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EVALUATION AND DETERMINATION OF THE SENSITIVITY AND SPECIFICITY OF A *Treponema pallidum* DRIED BLOOD SPOT METHOD FOR SEROLOGIC DIAGNOSIS OF SYPHILIS

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ABSTRACT

EVALUATION AND DETERMINATION OF THE SENSITIVITY AND SPECIFICITY OF A *Treponema pallidum* DRIED BLOOD SPOT (DBS) METHOD FOR SEROLOGIC DIAGNOSIS OF SYPHILIS

Background: Syphilis is a sexually transmitted infection (STI) caused by *Treponema pallidum subspecies pallidum*. Syphilis is known as the “great imitator" due to the similarity of clinical signs and symptoms to other infectious diseases. The primary diagnosis of syphilis relies on clinical findings, including the examination of treponemal lesions, and/or serologic tests. Serologic tests are divided into nontreponemal and treponemal tests. Nontreponemal tests are useful for screening, while treponemal tests are used as confirmatory tests.

Methods: A total of 200 serum and DBS specimens collected from patients at the Los Angeles Municipal Sexually Transmitted Disease Clinics were tested by the DBS and enzyme immunoassay (EIA) methods. These samples were sent to the Syphilis Diagnostics Laboratory, Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia for testing. Samples were blindly evaluated by the TREP-SPOT^{TM} DBS and the TREP-SURE^{TM} EIA methods for the detection of anti-treponemal IgG- and IgM-class antibodies.

Results: The sensitivity of the DBS method was 83% (95% CI, 73.89 - 89.50) and specificity was 100% (95% CI, 95.39 - 100)). The positive predictive value and negative predictive values were 100% (95% CI, 94.48 - 100) and 85% (95% CI, 77.43 - 91.0), respectively. The efficiency of the DBS method was 91.5%. The kappa value for the agreement between the DBS method and EIA assay was 0.83 (95% CI, 0.754 - 0.906). The correlation coefficient (r^2) between the anti-treponemal antibody assay results obtained from DBS and serum samples was 0.94.

Conclusion: DBS is an optimal choice to be used as a screening tool for the detection of anti-treponemal antibodies for the diagnosis of syphilis. The detection of anti-treponemal antibodies (TREP-SPOT^{TM} DBS EIA) compared favorably to the results of serum-base assay (TREP-SURE^{TM} EIA), with an overall concordance of 91.5%. Dried blood spots are technically easier to obtain and are suitable blood samples for primary health care centers.

Evaluation and Determination of the Sensitivity and Specificity of a *Treponema pallidum* Dried Blood Spot Method for Serologic Diagnosis of Syphilis

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A Thesis Submitted to the Graduate Faculty
of Georgia State University in Partial Fulfillment
of the Requirements for the Degree

MASTER OF PUBLIC HEALTH

ATLANTA, GEORGIA 30303

Evaluation and Determinations of the Sensitivity and Specificity of a *Treponema pallidum* Dried Blood Spot Method for Serologic Diagnosis of Syphilis

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Acknowledgements

I would like to thank my committee members, Dr. Christine Stauber and Dr. Lisa Casanova for their continued support and guidance throughout this project. In addition, I would like to acknowledge the support and assistance from Ms. Heather Jost, Dr. David Cox and Dr. Bharat Parekh at the Centers for Disease Control and Prevention. Finally, I would like to thank my family for all their continued support, encouragement, understanding, and motivation.

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- Turgeon, D. K. and T. R. Fritsche. Laboratory Approaches to Infectious Diarrhea. 2001. *Gastroenterol. Clin. North Am*. Sep;30(3): 693-707.
- Qin, Xuan, Turgeon, D**.,** Ingersoll, B., Monsaas, P., Lemonine, C., Tsosie, T., Stapp, L. and Abe, P. 2002. *Bordetella pertussis* PCR: Simultaneous Targeting of Signature Sequences. *Diagnostic Microbiol and Infec. Dis*. 43: 269-275, 2002.
- Turgeon, David K., Novicki, Thomas J., Quick, John, Carson, LaDonna, Miller, Pat, Ulness, Bruce, Cent, Anne, Ashley, Rhoda, Larson, Ann, Coyle, Marie, Limaye, Ajit, Cookson, Brad T. and Fritsche, Thomas R. 2003**.** Six Rapid Tests for Direct Detection of *Clostridium difficile* and its Toxins in Fecal Samples Compared with the Fibroblast Cytotoxicity Assay. *J of Clin Microbiol*. 41(2): 667-670.
- Lee, S, Turgeon, D.K., Ko, C. W., Fritsche, T. R. and Surawicz, C. M. 2003. Clinical Correlation of Toxin and Common Antigen Enzyme Immunoassay Testing in Patients with *Clostridium difficile* Disease. *Amer. J of Gastroenterol*. 98:1569-1572.
- **Additional publications on request**

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CHAPTER 1

INTRODUCTION

1.1 Background

Syphilis is a sexually transmitted infection (STI) caused by *Treponema pallidum* subspecies *pallidum*. Syphilis is known as the "great imitator" due to its similarity of clinical signs and symptoms to other infectious diseases. The World Health Organization (WHO) reports that more than 340 million new cases of STI occur worldwide (WHO, 2012b) . Globally, STIs are a major public health burden in developing countries. STIs account for one-third of the global burden of disease among women of reproductive age (WHO, 2012b). According to Centers for Disease Control and Prevention (CDC), there were over 36,000 documented cases of syphilis in 2006, including 9,756 cases of primary and secondary syphilis (CDC, 2010). The incidence of primary and secondary syphilis is highest in women 20 to 24 years of age and in men 35 to 39 years of age (CDC, 2012a). The prevalence of congenital syphilis in newborns also increased from 2005 to 2006, with 339 new cases reported in 2005, compared to 349 cases in 2006 (CDC, 2012a). The cases of syphilis among men who have sex with men (MSM) showed the largest increases since 2000 (CDC 2012a). MSM accounted for almost 62% of syphilis cases in 2009, up from 7% in 2000. CDC reported that the primary and secondary syphilis cases increased from 13,500 in 2008 to 13,997 in 2009, an increase of 3.7% (CDC 2012a; CDC, 2012b). The rate of the primary and secondary syphilis was highest among persons aged 20 to 24 years and 25 to 29 years in 2009 (CDC, 2012a).

The primary diagnosis of syphilis relies on clinical findings, including the examination of treponemal lesions, and/or serologic tests. Serologic tests are classified into two categories: nontreponemal and treponemal assays. Nontreponemal tests are useful for screening, while treponemal tests are used as confirmatory tests. Nontreponemal tests detect the host's antibody response to nontreponemal (cardiolipin-lecithin) antigen**.** Nontreponemal tests include the Venereal Disease Research Laboratory (VDRL), the rapid plasma reagin (RPR), the toludine red unheated serum (TRUS), and unheated serum reagin (USR) tests (Owusu-Eduse, 2011). In contrast, all treponemal tests allow for the detection of specific treponemal antibodies. Serologic treponemal tests include the microhemagglutination assay for *T. pallidum* (MHA-TP), *T. pallidum* particle agglutination (TPPA), *T. pallidum* hemagglutination assay (TPHA), fluorescent treponemal antibody absorption (FTA-ABS) test, and enzyme-linked immunosorbent assay (ELISA) (Wong, 2011). More recently, rapid and reliable nucleic acid amplification methods are available for the detection of *T. pallidum* in clinical samples (Heymans, 2010). These methods added great value for the diagnosis of congenital syphilis, neurosyphilis and early primary syphilis (Heymans, 2010).

Serum is the specimen of choice for both nontreponemal and treponemal serologic tests even though plasma can be used in some nontreponemal tests. Dried blood spots (DBS) have been used for rapid diagnosis of human immunodeficiency virus-1(Rottinghaus, 2012) and newborn screening for phenylketonuria (De Silva, 2010). Sample collection of DBS has never been routinely used for serologic confirmation of syphilis.

1.2 Purpose of Study

The aim of this study is to evaluate and determine the sensitivity and specificity of a *T. pallidum* DBS method for serologic diagnosis of syphilis. For the purpose of this study, TREP-SURE™ EIA (Phoenix Bio-Tech Corporation, 2010) was used for the *in vitro* diagnostic detection and confirmation of antitreponemal antibodies using a *T. pallidum* TREP-SPOT™ DBS method (Phoenix Airmid Biomedical Corporation, 2012).

1.3 Research Questions

This thesis will focus on the following questions: 1) Does a *T. pallidum* DBS method provide the sensitivity and specificity for the diagnosis and confirmation of syphilis? 2) Can a *T. pallidum* DBS method be used for the diagnosis and confirmation of syphilis? 3) Does a *T. pallidum* DBS method offer a convenient way to collect and transport blood samples for the diagnosis and confirmation of syphilis?

CHAPTER II

REVIEW OF THE LITERATURE

The purpose of this study is to evaluate and determine the sensitivity and specificity of a *T. pallidum* DBS method for serologic diagnosis of syphilis. This literature review will focus on the following major aspects: the global burden of STIs and syphilis, etiology, epidemiology, pathogenesis, clinical signs and symptoms, laboratory diagnosis of syphilis, and prevention and control.

2.1 Global burden of sexually transmitted infections

STIs are one of the leading causes of acute illness, long-term disability and death. Globally, STIs still remain a major public health threat in both developed and developing countries. According to the World Health Organization (WHO), over 340 million new cases of STIs reported worldwide in 1999 (WHO, 2012a). The highest incidence of STIs per 1000 population has occurred in sub-Saharan Africa (WHO, 2012a).

An estimated 12.22 million cases of syphilis occurred worldwide in 1999 according to WHO (WHO, 2012b). In contrast to Western Europe, the newly independent states of the former Soviet Union reported an alarming increase in the rates in syphilis (WHO, 2001). Syphilis incidence increased from 5 to 15 per 100,000 observed in 1990 to as high as 120 to 170 per 100,000 population in 1996 in these countries (WHO, 2001). The highest prevalence rate in pregnant women was 3.2% in Djibouti, followed by 3.0% in Morocco, and 1.1% in Qatar (WHO, 2001). CDC estimated that the number of cases

of primary and secondary syphilis in U.S. increased from 5,979 to 7,980; the rate increased from 2.1 to 2.7 cases per 100,000 population from 2000 to 2004 (MMWR, 2006).

STIs are caused by bacterial, parasitic, or viral pathogens. Some of the more common pathogens are *Chlamydia trachomatis, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Trichomonas vaginalis,* herpes simplex virus 2, human papillomavirus, hepatitis B virus, and human immunodeficiency virus (Ratnam, 2005).

2.2 Etiology and transmission of disease

Schaudinn and Hoffmann first identified *T. pallidum* as the causative agent for syphilis in 1905 (de Souza, 2005). More importantly, Schaudinn named this organism by using the Greek word *trepo* (I turn) and *nema* (thread) with the Latin word *pallida* (pale). *Treponema pallidum* means “a pale turning thread” (de Souza, 2005). The causative agent of syphilis is *Treponema pallidum* subspecies *pallidum*. *T. pallidum* is a member of the order *Spirochaetales*, family *Spirochaetaceae,* and genus *Treponemae*. The other three species of the genus *Treponema* cause diseases in humans. These include *T. pallidum* subspecies *pertenue,* causing yaws; *T. pallidum* subspecies e*ndemicum,* causing nonvenereal treponematosis (bejel); and *T. carateum*, causing pinta. Morphologically, *T. pallidum* is a small, unicellular, tightly coiled and helical organism measuring 6 - 15 µm in length and 14 - 0.2 µm in width (CDC, 2009a, 2009b). *T. pallidum* has not been grown successfully on artificial culture media. However, the organism has been cultivated in rabbit testicles (Brown, 1920).

Syphilis is transmitted primarily through sexual contact. The disease may be transmitted from mother to fetus during pregnancy or at birth resulting in congenital syphilis. Syphilis can occur through blood transfusion from the donor in early stages of infection (Gupta, 2012). Health care providers may acquire the primary lesions on the hands after examining unprotected clinical lesions of syphilis.

T. pallidum only infects humans. Humans are the only known natural reservoir for syphilis. The organism does not have a reservoir in animals or the environment (LaFond, 2006). The incubation period for syphilis is 10 days to 3 months. The average incubation period of primary syphilis is usually about 3 weeks. However, symptoms can appear as early as 9 days or as long as 3 months after exposure in rectal infections associated with homosexual men (LaFond, 2006).

2.3 Epidemiology of syphilis

STIs are the leading cause of morbidity and mortality among men, women and children (CDC, 2009). STIs still remain a major public health problem worldwide, especially in developing countries. Globally, about 340 million preventable new cases occur every year (WHO, 2001). Syphilis accounts for an estimated 12 million cases; 2 million of them are pregnant women (WHO, 2001). The 2000 Global Burden of Disease Estimates reported that 1.3% of deaths among children under the age of 5 years were due to congenital syphilis (WHO, 2001). The annual global number of cases of congenital syphilis is estimated to be between 575,000 and 713,600 (Hossain, 2007). The newborn

infants have a higher incidence of congenital syphilis than other infections caused by human immunodeficiency virus-1 and *Clostridium botulism* (Rodríguez-Cerdeira, 2012).

The total number of primary, secondary, early latent, late, late latent and congenital syphilis cases was 44,828 in 2009 in the United States according to CDC (CDC, 2010). During 2008 to 2009, primary and secondary syphilis rates increased 10% in the South, 2.7% in the Northeast, and 7.7% in the Midwest (CDC, 2010). In 2009, syphilis rates were highest in the South, 6.6 cases per 100,000 population (CDC, 2010).

According to CDC in 2009, Louisiana, Georgia, Arkansas, Alabama, Mississippi, Texas, Tennessee, North Carolina, New York, Illinois, Florida, Maryland, and California reported the highest rates of primary and secondary syphilis (CDC, 2010). These 13 states accounted for 75% of all U.S. cases of primary and secondary syphilis and exceed the national rate of 4.6 cases per 100,000 population (CDC, 2010). Of the 13 states with the highest rates of primary and secondary syphilis, 10 states were in the South (CDC, 2010).

During 2008 to 2009, the number of primary and secondary syphilis infections in men increased from 7.5 to 7.8 cases per 100,000 men, and decreased from 1.5 to 1.4 cases per 100,000 women during the same period (CDC 2009). In 2009, the overall rate of reported primary and secondary syphilis was highest in people aged 20 to 24 years and 25 to 29 years, 11.3 cases per 100,00 population, respectively (CDC, 2010). From 2008 to 2009, the reported incidence of primary and secondary syphilis increased in all ethnic groups except non-Hispanic whites and Hispanics. The rate of primary and secondary syphilis in non-Hispanics increased by 11.6% (from 17.2 cases per 100,000 population),

6.7% in Asian/Pacific Islanders (from 1.5 to 1.6 cases per 100,000 population), and 4.3% in American Indians/Alaska Natives (from 2.3% to 2.4 cases per 100,000) (CDC, 2009a). From 2008 to 2009, the incidence of primary and secondary syphilis decreased in non-Hispanic Whites and Hispanics, with a 4.5% decrease in non-Hispanic whites (from 2.2 to 2.1 per 100,000 population) and 2.2% in Hispanics (from 4.6 to 4.5 cases per 100,000 population) (CDC, 2009a).

2.4 Pathogenesis, clinical signs and symptoms

Syphilis remains a significant public health challenge. Mechanisms of *T. pallidum* pathogenesis are poorly understood for three reasons**:** 1) the inability to cultivate the causative agent *in vitro*; 2) the lack of suitable animal models; 3) the limited availability of information from human studies. Because of the paucity of human studies, the postulated pathogenesis of syphilis is primarily derived from animal models. Some early animal studies offered important insight into syphilis pathogenesis (Cameron, 1998). *T. pallidum* enters the body via skin and mucous membranes through small abrasions during sexual contact. Magnuson et al. (Magnuson, 1948) demonstrated a darkfield positive lesion when two organisms of *T. pallidum* were intracutaneously inoculated in rabbits. After initial entry, symptoms appear, within a few hours after inoculation, as *T. pallidum* navigates the lymphatic system and regional lymph nodes before traveling throughout the body via the blood stream (Singh, 1999). In some cases, the organism may remain at the site of entry, multiplying and sensitizing lymphocytes, while activating macrophages, resulting in the chancre at the site of transmission. Although the exact mechanism by

which *T. pallidum* enters the host cell has not been completely elucidated, it is most likely *T. pallidum* attaches to host cells by specific attachment ligands. It appears that penetration and damage of endothelial cells is critical for the pathogenicity and virulence of *T. pallidum* required (Riviere, 1989). Riviere et al. (Riviere, 1989) studied the invasive potential of *T. pallidum in vitro* and demonstrated the ability of the organism to penetrate the endothelial cell monolayer.

The route of transmission of syphilis is almost always through sexual contact however, it can also be acquired congenitally (transmitted transplacentally from an infected mother to her fetus). It can also be transmitted through blood transfusion.

Primary syphilis is usually characterized by one or more painless chancres of the skin or mucous membranes at the site of inoculation (Kent, 2008). These lesions are commonly seen on the genitalia. The changes appear within 3 to 4 weeks after exposure. These lesions heal spontaneously after 4 to 6 weeks. Induration is the most common sign and occurs in 47% to 92% of cases (Kent, 2008). Additionally, these lesions have numerous treponemal organisms and exudates from lesions are highly infectious.

There is no clear distinction between primary and secondary syphilis. However, secondary syphilis begins about 1 to 2 months after the appearance of the primary chancre (Kent, 2008). The secondary stage is characterized by rash, mucocutaneous lesions, and lymphadenopathy (Kent, 2008). A rash can develop over the body and commonly includes the palms of the hands and the soles of the feet (Euerle, 2011). The skin rash usually heals in 2 to 12 weeks on its own without scarring. Hypertrophic papular lesions can occur on the vulva or anus and can easily be confused with human

papilomavirus infection (Euerle, 2011). The other symptoms of secondary syphilis manifest as generalized lymphadenopathy, malaise, sore throat, headache, splenomegaly, and arthralgia (Euerle, 2011). This stage also heals spontaneously even without treatment in 3 to 12 weeks. Secondary syphilis can last from 2 to 6 weeks. The organisms continue to disseminate throughout the body via lymphatic and hematogenous spread.

Latent syphilis is referred to as the period after infection when *T. pallidum* continues in the body of the infected person without clinical manifestations of disease. Latent syphilis follows the resolution of secondary syphilis. Latent syphilis is subdivided into early, late, and unknown categories based on the duration of infection. Latent syphilis is classified as "early latent" if the infection is known to be less than one year in duration, "late latent" if the infection is known to be greater than one year in duration, or "latent syphilis of unknown duration" if the duration of infection is unknown (Euerle, 2011). Common signs and symptoms of latent syphilis include difficulty coordinating muscle movements, numbness, paralysis, dementia, and gradual blindness (Euerle, 2011).

Tertiary syphilis occurs 15 to 30 years after the initial infection**.** This stage is marked by long-term complication of the disease. Three major forms of tertiary syphilis include late benign (gummatous), cardiovascular syphilis, and neurosyphilis (Singh, 1999). The tertiary stage involves only a gumma formation and cardiovascular involvement without clinical manifestations of neurosyphilis. Late benign syphilis typically causes aortitis or gummatous changes of the skin, bone, or viscera. Cardiovascular syphilis occurs between 10 and 30 years after the initial infection. The most common manifestaton of cardiovascular syphilis is aortitis, typically involves the

ascending aorta (Karnath, 2009). Syphilitic aneurysms are the most common complication of syphilitic aortitis and complicated by aortic insufficiency and regurgitation (Karnath, 2009). Neurosyphilis is an infection of the central nervous system invaded by *T. pallidum*. Neurosyphilis can occur at almost any stage of syphilis. Early neurosyphilis presents with the involvement of meninges and menigovascular system after weeks to years of primary infection. Brain and spinal cord involvement usually indicates late syphilis and occurs years to decades after initial infection (Singh, 1999). As an untreated complication of progresses, it invades the spinal cord, impairs muscle function, and causes nerve damage. This condition is referred to as *Tabes dorsalis*. *Tabes dosalis* causes a slow degeneration of the covering of nerve cells and nerve fibers that relay sensory information to the brain (Singh, 1999). Without medical intervention, it can lead to mild to severe dementia, paralysis, and blindness. However, even with treatment, damage to nerve cells and fibers in the spinal cord cannot be reversed. The most common signs in the tertiary stage include fever, painful and, non-healing skin ulcers, and bone pain (Euerle, 2011; Karnath, 2009; Kent, 2008)

Congenital syphilis is a multisystem infection caused by *T. pallidum* and transmitted placentally to fetus. Transmission to the fetus in pregnancy can occur during any stage of syphilis. Untreated syphilis during pregnancy is associated with stillbirth, neonatal death, bone deformities, and neurologic complications (WHO, 2011). Early signs and symptoms of congenital syphilis are highly variable. Most clinical symptoms are not seen at birth but show within the 3 months of infant life. Clinical signs and symptoms of congenital syphilis in infants less than 2 years of age may manifest signs of

hepatosplenomegaly, rash, codyloma lata, jaundice, hemolytic anemia, hrombocytopenia, osteochondritis, and pseudoparalysis (Arnold, 2000; Rodriguez-Cerdeira, 2012). Late congenital syphilis in older children typically manifests after two years of life and causes gummatous ulcers, periosteal lesions, paresis, tabes, optic atrophy, interstitial keratitis, sensorineural deafness, and dental deformities (Rodriguez-Cerdeira, 2012).

Yaws is a highly contagious treponematosis. This disease is caused by *T. pallidum* subspecies pertenue. Yaws is not spread by sexual contact. Direct skin-to-skin contact is the main route for transmission. The disease is similar to syphilis and can persist for many years as a chronic, relapsing infection. Yaws is more common in children with a peak incidence in those 6 to 10 years of age. Yaws is endemic in the tropical and sub-tropical areas with hot temperatures, high humidity, and heavy rainfall. The prevalence of the disease is higher in areas with poor sanitation**,** overcrowding, and lack of public health surveillance system (Farnsworth, 2006). The primary stage of the disease begins 2 to 4 weeks after exposure. A small papule known as “mother yaws” develops at the site of inoculation. A papule gradually develops into a papilloma without treatment. Regional lymphadenopathy, fever and arthralgias may be seen with the primary stage of yaws (Farnsworth, 2006). The “mother yaws” may last from several weeks to months and may last into the secondary yaws. Secondary yaws tends to be more severe and involves in the skin, palmoplantar and bones. The characteristics of these lesions exhibit large raised papillomas and papules after a period of latency (about 6 to 16 weeks after the primary stage). Periostitis, osteitis, polydactylitis, arthralgias, generalized lymphadenopathy, headaches, and malaise may be associated with the secondary stage of yaws (Farnsworth,

2006). Five to ten years after infection, 10% of patients enter a tertiary stage of the disease which is typically characterized by destructive skin lesions and severely deformed bone and joint lesions (Farnsworth, 2006).

T. pallidum subspecies *endemicum* is the causative agent of bejel. The infection is very similar to syphilis but is not sexually transmitted. The primary route of transmission is through direct contract with infectious lesions on the skin and mucous membranes. However, the disease can spread by sharing common eating and drinking utensils. The main reservoir is children 2 to 15 years of age. Bejel is more common in dry, hot climates in the eastern Mediterranean region and in Saharan West Africa (Falabella, 1994; Farnsworth, 2006). In the early stage of infection, clinical features are rarely present but lesions are seen in the mouth, the back of the lips, and on the cheeks. Secondary lesions may persist in the mouth. The secondary lesions may be papules, macules, or various other rashes. A generalized lymphadenopathy is common at this stage (Farnsworth, 2006). Tertiary bejel can occur as early as 6 months or as late as several years after initial clinical manifestations resolve. Gangosa, uveitis, optic atrophy, and chorioretinits may occur in the late tertiary stage of infection (Falabella, 1994). The organism may infect the periosteal space, which leads to severe bone deformities.

Pinta is an acute and chronic nonvenereal treponematosis caused by *Treponema carateum.* Pinta occurs primarily in remote rural areas of Central and South America. Brazil, Venezuela, Columbia, Peru, Ecuador, and Mexico have the highest prevalence of the disease. Pinta is more common in early to late adulthood (Farnsworth, 2006). The

exact mode of transmission is unknown, but pinta is most likely transmitted by direct skin or, mucous membrane contact or by fomite contact (Farnsworth, 2006). In the early-stage disease, a papule appears on the uncovered part of the body. After a few weeks or months, the papules become small patches. These patches become hypochromic after several months and may persist for many years. The face and extremities are often the most affected sites. The secondary stage of pinta occurs between 1 and 12 months after the primary lesion stage. Many flat, red, scaly, lesions occur either near the primary lesion, or scattered around the body. The late stage generally develops 2 to 4 years after the onset of the disease and is characterized by the formation of colorless lesions. Unlike other endemic treponematosis, pinta has not been implicated in congenital, cardiac, and neurological abnormalities. Pinta is a rare infectious tropical disease affecting only the skin (Falabella, 1994; Farnsworth, 2006).

2.5 Laboratory diagnosis of syphilis and prevention

The diagnosis of syphilis remains more challenging than most other infections due to the inability of the organism to grow in culture. The laboratory diagnosis of syphilis has primarily relied on the host immunologic response or detection of the organism from active lesions. The laboratory tests for syphilis are divided into two broad classes: direct and indirect methods. Direct detection methods include darkfield microscopy, immunofluorescent staining, and nucleic acid amplication tests (NAATs). Darkfield microscopy is used for the detection of *T. pallidum* from lesions or aspirates collected at the primary stage of syphilis. Histological examination of infected tissues

using the polymerase chain reaction (PCR) is another example of direct method for the diagnosis of syphilis. These tests are most useful for the diagnosis of primary, secondary, and early congenital syphilis (Herremans, 2010). More recently, a number of NAATs have been used for the detection of *T. pallidium* in clinical specimens. These methods are not only useful for diagnosis of primary, secondary, and early congenital syphilis but also are suitable for monitoring the response to therapy (Ratnam, 2005).

Serological tests, the most common laboratory method, continue to play a significant role in the diagnosis and management of the disease. Indirect diagnosis is based on serological tests for the detection of specific *T. pallidum* antibodies. Serological tests fall into two classes: nontreponemal tests for screening, and treponemal tests for confirmation. Nontreponemal assays are widely used for syphilis screening, monitoring response to treatment, and determining the status of re-infection. Nontreponemal tests include the Venereal Research Laboratory (VDRL), the rapid plasma regain (RPR), the toluidine red untreated serum (TRUS), and untreated serum regain (USR) tests. The RPR and VDRL tests are the most commonly used nontreponemal tests for diagnosis of syphilis (Larsen, 1995). Known as cardiolipin tests, nontreponemal tests use an antigen, which contains standardized amounts of cardiolipin, cholesterol-lecithin. These assays detect both immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies formed by the host in response to the lipoidal substance released from either damaged host cells or infected treponemes. However, these nontreponemal tests have lower sensitivity and specificity (Larsen, 1995). Both RPR and VDRL tests are flocculation tests performed in a similar manner. The RPR tests uses a stabilized VDRL antigen mixed with charcoal

particles. When antigen-antibody complexes are formed with a positive serum, black clumps show up and remain as flocculates or clumps. A high rate of false-positives can occur in a low-prevalence population of patients. More importantly, false-positive results are usually due to autoimmune disease, drug addiction, acute viral infections, malaria, leprosy, mononucleosis, lupus erythematosus, and recent immunizations (Larsen, 1995). The advantage of nontreponemal tests is that they have a higher correlation with disease activity. Another advantage of the VDRL test is that it can be performed on cerebrospinal fluid (CSF) or serum and can be used to diagnose neurosyphilis. However, RPR cannot be used for testing CSF specimens due to lack of its sensitivity and specificity (Larsen, 1995). The USR test is another nontreponemal test that detects antilipoidal antibodies in patients with syphilis. A positive USR may indicate a past or present infection with *T. pallidum*. The TRUST is a macroscopic nontreponemal flocculation card test for screening syphilis. It also detects IgM and IgG antibodies to lipoid material from damaged host cells or cardiolipin released by the treponemes. With a positive test, a red flocculent forms when the TRUST antigen is mixed with a positive serum on a plastic-coated card. Biological false-positive reactions can occur in patients with autoimmune disease (Larsen, 1995). A prozone phenomenon can also cause false-negative reactions with these tests.

The second category of indirect tests is the treponemal tests. All treponemal tests use the *T. pallidum* antigen to detect specific anti-treponemal antibodies in serum. Treponemal assays are most often used to confirm the diagnosis of syphilis in patients with a reactive RPR or VDRL and rule out biological false positive results from

nontreponemal assays. Treponemal tests include the *T. pallidum* particle agglutination (TP-PA), microhemagglutination assay with *T. pallidum* antigen (MHA-TP), fluorescent treponemal antibody-absorption test (FTA-ABS), Western blot test (WB) and enzyme immunoassay (EIA). The TP-PA assay is a qualitative gelatin particle agglutination assay used for the detection of specific *T. pallidum* antibodies in human sera or plasma. The TP-PA is usually reactive in primary and secondary syphilis. The MHA-TP test is a passive agglutination test based on the agglutination of gel particles sensitized with *T. pallidum* antigens by antibodies found in the patient's serum (Larsen, 1995; Owusu-Eduse, 2011; Ratnam, 2005). This test is a confirmatory test to replace the (MHA-TP). The TP-PA test is used to confirm the diagnosis of syphilis in patients with a reactive RPR or VDRL. One advantage of this test is that the TP-PA test is as sensitive as the FTA-ABS test in the first stage of syphilis and is just as useful as the RPR in monitoring response to therapy. The FTA-ABS test is an indirect immunofluorescent antibody test. It detects antibodies to *T. pallidum* and can be used to detect syphilis infection at any stage of the disease. The test is mainly used to rule out biologic false-positives on the treponemal test reactions and to detect late syphilis in which nontreponemal test result may be nonreactive. The FTA-ABS test can be performed on either CSF or serum. A positive result of FTA-ABS-CSF assay can be used to confirm the diagnosis of neurosyphilis. The FTA-ABS-CSF assay has a sensitivity and specificity for neurosyphilis of 100% and 99.2%, respectively (LaFond, 2006). The WB test is also known as immunoblotting. In the treponemal WB, the resolved *T. pallidum* proteins are separated by gel-electrophoresis based on their molecular size (Byrne, 1992). These

proteins are transferred by electrophoresis to a nitrocellulose membrane, which is dried and cut into strips. These strips are incubated with patient's serum and antigen-antibody complexes are seen by adding enzyme-conjugated anti-human globulin followed by substrate, causing a color change. Lastly, EIAs are used for the detection of treponemal antibodies in serum. In these tests, the microtiter plate is coated with highly purified specific *T. pallidum* antigen. If the *T. pallidum*-specific antibodies are present in serum, they bind to and become immobilized by the antigen pre-coated on the microtiter wells. Only those microtiter wells that contain the *T. pallidum* IgG/IgM specific antibodies and horseradish peroxidase (HRPO) conjugate, and show a change in color. A change in color indicates a positive test. The ELISA tests can use various recombinant antigens to detect *T. pallidum* specific IgG, IgM, and IgA antibodies in serum or plasma samples (Larsen, 1995). The intensity of the color change is directly proportional to the amount of antibody concentration. This makes the test an effective diagnostic tool in all stages of syphilis with exception of early primary syphilis.

There are 4 major strategies to prevent and control syphilis: 1) education and counseling of persons at risk; 2) identification of symptomatic and asymptomatic persons not seeking treatment, 3) effective diagnosis and treatment; and 4) identification, evaluation, treatment, and counseling of sex partners who are infected with syphilis.

Educating the general public about the serious consequences of syphilis and reducing the risk of the disease transmission are important strategies in the prevention of syphilis. For example, condom education is effective in decreasing the transmission of syphilis and HIV. Condoms have played a significant role in preventing the sexual

transmission of HIV since the beginning of the epidemic (CDC, 2011). Many epidemiological studies cited that consistent condom use was found to reduce the transmission of HIV and STIs by 64% and 42%, respectively (CDC, 2010). According to the National Institute of Health and the National Institute of Allergy and Infectious Diseases, syphilis transmission is reduced by 29% with typical use (CDC, 2011)

Behavioral interventions for syphilis prevention are another integral component of the public education program. The public education program should also focus on "safer sex practices" and risk reduction strategies and risk behaviors. Counseling is an essential part of primary prevention strategies for reducing the transmission of syphilis. For example, trained counselors can discuss an individual's risky behaviors and provide the necessary information on how to reduce their risk for contracting syphilis.

According to the United States Preventive Services Task Force recommendation published in 1996, all pregnant women and persons at high risk for infection should be screened by routine serological testing for syphilis (Calonge, 2004). There is compelling evidence that screening for syphilis reduces morbidity or morality, the incidence of congenital syphilis in newborns, and disease transmission. A study evaluating the impact of serological screening testing in pregnant women revealed a decrease in the proportion of infants with clinical signs and symptoms syphilis, suggesting early detection and intervention can prevent the development of clinical disease with appropriate interventions (Calonge, 2004). Syphilis is a highly prevalent infection among MSM and transgender people, particularly among MSM living with HIV (WHO, 2010). An early diagnosis of syphilis in MSM and other high "risk group" would provide timely treatment

and benefit the individual and partners. Targeting the screening of the ‘risk group” is useful in the control of transmission of the disease.

There are many effective ways to prevent, diagnose, and treat syphilis. Prompt diagnosis and effective treatment of syphilis are necessary to avoid the serious complications and the spread of the disease. Treatment based on presumptive diagnosis of syphilis should be inexpensive, simple, safe, and effective. Targeting and treating the highly “risk group” have been very useful. For example, selective mass treatment of commercial sex workers prevented the spread of ongoing syphilis epidemic in Fresno, California in 1977 (Singh, 1999). WHO also adopted the same strategy to eradicate yaws. The effective use of this mass treatment strategy markedly lowered the prevalence of the disease in certain communities.

Prompt notification and treatment of sex partners after diagnosis of syphilis are essential to reduce the spread of disease and the risk of reinfection. This program can be effective if interventions focus on the importance of notification, improvement of communication skills, development of contact tracing programs and expedient partner therapy. The partner notification process involves a series of interrelated activities such as, interviewing the infected patient to obtain a complete list of exposed sex partners and locating these individuals in the community. Examination of partner notification data could provide valuable information regarding the social networks in a community and capture opportunities for targeted interventions. This allows for assistance in identifying and treating potentially infected sexual partners. All sexual partners need to be notified about an infection so they can be tested and, if necessary, treated. Partner services should

be carefully monitored and evaluated for completeness, timeliness, effectiveness, and cost-effectiveness.

Finally, penicillin is the drug of choice for treating all stages of syphilis according to the 2010 CDC STD Treatment Guidelines (MMWR, 2010). Additionally, penicillin remains the mainstay of treatment for neurosyphilis, congenital syphilis, or syphilis during pregnancy (MMWR, 2010). Alternate therapeutic agents are also available for treating penicillin-allergic patients with primary and secondary syphilis. Tetracycline, erythromycin, and ceftriaxone are effective for treating patients allergic to penicillin. The CDC 2010 STD Treatment Guidelines reports the effectiveness of azithromycin in treating early syphilis (CDC, 2010). However, there are some cases of treatment failure due to azithromycin-resistant mutations in *T pallidum* in several areas of the United States (MMWR, 2010). Additional studies are needed to confirm the efficacy of azithromycin before this treatment can be routinely used for treating syphilis. Despite efforts to develop a safe and effective syphilis vaccine, there is currently no vaccine to prevent syphilis. To this day, penicillin continues to be the drug of choice for the treatment of all stages of syphilis.

Chapter III

METHODOLOGY

The study evaluated the sensitivity and specificity of a *T. pallidum* DBS method for serologic diagnosis of syphilis in comparison to serum results.

3.1 Study population and enrollment

A total of 200 serum and DBS specimens collected from patients at the Los Angeles Municipal Sexually Transmitted Disease Clinics. Eligible subjects were those ≥20 years old with symptoms of a chancre on the genitals, rectum or mouth, swelling of the lymph nodes in the groin, fever, headache, achiness, loss of appetite, rash, painful non-healing skin ulcers and bone pain. Written informed consent was obtained from all study subjects, and signs and symptoms of syphilis, and medication history were assessed. DBS and serum specimens were collected from patients undergoing syphilis evaluation and testing in the STI clinics. These samples were sent to the Syphilis Diagnostics Lab, Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia testing. Samples were blindly evaluated by the DBS and the TREP- SURE™ EIA methods for the detection of anti-treponemal IgG- and IgM-class antibodies.

3. 2 Data sources and procedure

A total of 200 DBS and serum specimens were collected from participants in the study. The study was conducted during a 52 week period in September 2011 to September 2012. TREP- SURE™ was performed on serum at baseline (gold standard)

and was compared with DBS results. TREP- SURE™ is a qualitative EIA for the *in vitro* diagnostic detection of *T. pallidum* antibodies in human serum or plasma. This kit is commercially available and designed as a confirmatory test for *T. pallidum*. In this assay, the specific recombinant treponemal antigens are pre-coated on the microtiter plate wells. Patient sera (100 µl undiluted) and controls were added to the wells and incubated for 60 minutes at 37°C. The contents in the wells were discarded and washed 4 times with 300 µL per well of Wash Buffer Working Solution. Then, enzyme conjugated (100 µl) antigens were added to all wells and incubated for 30 minutes at 37°C. Again, the contents in the wells were discarded and washed 4 times with 300µL per well of Wash Buffer Working Solution after incubation. Tetramethylbenzidine (TMB) substrate solution (100 µl) was added to all wells and incubated the plate for15 (±2) minutes at 37°C, protected from light. Finally, Stop Solution (100 µl) was added to all wells. The plate microtiter plate wells were read within15 minutes with optical density at 450 nm and results were calculated. If specific anti-treponemal antibodies were present in the patient's serum, *T. pallidum* antibodies specifically were bound to the immunized antigens on the wells. The antigen-antibody complexes reacted with HRPO conjugated treponemal antigen. After a second washing step which removes the unbound conjugates, a color reaction takes place on the well as a result of addition of the peroxidase substrate, TMB. A color change is measured after adding stop solution. The amount of antibody present in the patient's serum is directly proportional to the color intensity in the microtiter wells. Results are expressed as an Optical Density (OD). If OD is greater than

1.2 it is considered positive and less than 0.8 as negative. If OD is between 0.8 and 1.1, it is considered an equivocal result. The complete assay takes about 3 hours.

For the DBS testing method (TREP-SPOT™ DBS) two 6.0 - 6.2 mm blood discs were punched from patient's dried blood spot card using a puncher and DBS discs were placed in 300 µl elution buffer, placed on a shaker on low speed and incubated for overnight at 4°C. The contents in the wells (100 µl) were transferred to the testing plate and 100 µl elution buffer was added to all wells in the plate and incubated for 30 minutes at 37°C. The contents in the wells were discarded and washed five times with 300µL per well of Wash Buffer Working Solution. Then, enzyme conjugated (100 µl) antigens were added to all wells and incubated for 30 minutes at room temperature (RT). Again, the contents in the wells were discarded and washed 4 times with 300µL per well of Wash Buffer Working Solution after incubation. TMB substrate solution (100 µl) was added to all wells and the plate was incubated for 10 minutes at RT. Finally, Stop Solution (100 µl) was added to all wells. The microtiter plate wells were read within 15 minutes with optical density at 450 nm and calculated results as same as the TREP- SURE™ method.

3.3 Data analysis

To evaluate performance characteristics, the following values were used: true positive (TP), false positive (FP), true negative (TN), and false negative (FN). A true positive is when the patient has the disease and the test is positive by the gold standard. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency (E) were calculated. The following formulas were used to calculate test indices:

1) sensitivity = [TP/(TP + FN)] × 100;
2) specificity = [TN/(TN + FP)] × 100;
3) PPV = [TP/(TP + FP)] x 100;
4) NPV = [TN/(TN + FN)] x 100;
5) E = [(TP + TN)/(TP + FP + FN + TN)] x 100.

All statistical calculations were performed using the Microsoft Office Excel 2010 and Clinical Calculator 1 - VassarStats. Statistical Computation Web. Kappa coefficients were calculated as a measure of agreement using the Chest X-ray Computation Web (http://www.graphpad.com/quickcalcs/kappa1/). The agreement of the results by kappa (κ) values is categorized as near perfect (0.81 to 1.0), substantial (0.61 to 0.8), moderate (0.41to 0.6), fair (0.21 to 0.4), slight (0 to 0.2), or poor (<0). Correlation coefficients (r^2) measure the strength of association between two values. The value of a correlation coefficient ranges between -1 and 1. The greater the absolute value of a correlation coefficient, the stronger the linear relationship. The strongest linear relationship is shown by a correlation coefficient of -1 or 1. The weakest linear relationship is shown by a correlation coefficient equal to 0.

Chapter IV

RESULTS

The study was designed to determine the sensitivity and specificity of a *T. pallidum* DBS method for serologic diagnosis of syphilis in comparison to serum results, by answering the following 3 questions:

1. **Does a *T. pallidum* dried blood spot method provide high sensitivity and specificity for the diagnosis and confirmation of syphilis?**
2. **Can a *T. pallidum* dried blood spot method be used for the diagnosis and confirmation of syphilis?**
3. **Does a *T. pallidum* dried blood spot method offer an easy and convenient way to collect and transport blood samples for the diagnosis and confirmation of syphilis?**

4.1 The performance characteristics of DBS method

A total of 200 serum and DBS specimens collected from patients at the Los Angeles Municipal Sexually Transmitted Disease Clinics were tested by the DBS and EIA methods. In all, the 100 specimens found to be reactive by the TREP-SURE™ EIA were tested by the TREP-SPOT™ DBS EIA. As shown in Figure 1, 83 were found to be reactive and 17 were found to be nonreactive by the TREP-SPOT™ DBS EIA. As shown in Table 1, sensitivity, specificity, positive predictive value, negative predictive value, efficiency were calculated using the formula below in Table 1.

The sensitivity of the DBS method was 83% (95% CI, 73.89 - 89.50) and specificity was 100% (95% CI, 95.39 - 100)). The positive predictive value and negative predictive values were

100% (95% CI, 94.48 – 100) and 85% (95% CI , 77.43 - 91.0), respectively. The efficiency of the DBS method was 91.5%. The kappa (κ) value for the agreement between the DBS method and EIA assay was 0.83 (95% CI, 0.754 - 0.906). The correlation coefficient (r^2) between the anti-treponemal antibody assay results obtained from DBS and serum samples is shown in Figure 1, demonstrating, $r^2 = 0.94$. Comparison of these results demonstrated a statistically significant positive correlation.

4.2 DBS method for the diagnosis of syphilis

These results indicate that DBS method can be used to detect anti-treponemal antibody in DBS samples. The DBS method demonstrated less sensitive (83% sensitivity) than the TREP-SURE™ EIA assay for the diagnosis of syphilis, but was significantly specific (100% specificity). The DBS method is an alternative to the use of combined VDRL and RPR tests for screening for syphilis. If the DBS method is used for screening alternative treponemal test, TPHA should be used for confirmatory testing. The method is particularly useful to surveillance in countries with limited resources settings.

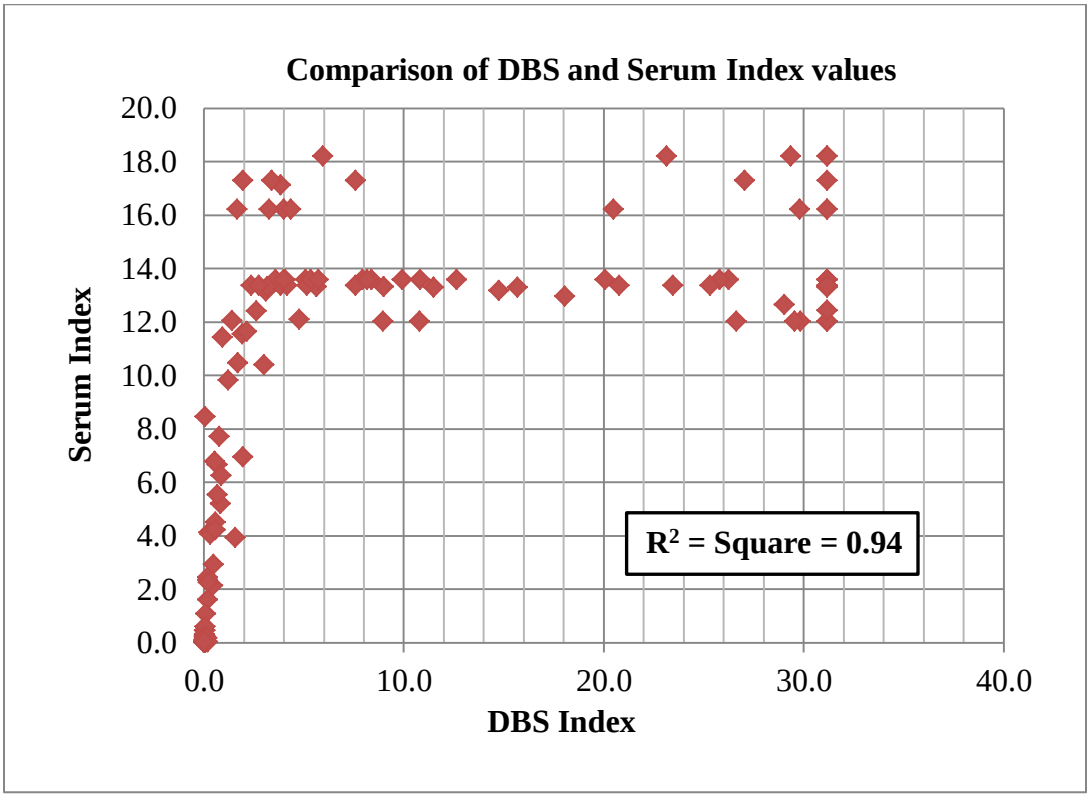
Table 1. Comparison of DBS And Serum Sample

		SERUM SAMPLE		
		POSTIVE	**NEGATIVE**	**TOTAL**
DBS SAMPLE	POSTIVE	TP 83	FP 0	83
	NEGATIVE	FN 17	TN 100	117
	TOTAL	100	100	200

Table 2. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, Efficiency, Kappa, and Correlation Coefficient On 200 Dried Blood Samples Against Gold Standard Of The TREP-SURE™ EIA In Serum

	Estimated Value	95% confidence Interval	
		Lower Limit	Upper Limit
Sensitivity	0.83	0.89506	0.73891
Specificity	1	0.95389	1
Positive predictive value	1	0.94487	1
Negative predictive value	0.854701	0.77473	0.9106
Efficiency	0.915		
Kappa	0.83	0.754	0.906
Correlation coefficient	0.94		

Figure 1. Correlation DBS And Serum Index Values



4.3 Collection and transport of DBS specimens

The *T. pallidum* dried blood spot method offers an easy and convenient way to collect and transport blood samples for the diagnosis of syphilis. Sample ccollection using the DBS technique is simple as shown Figure 2. As shown in Figure 3, dried blood spots can be used as an easy and inexpensive means for the collection and storage of peripheral blood specimens from infants, children and adults in settings where collection and storage of plasma is not optimal. No special handling is needed. DBS sampling would be a good choice to be used as a screening tool for the screening of syphilis. Some risks associated with transport and shipping are minimized because DBS cannot leak or be

broken in transit. Another advantage of DBS samples over routine serum/plasma samples is that only a small quality of blood, typically 50 µl, is required to make one dried blood spot. DBS specimens are easy to collect and store, and can be a convenient alternative to plasma or serum in resources-limited settings.

Figure 2. Dried Blood Spot Collection Card

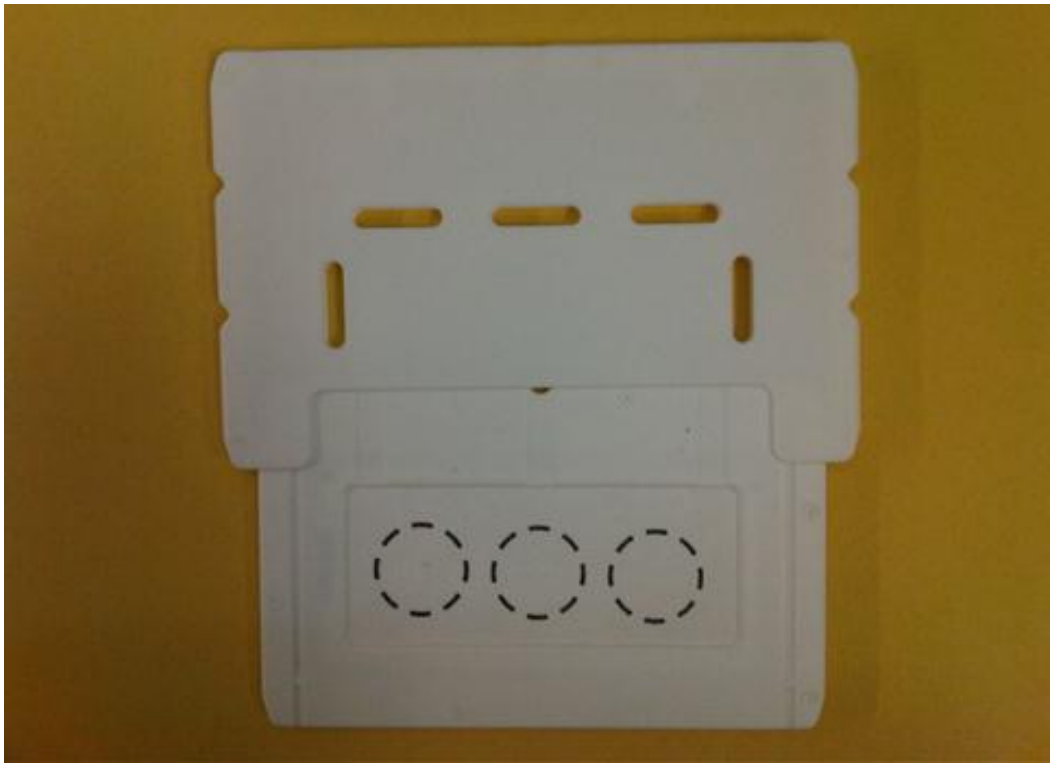


Figure 3. A Collected Blood Sample

4.4 Summary of results

To summarize, we found that the sensitivity of the DBS method was 83% ((95%, CI, 73.89 - 89.50) and specificity was 100% (95% CI, 95.39 - 100). The positive predictive value and negative predictive values were 100% (95% CI, 94.48 -100) and 85% (95% CI, 77.43 - 91.0), respectively. The efficiency of the DBS method was 91.5%. The kappa value for the agreement between the DBS method and EIA assay was 0.83 (95% CI, 0.745 - 0.906). The correlation coefficient (r^2) between the anti-treponemal antibody assay results obtained from DBS and serum samples was 0.94.

Chapter V

DISCUSSION AND CONCLUSION

5.1 Discussion

We evaluated the sensitivity, specificity, positive predictive value, negative predictive value, efficiency (overall agreement), kappa and correlation coefficient of the TREP-SPOT™ DBS assay, comparing it with the most commonly used reference test for the diagnosis of syphilis. The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency (overall agreement) were 83%, 100%, 100%, 85%, and 91.5%, respectively, when tested on 200 DBS and serum specimens. The ideal test is one that has very high sensitivity and specificity, so that most true cases are identified and most non-cases are excluded. The results obtained showed lower sensitivity and high specificity of the TREP-SPOT™ DBS EIA when compared with the TREP-SURE™ EIA. As shown in Table 1, a total of 200 specimens tested, 83 were found to be reactive by the TREP-SPOT™ DBS EIA and 100 were found to be nonreactive by the TREP-SURE™ EIA. Of the 100 TREP-SURE™ EIA-reactive specimens, 17 were nonreactive by the TREP-SPOT™ DBS EIA. These false negative results may be due to a very small concentration of antitreponemal antibodies present in the DBS samples.

The main factors influencing the low sensitivity (83%) could be due to the following three reasons: the amount of blood, dilution and cut-off values used. The amount of blood used for the preparation of the DBS samples was very minute using aliquots of only 5 µl of whole blood. Blood from the DBS punched disc was eluted with

100 µl of eluent buffer, further diluting the sample to 1:20 dilution. Therefore, the assay titers obtained from DBS samples were shown to be lower than the titers from the serum samples. Lastly, the same cut-off values (index values) used for the TREP-SURE™ EIA was used for the DBS samples, when they should not be.

In our example, the TREP-SPOT™ DBS EIA shows very high specificity (100%) for the detection of anti-treponemal antibodies. This demonstrates that the DBS sample method is unlikely to yield a false positive result. The specificity is the proportion of individuals without the disease who are correctly identified by the test as not having it. The positive predictive and negative predictive values were found to be 100% and 84%, respectively. When disease prevalence is relatively low, the positive predictive value (PPV) is low and false-positive test results are more likely. By contrast, when disease prevalence is low, the negative predictive value (NPV) is high, and negative results are more likely to be true. Sensitivity and specificity, the PPV and NPV will change with the prevalence of a disease. For rare diseases, the PPV will always be low or lower, even when a test is near perfect in terms of sensitivity and specificity.

The TREP-SPOT™ DBS showed efficiency (91%) with the TREP-SURE™ EIA for testing anti-treponemal antibodies. A high efficiency was measured between these two assays using DBS and serum specimens. A kappa value of 0.83 (95% CI, 0.754 - 0.906) was found for the agreement between the TREP-SPOT™ DBS and the TREP-SURE™, showing good agreement between these two tests. The TREP-SPOT™ DBS EIA showed near perfect correlation ($R^2 = 0.91$).

In summary, the findings indicate that dried blood spots are a feasible alternative to serum for serologic diagnosis of syphilis. DBS samples compared favorably to the results of the TREP-SURE^{TM} EIA, with an overall concordance of 91%. Dried blood spots are a convenient alternative to serum/plasma and are suitable for blood sampling in the primary health care setting. Studies shown on DBS samples in resource-limited settings have proven the usefulness and accuracy of these samples for the serological and molecular diagnosis of HIV and Hepatitis infections (Lee, 2011; Patton, 2006).

5.2 Study Limitations

This study has a number of limitations**.** First, we did not use a large study sample size. The small sample sizes (200 specimens) possibly reduced the power of the study to detect differences between the methods. The influence of sample size on the power of a test is critical. The larger the sample size in the future study will assure the greater statistical significance between the TREP-SURE^{TM} EIA and the TREP-SPOT^{TM} DBS EIA. Second, all patients enrolled in the study did not provide clinical information (underlying diseases, disorders, or conditions) that are likely to have a large bearing on the ability of the test to reliably rule out the possibly of false positive and negative results. It is well documented that Tuberculosis, Lyme disease, human immune deficiency virus (HIV) infections, and leptospirosis can cause biological false positives in syphilis (Ratnam, 2005). In contract, the latent syphilis and HIV co-infected with Tuberculosis can also cause biological false negatives in patients with syphilis (Ratnam, 2005). It is possible that seventeen false negative (17) results from the DBS samples

could have been attributed to pre-exiting or underlying medical conditions. It is also possible that false negative results might occasionally occur for another reason. Because all test results were not evaluated in concert with a clinical diagnosis and history due to unavailability of clinical diagnosis and history. Lastly, we did not use another confirmatory test in this study. DBS samples giving discrepant treponemal test results (17 false negatives) need to be further tested by another confirmatory method using either the FTA-ABS or PCR analysis.

5.3 Recommendations

Based on our findings, the study clearly showed DBS underestimated positive results mainly at low OD level, which could be due to the low blood volume in the spot. In particular, research should focus on further optimization of the assay using different cut-off values for the validation of the positivity and negativity in DBS samples because the small amount blood in the DBS specimens leads to lower assays titer**.**

The TREP - SPOT^{TM} DBS EIA may be suitable for the detection of antibodies *T. pallidum* and warrant further evaluation using a larger sample size in the study. A higher sample size gives more accurate results. Sample size is important primarily because of its effect on statistical power. Statistical power is the probability that a statistical test will indicate a significant difference when there truly is one. Attention to sample size will result in a more meaningful study, with results more representative of the population, which the research is geralizing. A high sample size of the study should be considered early in the planning phase of the study.

5.4. CONCLUSION

The use of dried blood spots is often the best sampling option for the detection of anti-treponemal antibodies for the diagnosis of syphilis in limited resource settings. The study findings indicate that DBS are a feasible alternative to serum/plasma for serological confirmation of syphilis. The detection anti-treponemal antibodies compared favorably to the results of serum-based assay (TREP-SURE^{TM}) EIA, with a specificity of 100% and an overall concordance of 91.5%.

Even though the results from DBS samples showed a lower relative sensitivity (83%), dried blood spots are technically easier to obtain and are suitable blood samples for primary health care centers. DBS can be collected from a heel, toe, and finger prick or prepared from venous blood**.** The relative ease of sample collection, transport, and storage are significant benefits.

REFERENCES

Arnold, SR and ford-Jones, EL. (2000). Congenital syphilis: a guide to diagnosis and management. *Pediatric Child Health,* 5(8), 463 - 469.

Brown, WH and Pearce, L (1920). Experimental syphilis in the rabbit: I. Primary infection in the testicle. *Jounral of Experimental Medicine,* 31(6), 749 - 64.

Byrne, R, Laska, S, Bell, M, Larson, D, Phillips, J and Todd, J (1992). Evaluation of a *Treponema pallidum* western immunoblot assay as a confirmatory test for syphilis. 115 -122.

Calonge, N. (2004). U.S. Preventive Services Task Force, screening for syphilis Infection: recommendation statement. *Annals of Family Medicine,* 2 (4), 362 - 365.

Cameron, C, Castro, C, Lukehart, SA and Van Voorhis. WC. (1998). Function and protective capacity of *Treponema pallidum* subsp.pallidum glycerophosphodiester phosphodiesterase. *Infection and Immunity,* 66 (12), 5763 - 5770.

Centers Disease Control and Prevention (CDC). (2009a). 2009. Sexually transmitted diseases surveillance. Retrieved October 23, 2012. http://www.cdc.gov/std/stats09/syphilis.htm.

Centers Disease Control and Prevention (CDC). (2009b). STD prevention. Self-study STD module. Pathogenesis and Microbiology. Retrieved October 23, 2012. http://www2a.cdc.gov/stdtraining/Self-Study/syphilis/syphilis3.asp.

Centers Disease Control and Prevention (CDC). (2010). 2010 sexually transmitted diseases surveillance. Retrieved October 23, 2012. http://www.cdc.gov/std/stats10/syphilis.htm.

Centers Disease Control and Prevention (CDC). (2011). Male latex condoms and sexually transmitted diseases. Retrieved October 25, 2012. http://www.cdc.gov/condomeffectiveness/latex.htm

Centers Disease Control and Prevention (CDC). (2012a). Sexually transmitted diseases (STDs). Syphilis & MSM (Men Who Have Sex With Men) - CDC Fact Sheet. Retrieved October 25, 2012. http://m.cdc.gov/en/HealthSafetyTopics/DiseasesConditions/STDs/syphilisMSM_FS.

Phoenix Airmid Biomedical Corporation. (2012). TREP-SPOT (DBS), Anti-treponemal EIA Screen, For Use with Dried Blood Spot Samples. Oakville, Ontario, Canada, Package Insert, 1 - 11.

Phoenix Bio-Tech Corporation. (2010). TREP-SURE™, Anti-treponema EIA Screen. Oakville, Oontaio, Canada, Package Insert. 1 - 16.

De Silva, V., Oldham, CD and May, SW. (2010). L-Phenylalanine concentration in blood of phenylketonuria patients: a modified enzyme colorimetric assay compared with amino acid analysis, tandem mass spectrometry, and HPLC methods. *Clincal Chemistry and Laboratory Medicine*, Sep;48(9), 1271- 1279.

de Souza, E. (2005). A hundred years ago, the discovery of *Treponema pallidum. Anais Brasileiros de Dermatologia*, 80(5), 547-548.

Euerle, B and Cunha, BA. (2011). Syphilis. Retrieved October 25, 2012. http://emedicine.medscape.com/article/229461-overview.

Falabella, R. (1994). Nonvenereal treponematoses: yaws, endemic syphilis, and pinta. *Journal of American Academy of Dermatology,* 31 (6), 1075 -1080.

Farnsworth, N and Rosen, T. (2006). Endemic treponematosis: review and update. *Clinics in Dermatology,* 24, 181 - 190.

Gupta, S, Bhattacharya, A, Singh, RR and Agarwal, VK. (2012). Syphilis D' Emblee. *Indian Journal of Dermatology,* 57(4), 296 - 298..

Herremans, T, Kortbeek, L, and Notermans DW. (2010). A review of diagnostic tests for congenital syphilis in newborns. *European Journal of Clinical Microbiology and Infectious Diseases,* 29 (5), 495 - 501.

Heymans, R, van der Helm, JJ, de Vries, Fennema, H S A, Coutinho, RA, and Bruisten, SM (2010). Clinical value of *Treponema pallidum* real-time PCR for diagnosis of syphilis. *Journal of Clinical Microbiology,* 48(2), 497 - 502.

Hossain, M., Broutet, N, and Hawkes, S. (2007). The elmination of congenital syphilis: a comparison of the proposed World Health Organization Action Plan for the elimination of congenital syphilis with existing National Maternal and Congenital Syphilis Policies. *Sexually Transmitted Diseases,* 34 (7), S22 - S30.

Karnath, B. (2009). Manifestations of syphilis. *Hospital Physician,* 43 - 48.

Kent, M. a. R., F. (2008). Reexamining syphilis: an update on epidemiology, clinical manifestations, and management. *The Annals of Pharmacotherapy,* 42, 226 -236.

LaFond, RE and Luckehart (2006). Biological basis for syphilis. *Clinical Microbiological Reviews,* 19 (1), 29 - 49.

Larsen, S, Steiner, BM and Rudolph, AH. (1995). Laboratory diagnosis and interpretation of tests for Syphilis. *Clinical Microbiological Reviews,* 8 (1), 1 - 21.

Lee, C., Sri Ponnampalavanar, S, Syed Omar, SF, Mahadeva, S and Ong, LY (2011). Evaluation of the dried blood spot (DBS) collection method as a tool for detection of HIV Ag/Ab, HBsAg, anti-HBs and anti-HCV in a Malaysian TAnnals Academy of Medicinetertiary Referral Hospital. *Annals Academy of Medicine, 40:10,* 448-452.

Magnuson, HJ, Eagle, H and Fleischman, R. (1948). The minimal infectious inoculum of Spirochaeta pallida (Nichols Strain) and a consideration of its rate of multiplication *in vivo*. *American Journal of Syphilis, Gonorrhea, and Venereal Diseases,* 32, 1 - 18.

Morbidity and Motality Weekly Report (MMWR). (2006). Primary and Secondary Syphilis --- United States, 2003--2004.

Morbidity and Motality Weekly Report (MMWR). (2006). Primary and Secondary (2010). Sexually transmitted diseases treatment guidelines, 2010.

Owusu-Eduse, K, Peterman, TA and Ballard, RC. (2011). Serologic testing for syphilis in the United States: A cost-effectiveness analysis of two screening algorithms. Sexually Transmitted Diseases. *Sexually Transmitted Diseases,* 38(1), 1 - 7.

Patton, J., Sherman, GG, Coovadia, AH, Stevens,WS, and Meyers, TM. . (2006). Ultrasensitive human immunodefi iency virus type 1 p24 antigen assay modified for use on dried whole

blood spots as a reliable, affordable test for infant diagnosis. *Clinical Vaccine Immunology, 13,* 152-1555.

Ratnam, S. (2005). The laboratory diagnosis of syphilis. *Canadian Journal of Infectious Diseases and Medical Microbiology,* 16 (1), 45 - 51.

Riviere, G R, Thomas, SS and Cobb, CM. (1989). In vitro model of *Treponema pallidum* invasiveness. *Infection and Immunity,* 57, 2267 - 2227.

Rodriguez-Cerdeira, C and Silami-Lopes, VG. (2012). Congenital syphilis in the 21st century. *Actas-sifiliograficas,* 103 (8), 679 -693.

Rottinghaus, E, Ugbena, R, Diallo, K, Bassey, O, Azeez, A, Devos, J, Zhang, G, Aberle-Grasse, J, Nkengasong, J, and Yang, C. (2012). Dried blood spot specimens are a suitable alternative sample type for HIV-1 viral load measurement and drug resistance genotyping in patients receiving first-line antiretroviral therapy.

Clinical Infectious Diseases, 54 (8), 1187-1195.

Singh, A and Romanowski, B. (1999). Reviews. Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features. *Clinical Microbiological Reviews,* 12 (2), 187 - 209.

The World Health Organization (WHO). (2001). Global prevalence and incidence of selected curable sexually transmitted infections. Retrieved October 26, 2012. http://www.who.int/reproductivehealth/publications/rtis/HIV_AIDS_2001_2/en/index.html.

The World Health Organization (WHO). (2010). A hidden epidemic: HIV, men who have sex with men and transgender people in Eastern Europe and Central Asia

Regional Consultation Kyiv, Ukraine 22-24 November 2010 Meeting Report. Retrieved October 26, 2012. http://www.euro.who.int/__data/assets/pdf_file/0010/140410/e94967.pdf.

The World Health Organization (WHO). (2011). Methods for surveillance and monitoring of congenital syphilis elimination within existing systems. Retrieved October 26, 2012. http://whqlibdoc.who.int/publications/2011/9789241503020_eng.pdf.

The World Health Organization (WHO). (2012a). Management of sexually transmitted infections. Report of an Intercountry Workshop Yangon, Myanmar, 16-20 July 2001) WHO Project. ICP RHR 001, World Health Organization Regional Offcie for South-East Asia New Delhi Decmeber 2011. Retrieved October 28, 2012. http://whqlibdoc.who.int/searo/2001/SEA_STD_42.pdf

The World Health Organization (WHO). (2012b). STDs: Global Estimates. Retrieved October 23, 2012. http://www.chastitycall.org/std.stats.html.

Wong, E, Klausner, JD, Caguin-Grygiel, GC, Madayag, CM, Barber, K, Qiu, JS, Liska, S and Pandori, M. (2011). Evaluation of an IgM/Igg sensitive enzyme immunoassay and the utility of index values for the screening syphilis infection in a high-risk population. *Sexually Transmitted Diseases, 38*(12), 1 - 5.