the presence of a pathogen is through an inflammatory response. During infection, host cells trigger the influx of serous fluids and the migration of leukocytes to the infected area, which stimulates an immune response. It is believed that through close association of *T. pallidum* with vascular endothelium, endothelial cells are triggered to induce inflammation, explaining the histopathologic features of syphilis that include perivasculitis and endothelial cell abnormalities (Riley *et al.*, 1994). Expression of cell adhesion molecules on capillary

endothelial cells helps to bind leukocytes, aiding their migration from the blood into the infected tissues (LaFond *et al.*, 2006). *T. pallidum* has been shown to promote increased adherence of lymphocytes and monocytes, immune cells prominent in the histopathology of syphilis, to human endothelial cells (Riley *et al.*, 1994). *T. pallidum* induces the expression of host cell adhesion molecules intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin, a process that acts by triggering inflammation, and facilitates the subsequent binding and migration of leukocytes to the site of infection (Lee *et al.*, 2000). The *T. pallidum* lipoprotein TpN47 was also found to stimulate production of these adhesion molecules, suggesting that this response is specifically triggered by *T. pallidum*, and is an important step in stimulating the host immune response.

One of the first immune cells to infiltrate infection sites during acute bacterial infection are the polymorphonuclear lymphocytes (PMNs). These granulocytes, specifically neutrophils, are a key part of the innate immune system response to acute infection and act by engulfing bacteria, releasing anti-microbials, and stimulating other cells of the immune system through the release of cytokines and chemokines (Ear *et al.*, 2008). PMNs are found very early in both natural and experimentally induced *T. pallidum* infection; however, the infiltration is transient and occurs in lower numbers than seen in other bacterial infections (LaFond *et al.*, 2006). Intradermal injection of the treponemal proteins TpN17 and TpN47 also resulted in increased infiltration by PMNs to the injection site (Sellati *et al.*, 2001). Neutrophils kill phagocytized pathogens by fusing bacteria-laden vacuoles with granules containing enzymes, superoxide radicals, and antimicrobial peptides (LaFond *et al.*, 2006). Defensins are a group of antimicrobial

cationic peptides that are present in neutrophil granules, and assist in the destruction of engulfed bacteria. Rabbit studies have identified the presence of neutrophil defensins NP-1, NP-2, and NP-5 at the site of *T. pallidum* infection within 24 hours of inoculation (Borenstein *et al.*, 1991a), and numerous defensins (NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5) have been shown to elicit strong antimicrobial activity against *T. pallidum* (Borenstein *et al.*, 1991b). These results indicate that neutrophils play an important role in the immune system's innate response to *T. pallidum* infection; however, the rapid dissemination and prevalence of latent infection indicates that their involvement is not sufficient to terminate infection.

Cells in the innate branch of the immune system contain pattern-recognition receptors (PRR), which include Toll-like receptors, retinoic acid-inducible gene I-like receptors, nucleotide oligomerization domain-like receptors, and C-type lectin receptors that recognize pathogen-associated molecular patterns (PAMP) (Takeuchi *et al.*, 2010). PAMPs are structures conserved among microbes, which include LPS, peptidoglycan and acylated moieties of lipoproteins (LaFond *et al.*, 2006). When a PRR recognizes a PAMP, intracellular signaling cascades trigger the release proinflammatory cytokines, interferons, chemokines, and antimicrobial proteins (Takeuchi *et al.*, 2010). The *T. pallidum* lipoprotein TpN47 has been shown to activate inflammation through the TLR-2 receptor (Sellati *et al.*, 2001, Lien *et al.*, 1999), further demonstrating the ability of *T. pallidum*, through proteins like TpN47, to induce an inflammatory response.

One of the major bridges between innate and adaptive immunity are dendritic cells (DCs). DCs are found in most nonlymphoid organs, including the epithelia, which is the major tissue involved in initiating *T. pallidum* infection (Reis e Sousa *et al.*, 1999).

When DCs engulf microbes they are activated and subsequently migrate to lymph nodes where they present their antigens via major histocompatibility complex II molecules to T cells (Reis e Sousa *et al.*, 1999, LaFond *et al.*, 2006). Studies have shown that *T. pallidum* is engulfed by DCs, which stimulates an increase in the surface expression of CD83, CD14, CD54, TLR-2 and TLR-4 – important signaling components for DC activation (Sellati *et al.*, 2001, Bouis *et al.*, 2001, Shin *et al.*, 2004). The treponemal protein TpN47 was also found to recruit and activate DCs (Sellati *et al.*, 2001, Shin *et al.*, 2004). *T. pallidum* and TpN47 also increased DC secretion of interleukin (IL)-2, IL-1β, tumor necrosis factor-α, and IL-6, further supporting DC stimulation (Bouis *et al.*, 2001).

The cell-mediated branch of the immune system has been shown to be responsible for the characteristic clearance of *T. pallidum* from early syphilis lesions (Lukehart, 1992). Lukehart *et al.* found that during rabbit testicular infection the cellular infiltrate included T cells and macrophages, which reached peak concentrations on day 13 and corresponded to the same day that *T. pallidum* reached maximum numbers (Lukehart *et al.*, 1980). This also triggered the rapid decline in the numbers of treponemes present at the sight of infection, with a striking absence of bacteria by day 17 (Lukehart *et al.*, 1980). Investigations into T cell involvement during infection used a panel of *T. pallidum* proteins to test T cell response over the course of rabbit syphilis infection and found that three proteins (TN17, TpN47, and TpN37) induced strong T cell proliferation, suggesting that these T cells had already been sensitized to *T. pallidum* (Arroll *et al.*, 1999). Subsequent experiments showed that the addition of sonicated *T. pallidum* induced T cell expression of IL-2 and INF-γ, but not IL10 – cytokine indicators of a cell-mediated response (Arroll *et al.*, 1999). Results seen in the rabbit model are also supported by

human investigations; exudates from human primary and secondary lesions showed the presence of CD8 cytotoxic lymphocytes, and mRNA analysis found transcripts for granzyme B and perforin – indicators of cytotoxic lymphocyte activation that indicate a cell-mediated response (van Voorhis *et al.*, 1996). It is believed that the main immune process involved in the removal of *T. pallidum* from primary chancres is antibody-mediated opsonization of treponemes and subsequent phagocytosis by host macrophages (Cameron *et al.*, 2000a). As previously mentioned, macrophages have also been shown to be present at the site of infection. Activation by T cells causes macrophages to migrate into the site of infection where they are further stimulated by INF-γ, and act by ingesting and killing treponemes (LaFond *et al.*, 2006). The humoral arm of the immune system functions by facilitating macrophage phagocytosis of *T. pallidum*. Research by Baker-Zander *et al.* found that when treponemes and macrophages were incubated with immune rabbit sera, phagocytosis of *T. pallidum* was drastically increased (Baker-Zander *et al.*, 1992). Further research in this laboratory found that significant opsonization of *T. pallidum* was not seen until day 10 of infection; however, it persisted till the end of the study (300 days) (Baker-Zander *et al.*, 1993). This timeline also coincides with the rapid decline of treponemes from the site of infection, which suggests that both antibody-mediated phagocytosis by macrophages and cytotoxic lymphocytes are important in the elimination of *T. pallidum*. One of the main problems associated with *T. pallidum* infection is the incomplete clearance of treponemes by the immune system, resulting in lifelong infection unless the patient is treated with antibiotics. Studies by Lukehart *et al.* found that after the majority of bacteria were removed from the lesions, a sub-population of *T. pallidum* remained that was resistant to phagocytosis by macrophages (Lukehart *et*

al., 1992). How these bacteria avoid phagocytosis and subsequent clearance is still unknown.

As a crucial part of the opsonization of treponemes necessary for bacterial clearance by macrophages, antibodies play a major role. Studies using the rabbit model found that after intratesticular infection, both immunoglobulin G (IgG) and immunoglobulin M (IgM) *T. pallidum*-specific antibodies were detectable by western blotting by day 6 (Lukehart *et al.*, 1986). Similar results were found using western blots on *T. pallidum* extracts using serum from patients infected with syphilis. IgG and IgM reactivity were seen for all stages of syphilis infection including one early-primary syphilis case that was positive only by dark-field microscopy (traditional serologic tests were nonreactive) (Baker-Zander *et al.*, 1985). In general, anti-treponemal IgM antibodies are produced approximately 2 weeks after initial exposure to *T. pallidum*, and IgG antibodies are detectable about 2 weeks after IgM production (Sena *et al.*, 2010). During secondary infection, IgG antibodies reach peak numbers, and studies indicated that IgG concentrations are increased in patients with longer duration of secondary symptoms (Sena *et al.*, 2010). *T. pallidum* specific IgG and IgM antibodies continued to be detectable in both humans and the rabbit model even after the symptoms have subsided and the disease has moved into the late-latent stage (11, 102). After the patient is treated for syphilis, IgM antibodies decrease quickly, and can become undetectable in 6-12 months. After successful treatment, *T. pallidum* specific IgG antibodies can persist anywhere from years to the lifetime of the patient (Sena *et al.*, 2010). Interestingly, approximately 33% of patients produce *T. pallidum* specific IgA antibodies, which are also seen to decrease over the course of treatment (Tanaka *et al.*, 1990).

In addition to macrophage-mediated killing through treponeme opsonization, studies indicate that antibodies are capable of inhibiting the development of syphilis lesions. Three studies published in 1973 found that, in rabbits, immunity to syphilis could be passively transferred using large volumes of serum from previously infected rabbits (Sepetjian *et al.*, 1973, Perine *et al.*, 1973, Turner *et al.*, 1973). As soon as treatment was discontinued lesions would develop, indicating that virulent treponemes were still present at the site of infection (Weiser *et al.*, 1976, Turner *et al.*, 1973). Further research found that this process likely involves the complement system; when IgG from immune rabbit serum and complement from nonimmune rabbit serum were added to virulent treponeme inoculations, lesions failed to develop at 80% of the sites. When only IgG was added, lesions were delayed yet developed at all sites (Blanco *et al.*, 1984). These results suggest that while antibodies are able to prevent chancre development, they are not sufficient to eradicate all of the treponemes. This incomplete immunity is further supported by the fact that patients who have been successfully treated for syphilis can succumb to reinfection (Fiumara, 1980).

1.5 Natural history of syphilis infection

Syphilis is a multi-stage disease, which includes primary, secondary, latent and tertiary symptoms. Commonly known as, “the great imitator,” syphilis can manifest in numerous ways, making it difficult to diagnose clinically – the presence or absence of any of the secondary, tertiary, or congenital symptoms listed below is in no way indicative of the presence of *T. pallidum* infection. Table 1 outlines the clinical manifestations and incubation periods characteristic of each stage of the disease.

Table 1: Common clinical manifestations of syphilis

Stage of syphilis	Clinical manifestations	Incubation period
Primary	Chancre, regional lymphadenopathy	3–90 days
Secondary	Rash, fever, malaise, lymphadenopathy, mucus lesions, condyloma lata, alopecia, meningitis, headaches	2 wk–6 months
Latent	Asymptomatic	Early, <1 yr; late, >1 yr
Tertiary		
Cardiovascular syphilis	Aortic aneurysm, aortic regurgitation, coronary artery ostial stenosis	10-30 years
Neurosyphilis		
Asymptomatic	None	
Acute syphilitic meningitis	Headache, meningeal irritation, confusion	<2 years
Meningovascular	Cranial nerve palsies	
General paresis	Prodrome: headache, vertigo, personality disturbances, followed by acute vascular event with focal findings	5-7 years
Tabes dorsalis	Insidious onset of dementia associated with delusional state, fatigue, intention tremors, loss of facial-muscle tone	10-20 years
	Lightning pains, dysuria, ataxia, Argyll Robertson pupil, areflexia, loss of proprioception	15-20 years
Gumma	Monocytic infiltrates with tissue destruction of any organ	1-46 years (most cases 15 years)
Congenital		
Early	Fulminant disseminated infection, mucocutaneous lesions, osteochondritis, anemia, hepatosplenomegaly, neurosyphilis	Onset <2 years
Late	Interstitial keratitis, lymphadenopathy, hepatosplenomegaly, bone involvement, condylomata, anemia, Hutchinsonian teeth, eight- nerve deafness, recurrent arthropathy, neurosyphilis	Persistence >2 years after birth

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1.5.1 Primary syphilis

Primary syphilis is characterized by the presence of an indurated papule called a "chancre" at the site of infection, which quickly enlarges and ulcerates (Lukehart, 1992, Singh *et al.*, 1999). The chancre, which presents 2-6 weeks after infection, ranges from 0.3-3.0 cm in diameter, is painless, and contains numerous treponemes making it highly infectious (Lukehart, 1992, DiCarlo *et al.*, 1997). Occasionally multiple lesions may

present, and the significant variability in morphologic presentation makes clinical diagnosis extremely difficult (DiCarlo *et al.*, 1997). Infection occurs when treponemes penetrate dermal microabrasions or intact mucous membranes (LaFond *et al.*, 2006). In heterosexual men the chancre is normally found on the penis, specifically the coronal sulcus and glans. Cases involving women typically present, in decreasing frequency, on the labia majora, labia minora, frenulum labiorum pudenda, and perineum. In men who have sex with men (MSM) chancres can present on the penis, anus or rectum. Chancres can also occur extragenitally, although this is believed to occur in less than 2% of cases (Singh *et al.*, 1999). Often localized, non-tender lymphadenopathy is also present (Lukehart, 1992). After 2-6 weeks the chancre heals spontaneously marking the end of primary syphilis (Lukehart, 1992).

1.5.2 Secondary syphilis

There may be no sharp division between primary and secondary syphilis. In approximately one third of cases a chancre may be present along with secondary symptoms; however, in some patients secondary symptoms may present as far as months after primary syphilis ends (Singh *et al.*, 1999). The diagnostic feature of secondary syphilis is a disseminated rash containing infectious *T. pallidum* (Lukehart, 1992). This rash is also difficult to diagnose clinically, since skin lesions range from macular to maculopapular, follicular, and even pustular (Singh *et al.*, 1999). Other symptoms include generalized lymphadenopathy, condylomata lata, mucous patches, headache, uveitis, alopecia, fever, and malaise (Lukehart, 1992, Singh *et al.*, 1999). Somewhat rare symptoms include lesions of the oral mucous membrane (5-22%), subclinical liver involvement (10%), bilateral tinnitus and deafness (Lukehart, 1992, Singh *et al.*, 1999).

As with primary syphilis secondary syphilis symptoms resolve spontaneously, although in some cases symptoms of secondary syphilis can be recurring (Lukehart, 1992, Singh *et al.*, 1999).

1.5.3 Latent syphilis

Latent syphilis is the period from the disappearance of secondary symptoms until the patient is either cured of disease or develops tertiary manifestations, although in most cases latency persists for the patient's lifetime (Singh *et al.*, 1999). The latent stage may also be interrupted by recurring secondary symptoms (Lukehart, 1992).

1.5.4 Tertiary Syphilis

Most of what we know about the progression of syphilis into the tertiary stage comes from two studies. The first study was started by Caesar Broek, who was chief of the venereal clinic at the University Hospital in Oslo from 1890-1910. Broek hospitalized 2181 patients with primary or secondary syphilis, and withheld mercury treatment from them believing that the toxic effects of mercury interfered with the body's natural ability to heal itself. When Salvarsan became available for treatment in 1910 and was believed to be effective, Broek administered the drug to the people in his study who could be found (Gjestland, 1955, Danbolt *et al.*, 1954, Clark *et al.*, 1955, Bruusgaard, 1929). In 1925 Broek's successor, E. Bruusgaard, tracked down 473 patients who had not been treated with Salvarsan in order to examine how syphilis progresses when no treatment has been administered (Bruusgaard, 1929). In 1955 Trygve Gjestland followed up on 1,404 of Boeck's patients, and published a retrospective review into the progression of untreated syphilis (Gjestland, 1955). This study has inherent limitations due to its retrospective nature, since it did not follow patients throughout the entire

duration of disease. Also, because there were no effective diagnostic tests at this time, Boeck relied on clinical diagnosis, which has been shown to be extremely unreliable (Gjestland, 1955, Danbolt *et al.*, 1954, Clark *et al.*, 1955, Bruusgaard, 1929).

After the development of the Wassermann test in 1906, syphilis infection could be more accurately diagnosed. In order to build off the Oslo study, a prospective study was undertaken by the United States Public Health Service in 1932 called, "The Tuskegee Study of Untreated Syphilis in the Negro Male." This study, conducted between 1932-1972, followed 600 African American men (399 who tested positive for syphilis infection) in order to monitor the progression of disease. Most of the information we have relating to the manifestations of tertiary syphilis stem from this study. This study is rightly criticized for being extremely unethical - the African American men in the study were not told they were positive for syphilis infection and penicillin, shown in the 1940's to be an effective cure for syphilis, was actively withheld from them (LaFond *et al.*, 2006, Kampmeier, 1974, Kampmeier, 1972, Hayden, 2003). When these experiments were made public in 1972 there was outrage that caused the immediate termination of the Tuskegee experiment and led the U.S. senate to begin hearings on human experimentation. Many of the ethical standards we have today in North America stem from the aftermath of the Tuskegee experiments (LaFond *et al.*, 2006, Hayden, 2003), yet these studies provide the basis of our understanding of tertiary syphilis.

Tertiary syphilis can appear years to decades after initial exposure to *T. pallidum* (Lukehart, 1992). Although most patients remain in the latent stage of infection, results from the Oslo study found that 1/3 of patients developed tertiary manifestations (Gjestland, 1955). This is during the pre-antibiotic era and the prevalence today is

believed to be much less, possibly due to coincidental antibiotic treatment (Lukehart, 1992). Tertiary syphilis may involve any organ system and, through chronic inflammation, lead to loss of function, destruction of tissue, or both (Lukehart, 1992). Traditionally tertiary syphilis is separated into 3 groups: cardiovascular syphilis, neurosyphilis, and gummatous syphilis. Cardiovascular syphilis can present 10-30 years after initial infection, and typically involves the ascending aorta but sometimes is present in the coronary arteries (Singh *et al.*, 1999). Many patients have *T. pallidum* present in their cerebrospinal fluid without developing neurological manifestations; however, some patients progress to symptomatic neurosyphilis, which is separated into four groups; acute syphilitic meningitis, meningovascular, general paresis, and tabes dorsalis (Singh *et al.*, 1999). Common symptoms of neurosyphilis can range from headaches to dementia, although there are also rare cases involving visual disturbances and numbness in the extremities (Singh *et al.*, 1999). Gummatous syphilis is characterized by granulomatous, nodular lesions that develop 2 or more years after initial infection. These lesions have variable central necrosis, and can lead to degradation of the skin, bones, liver, heart, brain, stomach, and upper respiratory tract (LaFond *et al.*, 2006). Tertiary syphilis, like all other stages of this disease, has a wide repertoire of symptoms making it difficult to diagnose clinically.

1.5.5 Congenital Syphilis

Congenital syphilis is recognized as the most significant disease affecting pregnancies and newborns worldwide. Approximately 2.1 million pregnant women are infected with active syphilis annually, and when untreated (or inadequately treated) there are adverse outcomes in 69% of cases. Complications include spontaneous abortion or

stillbirth (25%), prematurity or low birth weight (13%), infant mortality (11%) and neonatal complications (20%) (Hawkes *et al.*, 2011). *T. pallidum* can be transmitted from the bloodstream, through the placenta, to the developing fetus at any time during the pregnancy; however, the likelihood of infection is greater if the mother has early stage syphilis (Sheffield *et al.*, 2002). Penicillin treatment, if administered within the first two trimesters, has an overall treatment efficacy of >98% (Sheffield *et al.*, 2002).

Congenital syphilis is divided into two stages, early and late. Early congenital syphilis involves manifestations that occur during the first two years of life. One of the earliest symptoms of early congenital syphilis is persistent rhinitis, which occurs in 4-22% of infants and is characterized by purulent or blood tinged nasal discharge, which is highly infectious (Singh *et al.*, 1999). During this stage of infection, 33-50% of infants also present with a maculopapular rash similar to that of secondary infection in adults (Singh *et al.*, 1999, LaFond *et al.*, 2006). Generalized lymphadenopathy is also common, along with central nervous system involvement (Singh *et al.*, 1999). Osteochondritis of the infant's developing long bones can occur and is characterized by pain and lack of movement in the upper and lower extremities (LaFond *et al.*, 2006). Late congenital syphilis symptoms are numerous and include interstitial keratitis, deafness, neurosyphilis, arthropathy, and irregular childhood development (Singh *et al.*, 1999, LaFond *et al.*, 2006). Like the manifestation of disease progression seen with normal syphilis the symptoms of early and late congenital syphilis are quite numerous and varied (Table 1). Serologic diagnosis of congenital syphilis when a mother is positive, or recently treated, for *T. pallidum* infection is difficult due to the passive transfer of antibodies from a mother to her fetus. Since IgA does not cross the placental wall, diagnostic tests

identifying *T. pallidum*-specific IgA are being developed for the diagnosis of congenital syphilis (Sena *et al.*, 2010).

1.6 Treatment of *T. pallidum* infection

Over the last 500 years, numerous methods have been devised and tested in the attempt to cure syphilis. In 1497 the alchemist Paracelsus used mercury, which had been employed for centuries to combat leprosy and yaws, to treat syphilis. Over time, treatment with mercury took numerous forms including salves, pills, and even vapours. Mercury is now known to be extremely toxic, with side effects including extreme salivation, paralysis, shaking, anorexia, diarrhoea, nausea, and rotting or loosening of teeth. Even with its obvious toxicity, some physicians believed well into the 20th century that mercury was the best way to treat syphilis. In 1906, Paul Ehrlich developed an organic arsenic compound he called Salvarsan that, once modified to its active form in humans, could kill spirochetes. Although Salvarsan was eventually shown to be ineffective at clearing *T. pallidum* infection, it was this compound that was the first recorded use of chemotherapy to treat illness (Hayden, 2003). In 1943, Mahoney *et al.* showed that syphilis could be cured with penicillin, and almost 70 years later penicillin remains the treatment of choice (Mahoney *et al.*, 1943).

The length of treatment with penicillin G (gold standard) depends on the stage and clinical manifestations of the disease (Table 2) (2010). In primary and secondary cases, a single intramuscular injection of penicillin G is usually sufficient to eradicate infection, although in cases of neurosyphilis a strict two-week regimen of intravenous penicillin G may be required. For allergies to penicillin, patients exhibiting primary or secondary syphilis can be successfully treated with doxycycline (100 mg twice daily for

14 days) or tetracycline (500 mg four times daily for 14 days) (2010). Ceftriaxone (1 g daily for 10–14 days) has also been shown to effectively treat early syphilis (2010). Azithromycin should not be used due to recent evidence indicating *T. pallidum* has acquired resistance to azithromycin (Lukehart *et al.*, 2004, Stamm *et al.*, 2000, Mitchell *et al.*, 2006).

Table 2: Recommended treatment of syphilis by stage of disease.

Stage of syphilis	Treatment
Primary and Secondary	**Benzathine penicillin G** 2.4 million units IM in a single dose
Tertiary	**Benzathine penicillin G** 7.2 million units total, administered as 3 doses of 2.4 million units IM each at 1-week intervals
Early latent	**Benzathine penicillin G** 2.4 million units IM in a single dose
Late latent	**Benzathine penicillin G** 7.2 million units total, administered as 3 doses of 2.4 million units IM each at 1-week intervals
Neurosyphilis	**Aqueous crystalline penicillin G** 18–24 million units per day, administered as 3–4 million units IV every 4 hours or continuous infusion, for 10–14 days **Procaine penicillin** 2.4 million units IM once daily plus **Probenecid** 500 mg orally four times a day, both for 10–14 days (42)

IM – Intramuscular
IV – Intravenous

In cases of congenital syphilis, penicillin G is the only therapy documented to be effective. In instances where the mother has an allergy to penicillin, instead of using another antibiotic she should undergo desensitization (gradual introduction of penicillin) and then be treated with penicillin. The treatment for pregnant women infected with syphilis should correspond to the stage of disease, as listed in Table 2. Treatment of an infant born to a mother with positive serological tests for syphilis should vary depending on when/if the mother was treated, physical examination of the child for congenital syphilis symptoms, and the titre results of a non-treponemal test. Treatment can vary from intravenous penicillin G (every 12 hours for the first 7 days and 8 hours thereafter for a total of 10 days) to a single intramuscular injection of penicillin G (2010).

Patient follow-up is important to verify effective treatment. If signs or symptoms of infection persist, then treatment was most likely ineffective. Asymptomatic patients should still have the efficacy of treatment assessed by comparison of pre-treatment titres to their current non-treponemal (Venereal Disease Research Laboratory or the rapid plasma reagin) test titres. In general, after effective treatment of primary or secondary syphilis, there should be a four-fold reduction a patient's non-treponemal titre after 6 months. For effective treatment of latent syphilis, there should be a four-fold reduction in a patient's non-treponemal titre after 12-24 months (Romanowski *et al.*, 1991). In cases of retreatment, penicillin G should be administered by intramuscular injections weekly for 3 weeks (2010).

1.7 Direct detection methods for syphilis diagnosis

One of the first diagnostic tests developed for syphilis was the rabbit infectivity test (RIT), which involved the transfer of *T. pallidum* from human chancre exudate to the testicle of a rabbit (Larsen *et al.*, 1995). The RIT is extremely sensitive – active *T. pallidum* infection was identified in 47% of rabbits inoculated with only 1-2 treponemes (Magnuson *et al.*, 1948). This form of test was of course not practical for diagnosing a multitude of patients.

Due to its narrow width, *T. pallidum* cannot be observed using a normal light microscope. This delayed the identification of treponemes as the etiologic agent of syphilis until 1905 when Schaudinn and Hoffmann first visualized *T. pallidum* isolated from chancres using Giemsa stain (Schaudinn, 1905). In 1909, Coles published a paper outlining the use of dark field microscopy (DFM), which differs from conventional light microscopy by visualizing scattered instead of unscattered light. The increased sensitivity

of this method enabled Coles to not only visualize *T. pallidum*, but comment on its characteristic movement (Coles, 1909). When moist lesions are present, DFM is still the most specific test for diagnosing syphilis during primary, secondary, and early congenital stages (Larsen *et al.*, 1995). Care must be taken so that the sample is not contaminated with treponemes found in the normal genital (*T. refringens*) and oral (*T. denticola*) flora (Larsen *et al.*, 1995). DFM is especially sensitive during early primary cases of syphilis, when the antibodies necessary for identification via serological testing have not yet been produced. In special cases, DFM can also be used to identify treponemes in aspirations taken from lymph nodes (Larsen *et al.*, 1995).

The direct fluorescent-antibody test is a variation of DFM that uses FITC-labeled antibodies specific to *T. pallidum* and a dark field microscope (Kellogg, 1970). This allows the distinction between *T. pallidum* and non-pathogenic treponemes, and can be used in conjunction with histologic stains to visualize the presence of *T. pallidum* in tissue sections making it useful for biopsies and autopsies (Larsen *et al.*, 1995, Kellogg, 1970).

1.8 Indirect methods for syphilis diagnosis – non-treponemal tests

The first serological test for syphilis, developed by August Paul von Wassermann in 1906, was a complement fixation test that included liver extracted from newborns who had died of congenital syphilis (Wassermann, 1906). Although the Wassermann test was initially believed to be specific for *T. pallidum* infection, research by Landsteiner *et al.* demonstrated that other tissues, especially beef heart, could be used instead of human tissues from patients infected with syphilis (Larsen *et al.*, 1995). Further, the addition of cholesterol and lecithin was found to increase the sensitivity and specificity of testing

(Larsen *et al.*, 1995). Although the diagnosis of syphilis was drastically improved by these serological tests, some of the limitations included the large number of reagents needed for the test, and the length of time (24 hours) required to obtain results (Larsen *et al.*, 1995). In 1922, a flocculation test was developed that negated the necessity for complement fixation, therefore reducing the number of reactants and enabling the test to be read in only a few hours (Kahn, 1922). Standardization of these tests was another major limitation due to the inability to produce homogeneity between crude beef heart extracts. In 1941, cardiolipin was discovered to be the reactive component of beef heart extracts, and led to the production of pure cardiolipin – the use of which could be standardized (Pangborn, 1941). A new test was developed using a combination of pure cardiolipin, lecithin, and cholesterol. This test is the foundation of the currently used non-treponemal tests, which are still considered by many people to be the gold standard for syphilis diagnosis. The three main tests used today are the Venereal Disease Research Laboratory (VDRL) test, the rapid plasma reagin (RPR) test or the toluidine red unheated serum test (TRUST) (Larsen *et al.*, 1995). The VDRL is a microscopic test that is performed as follows: serum or cerebrospinal fluid (CSF) collected from a patient is added to a slide, and then a mixture of cardiolipin, lecithin, and cholesterol is added. The slide is placed on a rotator for 4 minutes, and then examined for the presence of flocculation under a light microscope. Tests are then defined as reactive (large clumps), weakly reactive (small clumps) or non-reactive (no clumps). In samples positive for syphilis, this test can be used quantitatively by performing serial dilutions of the patient's serum and recording the greatest dilution still giving a positive result (Larsen *et al.*, 1995).

The RPR and TRUST are macroscopic tests that employ the use of visualizing agents (charcoal for the RPR and toluidine red for TRUST) thus eliminating the need for microscopes. In these tests, patient serum and an emulsion of cardiolipin, lecithin, cholesterol, and the visualizing agent is added to a plastic-coated card and placed on a rotator for 8 minutes. The results are read as reactive or non-reactive depending on the presence or absence of visual clumping, which in positive reactions is due to the development of an antibody-antigen lattice made visible by trapping either carbon (RPR) or toluidine red (TRUST) within the lattice (Figure 2). These tests too can be used quantitatively by testing serial dilutions of patient serum and recording the greatest dilution still giving a positive result. However these macroscopic tests are not effective at testing CSF (Larsen *et al.*, 1995).

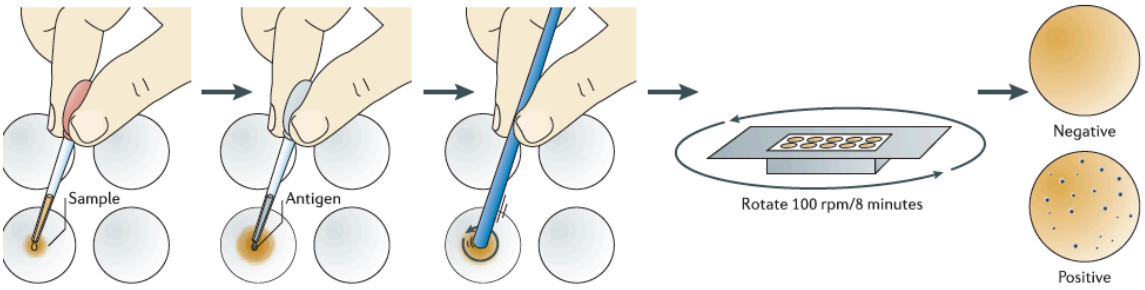

Figure 2: Procedure for the rapid plasma reagin test.
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These non-treponemal tests have many advantages; they are widely available, inexpensive to produce, and allow large throughput of serum samples. They are currently the only way physicians can monitor the efficacy of treatment – patient antibody reactivity with cardiolipin decreases during the course of treatment, whereas *T. pallidum*-specific antibodies identified by current treponemal diagnostic tests may persist for the

lifetime of the patient (LaFond *et al.*, 2006). Sensitivity ranges are high for diagnosing secondary and latent infection at 100 and 95-100% respectively. However the non-treponemal tests are suboptimal at diagnosing early and late latent infections having sensitivities of only 74-99, and 71-73% respectively (Sena *et al.*, 2010, Larsen *et al.*, 1995). In addition to their sensitivity in early stage infections, these non-treponemal tests have been shown to exhibit cross-reactivity against a multitude of diseases and health conditions, including chickenpox, rheumatoid arthritis, pregnancy and advanced age (Table 3) (Ratnam, 2005). Furthermore, these tests are susceptible to false negative results due to the prozone effect, which occurs when antibodies in excess block the normal antigen-antibody reaction. Some data predict that 1-2% of patients with secondary syphilis may exhibit false negatives with the non-treponemal tests due to the prozone effect (Larsen *et al.*, 1995).

1.9 Indirect methods for syphilis diagnosis – treponemal tests

1.9.1 The fluorescent treponemal antibody test

Although the Wasserman test was mistakenly believed to be specific, it was not until 1949 that the first serological diagnostic test was used to identify the presence of antibodies specific to *T. pallidum* (Nelson *et al.*, 1949). Nelson and Mayer developed the *T. pallidum* immobilization (TPI) test, which involved DFM observation of the ability of antibodies and the complement found in patient's serum to immobilize *T. pallidum* (which had been grown in rabbit testicles) (Nelson *et al.*, 1949). The TPI test was not ideal, due to the time required to complete each test, the difficulty in performing each test, and the cost of producing *T. pallidum* in the rabbit model. In 1957, D'Alessandro and Dardanoni tried to bypass this latter limitation by developing a test that used the non-pathogenic, cultivatable treponeme *T. phagedenis* instead of *T. pallidum*. However, their

test exhibited large amounts of false-positives and was found to be less sensitive than the TPI test (D'Alessandro *et al.*, 1953, Larsen *et al.*, 1995). In 1957, the first fluorescent treponemal antibody (FTA) test was developed, which used a combination of *T. pallidum*, serum collected from patients, fluorescein-labeled anti-human antibody, and a microscope with an ultraviolet light source (Deacon *et al.*, 1957). The specificity of this test was inadequate, which was believed to be due to the presence of shared antigens between *T. pallidum* and non-pathogenic treponemes present in the normal human flora. In 1962, the FTA test was adjusted to include an absorption step of patient serum with *T. phagedenis*, prior to its addition to *T. pallidum,* in order to remove antibodies against shared antigens (Deacon *et al.*, 1962). This work was the basis for the currently used FTA absorption (FTA-ABS) test, which was developed by Hunter *et al*. in 1964 (Hunter *et al.*, 1964).

When performing the current FTA-ABS test, patient serum is diluted in a suspension containing *T. phagedenis* in order to remove antibodies against antigens shared by *T. pallidum* and the non-pathogenic treponemes. The pre-absorbed serum is then added to a slide containing *T. pallidum*, allowing antibodies specific to *T. pallidum* to bind to the antigens found in the affixed treponemes. A FITC-labeled anti-human antibody is added, and the slide is examined under a fluorescence microscope. Tests are defined as being either reactive, reactive minimal, nonreactive or exhibiting atypical fluorescence. The FTA-ABS is believed to be one of the most sensitive and specific tests currently available (Table 4). However since it is a complex multi-component test requiring proper titration of conjugates, controls for each state of test reactivity, a

fluorescence microscope, and experienced technicians, variable results are often seen (Larsen *et al.*, 1995, Sena *et al.*, 2010).

Table 3: Causes of false-positive tests for syphilis.

Non-treponemal tests	Treponemal tests
Advancing age	Advancing age
Bacterial endocarditis	Brucellosis
Brucellosis	Cirrhosis
Chancroid	Drug addiction
Chickenpox	Genital herpes
Drug addiction	Hyperglobulinemia
Hepatitis	Immunizations
Idiopathic thrombocytopenic purpura	Infectious mononucleosis
Immunizations	Leptospirosis
Immunoglobulin abnormalities	Leprosy
Infectious mononucleosis	Lyme disease
Intravenous drug use	Malaria
Leprosy	Pinta
Lymphogranuloma venereum	Pregnancy
Malignancy	Relapsing fever
Measles	Scleroderma
Mumps	Systemic lupus erythematosus
Pinta	Thyroiditis
Pneumococcal pneumonia	Yaws
Polyarteritis nodosa	
Pregnancy	
Rheumatoid arthritis	
Rheumatic heart disease	
Rickettsial disease	
Systemic lupus erythematosus	
Thyroiditis	
Tuberculosis	
Ulcerative colitis	
Vasculitis	
Viral pneumonia	
Yaws	

This information was originally published in the *Can J Infect Dis Med Microbiol* 2005;16(1):45-51.

1.9.2 The microhemagglutination assay for antibodies to *T. pallidum* and *Treponema pallidum* particle agglutination tests

In 1967, Rathlev developed a haemagglutination test for syphilis that used formalinized tanned sheep erythrocytes, which had been sensitized with ultrasonicated extracts of the Nichols strain of *T. pallidum* (Rathlev, 1967). Although slight

modifications have been made, this was the basis for the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP) test, which was the main treponemal test until the *Treponema pallidum* particle agglutination (TP-PA) test was developed. The difference between the two tests is that the TP-PA test uses gelatin particles instead of erythrocytes, which makes the test more specific, less complicated (results can be visualized without the aid of a microscope) and less expensive than the MHA-TP test (Ratnam, 2005). As with the FTA-ABS test, the TP-PA test pre-absorbs serum from patients with *T. phagedenis* prior to addition to a microtitre plate. Pre-absorbed patient serum, and a solution containing gelatin particles sensitized with *T. pallidum* (Nichols strain) antigens, are added to microtitre wells, placed on a vibratory shaker for 30 seconds, and incubated at room temperature for 2 hours. The degree of agglutination is examined and a smooth mat of agglutinated cells will be present if the test is positive (Larsen *et al.*, 1995, Sena *et al.*, 2010). The TP-PA has comparable sensitivities and specificities to the FTA-ABS (Table 4) (Larsen *et al.*, 1995, Sena *et al.*, 2010).

Table 4: Sensitivity and specificity of serological tests for syphilis.

	Sensitivity (%) during stage of infection, average (range)				
Test	**Primary**	**Secondary**	**Latent**	**Late**	**Specificity**
Non-treponemal tests (Larsen *et al.*, 1995)					
VDRL	78 (74–87)	100	96 (88–100)	71 (37–94)	98 (96–99)
TRUST	85 (77–86)	100	98 (95–100)	NA	99 (98–99)
RPR	86 (77–99)	100	98 (95–100)	73	98 (93–99)
Early treponemal tests (Larsen *et al.*, 1995)					
MHA-TP	76 (69–90)	100	97 (97–100)	94	99 (98–100)
TP-PA	88 (86–100)	100	100	NA	96 (95–100)
FTA-ABS	84 (70–100)	100	100	96	97 (94–100)
EIA (Sena *et al.*, 2010)					
IgG-ELISA	100	100	100	NA	100
IgM-EIA	93	85	64	NA	NA
ICE	77	100	100	100	99
CIA (Sena *et al.*, 2010)					
CLIA	98	100	100	100	99

1.9.3 Enzyme immunoassays

The first enzyme immunoassay (EIA) for syphilis was developed in 1975 by Veldkamp *et al.*, in an attempt to decrease the cost and potential for technician error intrinsic to diagnostic testing at that time (Veldkamp *et al.*, 1975). Since then, a variety of EIAs (Table 5) have been developed for syphilis that utilize an array of treponemal antigens (some use crude extract of *T. pallidum*, while others use recombinant antigens), and target a variety of patient antibodies (IgM, IgG, and/or IgA) (Sena *et al.*, 2010). Although it is difficult to directly compare these tests because of the varied reference tests they used, it appears that these EIAs show promise due to their high sensitivities and specificities (some reports indicate that they are equal to or better than the FTA-ABS and TP-PA tests), and the potential for automation (Ratnam, 2005). One test, the Captia Syphilis-M EIA (Trinity Biotech) is also believed to be effective at diagnosing congenital syphilis, and potentially effective at monitoring treatment in patients with early primary syphilis (Sena *et al.*, 2010).

1.9.4 Polymerase chain reaction

Numerous polymerase chain reaction (PCR) diagnostic tests have been developed to aid in the diagnosis of syphilis. Due to the presence of multiple genes in *T. pallidum* that contain no homologues in other organisms, PCR can be highly effective at differentiating between *T. pallidum* and other non-pathogenic treponemes. Studies have shown the sensitivity to be quite high, with methods detecting as little as 1-10 organisms (Ratnam, 2005); however, this method still fails to reach sensitivities of those seen in the RIT (Larsen *et al.*, 1995). PCR may be effective in diagnosing congenital syphilis, a condition that is difficult to monitor with serologic tests because of the passive transfer of

antibodies from a mother to her child (Larsen *et al.*, 1995, Ratnam, 2005). PCR has also been shown to be highly effective at diagnosing neurosyphilis. Previous studies have indicated that *T. pallidum* may persist in the central nervous system for long periods even after adequate treatment (through evaluation with the VDRL test) with benzathine penicillin (Berry *et al.*, 1987). Although published sensitivities are quite low [only 7/29 patients with neurosyphilis detected in one study (Noordhoek *et al.*, 1991)], improvement of this method may allow increased efficacy of patient treatment in cases involving neurosyphilis.

1.9.5 Immunochemiluminescence assays

Chemiluminescence is the generation of energy in the form of light from a chemical reaction, the detection of which can be utilized in chemical assays. Chemiluminescence immunoassays (CIA) for detecting syphilis have been developed that are similar to EIAs, however, the reagents they use emit light, and results are produced in less than one hour (Wellinghausen *et al.*). These tests have been shown to exhibit high sensitivities and specificities (Table 5), and due to automated processing have high throughput and limit the human error associated with tests performed by technicians (Sena *et al.*, 2010). The sensitivity of these tests is likely lower than described since they are compared to diagnostic tests (RPR, TP-PA, FTA-ABS) that already have suboptimal sensitivities. Due to the recombinant *T. pallidum* antigens used in these tests, differentiating between current and past infections is currently not possible (Sena *et al.*, 2010).

Table 5: Treponemal antigens, antibody targets, and performance of several treponemal-based tests.

Test	Treponemal antigens	Treponemal antibody targets	Reference tests	Sensitivity %	Specificity %
Rapid tests					
Syphilis Fast	TpN15, TpN17, TpN47	IgM, IgG	VDRL, TPHA, FTA-ABS	95.6	99.9
Determine Syphilis TP	TpN47	IgM, IgG, IgA	TPHA, TPPA	97.2	94.1
Espline TP	TpN15, TpN17, TpN47	IgM, IgG, IgA	TPHA, TPPA	97.7	93.4
SD Bioline Syphilis 3.0	TpN15, TpN17, TpN47	IgM, IgG, IgA	TPHA, TPPA	95	94.9
EIA					
BioElisa Syphilis 3.0	Wild type	IgG	TPHA, FTA-ABS	99.5	99.4
CAPTIA Syphilis-G	Wild type	IgG	FTA-ABS	96.7	98.3
Eti-syphilis G	Wild type	IgG	RPR, MHA-TP, FTA-ABS	99.4	100
Trep-Check IgG EIA	Not specified	IgG	RPR, VDRL, TPPA, FTA-ABS	85.3	95.6
Syphilis EIA II	TpN15, TpN17, TpN47	IgM, IgG	TPHA, TPPA	99.1	100
Syphilis Total	TpN15, TpN17, TpN47	IgM, IgG	TPHA, TPPA	97.4	100
Enzywell Syphilis Screen Recombinant	TpN15, TpN17, TpN47	IgM, IgG	TPHA, TPPA	98.2	100
CIA					
LIASON CIA Assay	TpN17	IgM, IgG	RPR, TPPA	95.8	99.1
Architect CIA	TpN15, TpN17, TpN47	IgM, IgG	VDRL, TPPA	98.4	99.1

Sena, A. C., B. L. White, and P. F. Sparling. Novel *Treponema pallidum* serologic tests: a paradigm shift in syphilis screening for the 21st century. Clin Infect Dis, 2010, 51:700-8 by permission of Oxford University Press

1.9.6 Rapid point-of-care diagnostic tests

Of the 12 million people who are infected with syphilis every year, approximately 90% of cases occur in low-income countries, which lack the resources necessary to afford and perform the tests described above (Peeling *et al.*, 2006b). Due to the inequality between the burden of disease and diagnostic test availability, the World Health Organization is pushing for the development of effective rapid, point-of-care diagnostic

tests for syphilis, and has developed the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users) criteria to address these needs (Peeling *et al.*, 2006a). The majority of these tests are developed as either immunochromatographic strips (ICS) or latex agglutination (LA) tests.

Rapid ICS tests are the most common (there are >20 tests on the market), and involve one or more recombinant *T. pallidum* proteins, as the capture agent, applied to nitrocellulose strips. Most developed tests can use whole blood, plasma or serum, and detect IgG, IgM, and/or IgA (Sena *et al.*, 2010, Ratnam, 2005). These tests are quite simple to perform (Figure 3); patient serum/blood/plasma is applied to the test's sample well, followed by a diluent buffer, and then incubated for the specified time (all tests are <30 minutes). The presence of a visual line where the recombinant *T. pallidum* proteins are affixed indicates a positive test result (123, 133, 143). These tests are inexpensive (<$3 per test), simple to perform, do not require refrigeration, additional equipment, or highly trained personnel (Sena *et al.*, 2010, Ratnam, 2005, Tucker *et al.*, 2010). A recent meta-analysis of 15 independent studies showed current ICS tests have high specificity (98-99%) and a moderate to high range of sensitivities (75-94%) (Tucker *et al.*, 2010).

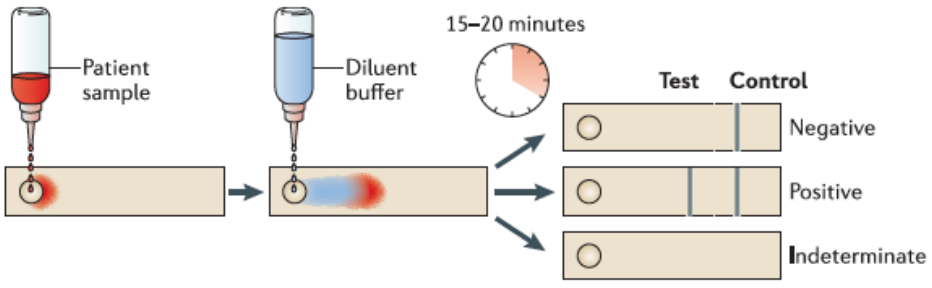

Figure 3: Rapid immunochromatographic strip tests for syphilis.
Patient's whole blood, serum or plasma, is added to the sample well, followed by diluent buffer. The test can then be read after the time specified by the manufacturer. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology **Peeling *et al***., 4:(12 Suppl):S7-19 copyright 2006

Latex agglutination tests use recombinant *T. pallidum* antigens bound to latex particles. Patient serum is combined with the sensitized latex particles, and examined for the presence of agglutination (which indicates a positive result). These tests are also inexpensive, and take only 3 minutes to perform (Fears *et al.*, 2001, Young *et al.*, 1998). Although extensive testing has not been performed on current LA tests, the preliminary results of one test (Syphilis Fast, Diesse Diagnostics) showed high sensitivities and specificities (95.6% and 99.9% respectively) (Young *et al.*, 1998).

A shared limitation between ICS and LA tests, since they both utilize recombinant *T. pallidum* proteins, is that they are ineffective at distinguishing between past and current infection, therefore they cannot be used to monitor the treatment of syphilis (Sena *et al.*, 2010, Ratnam, 2005). Furthermore, the sensitivity of these tests is likely lower than recorded since they are being compared to diagnostic tests (RPR, TP-PA, FTA-ABS) that already have suboptimal sensitivities.

1.10 Current algorithms for diagnosing syphilis

The current testing algorithm is outlined in Figure 4. If a patient presents with a chancre, the defining feature of primary syphilis, DFM can confirm infection by identifying the presence of *T. pallidum*. DFM is extremely limited due to a number of reasons. Most importantly, DFM requires the availability of a dark-field microscope, as well as an experienced technician, both of which are not normally available at the site of care. Second, in cases involving females or men who have sex with men, the chancre is painless and often internal, therefore going unnoticed by the patient and physician (Sena *et al.*, 2010, Larsen *et al.*, 1995).

Due to the limitations of DFM, serological tests are usually employed to diagnose infection. Traditional testing algorithms usually screen serum samples with a non-treponemal test (RPR or VDRL) followed by a treponemal test (MHA-TP, TP-PA, or FTA-ABS). Discordant results are often subjected to a second treponemal test (usually FTA-ABS) in order to confirm a syphilis diagnosis. The need for a two-step screening process is due to the high number of false positive results associated with the non-treponemal tests (section 1.7, Table 2), therefore requiring a specific treponemal test to confirm infection (Sena *et al.*, 2010, Larsen *et al.*, 1995, Ratnam, 2005). Recently, a reverse algorithm has been proposed that involves screening serum samples with an EIA/CIA that contains one or more of TpN15 (Tp0171), TpN17 (Tp0435), TpN47 (Tp0574), and TpN44 (Tp0768; TmpA), followed by a non-treponemal test, with discordant samples being re-screened with a treponemal test (Binnicker *et al.*, 2011, Binnicker *et al.*, 2012, Hoover *et al.*, 2011, Loeffelholz *et al.*, 2012, Sena *et al.*, 2010). This testing algorithm sequence has been shown to exhibit a higher false-positive rate than the traditional screening algorithm in populations with a low syphilis prevalence (Binnicker *et al.*, 2012), and two recent studies have shown discordant result rates of over 50% in these studies, with 17-32% of the discordant samples deemed to be non-reactive upon subsequent TP-PA or FTA-ABS screening. (MMWR, 2008, MMWR, 2011).

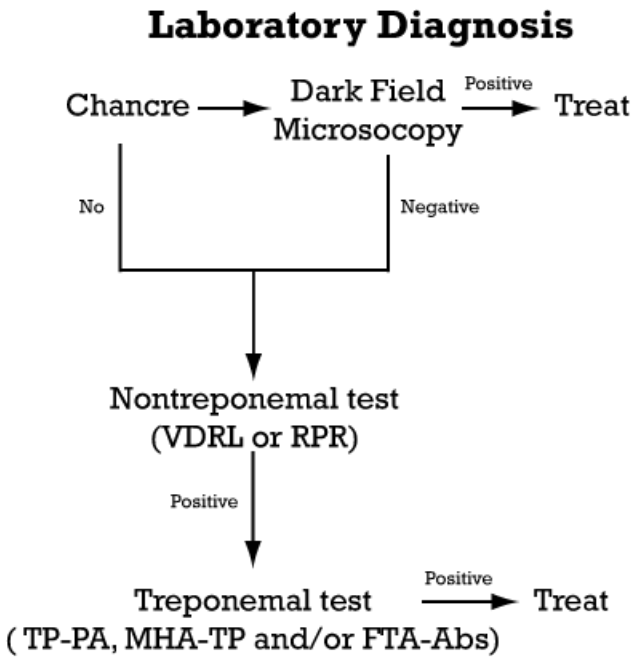

Figure 4: Traditional algorithm for laboratory diagnosis of syphilis infection.

1.11 Identification of diagnostic test antigens

Identification of individual *T. pallidum* proteins has been primarily driven by the desire to find virulence factors and outer membrane proteins (OMP) capable of eliciting a protective immune response; in other words, proteins useful for vaccine development. Elucidation of immunoreactive *T. pallidum* proteins began in 1975 and involved separating *T. pallidum* protein extracts using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) and subsequent western blotting with serum from previously infected rabbits or patients positive for syphilis (Norris, 1993). These studies identified a number of immunoreactive proteins, which were characterized by the strain of *T. pallidum*, and the size of the corresponding band (eg. TpN15 is a 15 kDa protein from *T. pallidum* Nichols strain). Murine monoclonal antibodies were produced in lymphocyte hybridoma cell lines (Robertson *et al.*, 1982), and these antibodies were screened to identify hybridomas producing monoclonal

antibodies against the major 2D SDS-PAGE immunoreactive protein spots (Jones *et al.*, 1984) (Marchitto *et al.*, 1986, Norris, 1993, Purcell *et al.*, 1989). In order for proteins to be useful in vaccine development, surface exposure is important. To identify where these proteins were located numerous methods were performed including density sedimentation with a sucrose gradient (Norris *et al.*, 1988, Houston *et al.*, 1990, Norris, 1993), surface radioiodination of OMPs (Penn *et al.*, 1985, Norris *et al.*, 1984, Moskophidis *et al.*, 1985, Alderete *et al.*, 1980), and selective solubilization with Triton X-100 or Triton X-114 (Radolf *et al.*, 1988a, Cunningham *et al.*, 1988, Penn *et al.*, 1985, Radolf *et al.*, 1988b). Although these studies further characterized the previously described immunoreactive proteins, the processes used are believed to disrupt the fragile outer membrane of *T. pallidum*, implying that the proteins thought to be surface exposed may reside on the cytoplasmic membrane, within the periplasm or be attached to the inner leaflet of the outer membrane.

Four proteins identified using the methods described above have been developed into EIAs, immunochemiluminescence assays, and rapid point-of-care syphilis diagnostic tests (Table 5): Tp0171 (TpN15), Tp0435 (TpN17), Tp0574 (TpN47) and Tp0768 (TpN44.5; TmpA). These proteins were some of the first immunoreactive proteins to be identified (Norgard *et al.*, 1986, Purcell *et al.*, 1989, Norris, 1993) and have been studied extensively for their role in immune system stimulation and pathogenicity. As outlined in the "Immune system response to *T. pallidum"* section, one or more of these proteins have been shown to increase infiltration of polymorphonuclear lymphocytes (Ear *et al.*, 2008), induce an inflammatory response (Sellati *et al.*, 2001, Lien *et al.*, 1999), recruit and activate dendritic cells (Sellati *et al.*, 2001, Shin *et al.*, 2004), and induce strong T cell

proliferation (Arroll *et al.*, 1999). These studies support the role of these proteins in the host immune response to *T. pallidum* infection and their use in diagnostic tests for syphilis. Using recombinant proteins for syphilis serology has great advantages: recombinant proteins are much less expensive to produce than crude *T. pallidum* extracts (bacteria must be grown in rabbit testicles), and they are more easily standardized. Tp0171, Tp0435, Tp0574, and Tp0768 have been developed into diagnostic tests, but unfortunately they have shown suboptimal sensitivities when tested in rapid point-of-care tests (75-94%) and EIAs (85-99%) (Tucker *et al.*, 2010, Sena *et al.*, 2010). This suggests that better antigens need to discovered and produced in recombinant form.

1.12 Immunoreactivity of Tp0326 and Tp0453

In 2003, Van Voorhis *et al.* screened 6 proteins that were predicted to reside on the outer membrane of *T. pallidum*; Tp0155, Tp0257, Tp0326 (Tp92), Tp0453, Tp0483, and Tp0751 (Van Voorhis *et al.*, 2003). Tp0257 and Tp0326 were previously discovered through the use of a *T. pallidum* genomic expression library screening with opsonic rabbit antiserum (Stebeck *et al.*, 1997, Cameron *et al.*, 2000b). The remaining proteins were identified to be potential OMPs through bioinformatic analysis and subsequent functional analyses (Houston *et al.*, 2011, Cameron *et al.*, 2005, Cameron, 2003, Cameron *et al.*, 2004). The genome of *T. pallidum* was examined using the program PSORT, which can assess the likelihood an open reading frames encodes an OMP. PSORT identified all 6 proteins screened in this study as having greater than 69% likelihood of being located on the outer membrane (Van Voorhis *et al.*, 2003).

Full or partial constructs of all 6 proteins were expressed recombinantly in *E. coli*. Each construct was expressed insolubly with an N-terminal six-histidine tag, purified and

subsequently renatured through dialysis. The resulting constructs were then screened against serum samples from patients with primary (n=14), secondary (n=13), latent (n=8), and neurosyphilis (n=8) in order to determine their sensitivity. Specificity was determined by screening the proteins against uninfected individuals (n=15), patients positive for leptospirosis (n=9), relapsing fever (n=8) and Lyme disease (n=8). Tp0257, Tp0326, and Tp0453 exhibited the greatest sensitivities and specificities: Tp0257 showed 91% sensitivity and 93% specificity, Tp0326 showed 98% sensitivity and 97% specificity, and Tp0453 showed 100% sensitivity and 100% specificity (Van Voorhis *et al.*, 2003). Tp0326 and Tp0453 were also shown to be more sensitive than the VDRL, identifying four patients with early primary syphilis that the VDRL had missed (Van Voorhis *et al.*, 2003). The insoluble nature of these proteins limited further development into commercial diagnostic tests since current test formats require soluble protein. The strong immunoreactivity seen for Tp0326 and Tp0453 in this study shows promise, which is why we have chosen to further develop them for the potential use in commercial diagnostic tests.

1.13 Tp0326

As mentioned above, Tp0326 was identified as a potential OMP by Cameron *et al.* through the use of a differential immunologic screening process. This technique uses the assumption that rabbit serum capable of *T. pallidum* opsonization contains antibodies that target surface exposed antigens, therefore by identifying antigens that are bound by opsonic antibodies you can assume that they are surface exposed. Briefly, a λ-phage *T. pallidum* genomic expression library was produced and used to infect *E. coli,* which enabled the λ-phage to express regions of the *T. pallidum* genome. Plaques formed by λ-

phage infection were then screened against opsonic and non-opsonic rabbit sera, and DNA was isolated from plaques showing reactivity to opsonic, but not non-opsonic antiserum. The DNA was then sequenced to identify which region of *T. pallidum* genome was expressed by the λ-phage (Stebeck *et al.*, 1997, Cameron *et al.*, 2000b).

Bioinformatics analysis has shown through sequence homology with Gram-negative outer proteins, and the presence of an N-terminal signal sequence indicative of protein translocation across the inner membrane, that Tp0326 is likely to reside on the surface of *T. pallidum* (Desrosiers *et al.*, 2011). Additional studies have found that *Tp0326* is a member of the highly conserved β-barrel assembly machinery protein A (BamA) family of proteins (Desrosiers *et al.*, 2011). Two highly characterized members of the BamA family include Omp85 of *Neisseria meningitidis* and YaeT of *Escherichia coli* (both Omp85 and YaeT are now referred to as BamA)(Wu *et al.*, 2005, Voulhoux *et al.*, 2003). These proteins are important in the biosynthesis of outer membranes of Gram-negative bacteria, mitochondria and chloroplasts (87, 147, 155). The structure of these bacterial BamA orthologues consist of an N-terminal region with five polypeptide transport-associated (POTRA) domains, and a C-terminal outer membrane spanning amphipathic β-barrel region (87, 147, 155). Although the process by which these BamA proteins function is not fully understood, it is believed that the β-strands of nascent proteins first associate with the β-sheets of the POTRA domains, and then the POTRA arm bends resulting in the formation of β-hairpins that are capable of insertion into the outer membrane (Knowles *et al.*, 2008).

The immunoreactivity of Tp0326 to serum from patients with syphilis and rabbits experimentally infected with *T. pallidum* has been shown in numerous studies, making it

a strong candidate for development into a recombinant antigen for diagnosis of syphilis (25, 33, 43, 51, 161). An interesting discovery in the paper by Desrosiers *et al.* was that the β-barrel region of Tp0326 was found to have limited reactivity in patients infected with syphilis, whereas the POTRA domain was found to be highly immunoreactive (Desrosiers *et al.*, 2011). Additionally, multiple studies indicate that the expression of full-length Tp0326 in *E. coli* is extremely unstable (Cameron *et al.*, 2000b, Tomson *et al.*, 2007), suggesting that a recombinant protein focusing on the POTRA region of Tp0326 would make the best diagnostic candidate. In 2010 Cox *et al.* described the expression of a recombinant version of Tp0326 consisting of amino acid residues Q22 - N434, which encodes POTRA1-5 (Genbank accession number NP_218766; note correction of start site) (Cox *et al.*, 2010). This construct was provided by Drs. Justin Radolf and Daniel Desrosiers at the University of Connecticut Health Center, and has been examined by serological screening in this study.

1.14 Tp0453

Through sequence analysis and the program PSORT, Van Voorhis *et al*. found that Tp0453 had no homologs in other bacteria, and a 78% probability of being an outer membrane protein (Van Voorhis *et al.*, 2003). Studies by Hazlett *et al.* attempted to identify trans-membrane proteins in *T. pallidum* through the use of a highly apolar probe previously shown to insert itself into the outer membrane of bacteria and, upon activation with ultraviolet radiation, bind to trans-membrane proteins. The probe bound to Tp0453, indicating that it was not only associated with the outer membrane, but may actually insert itself into the hydrophobic region of the outer membranes phospholipid bilayer. Further studies using the fluorescent marker calcein encapsulated within liposomes found

that Tp0453 promotes calcein release in a dose-dependent manner, suggesting that Tp0453 destabilizes cellular membranes. The authors speculated that Tp0453 is involved in transporting nutrients from outside the bacterium into the periplasm by disrupting the outer membrane via insertion of amphipathic α-helices. Treatment of whole treponemes with proteinase K led the researchers to conclude that Tp0453 is found on the inner leaflet of the outer membrane (Hazlett *et al.*, 2005).

Recently the crystal structure of Tp0453 has been elucidated. Tp0453 was found to have two amphipathic α-helices that transition between an open and closed conformation. These structures were produced by varying the concentration of detergents to mimic conditions inside and outside of the membrane. These results lead Luthra *et al.* to hypothesize that this conformational change is how Tp0453 acts to disrupt the outer membrane. This study also found that Tp0453 forms stable dimers upon membrane insertion. Although the sequence of Tp0453 has no homology to proteins found in other bacteria, the crystal structure was found to resemble lipoproteins found in *Mycobacterium tuberculosis* that are involved in translocating complex lipids. The authors concluded that Tp0453 inserts itself into the inner leaflet of the outer membrane, where it dimerizes and is involved in the transport of lipids, glycolipids and/or derivatives (Luthra *et al.*, 2011).

Tp0453 contains a signal peptide terminated by a lipobox, which directs it for lipid modification, and has been shown to be lipid modified when expressed in *E. coli* (Hazlett *et al.*, 2005). Although Tp0453 has not been identified in additional studies as a prominent immunoreactive protein, the results by Van Voorhis *et al.* (100% sensitivity and 100% specificity) indicate that it has great potential for the serodiagnosis of syphilis (Van Voorhis *et al.*, 2003).

1.15 Chimeric protein development

The development and use of chimeric proteins in diagnostics is a common practice that has many advantages. Cross-reactivity is a major concern, so by focusing on the key immunoreactive regions of antigenic proteins, a chimeric construct can be created that is highly immunoreactive, yet reduces the presence of cross-reactive epitopes (Gomes-Solecki *et al.*, 2000). This is also beneficial for the recombinant expression of immunoreactive regions of antigenic proteins that cannot be expressed solubly in full-length forms due to toxicity, size, or improper folding in the expression system. Additionally, there are cost-benefits to developing chimeras of antigenic proteins since only one protein needs to be expressed recombinantly, and the final diagnostic test (EIA, CIA, rapid point-of-care, etc) does not need to accommodate multiple proteins.

As described in section 1.10, previous studies identified Tp0326 and Tp0453 as potential candidates for development into serological diagnostic tests (Van Voorhis *et al.*, 2003). The major limitation in further examination of these in commercial diagnostic tests was the insoluble nature of the chimeric constructs. Early attempts in our laboratory to express soluble constructs of Tp0326 and Tp0453 were unsuccessful, so fragments of each protein were produced, and screened against serum from patients infected with syphilis to identify the regions of each protein contributing to their immunoreactivity (Cameron, unpublished results). Using the elucidated immunoreactive regions of each protein, chimeric constructs were designed using the MultiSite Gateway® Pro system (Invitrogen Inc., Burlington, ON, Canada). However, no soluble constructs could be produced (Smith, unpublished results). The development of soluble recombinant Tp0326 (Q22 - N434) (Cox *et al.*, 2010), and the expression of soluble Tp0453 (A32 - S287) in our laboratory, led to the development of a chimeric protein focusing on these two

regions. Numerous techniques were employed to successfully produce a chimera of Tp0326 and Tp0453 with soluble expression in *E. coli*, including codon harmonization, the inclusion of a glycine-serine linker, and variation of expression conditions.

1.15.1 Codon Harmonization

In previous attempts to produce soluble chimeric constructs of Tp0326 and Tp0453 it was apparent that truncated forms of these proteins were being produced. This indicated that there were problems with the complete translation of these constructs in *E. coli*. One reason that this may occur is codon usage bias, which is the difference in frequencies of synonymous codon use in the DNA of different organisms. For example, in Tables 6 and 7 the glycine codon GGA has a frequency (per thousand) of 22.8 in *T. pallidum*, but only 7.9 in *E. coli*. Synonymous codon usage has been shown to be correlated with the number of tRNA molecules present in organisms, so for codons of low frequency the complementary tRNA will also be in low abundance (Ikemura, 1985). Recombinant protein expression involves overexpression of the target protein, so if a *T. pallidum* gene expressed in *E. coli* contained the GGA codon, there is a strong possibility that the complimentary tRNA would be rapidly depleted, leading to a termination of translation and truncated proteins. Numerous studies have shown that when a single codon is synonymously changed, it can alter substrate specificity, protein structure, and/or protein expression (Kimchi-Sarfaty *et al.*, 2007, Lavner *et al.*, 2005, Adzhubei *et al.*, 1996, Coleman *et al.*, 2008). In order to increase the expression of recombinant proteins in heterologous expression systems, many researchers will optimize the DNA sequence of the target genes. With the advent and reduced cost of gene synthesis, it is now common practice to make synonymous substitutions in the target sequence to

exchange low frequency codons with those that have higher frequency. Numerous bioinformatic programs have been designed to this end and this technique has been shown to drastically increase the amount of protein expressed in heterologous expression systems (Puigbo *et al.*, 2007, Grote *et al.*, 2005).

Table 6: *Treponema pallidum* subsp. *pallidum* (Nichols strain) codon frequency table (http://www.kazusa.or.jp)

Fields: codon triplet, frequency per thousand, and (number of codon occurrence in genome)

UUU 26.8(464)	UCU 16.1(278)	UAU 28.0(485)	UGU 10.2(176)
UUC 18.7(324)	UCC 11.7(202)	UAC 14.7(255)	UGC 2.8(48)
UUA 7.6(131)	UCA 5.5(95)	UAA 0.1(1)	UGA 0.1(2)
UUG 17.7(306)	UCG 8.3(144)	UAG 1.7(30)	UGG 23.2(402)
CUU 9.8(169)	CCU 8.4(145)	CAU 7.5(130)	CGU 16.1(278)
CUC 16.3(282)	CCC 7.9(136)	CAC 10.2(176)	CGC 8.5(148)
CUA 4.3(75)	CCA 7.8(135)	CAA 9.8(170)	CGA 3.9(67)
CUG 22.9(397)	CCG 15.3(265)	CAG 21.4(370)	CGG 11.7(202)
AUU 23.2(402)	ACU 11.1(193)	AAU 17.3(300)	AGU 10.9(188)
AUC 10.6(183)	ACC 19.8(342)	AAC 20.0(346)	AGC 12.9(224)
AUA 6.2(108)	ACA 5.9(102)	AAA 17.8(309)	AGA 2.5(43)
AUG 13.5(234)	ACG 22.1(382)	AAG 48.3(836)	AGG 3.6(63)
GUU 16.3(282)	GCU 16.8(291)	GAU 27.6(478)	GGU 22.3(386)
GUC 12.6(218)	GCC 28.6(496)	GAC 19.3(335)	GGC 24.2(419)
GUA 14.8(257)	GCA 26.4(458)	GAA 16.7(290)	GGA 22.8(395)*
GUG 36.1(625)	GCG 23.8(412)	GAG 39.0(676)	GGG 32.1(555)

***GGA is underlined as a demonstration of the codon frequency differences that exist between *T. pallidum* and *E. coli*, see text for further details.**

Table 7: *Escherichia coli* (W3110 strain) frequency table (http://www.kazusa.or.jp)

Fields: codon triplet, frequency per thousand, and (number of codon occurrence in genome)

UUU 22.2(30462)	UCU 8.4(11512)	UAU 16.1(22037)	UGU 5.1(7016)
UUC 16.5(22705)	UCC 8.6(11802)	UAC 12.2(16795)	UGC 6.4(8797)
UUA 13.8(18894)	UCA 7.0(9620)	UAA 2.0(2765)	UGA 0.9(1249)
UUG 13.6(18664)	UCG 8.9(12210)	UAG 0.2(321)	UGG 15.2(20889)
CUU 11.0(15082)	CCU 7.0(9540)	CAU 13.0(17791)	CGU 21.0(28866)
CUC 11.1(15272)	CCC 5.5(7490)	CAC 9.8(13399)	CGC 22.3(30530)
CUA 3.8(5266)	CCA 8.4(11569)	CAA 15.4(21121)	CGA 3.5(4810)
CUG 53.1(72898)	CCG 23.4(32080)	CAG 29.0(39835)	CGG 5.4(7401)
AUU 30.4(41644)	ACU 8.8(12119)	AAU 17.6(24106)	AGU 8.7(11924)
AUC 25.2(34568)	ACC 23.5(32265)	AAC 21.6(29581)	AGC 16.1(22067)
AUA 4.2(5733)	ACA 6.9(9452)	AAA 33.6(46116)	AGA 2.0(2771)
AUG 27.8(38167)	ACG 14.4(19820)	AAG 10.3(14174)	AGG 1.1(1496)
GUU 18.2(24991)	GCU 15.2(20813)	GAU 32.2(44217)	GGU 24.7(33875)
GUC 15.3(21050)	GCC 25.7(35252)	GAC 19.1(26270)	GGC 29.8(40849)
GUA 10.9(14901)	GCA 20.1(27567)	GAA 39.7(54431)	GGA 7.9(10774)*
GUG 26.3(36108)	GCG 33.9(46524)	GAG 18.0(24629)	GGG 11.0(15115)

***GGA is underlined as a demonstration of the codon frequency differences that exist between *T. pallidum* and *E. coli*, see text for further details.**

In order to be compatible with commercial diagnostic tests, a Tp0326-Tp0453 chimera needs to be expressed in a soluble form. An inherent difficulty in producing soluble heterologous protein is the formation of inclusion bodies – aggregates of proteins that precipitate out of solution. This is thought to be due to improper folding, which allows hydrophobic interactions between proteins, causing them to aggregate (Wetzel, 1994). It is hypothesized that in native protein coding sequences, the presence of rare codons may promote proper folding by delaying translation – the additional time it takes for a rare tRNA to bind to its complementary mRNA sequence allows chaperones to successfully fold the protein (Angov *et al.*, 2008, Adzhubei *et al.*, 1996). Instead of codon optimization Angov *et al.* used a technique they called, "codon harmonization," which involved making synonymous substitutions to the target gene with codons of similar frequencies in *E. coli*. They found that codon harmonization produced not only increased expression, but proper folding of proteins (Angov *et al.*, 2008). In order to promote proper folding, and therefore soluble production of the Tp0453-Tp0326 chimeric protein, the sequence was manually codon harmonized prior to gene synthesis.

1.15.2 Glycine-serine linkers

Another problem associated with creating a chimeric protein is the potential for the different protein domains to interact with each other causing misfolding, insolubility, and loss of topographically assembled epitopes. For this reason, a linker was employed. Natural linkers are present in many proteins and function by joining separate domains. A study published by Argos examined the residues found in linker-regions of all elucidated tertiary protein structures and concluded that glycine (Gly), serine (Ser) and threonine would make the best residues for linkers fusing independent protein regions (Argos,

1990). Research on antibody fusion proteins has shown that the inclusion of a linker region can increase expression efficiency (Trinh *et al.*, 2004). Furthermore, synthetic linkers can promote proper protein folding, which was confirmed by Zhang *et al.* through measurement of the biological activities of the separate protein domains (Zhang *et al.*, 2009). Furthermore, as the length of the linker increases, there is a measurable increase in the biological activities of the two protein domains, suggesting that the increased length allows each domain to fold properly (Zhang *et al.*, 2009). If linkers are too long, however, they may be susceptible to proteolytic cleavage during protein synthesis (Gustavsson *et al.*, 2001). The linker chosen for this study was $(Gly_4Ser)_3$, which has previously been shown to be effective at increasing expression and maintaining function in antibody fusion experiments (63). Furthermore, nuclear magnetic resonance analysis indicates that it is highly flexible and does not disrupt folding (Trinh *et al.*, 2004, Freund *et al.*, 1993).

1.16 Research hypothesis and objectives

I hypothesize that the development of a diagnostic test based on immunodominant regions of multiple *Treponema pallidum* subsp. *pallidum* proteins will enhance early detection of syphilis infections and improve overall syphilis diagnosis.

The objectives of my research were to: (1) create a soluble chimeric construct of Tp0326 and Tp0453 and (2) elucidate its sensitivity and specificity for diagnosis of syphilis in comparison to soluble forms of the single antigens Tp0326 and Tp0453, through subsequent screening against an extensive collection of characterized serum samples.

Chapter 2: Syphilis diagnosis
New proteins for a new perspective

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The work contained in this paper constitutes the manuscript submitted to and accepted by the Journal of Clinical Microbiology.

Dr. Muhammad G. Morshed and Yvonne Simpson assisted with development of the ELISA assay, and blinding of the study, as well as provided the characterized serum.
Charmaine Wetherell expressed the Tp0453 construct used in this study.
Dr. Laura L.E. Cowen assisted with the statistics used for determining the cut-off values.
Dr. Martin Boulanger assisted with the delineation of the region of Tp0453 expressed in this study.
Dr. Justin Radolf and Dr. Dan Desrosiers provided the Tp0326 construct.

2.1 Abstract

Syphilis is a sexually transmitted disease caused by the bacterium *Treponema pallidum* subsp. *pallidum* that is effectively treated with penicillin yet remains prevalent worldwide, due in part to the shortfalls of current diagnostic tests. Traditional serological testing algorithms screen for non-treponemal antibodies with subsequent screening of reactive samples for treponemal-specific antibodies. Reverse testing algorithms have recently been implemented that use recombinant *T. pallidum* proteins in automated assays to detect treponemal-specific antibodies, with reactive samples subsequently confirmed using non-treponemal tests and discordant results re-analyzed using treponemal-specific tests. Limitations exist with both the sensitivity and specificity of non-treponemal and treponemal tests, highlighting the need for identification of novel syphilis diagnostic candidates. Here we report the production of soluble recombinant versions of three diagnostic candidates: Tp0326, Tp0453, and a novel Tp0453-Tp0326 chimera. The sensitivity of these recombinant proteins was assessed by screening characterized serum samples from primary, secondary, and latent stages of infection (n=169). The specificity was assessed by screening false positives identified via the standard testing algorithm (n=19), using sera from patients with potentially cross-reactive infections (*Leptospira*, *Borrelia burgdorferi, H. pylori,* Epstein-Barr virus, hepatitis B virus, hepatitis C virus, and cytomegalovirus) (n=38), and from uninfected individuals (n=13). The sensitivities and specificities for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera were found to be 86%, 98% and 98% (sensitivity) and 99%, 100% and 99% (specificity), respectively. These findings identify Tp0453 and the Tp0453-Tp0326 chimera as novel syphilis-

specific diagnostic candidates for accurate detection of all stages of infection and for future development into point-of-care and automated diagnostic test formats.

2.2 Introduction

Syphilis is a chronic infection caused by the spirochete *Treponema pallidum* subsp. *pallidum* that is generally transmitted either through sexual contact or vertically from an infected mother to her fetus. Syphilis is a global health concern with an estimated burden of 25 million people worldwide and an estimated annual incidence rate of 12 million cases (Gerbase *et al.*, 1998). Further, infection with *T. pallidum* has been shown to increase the likelihood of contracting HIV, with risk estimates ranging from 2.3-8.6% (Fleming *et al.*, 1999). The incidence rate of syphilis in men who have sex with men has also been increasing throughout North America and Europe (Ciesielski, 2003, Simms *et al.*, 2005). In addition, congenital syphilis is recognized as the most significant disease affecting pregnancies and newborns worldwide, with over 2 million pregnant women estimated to be infected with syphilis annually (Schmid *et al.*, 2007). Without treatment, there are adverse outcomes in 69% of cases, including spontaneous abortion or stillbirth, neonatal complications, and infant mortality (Hawkes *et al.*, 2011).

Primary syphilis is characterized by a painless open sore called a chancre, which develops on average 3 weeks after infection at the site of inoculation (LaFond *et al.*, 2006). The chancre spontaneously resolves and 1-3 months later secondary symptoms may present. Secondary infection typically manifests as a generalized rash, often localizing to the trunk of the body, palms of the hands, and soles of the feet. After 1-3 months, secondary symptoms resolve and the disease enters an asymptomatic latent phase (Baughn *et al.*, 2005, LaFond *et al.*, 2006). In some instances the disease can progress

from latency to a tertiary stage, which can involve the development of gummas, central nervous system complications or cardiovascular disease (LaFond *et al.*, 2006, Kampmeier, 1972, Clark *et al.*, 1955, 2010). Since the symptoms of syphilis infection are so similar to other diseases, and resolve on their own, syphilis has always been a challenging disease to diagnose clinically.

During primary infection when a chancre is present, dark-field microscopy and/or PCR can be performed to identify spirochetes present at the site of infection. The chancre normally resolves in 4-6 weeks, and often goes unnoticed if internally located in either the anus or vagina, making diagnosis by dark-field microscopy extremely limited. This method of diagnosis also requires the presence of both a dark-field microscope and a trained microscopist, which further limits the usefulness of this laboratory-based diagnostic method (Ratnam, 2005).

The gold standard for syphilis diagnosis relies on the use of a series of serological testing regimes. Traditional serological testing algorithms for diagnosis of *T. pallidum* infection comprise detection of non-treponemal antibodies using the rapid plasma reagin (RPR) test or Venereal Disease Research Laboratory (VDRL) test, followed by further screening of reactive samples for detection of treponemal-specific antibodies using the fluorescent treponemal antibody-absorption (FTA-ABS) test, the microhemagglutination assay for *T. pallidum* (MHA-TP) test or the *T. pallidum* particle agglutination (TP-PA) test. The necessity for multiple tests is due to the inadequacies of current syphilis diagnostic tests. The RPR and VDRL show median sensitivities of only 86% and 78%, respectively, during primary-stage infections and 73% and 71%, respectively, for late-stage infections (Sena *et al.*, 2010, Larsen *et al.*, 1995). Further, these non-treponemal

tests have been shown to exhibit cross-reactivity against a multitude of diseases and health conditions, including chickenpox, rheumatoid arthritis, pregnancy and advanced age (Larsen *et al.*, 1995, Ratnam, 2005). The confirmatory tests (MHA-TP, TP-PA and FTA-ABS) are expensive and have major limitations due their reliance on experienced technicians and adequate testing facilities, factors that are especially restricting in rural areas and developing countries (Ratnam, 2005). These confirmatory tests also have poor sensitivities for detecting early infection, with 88% reported for the MHA-TP (Sena *et al.*, 2010, Castro *et al.*, 2003), 88% for the TP-PA, and 84% for the FTA-ABS (Ratnam, 2005, Sena *et al.*, 2010).

The relatively recent introduction of automated enzyme immunoassays (EIAs) and chemiluminescence immunoassays (CIAs) for diagnosing syphilis infection has prompted implementation of a reverse algorithm screening protocol, whereby samples are first screened via EIA/CIA against a panel of recombinant treponemal proteins [one or more of TpN15 (Tp0171), TpN17 (Tp0435), TpN47 (Tp0574), and TpN44 (Tp0768; TmpA)]. Positive samples are subsequently re-screened with a non-treponemal test and discordant samples (e.g. EIA/CIA reactive and RPR/VDRL nonreactive) with a treponemal test (Binnicker *et al.*, 2011, Binnicker *et al.*, 2012, Hoover *et al.*, 2011, Loeffelholz *et al.*, 2012, Sena *et al.*, 2010). This testing algorithm sequence has been observed to lead to a higher false-reactive rate than traditional screening in a population with a low prevalence of syphilis (Binnicker *et al.*, 2012), and in two studies to lead to discordant result rates of over 50% (MMWR, 2008, MMWR, 2011). Rapid point-of-care (POC) tests based upon the same panel of recombinant treponemal proteins have also become available, although a recent review of results from 15 studies, undertaken at both

sexually transmitted infection and antenatal clinics, showed an average sensitivity of only 86% (Tucker *et al.*, 2010). The poor performance of syphilis EIA, CIA and POC tests is thought to be primarily due to inadequacies associated with the recombinant proteins currently being used in these diagnostic assays.

Due to the aforementioned limitations associated with the currently used syphilis diagnostic tests, identification of novel diagnostic protein candidates with enhanced sensitivities and specificities is needed. Previous research identified two antigens, Tp0326 (Tp92) and Tp0453, which exhibited high sensitivity and specificity when insoluble preparations of these proteins were screened against a panel of serum from patients positive for syphilis and negative controls, respectively (Van Voorhis *et al.*, 2003). To ensure reliable results when incorporated into commercial diagnostic tests, it is essential for recombinant proteins to be expressed in a soluble form. The purpose of this study was to further investigate the diagnostic potential of these proteins by producing soluble, recombinant versions of Tp0326 and Tp0453, as well as a Tp0453-Tp0326 chimera, with subsequent screening of these soluble diagnostic candidates against a more extensive serum bank. These investigations revealed sensitivities and specificities of 98% and 99-100%, respectively, for Tp0453 and the Tp0453-Tp0326 chimera, and identified these proteins as promising new candidates for improved syphilis diagnosis.

2.3 Materials and methods

2.3.1 Preparation of *Treponema pallidum* subsp. *pallidum* (Nichols strain) genomic DNA

T. pallidum subsp. *pallidum* (Nichols strain) was propagated in New Zealand White rabbits as described elsewhere (Lukehart *et al.*, 2007). All animal studies were approved by the University of Victoria Institutional Review Board and conducted in strict

accordance with standard accepted principles as set forth by the Canadian Council on Animal Care, National Institutes of Health and the United States Department of Agriculture in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care and the Canadian Council on Animal Care.

2.3.2 Synthesis of the *tp0326* and *tp0453* constructs and the *tp0453-tp0326* chimera construct

The *tp0326* construct used in this study encodes amino acid residues Q22 - N434 (Genbank accession number NP_218766; note correction of start site) and has been previously described by Cox *et al.* (*tp0326 POTRA1-5*/pET28a, hereafter referred to as *tp0326*) (Cox *et al.*, 2010). The *tp0453* construct (encoding residues A32 - S287) was amplified from *T. pallidum* subsp. *pallidum* genomic DNA using the sense (5'-CTAGACCATATGCACGTTCCTCCACGCAGAATTC-3'; NdeI site underlined) and antisense (5'-GTCAGCTCGAGTCAGCTGCCAATTTTGAGCCGTG-3'; XhoI site underlined) primer pair. The resulting amplicon was ligated into the cloning vector pJET1 (Fermentas, Burlington, ON, Canada), digested with the restriction enzymes NdeI and XhoI and ligated into a similarly digested pET28a expression vector (EMD Inc., Mississauga, ON, Canada). The *tp0453-tp0326* chimera construct includes the regions used in the *tp0326* and *tp0453* constructs encoding amino acids Q22 - N434 for Tp0326 and A32 - S287 for Tp0453. The *tp0453* sequence was placed 5' of the *tp0326* sequence, with a linker sequence positioned between the two constructs (5'-GGTGGAGGTGGCAGTGGTGGAGGAGGAAGCGGCGGGGGTGGGTCA). This intervening sequence encodes a glycine-serine linker [$(GGGGS)_3$] to provide a flexible spacer between the regions, as previously described by Trinh *et al.* (Trinh *et al.*, 2004). The chimeric DNA sequence was manually codon harmonized, as described by Angov *et*

al. (Angov *et al.*, 2008), in order to optimize soluble expression in *E. coli*. Briefly, synonymous changes were made to the codons of the native *T. pallidum* subsp. *pallidum* sequences using codon frequency tables for *T. pallidum* and *E. coli* strain W3110 (Codon Usage Database, Kazusa DNA Research Institute, Chiba, Japan) in order to match the codon frequencies of the *E. coli* expression strain, BL21 Star™ (DE3) (Invitrogen Inc., Burlington, ON, Canada). The restriction site sequences NdeI and XhoI were added to the 5' and 3' ends, respectively, of the chimera construct. The final sequence (Figure 5) was created through DNA synthesis by Integrated DNA Technologies (Coralville, IA, USA). The synthesized sequence, which was received in the pIDTSMART-Kan vector, was transformed into One Shot® Mach1™ T1 chemically competent *E. coli* cells (Invitrogen) as per the manufacturer's instructions. Transformed cells were grown overnight in a 5ml aliquot of Luria Broth (LB)-50 μg/ml kanamycin, and the chimeric *tp0453-tp0326* pIDTSMART-Kan construct was isolated using the GeneJET™ Plasmid Miniprep kit (Fermentas). The isolated plasmid was digested with NdeI and XhoI and ligated into a similarly digested pET28a vector (EMD Inc.). The sequences of the *tp0326* and *tp0453* constructs, as well as the *tp0453-tp0326* chimera construct, within the pET28a vector were verified through DNA sequencing.

1 **CATATG**GCAT CAGTTGATCC GTTGGGTGTT GTGGGATCTG GAGCTGATGT GTATCTCTAC TTCCCTGTCG CTGGGAACGA GAATTTGATT TCAAGGATCA

101 TCGAAAATCA CGAATCAAAA GCGGACATCA AGAAGATAGT CGATCGAACA ACTGCGGTCT ACGGTGCCTT CTTTGCAAGA TCCAAAGAAT TCAGACTCTT

201 TGGATCTGGA TCTTACCCCT ATGCTTTCAC AAATCTTATT TTCTCGCGTT CAGATGGGTG GGCGAGTACA AAAACCGAAC ACGGTATCAC ATATTACGAG

301 AGCGAGCATA CTGATGTCTC TATTCCTGCT CCTCATTTCT CTTGTGTGAT TTTTGGATCG TCTAAAAGGG AACGAATGAG CAAGATGTTG AGCAGATTAG

401 TTAACCCTGA CAGACCGCAA TTGCCTCCTA GATTTGAAAA GGAATGCACG TCAGAAGGAA CATCACAAAC TGTCGCTTTA TACATAAAAA ATGGAGGTCA

501 TTTCATCACT AAACTGTTAA ATTTTCCACA GTTGAACCTC CCTTTAGGTG CTATGGAACT CTATCTCACA GCTCGTCGAA ATGAGTACCT CTACACACTA

601 AGCCTCCAAC TAGGAAATGC CAAGATTAAC TTCCCGATAC AGTTCCTGAT TTCACGAGTT TTGAATGCTC ATATTCATGT GGAGGGAGAC AGACTGATCA

701 TTGAGGATGG AACTATCTCG GCGGAACGTT TGGCCTCGGT AATCTCCTCC CTGTACTCTA AAAAGGGCTC TTCTGGTGGA GGTGGCAGTG GTGGAGGAGG

801 AAGCGGCGGG GGTGGGTCAC AAGCAAATGA TAACTGGTAC GAAGGTAAAC CTATATCAGC AATATCATTC GAGGGGCTCG AATACATCGC ACGTGGGCAG

901 TTAGATACGA TCTTCTCCCA GTATAAAGGG CAGAAGTGGA CCTACGAACT TTACCTGGAG ATTCTTCAAA AGGTTTACGA TTTGGAATAT TTTTCTGAAG

1001 TGTCTCCCAA AGCTGTTCCC ACTGATCCTG AATACCAATA CGTTATGTTA CAATTCACTG TGAAAGAAAG GCCCTCAGTT AAAGGAATTA AGATGGTTGG

1101 GAATTCTCAG ATTAGGAGTG GAGATTTGCT CAGTAAAATT CTTCTTAAAA AGGGTGATAT CTATAATGAG GTTAAGATGA AAGTGGATCA AGAAAGTCTT

1201 AGAAGACATT ACTTGGACCA GGGATACGCT GCAGTGAAAA TTTCTTGTGA AGCTAAGACA GAGGCTGGCG GCGTTGTTGT CCAGTTCACC ATCCAAGAAG

1301 GGAAGCAAAC GGTTGTCAGT AGAATCCAGT TCAAAGGAAA CAAGGCTTTC ACCGAAAGTG TCCTTAAGAA GGTACTAAGC ACTCAAGAAG CTAGATTTCT

1401 AACCTCTGGT GTGTTTAAGG AGAATGCTCT AGAGGCCGAC AAAGCTGCAG TGCATTCTTA CTATGCTGAA AGAGGATATA TCGATGCTAG GGTTGAGGGT

1501 GTGGCTAAGA CGGTTGATAA GAAGACAGAT GCATCTAGAA ATCTCGTAAC CTTGACCTAC ACTGTGGTCG AGGGAGAGCA ATACCGATAC GGTGGAGTCA

1601 CTATAGTGGG AAACCAAATA TTCAGCACAG AAGAACTACA AGCTAAAATT AGACTTAAAC GAGGAGCTAT TATGAATATG GTTGCTTTTG AGCAGGGCTT

1701 TCAAGCCCTC GCTGACGCAT ACTTTGAAAA CGGTTACACA AGTAACTACT TGAACAAGGA AGAACACAGG GACACAGCTG AAAAAACGTT ATCTTTTAAA

1801 ATTACAGTAG TGGAGAGAGA AAGATCTCAT GTTGAACATA TCATTATTAA AGGAACTAAG AATACTAAAG ACGAGGTTAT CCTAAGAGAG ATGCTTTTGA

1901 AACCCGGGGA TGTGTTTAGT AAATCTAAAT TTACTGATAG TTTACGTAAC CTTTTCAATT TGAGATACTT CAGTAGCCTG GTTCCAGATG TAAGGCCAGG

2001 CAGCGAGCAA GATTTAGTCG ATATTATACT AAACGTTGAA GAACAATCTA CAGCAAACTG A**CTCGAG**

Figure 5: Nucleotide sequence of the *tp0453-tp0326* chimera.

2.3.3 Recombinant protein expression and purification

Expression constructs *tp0326, tp0453* and the *tp0453-tp0326* chimera were transformed into the *E. coli* expression strain BL21 Star™ (DE3) (Invitrogen) as per the manufacturer's instructions. Recombinant expression of Tp0326 and Tp0453 was performed according to the following protocol. Transformants were grown in two (Tp0453) or three (Tp0326) test tubes containing 5ml aliquots of LB-50 μg/ml kanamycin for 5-7 hours at 37˚C on a rotator with aeration. These cultures were pooled and transferred to three (Tp0326) or four (Tp0453) 250ml Erlenmeyer flasks (1ml of pooled culture per flask) containing 50ml each of LB-50 μg/ml kanamycin and incubated overnight for 16-18 hrs at 37˚C with aeration. The cultures were pooled, then 25 ml (Tp0326) or 30 ml (Tp0453) of pooled culture were transferred to each of six baffled 4L Erlenmeyer flasks containing 1 L of LB-50 μg/ml kanamycin. These flasks were incubated at 37°C with aeration for 4-5 hrs until the optical density (OD) at 600nm

reached 1.5-1.6 (Tp0326) or 0.7-1.0 (Tp0453). Recombinant expression of the Tp0453-Tp0326 chimera was performed according to the following protocol. Transformants were grown in six test tubes containing 5ml aliquots of LB-50 μg/ml kanamycin for 5-7 hours at 37˚C on a rotator with aeration. These cultures were pooled and transferred to six 250ml Erlenmeyer flasks (1ml of pooled culture per flask) containing 50ml each of LB-50 μg/ml kanamycin and incubated overnight for 16-18 hrs at 37˚C with aeration. The cultures were transferred to twelve 4L Erlenmeyer flasks containing 1.5 L of LB-50 μg/ml kanamycin (20ml pooled culture per flask) and cultured at 37°C with aeration for 4-5 hrs until an OD of 1.4-1.5 was reached. For all constructs the temperature was dropped to 25˚C for 0.5-1 hour until an OD of 1.2 (Tp0453) or 1.7 (Tp0326 and the Tp0453-Tp0326 chimera) was reached, after which the temperature was subsequently dropped to 16˚C. After 30 minutes at 16˚C, recombinant expression was induced for all constructs using a final concentration of 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) (Invitrogen) and cultures were grown for an additional 16-18 hrs at 16°C post-induction. Bacteria were harvested by centrifugation and resuspended in 20 ml of 20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole (Tp0326 and the Tp0453-Tp0326 chimera) or 25 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole (Tp0453), in the presence of a protease inhibitor cocktail (Product number 539134, EMD Inc.). The suspension of cells expressing Tp0453 was stored at -20˚C. The suspensions of cells expressing Tp0326 and the Tp0453-Tp0326 chimera were frozen in liquid nitrogen and then stored at -20˚C. After thawing overnight at 4˚C, lysis buffer was added to the suspensions of cells expressing Tp0326 and the Tp0453-Tp0326 chimera to give a final concentration of 5 mg/ml CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)

(Sigma-Aldrich, Oakville, Ontario, Canada), 5mM $MgCl_2$, and 1.6 U/ml DNaseI (Invitrogen). The suspensions were incubated for 30 minutes at room temperature to allow for lysis, after which the lysates were sonicated (3 x 10 s) at 10 W. The suspension of cells expressing Tp0453 was lysed in a French press using two passes at 18,000 psi. All resulting lysates were centrifuged at 20,000 *x g* at 4^{o}C for 45 minutes. The supernatants containing the soluble recombinant proteins were removed and subjected to immobilized metal ion affinity chromatography (IMAC) purification, as outlined below.

Soluble histidine-tagged recombinant proteins were isolated using fast protein liquid chromatography (FPLC) and IMAC methodologies. Briefly, using an AKTA Prime Plus FPLC system (GE Healthcare, Baie D'Urfe, QC, Canada), cell-lysis supernatants were applied to 1mL HisTrapp FF affinity columns (GE Healthcare) containing Ni^{2+}-ions immobilized on agarose beads. The columns were washed with cold Tris buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole) (Tp0326 and the Tp0453-Tp0326 chimera) or HEPES buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole) (Tp0453) and Ni^{2+}-bound proteins were eluted using an imidazole gradient (20-500 mM imidazole). Fractions were monitored with the AKTA prime plus spectrophotometer by measuring the OD at 280 nm, and the purity of the eluted proteins was verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining.

Following Ni^{2+} purification, proteins were concentrated to 2-5 mL using a 10 kDa molecular mass cut-off centrifugal filter unit (Millipore, Billerica, MA, USA). Using the AKTA Prime Plus FPLC system, a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) was equilibrated with cold Tris buffer (20 mM Tris pH 7.5, 150 mM NaCl)

(Tp0326 and the Tp0453-Tp0326 chimera) or room temperature HEPES buffer (20 mM HEPES pH 7.5, 150 mM NaCl), and the concentrated Ni^{2+} purification solution was injected onto the column. The same buffers were used to elute the recombinant proteins off the column, and fractions were monitored by measuring the OD at 280 nm. Fractions corresponding to the elution peaks were examined via SDS-PAGE, pooled, and concentrated (as described above). Protein concentrations were determined using the Thermo Scientific bicinchoninic acid (BCA) protein assay kit (Fischer Scientific Limited, Ottawa, ON, Canada), and pooled protein solutions were flash frozen in liquid nitrogen for 3 minutes and stored at -80ºC.

2.3.4 Enzyme-linked immunosorbent assays

Frozen protein aliquots were thawed in a water bath at 25°C for 90 seconds. Ninety-six well plates (Nunc-Immuno™ MaxiSorp™; Sigma) were incubated at 4°C overnight with 50 µl of 6.0 µg/ml recombinant protein solution in phosphate buffered saline (PBS), pH 7.4. Plates were blocked for 2 hours at room temperature with 1 X PBS, 4% milk powder. Wells were washed 3 times with wash buffer (1X PBS, 0.05% Tween-20). Human sera was diluted 1:400 in dilution buffer (1 X PBS pH 7.4, 4% milk powder, 0.2% Triton X-100), and 50 µl of diluted serum were added to plates in triplicate and incubated for 1 hour at room temperature. Plates were washed with 3 quick washes and 3 x 10 minute washes. Goat anti-human IgG (Fab specific) peroxidase (Sigma) was diluted to 1:3,000 in dilution buffer and 50 µl were added to each well and incubated for 1 hour at room temperature. Plates were washed with 3 quick washes and 3 x 10 minute washes. All wash steps, as well as the primary and secondary antibody incubations, were done on a rotator at 80 RPM. Plates were developed by adding 100 µl of tetramethylbenzidine-H-

$_2O_2$ substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) to each well for 30 minutes at room temperature. Absorbance was read at 630 nm.

2.3.5 Statistical analysis

Immunoassay cut-off determination was done using serum samples from 24 patients negative for syphilis infection. The samples used for this part of the study were from patients with negative RPR and TP-PA, and differed from the samples used in the final immunological screening. Each sample was run in triplicate in three independent assays. The average absorbance for the negative serum samples over three runs was calculated using the equation:

$$\bar{y}_{\cdot} = \sum_{i=1}^{n} \bar{y}_i$$

where n is the number of replicates for each protein, and $y_{\cdot}$ represents the average absorbance for each run (over the 24 samples).

The empirical standard error was estimated using the equation:

$$\hat{se}(\bar{y}_{\cdot}) = \left\{ \sum_{i=1}^{n} \frac{(\bar{y}_i - \bar{y}_{\cdot})^2}{n(n-1)} \right\}^{1/2}$$

Cut-off values using a 98% confidence interval were calculated using the equation:

$$\bar{y}_{\cdot} \pm 6.965 \times \hat{se}(\bar{y}_{\cdot})$$

where 6.965 is the critical value of the T-distribution for a 98% confidence interval, with 2 degrees freedom. The upper-limit of the 98% confidence interval defines the cut-off values for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera giving values of 0.138, 0.116, and 0.148, respectively. An equivocal range of greater or less than 10% was calculated, giving ranges of 0.124-0.151, 0.104-0.127 and 0.133-0.163 for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera, respectively (Burd, 2010). Positive samples were defined as being greater than the equivocal range, and negative samples were defined as being less than the equivocal range. Values falling within the equivocal range were discarded from the study.

2.3.6 Serum panel

The serum samples for this study were provided by the British Columbia Centre for Disease Control Public Health Microbiology and Reference Laboratory, and ethics approval was obtained from the University of British Columbia and the University of Victoria Human Ethics Boards. The positive sera contained 169 characterized samples from patients with confirmed primary (n=70), secondary (n=47), and early latent (n=52) syphilis infection. Positive samples were defined as having both a positive RPR and TP-PA assay, or in cases where only the RPR or TP-PA test was positive, a positive FTA-ABS test. The serum bank also contained 70 samples from patients negative for syphilis infection including non-reactive (n=11), false positive (positive RPR or TP-PA but a negative FTA-ABS) (n=21), and potentially cross-reactive (n=38) samples. The latter samples included serum samples from patients infected with *Borrelia burgdorferi* (n=4), *Leptospira* (n=4), *Helicobacter pylori* (n=5), Cytomegalovirus (n=5), Epstein-Barr virus

(n=5), Hepatitis B virus (n=10) and Hepatitis C virus (n=5). The diagnostic tests used to confirm each potentially cross-reactive sample are listed in Table 1. All potentially cross-reactive samples were screened with the RPR and TP-PA and found to be negative. All serum samples were stored at -20^{o}C and had undergone only 2 freeze-thaw cycles. All serum samples were blinded prior to screening.

Table 8: Diagnostic tests used in this study to assess positive or negative infections.

Bacterium/Virus	Diagnostic Test	Manufacturer
T. pallidum	Rapid plasma reagin	Becton-Dickinson, Mississauga, ON, Canada
T. pallidum	*Treponema pallidum* particle agglutination assay	Fujirebio Inc., Malvern, PA, USA
T. pallidum	Fluorescent treponemal antibody absorption assay	Zeus Scientific, Branchburg, NJ, USA
B. burgdorferi	Western blot IgG	MarDx Diagnostics, Inc., California, USA
Leptospira	Panbio® *Leptospira* IgM	PanBio, Queensland, Australia
H. pylori	*H. pylori* IgG	Siemens Healthcare, Ontario, Canada
Cytomegalovirus	VIDAS® CMV IgG	bioMérieux, Quebec, Canada
Epstein-Barr virus	Enzygnost® Anti-EBV IgG/IgM	Dade Behring/Siemens Healthcare, Ontario, Canada
Hepatitis B virus	Anti-HBs or Anti-HBc Total	Siemens Healthcare, Ontario, Canada
Hepatitis C virus	HCV	Siemens Healthcare, Ontario, Canada

2.4 Results

2.4.1 Production of soluble recombinant proteins.

Previous investigations conducted using insoluble, recombinant versions of the *T. pallidum* proteins Tp0326 (Tp92) and Tp0453 identified these proteins as being highly reactive with serum samples collected from syphilis patients (Van Voorhis *et al.*, 2003). The insoluble nature of these recombinant proteins prevented further analysis of their potential for the serodiagnosis of syphilis, since commercial diagnostic tests require soluble proteins in order to facilitate standardization. To fully explore the diagnostic potential of these proteins, in this study we produced soluble versions of Tp0326 and Tp0453. The soluble Tp0326 recombinant protein, previously described by Cox *et al.* (Cox *et al.*, 2010), encompasses amino acid residues Q22 - N434 of the full-length

protein sequence and is produced as a single protein that is estimated to have a purity of >98% by SDS-PAGE analysis (Figure 2A). The production of soluble recombinant Tp0453 was achieved through expression of amino acids residues A32 - S287 using the pET28a expression vector and the *E. coli* expression strain BL21 Star™ (DE3). This recombinant protein preparation was also judged to be >98% pure via SDS-PAGE analysis (Figure 2B). Numerous attempts were made to produce a soluble chimeric construct of Tp0326 and Tp0453 (data not shown), with only one construct (Tp0453-Tp0326 chimera) resulting in successful soluble expression in *E. coli*. This chimera includes the amino acid residues contained within the individual Tp0326 and Tp0453 recombinant proteins (amino acids Q22 - N434 for Tp0326 and A32 - S287 for Tp0453), with the Tp0453 region being positioned N-terminal to the Tp0326 fragment (Figure 3). Prior attempts to create a Tp0453-Tp0326 chimera consistently resulted in expression of a truncated protein product, which was hypothesized to be due to the difference in codon usage between *T. pallidum* and the *E. coli* expression strain utilized in this study. Numerous studies have suggested that species to species differences in codon usage can account for difficulties encountered in recombinant expression including lack of expression, insoluble protein production, and premature translation termination (4, 86, 88, 90, 97, 171). Codon harmonization is a technique that matches the codons used in the gene encoding the protein of interest with that of the codon preferences used by the bacterial expression strain. Using codon frequency tables for *T. pallidum* subsp. *pallidum* and *E. coli* the sequences of *tp0326* and *tp0453* were codon harmonized for expression in *E. coli*. A flexible linker consisting of three repeats of GGGGS, as previously described by Trinh *et al.* (Trinh *et al.*, 2004), was used to increase the distance between the *tp0326*

and *tp0453* regions. Linker regions have been shown to promote proper folding and increased solubility through reducing the interactions between separate chimeric regions (Trinh *et al.*, 2004). Collectively these optimization techniques resulted in the successful soluble expression and purification of a Tp0453-Tp0326 chimera that appeared as a single band upon SDS-PAGE analysis and was judged to be >98% pure (Figure 2C).

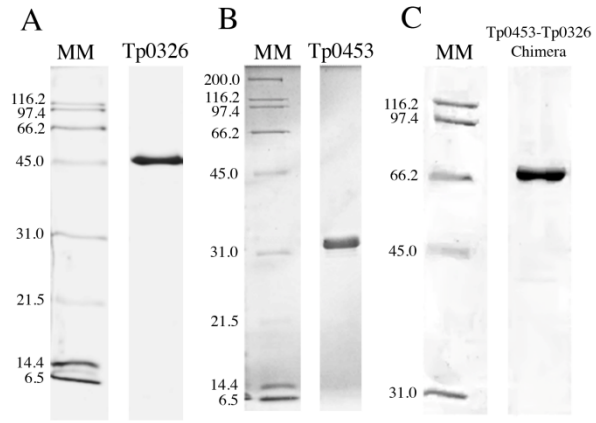

Figure 6: SDS-PAGE analysis of the gel filtration elutions of soluble recombinant proteins
(**A**) Tp0326 (49.4 kDa), (**B**) Tp0453 (30.8 kDa), and (**C**) the Tp0453-Tp0326 chimera (78.7 kDa).

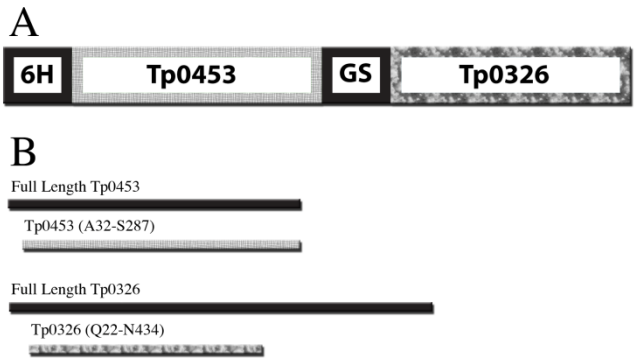

Figure 7: Schematics of soluble proteins used in study.

(A) Schematic of the Tp0453-Tp0326 chimera. 6H represents the histidine tag, GS represents the incorporated glycine-serine repeat linker region. (**B**) The portion of each of full-length Tp0453 and Tp0326 covered in the chimera.

3.4.2 Diagnostic performance of the recombinant proteins.

The three soluble recombinant proteins (Tp0326, Tp0453, and the Tp0453-Tp0326 chimera) were tested against a well-characterized serum panel from syphilis patients to determine the sensitivity of these proteins for diagnosing syphilis infections (Table 2). Positive serum samples (n=169) included patients with confirmed primary (n=70), secondary (n=47), and early latent (n=52) syphilis infection. The overall sensitivity of Tp0326 was determined to be 86%, with sensitivities of 69%, 98% and 94% for detecting primary, secondary, and early latent infection, respectively. Tp0453 had an overall sensitivity of 98%, with sensitivities of 96%, 100% and 100% for detecting primary, secondary, and early latent infection, respectively. Finally, the Tp0453-Tp0326 chimera had an overall sensitivity of 98%, with sensitivities of 94%, 100% and 100% for detecting primary, secondary, and early latent infection, respectively.

Table 9: Sensitivity of Tp0326, Tp0453 and Tp0453-Tp0326 chimera.

Summary of the sensitivity of the Tp0326, Tp0453 and Tp0453-Tp0326 chimera recombinant proteins as determined by ELISA analysis of sera from patients positive for primary, secondary or early latent syphilis.

Protein screened and stage of syphilis infection	ELISA result			Sensitivity (%)
	Positive	Negative	Equivocal	
Tp0326				
Primary	41	18	11	69
Secondary	45	1	1	98
Early Latent	46	3	3	94
All stages	132	22	15	86
Tp0453				
Primary	64	3	3	96
Secondary	47	0	0	100
Early Latent	52	0	0	100
All stages	163	3	3	98
Tp0453-Tp0326 Chimera				
Primary	58	4	8	94
Secondary	47	0	0	100
Early Latent	52	0	0	100
All stages	157	4	8	98

To determine the specificity of the recombinant proteins, the Tp0326, Tp0453, and Tp0453-Tp0326 chimera were tested against negative serum samples (n=70), including non-reactive (n=11), false positive (positive RPR or TP-PA but a negative FTA-ABS) (n=21), and potentially cross-reactive (n=38) samples (Table 3). The overall specificities for Tp0326, Tp0453, and the Tp0453-Tp0326 chimera were 99%, 100%, and 99%, respectively. All three proteins exhibited 100% specificity when tested against the non-reactive and false positive serum samples. The potentially cross-reactive sera included samples from patients infected with *B. burgdorferi* (n=4), *Leptospira* (n=4), *H. pylori* (n=5), Cytomegalovirus (n=5), Epstein-Barr virus (n=5), hepatitis B virus (n=10), and hepatitis C virus (n=5). Tp0326 exhibited a positive reaction to one serum sample from a patient infected with *B. burgdorferi*, while the Tp0453-Tp0326 chimera similarly

exhibited a positive reaction to one serum sample from a patient infected with the Epstein-Barr virus. Tp0453 showed no reactivity to any of the potentially cross-reactive sera.

Table 10: Specificity of the Tp0326, Tp0453 and Tp0453-Tp0326 chimera.

Summary of the specificity of the Tp0326, Tp0453 and Tp0453-Tp0326 chimera recombinant proteins as determined by ELISA analysis of sera from individuals negative for syphilis infection, including analysis of sera from patients with potentially cross-reactive infections.

Protein screened and negative/cross-reactive serum used	ELISA result			Specificity (%)
	Positive	Negative	Equivocal	
Tp0326				
Non-reactive	0	11	0	100
False positives	0	21	0	100
Borrelia burgdorferi	1	2	1	67
Leptospira	0	4	0	100
Helicobacter pylori	0	5	0	100
Cytomegalovirus	0	4	1	100
Epstein-Barr virus	0	5	0	100
Hepatitis B virus	0	10	0	100
Hepatitis C virus	0	5	0	100
All negative samples	1	67	2	99
Tp0453				
Non-reactive	0	11	0	100
False positives	0	20	1	100
Borrelia burgdorferi	0	4	0	100
Leptospira	0	4	0	100
Helicobacter pylori	0	5	0	100
Cytomegalovirus	0	5	0	100
Epstein-Barr virus	0	5	0	100
Hepatitis B virus	0	10	0	100
Hepatitis C virus	0	5	0	100
All negative samples	0	69	1	100
Tp0453-Tp0326 chimera				
Non-reactive	0	11	0	100
False positives	0	21	0	100
Borrelia burgdorferi	0	4	0	100
Leptospira	0	4	0	100
Helicobacter pylori	0	5	0	100
Cytomegalovirus	0	5	0	100
Epstein-Barr virus	1	3	1	75
Hepatitis B virus	0	10	0	100
Hepatitis C virus	0	5	0	100
All negative samples	1	68	1	99

2.5 Discussion

Out of the three diagnostic protein candidates analyzed in this study, Tp0326 was found to perform the least well in exhibiting a sensitivity of 86% and a specificity of 99%. Tp0326 is an ortholog of the BamA molecule found throughout Gram-negative bacteria (Desrosiers *et al.*, 2011), and the polypeptide transport-associated (POTRA) region used in this study is highly conserved (33, 51, 138). This may affect its specificity, and explain the reactivity seen in one serum sample from a patient positive for infection with *B. burgdorferi*. Further, the moderate observed sensitivity of Tp0326 suggests that this protein on its own may not constitute an effective diagnostic candidate.

Tp0453 was determined to be the best diagnostic candidate in this study, exhibiting a sensitivity of 98% and a specificity of 100%. These results match the previous results of Van Voorhis and coworkers who showed that an insoluble preparation of Tp0453 exhibited 100% sensitivity and 100% specificity upon screening of n=83 serum samples (Van Voorhis *et al.*, 2003). No serum samples from patients negative for syphilis infection showed reactivity against Tp0453, even those from patients infected with the related spirochetes *B. burgdorferi* and *Leptospira.* The high specificity seen in this study may be explained by the sequence of Tp0453, which has no homologues in other bacteria (62, 73, 111). The coding region used in the *tp0453* construct is identical across all sequenced strains of *T. pallidum*, suggesting that patients infected with any strain of *T. pallidum* will likely have antibodies against this protein (73, 111).

The strong immunoreactivity exhibited by Tp0326 and Tp0453 suggested that creating a chimera of these two proteins that contains epitopes from both proteins could potentially increase sensitivity. The soluble Tp0453-Tp0326 chimera created for this study shows high sensitivity and specificity (98% and 99% respectively), making this

construct another potential candidate for use in syphilis diagnosis. However, our hypothesis that the Tp0453-Tp0326 chimera would identify more positive syphilis infections has not yet been confirmed in the sample set we have analyzed to date, since the sensitivities of Tp0453 and the Tp0453-Tp0326 chimera are approximately equal. The difference in specificity is also not significant, indicating that further screening with a larger sample set needs to be conducted to determine if the Tp0453-Tp0326 chimera does indeed perform better in accurately detecting syphilis infections due to the incorporation of the dual protein regions.

It should be emphasized that in this study there is a reliance upon the RPR, TP-PA, and FTA-ABS assays to define a positive syphilis infection. In this regard, although the sera used in this study were well characterized, the inherent propensity for all three tests to generate false-positives allows for speculation that some of the false-negative results received in these investigations may actually be true negatives. Furthermore, using traditional screening algorithms false-negative results can arise in samples collected from patients with early primary infection due to the frequent lack of non-treponemal antibodies in these patients (Hoover *et al.*, 2011). A future area of investigation with the Tp0453 protein and the Tp0453-Tp0326 chimera is to determine if these diagnostic candidates can accurately diagnose RPR- or VDRL-negative serum samples from patients deemed to be positive for early primary syphilis infection via dark-field microscopy. Demonstration of a positive result in such a study would serve to verify prior results reported by Van Voorhis *et al.* (Van Voorhis *et al.*, 2003) who found that four patients with dark-field confirmed early primary syphilis infection exhibited a positive reaction to

Tp0453 and Tp0326 but were VDRL-negative, suggesting these two diagnostic candidates may be particularly adept at identifying early cases of syphilis infection.

In summary, in this study we have identified the novel recombinant diagnostic candidates Tp0453 and the Tp0453-Tp0326 chimera which exhibit high sensitivity (98%) for detection of all stages of infection, including early primary cases, and are extremely specific (100% and 99% respectively) even when tested against potentially cross-reactive sera. Thus, the use of these proteins in a diagnostic test format could alleviate some of the major limitations currently confronting the syphilis diagnostic field. Further, the ability to produce these proteins in a soluble format will facilitate their incorporation into multiple diagnostic test formats, including automated assays and rapid POC tests.

2.6 Acknowledgments

The authors would like to thank Drs. Justin Radolf and Daniel Desrosiers, University of Connecticut Health Center, for their gift of the *tp0326 POTRA1-5*/pET28a construct, Ms. Quantine Wong and all the staff at the Zoonotic Diseases and Emerging Pathogens Section of British Columbia Center for Disease Control Public Health Microbiology and Reference Laboratory for their assistance with screening, Drs. Sheila Lukehart and Wesley Van Voorhis, University of Washington, for helpful discussions relating to this project, and Dr. Martin Boulanger, University of Victoria, for his assistance with the *tp0453* construct design. This work was funded by an operating grant from the Canadian Institutes of Health Research (C.E.C.; HPR 85530) and a Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship to Brenden C. Smith.

Chapter 3: Conclusions and future directions

The specificity of Tp0326 was high at 99%, with reactivity to only one serum sample from a patient positive for infection with *B. burgdorferi*. Tp0326 is an ortholog of the BamA molecule found throughout Gram-negative bacteria (Desrosiers *et al.*, 2011), and the polypeptide transport-associated (POTRA) region used in this study is the most highly conserved region of this protein (Desrosiers *et al.*, 2011). This may affect its specificity, and explain the reactivity seen in one serum sample from a patient positive for infection with *B. burgdorferi*. Basic Logical Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) analysis of sequence similarities between Tp0326 of *T. pallidum* and its homologue in *B. burgdorferi* indicate that they share only 33% of residues in this region. Taking into consideration the positive substitutions (aligned residues with similar chemical properties) and low amount of introduced gaps, and especially the conserved structural homology of the POTRA domains across Gram negative bacteria, one can predict that these two proteins may share some conformational epitopes that could lead to the production of antibodies that are reactive to both proteins. The low sequence similarity makes it unlikely that the cross-reactivity is due to a linear epitope shared between the two proteins. Interestingly, two other serum samples from patients infected with *B. burgdorferi* were found to be unreactive to Tp0326. Human serum contains an extremely complex mixture of antibodies, produced by the immune system to bind numerous antigens – antigens found on bacteria, viruses, allergens, and in some cases, patients own tissues. The presence of cross-reactivity in only one patient positive for infection with *B. burgdorferi* suggests that further screening of additional serum from patients infected with *B. burgdorferi* should be done to determine if the reactivity is due

to *B. burgdorferi* infection, or some other antigen source. Regardless of the presence of one cross-reactive sample, the low sensitivity seen for diagnosing primary syphilis indicates that Tp0326 would not constitute an effective diagnostic antigen on its own. Many diagnostic tests use multiple antigens in a single tests in order to increase the sensitivity, however, none of the false-negative results seen with Tp0453 were positive with Tp0326, therefore at this time there is no indication that the addition of Tp0326 on its own would contribute to diagnostic tests sensitivity.

Tp0435 was found to be the best diagnostic candidate in this study exhibiting a sensitivity of 98% and a specificity of 100%. None of the studies undertaken to identify immunoreactive antigens in *T. pallidum*, from immunoblotting *T.pallidum* extracts on 2D gels to assaying the entire proteome with serum from patients infected with syphilis, uncovered Tp0453 as a potential diagnostic antigen (25, 119). Studies in our laboratory aiming to identify potential virulence factors within *T. pallidum* identified Tp0453 as a putative outer membrane protein, and subsequent research showed that it is highly immunoreactive. None of the experiments examining the immune system's response to previously described *T. pallidum* proteins have been performed with Tp0453. The proteins TpN17, TpN37 and/or TpN47, stimulate increased infiltration of polymorphonuclear lymphocytes (Sellati *et al.*, 2001), recruitment and activation of dendritic cells (Sellati *et al.*, 2001, Shin *et al.*, 2004), sensitization of T cells, and a cell mediated response through cytokine and chemokine secretion (Sellati *et al.*, 2001, Lien *et al.*, 1999) (Bouis *et al.*, 2001) (Arroll *et al.*, 1999). Considering we have shown Tp0453 to be highly immunoreactive, it is likely that it will elicit similar effects to TpN17,

TpN37 and TpN47 on the host immune system. Further experimentation will be required to test this hypothesis.

High sensitivity of Tp0453 was seen for all stages of syphilis infection. Patients with primary syphilis exhibited a sensitivity of 96%, and patients with secondary and early-latent stage syphilis exhibited 100% sensitivity. It is important to highlight the sensitivity of Tp0453 for diagnosing primary syphilis since the major limitation of current diagnostic tests is low sensitivity in early cases, the stage at which *T. pallidum* is most contagious, and causes the greatest transmission rate from a mother to her fetus in congenital cases. The specificity for Tp0453 was found to be 100%, with no cross-reactivity to the related infections *Borrelia burgdorferi*, *Leptospira*, *Helicobacter pylori*, Cytomegalovirus, Epstein-Barr virus, Hepatitis B virus and Hepatitis C virus. Tp0453 also did not react positively to serum exhibiting false positives with the RPR or TP-PA tests. The reason for this high sensitivity is likely due to the fact that Tp0453 is unique to the genus *Treponema*, and has only 40% shared residues with its closest homologue in *Treponema phagedenis*. The lack of reactivity to all of the negative serum suggests that the sequence of Tp0453 in *T. pallidum* has diverged enough from is homologues in normal human flora (*T. refringens* and *T. denticola*) so as to not exhibit any cross-reactivity. A recent paper by Luthra *et al.* suggested that Tp0453 has structural homology to the proteins LprG and LppX in *M. tuberculosis*, however, these proteins share no sequence homology with Tp0453. The authors suggest that the similarity between these proteins is apparent when visually comparing the crystal structures of these three molecules (Luthra *et al.*, 2011). Although these proteins may be eventually found to serve similar functions, any structural homology is distant at best and it is therefore unlikely

that they share any conformational or linear epitopes that would contribute to cross-reactivity.

The high sensitivity, especially in early stage infections, and specificity, even when tested against potentially cross-reactive sera, indicate that Tp0453 has great potential for further development into commercial serological diagnostic tests.

The creation of a chimeric construct of Tp0326 and Tp0453 was undertaken for two reasons. First, when this project began neither Tp0326 or Tp0453 had been expressed solubly by our lab or in the literature, so we hypothesized that creating a chimera of shorter immunoreactive regions from each protein might produce a soluble construct. Second, because of the strong immunoreactivity exhibited by Tp0326 and Tp0453 in the Van Voohris *et al.* study (Van Voorhis *et al.*, 2003), the creation of a chimera that would contain epitopes from both proteins could potentially increase overall sensitivity. Through the course of this study 12 chimeric constructs were created, and extensive protein expression screenings were performed for each construct in an attempt to produce soluble protein. Only one construct (the Tp0453-Tp0326 chimera) yielded soluble protein when expressed in *E. coli*. This construct was condon harmonized for expression in *E. coli* and consists of an N-terminal histidine tag, residues A32 - S287 from Tp0453, a glycine-serine linker, and residues Q22 - N434 from Tp0326.

It can be hypothesized that the use of a glycine-serine linker in the final construct contributed to the solubility of the Tp0453-Tp0326 chimera. Due to flexibility and the increased length between regions, the addition of a linker has been shown to increase expression efficiency, and proper folding in heterologously expressed proteins (Zhang *et al.*, 2009, Trinh *et al.*, 2004, Freund *et al.*, 1993). Both regions of Tp0326 and Tp0453

used in the chimera were expressed independently in soluble form, so in order to create a soluble chimeric construct of these regions, it was important to maintain their proper folding. Previous research has shown that directly combining regions of independent proteins can lead to insoluble heterologous expression due to unfavourable interactions between the separate domains, which can be resolved through the use of a flexible linker (Zhang *et al.*, 2009). The inclusion of the glycine-serine linker between Tp0326 and Tp0453 therefore may have allowed the two separate domains to fold properly. Optimal folding of the Tp0453-Tp0326 chimera is further supported by the similarity in sensitivities between the Tp0453-Tp0326 chimera and Tp0453, which suggests that the epitopes in Tp0453 are maintained in its respective region of the Tp0453-Tp0326 chimera.

Codon harmonization was another method employed during the creation of the Tp0453-Tp0326 chimera. Early attempts to produce soluble chimeric constructs of Tp0326 and Tp0453 yielded only truncated proteins indicating that there were problems with the completion of translation in the *E. coli* host (Smith, unpublished results). Although these early constructs included slightly different regions of Tp0326 and Tp0453, these results suggested that the creation of new chimeric proteins may also yield truncated proteins. Codon usage bias has been implicated in premature termination of translation, and studies have shown that a single synonymous change can alter substrate specificity, protein structure, and/or protein expression (Kimchi-Sarfaty *et al.*, 2007, Lavner *et al.*, 2005, Adzhubei *et al.*, 1996, Coleman *et al.*, 2008). These studies inspired the decision to make synonymous changes to the chimeric coding sequence in order to reflect the codon frequencies found in the expression strain of *E. coli* (codon

harmonization). Since the purpose of this study was not to examine the benefits of using condon harmonization to express *T. pallidum* proteins, we cannot extrapolate a claim that codon harmonization is responsible for soluble expression of the Tp0453-Tp0326 chimera. Experiments could be designed to assess the usefulness of codon harmonization during heterologous expression of *T. pallidum* proteins in *E. coli* by creating constructs of a single *T. pallidum* gene with varied synonymous substitutions and comparing the amount of soluble protein produced, and examining for the presence of truncated proteins. Considering the evidence presented by other researchers and the results of this study there is evidence to suggest that the use of codon harmonization should be further examined for the expression of heterologous proteins in organisms highly diverged from the proteins original host.

Our hypotheses that the creation of a Tp0453-Tp0326 chimera would identify more positive syphilis infections than Tp0453 or Tp0326 individually has not yet been confirmed in the sample set we have analyzed to date, since the sensitivities of Tp0453 and the Tp0453-Tp0326 chimera are approximately equal. Furthermore, no serum samples that were found to exhibit false-negatives in Tp0453 were found to be positive by the Tp0453-Tp0326 chimera, suggesting that Tp0453 is the major contributor to the immunoreactivity of the Tp0453-Tp0326 chimera. Our results, however, show that both Tp0453 and the Tp0453-Tp0326 chimera have promise for incorporation into commercial diagnostic tests. Further screening with a larger sample set needs to be conducted to determine if the Tp0453-Tp0326 chimera is better than Tp0453 due to its addition of the Tp0326 region.

One of the biggest limitations in this study was the reliance on the RPR, TP-PA, and FTA-ABS tests for defining positive and negative infections. These tests have been shown to exhibit false-positives in numerous conditions including pregnancy and advanced age (Table 3) (Ratnam, 2005). This suggests that some of the false-negative results seen in this study may be true negatives that were incorrectly identified as positive by the RPR and TP-PA. The three samples identified to be false-negative in this screening by Tp0326, Tp0453 and the Tp0453-Tp0326 chimera all exhibited a positive TP-PA, but low titres for the RPR (dilutions ranging from 1:1-1:4). The low RPR titres may indicate that these samples were true negatives, and incorrectly characterized as a positive infection. We were unable to view the patient files for these cases, and therefore could not determine how primary infection was classified (presence of primary chancre, sexual history, etc.), or confirm that these samples were true positives.

In the Van Voorhis *et al.* study, Tp0326 and Tp0453 identified 4 serum samples from patients with early primary syphilis that had been deemed negative by the non-treponemal VDRL test, indicating that antibodies against Tp0326 and Tp0453 may be produced before the anti-cardiolipin antibodies identified by the VDRL (Van Voorhis *et al.*, 2003). For this study we were unable to obtain serum from patients with early primary syphilis that exhibited negative results in the RPR and/or TP-PA tests, so unfortunately we could not examine if our diagnostic antigens were more sensitive than these tests in detecting early stage infections. Further studies need to be done to examine the efficacy of Tp0326, Tp0453, and the Tp0453-Tp0326 chimera in identifying early primary infections, however, the results presented here indicate that both proteins are

highly sensitive and specific, and may be more effective at diagnosing syphilis than current diagnostic tests.

One area which was not examined through the course of this study, was the ability of Tp0453 and the Tp0453-Tp0326 chimera to distinguish between current and previous infections. Current treponemal tests can remain positive, even after effective treatment, for years to the lifetime of a patient (Sena *et al.*, 2010). This is why non-treponemal tests are used to examine if a patient has been re-infected with *T. pallidum*, as well as to monitor the efficacy of treatment. Tp0453 and the Tp0453-Tp0326 chimera need to be tested against serum samples from patients previously infected with syphilis, who have received adequate treatment, in order to determine if these samples remain positive or are negative. Although an inability to distinguish between past and current infections would not negate the anticipated improvement in sensitivity and specificity, Tp0453 and the Tp0453-Tp0326 chimera can differentiate between these two states, we may no longer need to rely on the non-treponemal tests for monitoring the treatment of syphilis.

One aspect that contributes to the cost of production of recombinant proteins for diagnostic test purposes, is the yield of protein per expression trial. Tp0453 has a large yield, giving approximately 18 mg of protein per 6 litres of growth medium. The Tp0453-Tp0326 chimera does not have as large a yield in expression, giving approximately 1 mg of protein per 18 litres of growth medium. Further studies will need to be performed to assess if the current expression of the Tp0453-Tp0326 chimera is adequate for development into large-scale commercial production, or if further optimization needs to be done to increase its yield.

A provisional patent is in place for both Tp0453 and the Tp0453-Tp0326 chimera, and we are currently in discussions with a number of diagnostic companies who are interested in testing these constructs in already developed diagnostic test formats. Due to the soluble nature of Tp0453 and the Tp0453-Tp0326 chimera, we anticipate that they will be incorporated into numerous diagnostic tests including enzyme immunoassays, chemiluminescence assays, and rapid point-of-care diagnostic tests. The majority of these tests currently use other *T. pallidum* proteins in a similar format, so incorporating Tp0453 and the Tp0453-Tp0326 chimera is not likely to be difficult, expensive, or overly time consuming. Subsequent screening of these tests against even larger collections of serum will provide further insight into the sensitivity and specificity of each protein and their usability in different test formats. Finally, if the high sensitivities and specificities of Tp0453 and the Tp0453-Tp0326 chimera continue to be seen when they are included in the newly developed diagnostic tests, we anticipate that these tests will become commercially available and help to improve overall syphilis diagnosis.

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Appendix: Raw data and calculations

Supplemental Table 1: Preliminary ELISA based screening of negative serum samples to determine cut-off values.

Sample	Absorbance values								
	Tp0453-Tp0326 chimera			Tp0453			Tp0326		
	Aug. 23	Aug. 29	Sept. 14	Aug. 29	Sept. 14	Sept. 29	Aug.29	Sept. 14	Sept. 29
QA06	0.132	0.153	0.149	0.097	0.088	0.093	0.114	0.099	0.077
QA07	0.220	0.211	0.249	0.096	0.100	0.125	0.205	0.228	0.255
QA08	0.119	0.174	0.135	0.106	0.089	0.061	0.134	0.101	0.076
QA18	0.083	0.101	0.081	0.077	0.065	0.057	0.086	0.069	0.052
QA19	0.151	0.227	0.179	0.090	0.075	0.076	0.123	0.082	0.080
QA20	0.109	0.128	0.104	0.094	0.067	0.070	0.105	0.082	0.079
QA21	0.128	0.148	0.160	0.098	0.094	0.091	0.108	0.093	0.072
QA22	0.128	0.134	0.134	0.082	0.069	0.066	0.092	0.087	0.067
QA23	0.100	0.104	0.102	0.092	0.073	0.068	0.091	0.081	0.069
QA24	0.134	0.145	0.146	0.106	0.082	0.083	0.106	0.089	0.071
QA25	0.112	0.110	0.101	0.083	0.076	0.061	0.097	0.077	0.068
QA26	0.130	0.120	0.119	0.091	0.063	0.065	0.092	0.070	0.068
QA27	0.087	0.120	0.093	0.099	0.069	0.071	0.092	0.076	0.073
QA28	0.089	0.106	0.116	0.082	0.071	0.056	0.086	0.076	0.058
QA29	0.203	0.161	0.178	0.088	0.077	0.093	0.127	0.115	0.107
QA30	0.119	0.098	0.100	0.089	0.071	0.062	0.102	0.077	0.067
QA31	0.097	0.101	0.079	0.091	0.070	0.077	0.089	0.066	0.088
QA32	0.122	0.127	0.132	0.086	0.069	0.100	0.111	0.116	0.096
QA33	0.132	0.138	0.133	0.101	0.082	0.071	0.107	0.096	0.072
QA34	0.103	0.117	0.122	0.096	0.075	0.075	0.097	0.088	0.078
QA35	0.128	0.119	0.138	0.086	0.082	0.111	0.096	0.098	0.094
QA36	0.092	0.098	0.098	0.079	0.074	0.067	0.078	0.076	0.072
QA37	0.103	0.119	0.115	0.099	0.083	0.073	0.095	0.088	0.096
QA38	0.090	0.109	0.103	0.088	0.077	0.060	0.090	0.069	0.057
Average	**0.121**	**0.132**	**0.128**	**0.091**	**0.077**	**0.076**	**0.105**	**0.092**	**0.083**

Supplemental Table 2: Determination of cut-off values and equivocal ranges.

	Tp0453-Tp0326 chimera	Tp0453	Tp0326
Overall average	0.127	0.081	0.093
Empirical standard error	0.003	0.005	0.006
98% CI Cutoff	0.148	0.116	0.138
Equivocal range	0.133-0.163	0.104-0.127	0.124-0.151

Supplemental Table 3: Absorbance values and results of ELISA based screening of positive serum samples from patients with primary (P), secondary (S), and early latent (EL) syphilis. Samples have also been assessed with the RPR and TP-PA tests, as well as the FTA-Abs when required to confirm a positive infection. Results are recorded as positive (P), negative (N) or equivocal (E).

					Tp0326		Tp0453		Chimera	
Sample	Stage	RPR	TP-PA	FTA-Abs	Abs	Result	Abs	Result	Abs	Result
TP001	P	Reactive 1:4	Reactive...		0.152	P	0.135	P	0.158	E
TP002	P	Reactive 1:32	Reactive...		0.787	P	0.667	P	0.930	P
TP003	P	Reactive 1:32	Reactive...		0.171	P	0.319	P	0.391	P
TP004	S	Reactive 1:64	Reactive...		2.301	P	2.428	P	2.451	P
TP005	P	Reactive 1:1	Reactive.	Reactive	0.203	P	0.206	P	0.158	E
TP006	P	Reactive 1:4	Reactive.	Reactive..	0.142	E	0.201	P	0.227	P
TP007	P	Reactive 1:2	Reactive...		0.128	E	0.191	P	0.223	P
TP008	P	Reactive 1:8	Reactive...		0.119	N	0.189	P	0.202	P
TP010	P	Reactive 1:8	Reactive...		0.251	P	1.674	P	1.718	P
TP011	P	Reactive 1:64	Reactive...		0.285	P	1.111	P	1.321	P
TP012	P	Reactive 1:32	Reactive...		0.093	N	0.562	P	0.589	P
TP013	P	Reactive 1:16	Reactive...		1.878	P	1.478	P	2.015	P
TP015	P	Reactive 1:64	Reactive...		2.373	P	2.538	P	2.545	P
TP017	P	Reactive 1:4	Reactive..		0.500	P	0.608	P	0.810	P
TP018	P	Reactive 1:1	Reactive..		0.079	N	0.087	N	0.094	N
TP019	P	Reactive 1:4	Reactive...		1.638	P	0.926	P	1.698	P
TP020	P	Reactive 1:64	Reactive....		0.779	P	2.680	P	2.620	P
TP021	P	Reactive 1:32	Reactive....		0.116	N	0.553	P	0.405	P
TP022	P	Reactive 1:2	Reactive...		0.189	P	0.220	P	0.295	P
TP024	P	Reactive 1:64	Reactive....		2.510	P	2.529	P	2.552	P
TP025	P	Reactive 1:128	Reactive....		0.867	P	2.093	P	2.183	P
TP026	P	Reactive 1:2	Reactive.		0.130	E	1.582	P	2.015	P
TP027	P	Reactive 1:4	Reactive...		0.689	P	0.316	P	0.767	P
TP028	P	Reactive 1:16	Reactive...		1.200	P	1.310	P	1.524	P
TP030	P	Reactive 1:32	Reactive...		0.082	N	0.170	P	0.222	P
TP032	P	Reactive 1:256	Reactive.		0.940	P	2.027	P	2.179	P
TP033	P	Reactive 1:16	Reactive...		0.771	P	0.525	P	0.905	P
TP034	P	Reactive 1:2	Reactive...		1.154	P	0.703	P	1.309	P
TP035	P	Reactive 1:16	Reactive...		0.119	N	0.182	P	0.331	P
TP037	P	Reactive 1:4	Reactive...		0.703	P	0.909	P	1.069	P
TP038	P	Non-Reactive	Non-Reactive	Reactive.	0.131	E	0.115	E	0.151	E

TP040	P	Reactive 1:16	Reactive...	0.141	E	0.227	P	0.492	P
TP041	P	Reactive 1:16	Reactive.	0.202	P	0.696	P	0.795	P
TP042	P	Reactive 1:8	Reactive...	0.144	E	0.269	P	0.245	P
TP043	P	Reactive 1:64	Reactive...	2.285	P	2.444	P	2.431	P
TP044	P	Reactive 1:8	Reactive...	0.105	N	0.163	P	0.150	E
TP045	P	Reactive 1:8	Reactive...	0.955	P	0.910	P	1.423	P
TP046	P	Reactive 1:8	Reactive..	0.151	E	0.159	P	0.327	P
TP047	P	Reactive 1:128	Reactive.	0.707	P	2.480	P	2.343	P
TP049	P	Reactive 1:4	Reactive...	0.785	P	0.716	P	1.133	P
TP050	P	Reactive 1:2	Reactive..	0.287	P	0.570	P	0.771	P
TP051	EL	Reactive 1:32	Reactive..	0.412	P	1.588	P	1.895	P
TP052	S	Reactive 1:512	Reactive...	1.107	P	2.273	P	2.162	P
TP053	EL	Reactive 1:128	Reactive..	1.055	P	1.086	P	1.576	P
TP054	EL	Reactive 1:32	Reactive....	0.118	N	1.182	P	1.240	P
TP055	EL	Reactive 1:64	Reactive...	1.996	P	2.447	P	2.279	P
TP056	EL	Reactive 1:64	Reactive...	2.330	P	2.248	P	2.401	P
TP057	P	Reactive 1:8	Reactive...	1.544	P	0.671	P	1.734	P
TP058	EL	Reactive 1:64	Reactive....	1.819	P	2.530	P	2.368	P
TP059	P	Reactive 1:1	Reactive..	0.308	P	0.169	P	0.376	P
TP060	EL	Reactive 1:64	Reactive...	2.097	P	2.631	P	2.502	P
TP061	EL	Reactive 1:2	Reactive...	0.130	E	0.157	P	0.190	P
TP062	S	Reactive 1:512	Reactive...	0.397	P	2.254	P	1.960	P
TP063	S	Reactive 1:8	Reactive...	0.981	P	0.454	P	1.025	P
TP064	EL	Reactive 1:256	Reactive...	0.303	P	2.256	P	2.294	P
TP065	P	Reactive 1:64	Reactive...	0.130	E	0.157	P	0.190	P
TP066	P	Reactive 1:16	Reactive...	0.397	P	2.254	P	1.960	P
TP067	EL	Reactive 1:128	Reactive.	0.981	P	0.454	P	1.025	P
TP068	S	Reactive 1:16	Reactive...	0.303	P	2.256	P	2.294	P
TP069	P	Reactive 1:32	Reactive.	0.130	E	0.157	P	0.190	P
TP070	S	Reactive 1:64	Reactive...	1.885	P	2.331	P	2.341	P
TP071	EL	Reactive 1:32	Reactive...	1.459	P	2.070	P	2.148	P
TP072	EL	Reactive 1:512	Reactive.	0.333	P	2.221	P	2.137	P
TP073	S	Reactive 1:64	Reactive...	1.004	P	1.367	P	1.654	P
TP074	EL	Reactive 1:4	Reactive...	0.148	E	0.161	P	0.231	P
TP075	S	Reactive 1:512	Reactive...	1.233	P	2.555	P	2.395	P
TP077	EL	Reactive 1:64	Reactive....	1.199	P	2.331	P	2.308	P

TP078	EL	Reactive 1:8	Reactive...	0.188	P	1.141	P	1.405	P
TP079	P	Reactive 1:8	Reactive...	0.841	P	2.095	P	2.134	P
TP080	S	Reactive 1:128	Reactive....	1.822	P	2.406	P	2.315	P
TP082	EL	Reactive 1:256	Reactive....	0.470	P	2.533	P	2.372	P
TP083	P	Reactive 1:2	Reactive...	0.119	N	0.202	P	0.201	P
TP085	EL	Reactive 1:128	Reactive...	0.571	P	2.261	P	2.299	P
TP087	EL	Reactive 1:8	Reactive...	1.374	P	2.390	P	2.437	P
TP088	EL	Reactive 1:8	Reactive...	0.390	P	1.645	P	1.769	P
TP090	P	Reactive 1:128	Reactive....	2.281	P	1.753	P	2.417	P
TP092	EL	Reactive 1:1024	Reactive....	1.664	P	2.571	P	2.530	P
TP096	EL	Reactive 1:64	Reactive....	0.812	P	2.363	P	2.368	P
TP097	P	Reactive 1:32	Reactive....	0.401	P	0.576	P	0.727	P
TP098	S	Reactive 1:128	Reactive....	1.620	P	1.852	P	2.236	P
TP099	EL	Reactive 1:16	Reactive....	0.822	P	1.149	P	1.452	P
TP100	EL	Reactive 1:16	Reactive....	0.460	P	1.786	P	1.763	P
TP101	EL	Reactive 1:128	Reactive...	0.482	P	1.463	P	1.520	P
TP102	S	Reactive 1:2048	Reactive....	0.146	E	2.422	P	2.245	P
TP106	P	Reactive 1:1	Reactive..	0.088	N	0.255	P	0.223	P
TP107	EL	Reactive 1:64	Reactive....	1.079	P	2.253	P	2.229	P
TP108	P	Reactive 1:8	Reactive...	0.121	N	0.350	P	0.283	P
TP109	S	Reactive 1:64	Reactive....	0.595	P	2.372	P	2.267	P
TP110	P	Reactive 1:1	Reactive....	0.072	N	0.083	N	0.093	N
TP111	EL	Reactive 1:16	Reactive.	0.197	P	0.295	P	0.482	P
TP112	EL	Reactive 1:64	Reactive....	0.678	P	2.306	P	2.107	P
TP113	S	Reactive 1:128	Reactive...	1.686	P	2.593	P	2.404	P
TP114	EL	Reactive 1:4	Reactive.	0.220	P	0.478	P	0.714	P
TP115	S	Reactive 1:256	Reactive.	0.509	P	1.474	P	1.582	P
TP116	S	Reactive 1:1024	Reactive...	0.911	P	2.673	P	2.329	P
TP117	EL	Reactive 1:64	Reactive....	1.338	P	2.282	P	2.148	P
TP119	S	Reactive 1:32	Reactive....	0.700	P	0.716	P	1.121	P
TP120	EL	Reactive 1:16	Reactive..	1.060	P	0.726	P	1.398	P
TP121	EL	Reactive 1:64	Reactive....	2.043	P	2.551	P	2.435	P
TP122	S	Reactive 1:128	Reactive....	0.326	P	1.379	P	1.340	P
TP123	S	Reactive 1:512	Reactive....	1.689	P	2.660	P	2.578	P
TP124	S	Reactive 1:64	Reactive....	1.893	P	2.455	P	2.401	P
TP125	S	Reactive 1:2048	Reactive....	1.268	P	2.618	P	2.526	P

TP126	S	Reactive 1:128	Reactive....	1.116	P	2.276	P	2.380	P
TP127	P	Reactive 1:16	Reactive.	0.436	P	0.481	P	0.913	P
TP129	P	Reactive 1:2	Reactive...	0.112	N	0.230	P	0.181	P
TP130	S	Reactive 1:128	Reactive...	1.382	P	2.449	P	2.241	P
TP131	P	Reactive 1:4	Reactive....	0.105	N	0.109	E	0.143	E
TP132	EL	Reactive 1:16	Reactive....	0.419	P	0.921	P	1.032	P
TP133	EL	Reactive 1:256	Reactive....	1.072	P	2.418	P	2.360	P
TP134	S	Reactive 1:64	Reactive....	0.472	P	1.869	P	1.687	P
TP136	EL	Reactive 1:128	Reactive....	1.468	P	2.251	P	2.270	P
TP137	EL	Reactive 1:2	Reactive...	0.114	N	0.200	P	0.276	P
TP138	EL	Reactive 1:128	Reactive....	0.516	P	2.645	P	2.497	P
TP140	EL	Reactive 1:2048	Reactive.	0.619	P	2.397	P	2.206	P
TP142	EL	Reactive 1:4	Reactive...	0.539	P	1.598	P	1.693	P
TP144	EL	Reactive 1:2	Reactive...	0.478	P	0.644	P	0.931	P
TP145	EL	Reactive 1:512	Reactive.	0.473	P	2.632	P	2.535	P
TP146	EL	Reactive 1:16	Reactive....	0.200	P	1.027	P	1.188	P
TP147	S	Reactive 1:16	Reactive...	1.970	P	2.027	P	2.289	P
TP149	S	Reactive 1:256	Reactive...	0.555	P	1.681	P	1.896	P
TP150	EL	Reactive 1:2	Reactive...	0.187	P	0.707	P	0.596	P
TP151	S	Reactive 1:256	Reactive...	1.165	P	2.509	P	2.353	P
TP152	S	Reactive 1:32	Reactive...	1.396	P	2.167	P	2.120	P
TP153	S	Reactive 1:64	Reactive...	1.953	P	2.361	P	2.380	P
TP154	P	Reactive 1:8	Reactive...	0.860	P	1.314	P	1.477	P
TP155	S	Reactive 1:128	Reactive....	2.117	P	2.585	P	2.384	P
TP156	P	Reactive 1:32	Reactive....	1.217	P	1.969	P	2.005	P
TP157	P	Reactive 1:32	Reactive...	0.292	P	1.656	P	1.671	P
TP158	EL	Reactive 1:16	Reactive..	0.546	P	0.648	P	0.928	P
TP159	S	Reactive 1:16	Reactive...	0.279	P	1.137	P	1.006	P
TP160	S	Reactive 1:512	Reactive....	2.297	P	2.650	P	2.560	P
TP161	EL	Reactive 1:16	Reactive...	0.115	N	0.336	P	0.444	P
TP162	EL	Reactive 1:64	Reactive...	0.516	P	2.311	P	2.179	P
TP163	S	Reactive 1:128	Reactive...	2.024	P	2.632	P	2.525	P
TP164	P	Reactive 1:8	Reactive...	0.905	P	1.359	P	1.477	P
TP165	S	Reactive 1:64	Reactive....	1.767	P	2.420	P	2.183	P
TP166	EL	Reactive 1:256	Reactive....	0.960	P	2.548	P	2.314	P
TP167	EL	Reactive 1:32	Reactive.	0.166	P	0.932	P	0.996	P

TP168	EL	Reactive 1:32	Reactive....	0.276	P	1.691	P	1.854	P
TP169	EL	Reactive 1:4	Reactive....	0.450	P	1.703	P	1.679	P
TP170	EL	Reactive 1:128	Reactive.	0.133	E	0.441	P	0.572	P
TP171	S	Reactive 1:512	Reactive....	2.297	P	2.497	P	2.369	P
TP172	S	Reactive 1:32	Reactive...	0.770	P	1.945	P	1.543	P
TP173	P	Reactive 1:8	Reactive.	0.092	N	0.165	P	0.143	E
TP174	EL	Reactive 1:16	Reactive....	1.103	P	1.736	P	1.526	P
TP177	P	Reactive 1:32	Reactive.	0.147	E	0.688	P	0.851	P
TP178	P	Reactive 1:16	Reactive...	0.836	P	1.818	P	1.351	P
TP179	S	Reactive 1:128	Reactive...	2.137	P	2.544	P	2.394	P
TP180	EL	Reactive 1:16	Reactive....	0.382	P	0.509	P	0.573	P
TP181	S	Reactive 1:32	Reactive...	1.424	P	2.481	P	2.069	P
TP182	S	Reactive 1:256	Reactive...	2.261	P	2.645	P	2.413	P
TP183	S	Reactive 1:256	Reactive...	2.215	P	2.587	P	2.294	P
TP184	S	Reactive 1:128	Reactive...	2.212	P	2.497	P	2.124	P
TP185	S	Reactive 1:128	Reactive....	1.084	P	2.611	P	2.214	P
TP186	S	Reactive 1:256	Reactive...	0.576	P	1.167	P	1.158	P
TP187	P	Reactive 1:4	Reactive...	0.069	N	0.065	N	0.091	N
TP189	S	Reactive 1:256	Reactive...	1.802	P	2.575	P	2.299	P
TP191	P	Reactive 1:64	Reactive....	1.006	P	1.143	P	1.339	P
TP192	S	Reactive 1:1024	Reactive....	0.116	N	2.611	P	2.235	P
TP193	S	Reactive 1:128	Reactive....	1.972	P	2.461	P	2.186	P
TP194	P	Reactive 1:2	Reactive....	0.101	N	0.200	P	0.172	P
TP195	P	Reactive 1:64	Reactive....	0.161	P	0.146	P	0.739	P
TP196	P	Reactive 1:1	Reactive..	0.098	N	0.158	P	0.159	E
TP197	P	Reactive 1:128	Reactive...	0.401	P	2.018	P	2.052	P
TP198	S	Reactive 1:128	Reactive..	1.602	P	2.126	P	2.017	P
TP199	S	Reactive 1:256	Reactive...	1.521	P	2.288	P	1.935	P
TP200	P	Reactive 1:1	Reactive...	0.127	E	0.159	P	0.158	E
TP202	P	Reactive 1:1	Reactive...	0.086	N	0.117	E	0.098	N

Supplemental Table 4: Absorbance values and results of analytical false positive serum sample screening. Samples have also been assessed with the RPR, TP-PA, and FTA-Abs tests. Results are recorded as positive (P), negative (N) or equivocal (E).

				Tp0326		Tp0453		Tp0453-Tp0326 Chimera	
Sample	**RPR**	**TP-PA**	**FTA-Abs**	**Abs**	**Result**	**Abs**	**Result**	**Abs**	**Result**
TP036	Non-Reactive	Reactive.	Non-reactive	0.112	N	0.111	E	0.121	N
TP039	Non-Reactive	Non-Reactive	Non-reactive	0.081	N	0.076	N	0.091	N
TP148	Non-Reactive	Reactive..	Non-reactive	0.068	N	0.069	N	0.081	N
TP176	Non-Reactive	Reactive..	Non-reactive	0.083	N	0.078	N	0.085	N
TP201	Non-Reactive	Reactive.	Non-reactive	0.080	N	0.067	N	0.089	N
TP203	Non-Reactive	Non-Reactive	Non-reactive	0.069	N	0.061	N	0.083	N
TP143	Reactive 1:16	Reactive.	Non-reactive	0.081	N	0.103	N	0.110	N

Supplemental Table 5: Absorbance values and results of ELISA based screening of biological false positive serum samples. Samples have also been assessed with the RPR, TP-PA, and FTA-Abs tests. Results are recorded as positive (P), negative (N) or equivocal (E).

				Tp0326		Tp0453		Tp0453-Tp0326 Chimera	
Sample	**RPR**	**TP-PA**	**FTA-Abs**	**Abs**	**Result**	**Abs**	**Result**	**Abs**	**Result**
TP205	Reactive 1:1	Non-Reactive	Non-Reactive	0.091	N	0.079	N	0.097	N
TP206	Reactive 1:1w	Non-Reactive	Non-Reactive	0.069	N	0.064	N	0.085	N
TP207	Reactive 1:4	Non-Reactive	Non-Reactive	0.058	N	0.063	N	0.064	N
TP208	Reactive 1:2	Non-Reactive	Non-Reactive	0.068	N	0.071	N	0.071	N
TP209	Reactive 1:1	Non-Reactive	Non-Reactive	0.070	N	0.068	N	0.070	N
TP210	Reactive 1:1	Non-Reactive	Non-Reactive	0.056	N	0.059	N	0.071	N
TP211	Reactive 1:32	Non-Reactive	Non-Reactive	0.071	N	0.074	N	0.103	N
TP212	Reactive 1:16	Non-Reactive	Non-Reactive	0.064	N	0.066	N	0.074	N
TP213	Reactive 1:1	Non-Reactive	Non-Reactive	0.076	N	0.087	N	0.086	N
TP214	Reactive 1:2	Non-Reactive	Non-Reactive	0.065	N	0.061	N	0.065	N
TP215	Reactive 1:8	Non-Reactive	Non-Reactive	0.077	N	0.060	N	0.074	N
TP216	Reactive 1:8	Non-Reactive	Non-Reactive	0.079	N	0.072	N	0.079	N
TP009	Reactive 1:2	Non-Reactive	Non-Reactive	0.078	N	0.069	N	0.088	N
TP128	Reactive 1:1	Non-Reactive	Non-Reactive	0.106	N	0.075	N	0.110	N

Supplemental Table 6: Absorbance values and results of ELISA based screening of negative serum samples. Samples have also been assessed with the RPR, TP-PA, and FTA-Abs tests. Results are recorded as positive (P), negative (N) or equivocal (E).

				Tp0326		Tp0453		Tp0453-Tp0326 Chimera	
Sample	**RPR**	**TPPA**	**FTA-Abs**	**Abs**	**Result**	**Abs**	**Result**	**Abs**	**Result**
TP217	Non-Reactive	Non-Reactive	Non-Reactive	0.111	N	0.066	N	0.111	N
TP218	Non-Reactive	Non-Reactive	Non-Reactive	0.092	N	0.074	N	0.104	N
TP219	Non-Reactive	Non-Reactive	Non-Reactive	0.064	N	0.063	N	0.080	N
TP220	Non-Reactive	Non-Reactive	Non-Reactive	0.062	N	0.059	N	0.058	N
TP222	Non-Reactive	Non-Reactive	Non-Reactive	0.086	N	0.073	N	0.076	N
TP224	Non-Reactive	Non-Reactive	Non-Reactive	0.065	N	0.064	N	0.069	N
TP225	Non-Reactive	Non-Reactive	Non-Reactive	0.065	N	0.058	N	0.070	N
TP226	Non-Reactive	Non-Reactive	Non-Reactive	0.074	N	0.065	N	0.095	N
TP227	Non-Reactive	Non-Reactive	Non-Reactive	0.069	N	0.065	N	0.071	N
TP228	Non-Reactive	Non-Reactive	Non-Reactive	0.076	N	0.062	N	0.087	N
TP229	Non-Reactive	Non-Reactive	Non-Reactive	0.068	N	0.066	N	0.075	N

Supplemental Table 7: Absorbance values and results of ELISA based screening of *Borrelia burgdorferi* (BBWB), *Leptospira*, *Helicobacter pylori* (HPG), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Hepatitis B virus (HBs) and Hepatitis C virus (HCV).

		Tp0326		Tp0453		Tp0453-Tp0326 Chimera	
Sample	Organism/diagnostic test used to confirm infection	Abs	Result	Abs	Result	Abs	Result
TP231	BBWB IgG Reactive	0.083	N	0.095	N	0.094	N
TP230	BBWB IgG Reactive	0.198	P	0.072	N	0.091	N
TP232	BBWB IgG Reactive	0.120	N	0.083	N	0.086	N
TP233	BBWB IgG Reactive	0.145	E	0.080	N	0.092	N
TP234	*Leptospira* IgM Reactive	0.075	N	0.069	N	0.074	N
TP235	*Leptospira* IgM Reactive	0.098	N	0.098	N	0.104	N
TP236	*Leptospira* IgM Reactive	0.091	N	0.081	N	0.085	N
TP237	*Leptospira* IgM Reactive	0.087	N	0.073	N	0.099	N
TP238	HPG Reactive	0.080	N	0.072	N	0.087	N
TP239	HPG Reactive	0.092	N	0.082	N	0.095	N
TP240	HPG Reactive	0.091	N	0.097	N	0.100	N
TP241	HPG Reactive	0.076	N	0.072	N	0.080	N
TP242	HPG Reactive	0.077	N	0.073	N	0.094	N
TP243	CMV IgG/IgM Reactive	0.134	E	0.097	N	0.122	N
TP244	CMV IgG/IgM Reactive	0.078	N	0.078	N	0.103	N
TP245	CMV IgG/IgM Reactive	0.078	N	0.074	N	0.084	N
TP246	CMV IgG/IgM Reactive	0.088	N	0.082	N	0.095	N
TP247	CMV IgG/IgM Reactive	0.088	N	0.077	N	0.109	N
TP248	EBV IgG/IgM Reactive	0.080	N	0.073	N	0.125	N
TP249	EBV IgG/IgM Reactive	0.084	N	0.075	N	0.449	P
TP250	EBV IgG/IgM Reactive	0.077	N	0.071	N	0.150	E
TP251	EBV IgG/IgM Reactive	0.080	N	0.074	N	0.088	N
TP252	EBV IgG/IgM Reactive	0.092	N	0.088	N	0.123	N
TP253	HBs IgG Reactive	0.054	N	0.052	N	0.056	N
TP254	HBs IgG Reactive	0.073	N	0.068	N	0.084	N
TP255	HBs IgG Reactive	0.063	N	0.059	N	0.067	N
TP256	HBs IgG Reactive	0.090	N	0.057	N	0.090	N
TP257	HBs IgG Reactive	0.056	N	0.055	N	0.058	N
TP258	HBc Reactive	0.063	N	0.059	N	0.070	N
TP259	HBc Reactive	0.067	N	0.071	N	0.084	N
TP260	HBc Reactive	0.085	N	0.088	N	0.090	N
TP261	HBc Reactive	0.067	N	0.069	N	0.072	N
TP262	HBc Reactive	0.072	N	0.074	N	0.089	N
TP263	HCV IgG Reactive	0.078	N	0.064	N	0.072	N
TP264	HCV IgG Reactive	0.062	N	0.061	N	0.104	N
TP265	HCV IgG Reactive	0.078	N	0.068	N	0.102	N
TP266	HCV IgG Reactive	0.056	N	0.056	N	0.072	N
TP267	HCV IgG Reactive	0.075	N	0.071	N	0.068	N