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Innovative Testing Strategies for the Diagnosis of Syphilis

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Publication Date
2013

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Innovative Testing Strategies for the Diagnosis of Syphilis

By

Anthony Tran

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Public Health in the Graduate Division of the University of California, Berkeley

Committee in charge:

Professor George Sensabaugh, Chair
Professor Gertrude Buehring
Professor Amy Herr
Associate Clinical Professor Mark Pandori

Spring 2013
Innovative Testing Strategies for the Diagnosis of Syphilis

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by

Anthony Tran
Abstract

Innovative Testing Strategies for the Diagnosis of Syphilis

by

Anthony Tran

Doctor of Public Health

University of California, Berkeley

Professor George Sensabaugh, Chair

Traditional serological syphilis testing in the US currently includes screening with a nontreponemal assay that detects antibodies to lipoidal material released into the blood from host cells damaged by *Treponema pallidum* as well as lipids on the surface of nonspecific treponemes itself. Reactive nontreponemal tests are subsequently confirmed with a test that detects antibody specific for *T. pallidum* (termed “treponemal”). It has been suggested that this traditional serological algorithm could be reversed so that screening begins with a treponemal test, such as an enzyme immunoassay (EIA), and if reactive, infection would be confirmed with a nontreponemal test. In cases of discordance between the screening treponemal test and the confirmatory non-treponemal test, the CDC suggests that a second treponemal test (such as a TPPA) could be utilized to serve as a supplemental test.

The reverse sequence testing algorithm offers several advantages over the traditional algorithm for laboratories. Screening with an immunoassay (IA) (1) allows for the use of automation, thus potentially increasing laboratory efficiency; (2) provides objective instead of subjective results; (3) reduces the amount of manual manipulation that is involved with nontreponemal assays. However, the algorithm is not without issues. Perhaps greatest amongst them is that such tests will be reactive in cases where the patient has previously been treated for syphilis. This may be particularly problematic in a setting with a high prevalence of syphilis where many patients being tested may have had a previous case of the disease.

This dissertation is formatted as three individual, self-contained reports of research investigations with an overall introduction and conclusion. Chapter 2 reports on the performance of a reverse sequence algorithm in San Francisco and shows that this algorithm would cause a large amount of additional work for the laboratory. Chapter 3 describes a reduction in the number of supplemental treponemal tests that needs to be run if EIA signal-to-cutoff ratios were considered as part of the algorithm. Chapter 4 describes how a rapid syphilis test could potentially replace the TPPA as the second supplemental test in the reverse sequence algorithm, thus decreasing the turnaround time to report positive syphilis results.
# Table of Contents

List of Figures .......................................................... ii

List of Tables .......................................................... iii

Major Abbreviations Listing .......................................... iv

1 Introduction .......................................................... 1
  1.1 Clinical Stages of Syphilis ......................................... 1
  1.2 Epidemiology of Syphilis ........................................... 3
  1.3 Current Testing Methodologies ..................................... 5
  1.4 APHL/CDC Expert Consultation Meeting .......................... 14
  1.5 Research Study Papers .............................................. 14
  1.6 Institutional Review Board Approval .............................. 14
  1.7 Funding Source ..................................................... 15

2 Evaluation of an Enzyme Immunoassay in an Alternative Syphilis Diagnostic Algorithm: Implications for a High Prevalence Setting .......................... 16
  2.1 Introduction .......................................................... 16
  2.2 Materials and Methods ............................................. 18
  2.3 Results .............................................................. 20
  2.4 Discussion ........................................................ 24

3 The Role of Enzyme Immunoassay Signal-to-Cutoff Ratios in Predicting Other Treponemal Assay Results .................................................. 27
  3.1 Introduction .......................................................... 27
  3.2 Materials and Methods ............................................. 28
  3.3 Results .............................................................. 30
  3.4 Discussion ........................................................ 34

4 The Role of a Rapid Treponemal Syphilis Test in Various Syphilis Serological Diagnostic Algorithms in a High Prevalence Setting .......................... 36
  4.1 Introduction .......................................................... 36
  4.2 Materials and Methods ............................................. 37
  4.3 Results .............................................................. 39
  4.4 Discussion ........................................................ 41

5 Conclusion ........................................................... 43

Bibliography ........................................................... 46
# List of Figures

1.1 Syphilis—Reported Cases by Stage of Infection, United States, 1941–2011 .................................................................................................................. 3
1.2 Traditional Syphilis Testing Algorithm .......................................................... 6
2.1 Potential Reverse Sequence Syphilis Testing Algorithm ............................... 17
2.2 Results from the traditional syphilis serological algorithm ....................... 20
2.3 Results from the reverse syphilis serological algorithm ......................... 21
3.1 Receiver Operating Characteristic (ROC) curve demonstrating the discrimination of the TS-EIA in predicting TPPA positivity. ...................... 31
3.2 Relationship between TREP-SURE™ EIA S/CO ratios and corresponding positive TPPA results for all TS-EIA specimens (n=478) from the reverse sequence algorithm performed at the SFDPHL ......................................................................................................................... 33
3.3 Relationship between TREP-SURE™ EIA S/CO ratios and corresponding positive TPPA results for the subset of discordant TS-EIA positive, VDRL nonreactive specimens (n=292) from the reverse sequence algorithm performed at the SFDPHL ......................................................................................................................... 34
List of Tables

1.1 Cases of Syphilis Reported by State Health Departments and Rates per 100,000 Population by decade........................................... 4
1.2 Cases and Rates of Primary and Secondary Syphilis in the US, 2011................................................................................................. 5
1.3 Performance of VDRL, RPR and TPPA Serological Tests 7
1.4 Types of diagnostic tests utilized according to stage of syphilis infection.................................................................................. 12
1.5 Summary characteristics of various diagnostic syphilis assays... 13
2.1 Results from CAPTIA Syphilis-IgM Capture EIA on 292 discordant TREP-SURE™ EIA positive and VDRL nonreactive serum specimens................................................................. 22
2.2 Summary of overall testing outcomes for VDRL, TPPA and TS-EIA. 23
2.3 Sensitivity, specificity and PPV of two reverse sequence algorithm scenarios compared to “gold standard” traditional algorithm........................................................................................................ 23
3.1 Kappa classifications .................................................................................. 31
3.2 Relationship of TPPA results to corresponding TREP-SURE™ EIA S/CO ratios.................................................................................. 32
4.1 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Syphilis Health Check rapid test.................................................................................................................. 39
4.2 Performance of Syphilis Health Check rapid test against specimens of varying categories......................................................... 40
4.3 Comparison of Syphilis Health Check Rapid Test to TPPA Assay........................................................................................................ 41
5.1 Estimated costs for each type of test performed at SFDPHL................. 44
## Major Abbreviations Listing

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APHL</td>
<td>Association of Public Health Laboratories</td>
</tr>
<tr>
<td>AUROC</td>
<td>Area Under the Receiver Operating Characteristic</td>
</tr>
<tr>
<td>BFP</td>
<td>Biological false positive</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDPH</td>
<td>California Department of Public Health</td>
</tr>
<tr>
<td>CIA</td>
<td>Chemiluminescence Immunoassay</td>
</tr>
<tr>
<td>CLIA</td>
<td>Clinical Laboratory Improvements Amendments</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DFA</td>
<td>Direct Fluorescent Antibody</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>FTA-ABS</td>
<td>Fluorescent Treponemal Antibody Absorption</td>
</tr>
<tr>
<td>GC</td>
<td><em>Neisseria gonorrhea</em> or gonococcal disease</td>
</tr>
<tr>
<td>HC</td>
<td>Syphilis Health Check</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>LIS</td>
<td>Laboratory Information System</td>
</tr>
<tr>
<td>MHA-TP</td>
<td>Microhemagglutination-<em>Treponema pallidum</em></td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
</tr>
<tr>
<td>NOI TR</td>
<td>Notice of Intent to Rely</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>POC</td>
<td>Point-of-contact</td>
</tr>
<tr>
<td>P&amp;S</td>
<td>Primary and Secondary (cases of syphilis)</td>
</tr>
<tr>
<td>PHM</td>
<td>Public Health Microbiologist</td>
</tr>
<tr>
<td>PHS</td>
<td>Public Health Service</td>
</tr>
<tr>
<td>PNA</td>
<td>Percent Negative Agreement</td>
</tr>
<tr>
<td>POA</td>
<td>Percent Overall Agreement</td>
</tr>
<tr>
<td>PPA</td>
<td>Percent Positive Agreement</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>RPR</td>
<td>Rapid Plasma Reagin</td>
</tr>
<tr>
<td>RST</td>
<td>Rapid syphilis test</td>
</tr>
<tr>
<td>SFDPHL</td>
<td>San Francisco Department of Public Health Laboratory</td>
</tr>
<tr>
<td>S/CO</td>
<td>Signal-to-Cut-off</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually Transmitted Disease</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TAT</td>
<td>Turnaround Time</td>
</tr>
<tr>
<td>TPPA</td>
<td><em>Treponema pallidum</em> Passive Particle Agglutination</td>
</tr>
<tr>
<td>TS</td>
<td>TREP-SURE™</td>
</tr>
<tr>
<td>VDRL</td>
<td>Venereal Disease Research Laboratory</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

To many, the word syphilis invokes strong feelings due to its unknown origins and controversial history. One of the most infamous and unethical public health experiments in the history of the United States (US) involved 399 African-American sharecroppers in Macon, Georgia [1]. This 40-year study, called the “Tuskegee Study of Untreated Syphilis in the Negro Male,” was sponsored by the US Public Health Service (PHS) to observe the effects of untreated syphilis. Beginning in 1932, the study monitored participants’ development of syphilis yet provided no treatment options, despite penicillin being available and the drug of choice to treat syphilis in 1947 [2,3]. A debate still remains over the origins of this scourge called *morb*us *g*allicus in the 15th century; was it brought over by Christopher Columbus or was it here in the Americas and brought back to Europe by Columbus [3,4]?

Syphilis is a sexually transmitted disease (STD) that can also be transmitted from mother to child. It is caused by a small spirochete organism called *Treponema pallidum* subspecies *pallidum* (or simply referred to as *T. pallidum*). It has many unique features that definitively separate it from other pathogenic organisms found in humans. Unlike typical bacteria that are bacilli (rod) or cocci (round)-shaped, spirochetes have a spiral-like or corkscrew form. Also distinct is the fact that pathogenic treponemes like *T. pallidum* cannot be cultured in vitro, so it is difficult to study their metabolic, physical and pathogenic makeup [5]. Fortunately, syphilis is still highly susceptible to penicillin, even after 70 years of use, and thus does not contribute to the growing public health concern of antimicrobial resistance. This is most likely because the genome of *T. pallidum* seemingly lacks transposable elements thus suggesting that the genome is very conserved and stable [5].

1.1 Clinical Stages of Syphilis

Often called “the great imitator” because signs, symptoms and clinical presentation mimic many other diseases, syphilis has four distinct phases of clinical manifestations: primary, secondary or disseminated, latent and tertiary (late) stage.

Primary Stage
After about a three-week incubation phase from time of exposure (range three to 90 days) the first or primary stage of syphilis presents [5]. This usually consists of a firm, round, small and painless ulcerative lesion or chancre that spontaneously resolves within three to six weeks without treatment [5,6]. This chancre is teeming with *T. pallidum* spirochetes, making the primary stage the most infectious. Because open lesions can serve as a portal of entry for other bacteria and viruses, primary syphilis has also been linked to HIV infection and other STDs [7]. Furthermore, infection with *T. pallidum* has also been shown to directly facilitate HIV infection and progression because the immune cells that are susceptible to HIV are found in
abundance in syphilis lesions [7]. Therefore, preventing syphilis has implications for stemming HIV infection as well.

**Secondary Stage**
Without treatment, syphilis can progress to the secondary stage, which consists of maculopapular rash and skin lesions that may appear while the primary chancre is healing or several weeks later [5,6]. The rough, red or reddish brown rashes are usually not itchy and characteristically appear on the palms and soles of the feet, although they can also emerge all over the body [5]. Sometimes the spots are so faint that they are not noticeable. During this stage, rashes and lesions contain *T. pallidum* spirochetes and therefore these individuals are also infectious. Other signs and symptoms of secondary syphilis are nondescript and include fever, swollen lymph glands, sore throat, patchy hair loss, headaches, weight loss, muscle aches and fatigue [5]. In some instances, secondary syphilis can affect the central nervous system (CNS) causing headaches, impaired vision and vertigo, as well as other organs such as the kidneys and gastrointestinal tract [6]. Signs and symptoms of secondary syphilis resolve on their own. However if left untreated, it can progress to latent and possibly tertiary or late stage syphilis [5].

**Latent Stage**
This stage of syphilis can be problematic as there are no apparent signs or symptoms of disease. By definition, latent syphilis is characterized by a reactive or positive serological treponemal antibody test such as the fluorescent treponemal antibody absorption (FTA-ABS) or *Treponema pallidum* passive particle agglutination (TPPA) test, but lacks any clinical manifestations of syphilis [6]. The first four years after infection are designated the early latent period where clinical relapse may occur and the patient can become infectious again. Late latent syphilis follows this and is associated with host resistance to reinfection and infectious relapse, however pregnant women can still infect her fetus in utero causing congenital syphilis [6].

**Tertiary Stage**
Without treatment, approximately 15% of individuals progress to the late stage of syphilis five to 30 or more years after initial infection [5,6]. This stage represents the most severe disease and can include neurological, cardiovascular, gumma formation on and in bones and even death. Neurosyphilis can cause slurring of speech, seizures, dementia and mimic many other CNS diseases, thus it can be difficult to diagnose syphilis at this late stage. However, cardiovascular syphilis and gumma formation are considered rare findings now.
1.2 Epidemiology of Syphilis

Overall, syphilis has been on a downward trend since the introduction of penicillin and increased public health measures to combat it [4]. There has been a dramatic decrease in the number and rate of primary and secondary (P&S) cases of syphilis between 1941 (68,231 cases, 51.7 cases per 100,000 persons) when records began to be kept and 2011 (13,970 cases, 4.5 cases per 100,000 persons) (Figure 1.1) [8]. Recently, however, there have been signs of re-emergence of syphilis. In the 1990s the increase was linked to exchanges of sex for drugs. Over the last decade outbreaks among men who have sex with men (MSM) has resulted in a small but steady increase in rates of P&S syphilis [5,9,46] and lately that increase has accelerated. In 2006, MSM accounted for nearly 60% of new syphilis infections in the United States [10] and with the latest CDC reports in 2011; this has increased to 72% [8,17]. Despite the recent upward trend, rates of syphilis still remain relatively low with overall national prevalence estimated at 0.7% in 2004 among 14-49 year olds and overall just fewer than 14,000 total P&S cases reported in 2011 (Table 1.1) [8,11]. Compare this with over 1.3 million cases of Chlamydia and 300,000 cases of gonorrhea reported in 2011 [8].

![Figure 1.1 Syphilis—Reported Cases by Stage of Infection, United States, 1941–2011](image_url)
Table 1.1 70 Years of Syphilis Surveillance: Cases of Syphilis Reported by State Health Departments and Rates per 100,000 Population by decade since syphilis surveillance began, United States, 1941-2010 [55].

<table>
<thead>
<tr>
<th>Year</th>
<th>All Stages</th>
<th>Primary &amp; Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Rate</td>
</tr>
<tr>
<td>1941*</td>
<td>485,560</td>
<td>368.2</td>
</tr>
<tr>
<td>1951</td>
<td>174,924</td>
<td>116.1</td>
</tr>
<tr>
<td>1961</td>
<td>124,658</td>
<td>68.8</td>
</tr>
<tr>
<td>1971</td>
<td>95,997</td>
<td>46.4</td>
</tr>
<tr>
<td>1981</td>
<td>72,799</td>
<td>31.7</td>
</tr>
<tr>
<td>1991</td>
<td>128,719</td>
<td>50.9</td>
</tr>
<tr>
<td>2001</td>
<td>32,284</td>
<td>11.2</td>
</tr>
<tr>
<td>2011</td>
<td>46,042</td>
<td>14.9</td>
</tr>
</tbody>
</table>

*First year CDC began syphilis surveillance

Unfortunately, as with many other diseases in the US, disparities among race/ethnicities and geographic localities exist. Blacks still account for the highest rates in the country at nearly four times the national average in 2011 despite an overall 6.6% decrease in P&S syphilis rates from 2010 to 2011 [47]. In addition, rates for Asians and Pacific Islanders had the steepest rise, by nearly 25%, in the same time frame [47]. Geographic differences are also observed. Southern states still account for nearly half of all syphilis infections in the US, despite a 1.8% decrease in P&S syphilis rates from 2010-2011. Furthermore only 15 states, including Washington, DC, account for about 71% of all primary and secondary infections in the US [8].

Despite the low prevalence of syphilis nationally, it is increasingly a significant problem in California. In 2011, California had 2,443 cases of P&S syphilis, which is nearly double the number of cases of any other state [12]. This also represents a 15.5% increase from 2010 [13]. California also ranks as tied for the sixth highest incidence of syphilis in 2011 (up from ninth in 2010) [12,14]. As expected, some of the larger metropolitan areas in the state produce the most disproportionate amount of P&S syphilis cases.

The picture in San Francisco looks even worse. In 2011, San Francisco County had the third highest number of cases (n=388) and second highest incidence rate (48.2 cases per 100,000 population) of P&S syphilis cases in the country (Table 1.2) [15]. This is over ten times the national case rate of 4.5/100,000 population. Nearly all of the syphilis cases in San Francisco are from MSM who are also mostly co-infected with HIV [16]. The San Francisco Department of Public Health (SFDPH)
recommends a syphilis test every three to six months for sexually active MSM and at even more frequent intervals if they have been previously diagnosed with syphilis [16]. This has led to a large volume of syphilis tests performed at the San Francisco Department of Public Health Laboratory (SFDPHL) with over 23,000 nontreponemal screening tests performed annually [48]. With such a large number of syphilis tests being conducted, it is imperative to have the most accurate and timely testing strategies in place. This dissertation will examine several innovative and alternative testing strategies for the prompt and proper diagnosis of syphilis in San Francisco.

Table 1.2 Cases and Rates of Primary and Secondary Syphilis in the US, 2011

<table>
<thead>
<tr>
<th>Region</th>
<th>Cases</th>
<th>Rates per 100,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States, Total</td>
<td>13,970</td>
<td>4.5</td>
</tr>
<tr>
<td>United States, Metro</td>
<td>10,702</td>
<td>6.4</td>
</tr>
<tr>
<td>California</td>
<td>2,443</td>
<td>6.6</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>810</td>
<td>8.2</td>
</tr>
<tr>
<td>San Diego</td>
<td>293</td>
<td>9.5</td>
</tr>
<tr>
<td>San Francisco</td>
<td>388</td>
<td>48.2</td>
</tr>
</tbody>
</table>

1.3 Current Testing Methodologies

As syphilis resembles many other STDs, it is vital to have accurate tests to aid in diagnosis. A number of serological methods are FDA approved in the US to accomplish this goal. The current CDC STD Treatment Guidelines recommend a sequential testing strategy involving an initial screen with a nontreponemal assay, such as the rapid plasma reagin (RPR) or Venereal Disease Research Laboratory (VDRL), followed by a treponemal specific assay, such as an enzyme immunoassay (EIA) or TPPA, for confirmation (Figure 1.2) [17]. Nontreponemal tests detect antibodies to lipoidal material released by host cells damaged by T. pallidum, while treponemal tests directly detect antibodies specific to T. pallidum. In this dissertation, this strategy will be referred to as the “traditional” or “gold standard” algorithm. In addition to serological tests, direct detection of T. pallidum from primary lesions provides the most definitive diagnosis of syphilis. Unfortunately, due to the lack of availability of reagents and trained personnel, few laboratories still offer this test [17,18].

The following sections will describe the serological testing methodologies analyzed in this investigation. One nontreponemal assay (VDRL) and three treponemal assays (EIA, TPPA and a rapid syphilis test) will be evaluated. Tables 1.4 and 1.5 at the end of this section summarize the hallmarks of the types of diagnostic tests that are utilized to diagnosis syphilis at each stage of infection.
1.3.1 Nontreponemal Serologic Tests

Nontreponemal tests for *T. pallidum* have traditionally been used as screening tools to aid in the diagnosis of syphilis. That paradigm is now shifting as some laboratories are beginning to utilize a treponemal test to screen and a nontreponemal test to confirm the diagnosis of syphilis [17]. Nontreponemal tests become positive one to four weeks after the appearance of a primary chancre and about six weeks after exposure to *T. pallidum* [3,6,19]. These tests can be considered tests of surrogate markers as they detect antibodies to lipoidal material released from damaged host cells caused by *T. pallidum* in the blood as well as lipids on the surface of nonspecific treponemes itself [3,6,19]. They do not recognize antibodies directed toward *T. pallidum*. Nontreponemal tests are also the only tests that are recommended to monitor the course of syphilis during and after treatment [19]. They are widely utilized as screening tools because they are rapid, easy to use and inexpensive [19]. The two most commonly utilized nontreponemal tests are the rapid plasma reagin (RPR) and Venereal Disease Research Laboratory (VDRL) tests. Only the VDRL assay was evaluated since it is the nontreponemal test currently used by the SFDPHL.

Rapid Plasma Reagin Test
The rapid plasma reagin (RPR) is an eight-minute macroscopic flocculation card test
that is FDA approved for the qualitative and semiquantitative screening of syphilis [20]. Each plastic coated card contains ten 18-mm circles where each individual reaction will occur. Serum or plasma specimens can be utilized for the RPR test and up to ten samples can be read on one test card (not including controls). The assay measures IgM and IgG antibodies released by the body in response to lipoidal material discharged from damaged host cells and cardiolipin released from treponemes [21,22]. The presence of antibodies is indicated by agglutination of carbon particles containing cardiolipin, lecithin and cholesterol. Carbon particles in the antigen mixture provide visualization of a reactive reaction and show up as small black clumps on the white card. Nonreactive specimens will be uniformly gray or form a button in the middle of the card.

The RPR is a commonly utilized nontreponemal test and a relatively accurate screening tool, although sensitivity and specificity are dependent on the stage of untreated syphilis with the highest sensitivity being reported in secondary syphilis [23]. For example, sensitivity in primary syphilis is 86% and spikes to 100% in the secondary stage. Specificity of the RPR is around 98% [18,21]. Table 1.3 illustrates the sensitivities of various commonly utilized serological assays at different stages of syphilis infection. Unfortunately, antilipoidal antibodies are not only produced against *T. pallidum* affected cells, as cross-reactivity with other treponemal species (i.e., yaws, pinto and bejel), and in autoimmune diseases (e.g., systemic lupus erythematosus), malaria, vaccinia, mononucleosis, leprosy, viral pneumonia, malignant neoplasms, and even pregnancy and advanced age [18,20].

**Table 1.3** Performance of VDRL, RPR and TPPA serological tests [21]

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity by stage of untreated syphilis (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
<td>Secondary</td>
</tr>
<tr>
<td>VDRL</td>
<td>78 (74-87)*</td>
<td>100</td>
</tr>
<tr>
<td>RPR</td>
<td>86 (77-99)</td>
<td>100</td>
</tr>
<tr>
<td>TPPA</td>
<td>88 (86-100)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Range in Centers for Disease Control and Prevention studies

**Venereal Disease Reference Laboratory Test**
The Venereal Disease Reference Laboratory (VDRL) test is a rapid flocculation test that is FDA-approved for qualitative and semiquantitative detection of reagin antibodies in human sera [24]. There are several key ways that VDRL differs from the RPR test:

- VDRL utilizes glass slides with reaction circles instead of a plastic coated card;
- Each glass card has twelve reaction circles instead of ten;
- Serum is the only acceptable specimen type;
- Sera need to be inactivated for 30 minutes at 56°C prior to conducting the
test to inactivate complement;
- Results are read under a microscope instead of macroscopically;
- No carbon particles are required in the antigen solution for visualization;

Otherwise, the principles and basis of the VDRL are very similar to the RPR.

The VDRL assay measures IgM and IgG antibodies released by the body in response to lipoidal material discharged from damaged host cells and reagin released from treponemes [21,24]. As with the RPR test, if antibodies are present, they agglutinate to the antigen containing cardiolipin, lecithin and cholesterol in a saline solution. The reaction must be read under a light microscope at 10x magnification [21]. Reactive results will have some degree of clumping and clusters, while nonreactive specimens will have no clumping.

The VDRL is also a relatively accurate screening tool for syphilis; however, sensitivity and specificity are dependent on clinical stage of disease with the highest sensitivity being reported during secondary syphilis (Table 1.3) [23]. Overall sensitivity and specificity match that of the RPR, with the exception of sensitivity during primary syphilis where the VDRL is slightly lower than that of the RPR (78% vs. 86%) (Table 1.3) [18,21]. Also similarly to the RPR, the antilipoidal antibodies produced react not only against *T. pallidum* affected cells, but with other treponemal species (i.e., causing yaws, pinto and bejel), and are common in nontreponemal disorders and diseases [18,21].

### 1.3.2 Treponemal Serologic Tests

Treponemal tests for *T. pallidum* have traditionally been used as a confirmatory tool to aid in the diagnosis of syphilis. That paradigm is now shifting as some laboratories are beginning to utilize a treponemal test to screen and a nontreponemal test to confirm the diagnosis of syphilis [17]. Unlike the RPR and VDRL nontreponemal tests discussed previously, treponemal tests detect antibodies directly formed against *T. pallidum*. Two limitations with many treponemal assays are that they remain reactive for years with or without treatment (specifically assays that detect IgM and IgG antibodies) and that titers correlate poorly with disease activity, thus these tests should not be performed to examine response to treatment, relapse or reinfection of syphilis in previously treated populations [19]. There are a number of treponemal tests currently available in the US. This dissertation discusses the three treponemal tests that were evaluated in the studies: the *Treponema pallidum* Passive Particle Agglutination (TPPA), TREP-SURE™ enzyme immunoassay (EIA) and Syphilis Heath Check rapid test.

*Treponema pallidum* Passive Particle Agglutination

The TPPA test is a qualitative assay used for the detection of *T. pallidum* antibodies in human serum and plasma to aid in the diagnosis of syphilis [25]. The principle of the test is based on agglutination of antibodies to colored gelatin particles sensitized
with the Nichols strain of \emph{T. pallidum} antigen. The antigen is the same as the Microhemagglutination \emph{T. pallidum} (MHA-TP) assay, however, as it utilizes colored gelatin particles instead of erythrocytes for sensitization, nonspecific reactions with plasma are eliminated [19]. The reaction is carried out in a 96-well “U” shaped microplate, which allows a maximum of 24 specimens to be run at once (not including controls). Results are read macroscopically after a two-hour incubation at room temperature to allow the antigen-antibody complexes to form. If specific \emph{T. pallidum} antibodies are present in the serum or plasma specimen, a smooth mat of agglutinated particles will form in the microplate tray indicating a reactive result. A compact button formed by the settling of non-agglutinated particles is indicative of a nonreactive reaction [25].

The TPPA assay is an accurate diagnostic test to aid in the diagnosis of syphilis. However, like other serological tests for syphilis, its sensitivity and specificity are affected by clinical stage of disease [18]. Sensitivity of the assay is highest (100%) during secondary and early latent syphilis and lowest during primary syphilis (88%) (Table 1.3) [21]. As the case with all laboratory tests, there are limitations of the methodology. These include some cross-reactivity with other treponemal species, specifically yaws and pinta and false-positive results from patients with HIV, leprosy, toxoplasmosis, \emph{Helicobacter pylori} and drug addiction [25]. Like many other treponemal assays that detect both IgM and IgG antibodies to \emph{T. pallidum}, the TPPA cannot differentiate between current and past treated infection. A structural consideration of the test is that once the sensitized and unsensitized particles are reconstituted, they expire in seven days so laboratories will usually batch specimens and perform the TPPA at certain intervals (e.g., weekly or bi-weekly) so that reagents are not wasted.

\textbf{TREP-SURE\textsuperscript{TM} Enzyme Immunoassay}

Due to the ease of automation and the possibility of detecting a large range of antibodies and antigens, enzyme immunoassays (EIAs) are commonly utilized in infectious diseases testing [23]. With the resurgence of syphilis in recent years, many EIAs have been developed to aid in the diagnosis of syphilis [27]. Enzyme immunoassay tests have been demonstrated to provide at least equal performance with regards to sensitivity and specificity when compared to other treponemal tests, with the added advantage of objective interpretation of results [19]. Although the basic principle of each method is largely the same, many EIAs vary in antigenic makeup and the specific antibodies that are identified (i.e., IgM and IgG). The majority of EIA tests utilize an enzyme-linked immunosorbent assay (ELISA) format with various antigens including nonspecific (cardiolipin/lecithin/cholesterol mixtures), specific purified \emph{T. pallidum} extracts (Nichols strain) and individual or combination recombinant \emph{T. pallidum} proteins [27].

The advantages of screening with EIAs are that they can be fully automated, thus reducing the amount of manual labor required for performing the test and freeing up laboratory staff time to perform other activities and provide objective qualitative and quantitative results. These quantitative (i.e., optical density values and signal-
to-cutoff ratios) results offer an additional dimension to help identify how much antibody to *T. pallidum* is present in the specimen. Unfortunately, EIA platforms that detect IgM and IgG antibodies also suffer from the same limitations as other treponemal assays, mainly the inability to differentiate between current and past treated infection.

The EIA assay that was evaluated for the purposes of this dissertation is the TREPSURE™ EIA (TS-EIA) (Phoenix Bio-Tech Corp, Jamestown, NY). This test utilizes the typical ELISA sandwich format. Specific recombinant treponemal antigens are coated onto a standard 96-well microtiter plate. Anti-treponemal antibodies found in patient serum or plasma will bind to the specific antigen-coated wells with all unbound proteins removed during an initial wash step. A Horseradish Peroxidase (HRP) conjugated treponemal antigen is subsequently added to the antigen-antibody complex and a second wash step is performed to remove any unbound conjugate. A chromogenic reaction occurs after a substrate for the peroxidase, tetramethyl benzidine (TMB), is added. The resulting color is read spectrophotometrically at 450nm after a stop solution is added to halt the reaction. Color intensity correlates with the amount of antibody present in the patient sample. For this assay, an index value of <0.80 indicates a ‘negative’ result and an index value of >1.20 is considered ‘positive’ [26].

**Syphilis Health Check Rapid Test**

With the advent of HIV rapid tests in the US in 2003 and wide utilization by community based organizations and publicly funded testing sites, access to HIV testing services are now easier to obtain than ever before [28]. Many rapid tests have been developed for other infectious diseases as well. A number of these rapid point-of-contact (POC) syphilis tests are commercially available internationally and exist in either the traditional lateral flow immunochromatographic strip test or agglutination tests of latex particles coated with treponemal antigen [19]. However, the use of rapid tests for syphilis in high prevalence settings may be difficult, as it is assumed that a high number of the infected population will have antibodies from previous syphilis infections [29]. The World Health Organization (WHO) suggests that rapid POC syphilis tests can be a great aid in areas where access to a laboratory is difficult and low patient return rates are high [30]. For these very reasons, a rapid syphilis testing strategy in the US would provide immediate results, thus reducing loss of clients to follow-up and provide instant referral to treatment and care.

In addition to the use of rapid tests in POC venues, these assays are also utilized in traditional laboratory settings as well. Rapid influenza and HIV tests are routinely utilized in the laboratory as a way to provide prompt diagnosis of infection [41,49,50]. This dissertation explores the potential use of a rapid syphilis test (RST) in the laboratory as an aid in the prompt diagnosis of syphilis.

On August 1, 2011, the FDA announced the approval of the first POC test for syphilis [51] in the US. The Syphilis Health Check (Diagnostics Direct LLC, Stone Harbor, NJ) RST is a membrane-based immunochromatographic assay for the qualitative
detection of *T. pallidum* IgM and IgG antibodies in human serum, plasma and whole blood [31]. It can be used as an initial screening test or in conjunction with a nontreponemal assay (e.g., RPR or VDRL) to aid in the diagnosis of syphilis. It has high agreement with other treponemal tests (98%) and results are available in only ten minutes [31]. The principles of the test are the same as other lateral flow rapid tests. Depending on specimen type utilized one (serum/plasma) or two (whole blood) drops of specimen are placed into the sample well followed by four drops of a wash buffer. The specimen travels along the immunochromatographic membrane and if antibodies to *T. pallidum* are present, they will bind to a specific anti-human immunoglobulin and synthetic protein combination on the membrane forming a pink line on the test area of the strip indicating a reactive result. A nonreactive result will have no pink line [31]. The test is available as a Clinical Laboratory Improvement Amendments (CLIA) moderate complexity assay. No evaluations of this assay have been conducted in the US due to its recent introduction; however, assessments of similar tests internationally have yielded promising results with performance similar to other treponemal tests [30,72].

### 1.3.3 IgM Specific Treponemal Serologic Tests

In addition to the five main tests that were described previously, a follow-up assay was performed to assist in the determination of syphilis status of the specimens in the study. This method detects IgM-only specific antibodies in human sera so they were utilized to examine whether the individual being tested had recently acquired syphilis infection. The assay was used only to further explain discordant test results and was not intended to be part of standard of care or used in a multi-test algorithm. The addition of this test would add cost and time to the diagnosis of syphilis.

**CAPTIA Syphilis-IgM Enzyme Immunoassay (EIA)**

Unlike the TREP-SURE™ EIA discussed earlier, the CAPTIA Syphilis-IgM EIA test (Trinity Biotech, Jamestown, NY) is an antibody capture assay for the detection of only IgM antibodies to *T. pallidum*. Therefore, it is designed to detect untreated syphilis infection during the primary and secondary stages of disease. During early primary syphilis, IgM antibodies are the first to appear [52]. IgG class antibodies reach significant titers later in the primary stage [54]. As disease progresses into the secondary stage of infection, IgM and IgG antibodies reach peak titers [52]. The test is also susceptible to treated syphilis infection as IgM levels decline, eventually to negativity. The rate of decline depends on the stage of infection when treatment commenced. Therefore syphilis treated in the primary stage will demonstrate a more rapid IgM antibody decline than patients treated at a late stage of infection [53].

The CAPTIA Syphilis-IgM EIA test is an FDA-approved IgM antibody detection assay for *T. pallidum* in the US. Goat antihuman IgM antibodies are coated on the inner surface of a microtiter plate well. Patient serum is diluted and added to the well. During the one-hour incubation stage, a proportion of human IgM antibodies to *T. pallidum* are captured and bound to the well. Unbound human antibodies are
removed during the wash step. A conjugate comprised of purified *T. pallidum* antigen labeled with HRP is then added to each well and binds to the antibody-antibody complex. Any unbound conjugate is subsequently removed during the next wash step. Surface-bound HRP is detected by reaction with a chromogenic substrate. The resulting color is read spectrophotometrically at 450nm after a stop solution is added to halt the reaction. The intensity of color from the reaction is directly proportional to total serum IgM found in the sample. The assay is performed in a typical 96-well microtiter plate [52].

**Table 1.4** Types of diagnostic tests used according to stage of syphilis infection [5]

<table>
<thead>
<tr>
<th>Stage of Infection</th>
<th>Approximate Time from Infection</th>
<th>Antibody Type Produced</th>
<th>Optimal Diagnostic Test Type Utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>3-90 days</td>
<td>IgM &amp; IgG</td>
<td>Nontreponemal &amp; Treponemal</td>
</tr>
<tr>
<td>Secondary</td>
<td>2-12 weeks</td>
<td>IgM &amp; IgG</td>
<td>Nontreponemal &amp; Treponemal</td>
</tr>
<tr>
<td>Early Latent</td>
<td>&lt;1-4 years</td>
<td>IgG</td>
<td>Nontreponemal &amp; Treponemal</td>
</tr>
<tr>
<td>Late Latent</td>
<td>4+ years</td>
<td>IgG</td>
<td>Treponemal</td>
</tr>
<tr>
<td>Tertiary</td>
<td>5-30+ years</td>
<td>IgG</td>
<td>Treponemal</td>
</tr>
<tr>
<td>Test</td>
<td>Type</td>
<td>Assay Format</td>
<td>Result Type</td>
</tr>
<tr>
<td>------------</td>
<td>------</td>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>RPR</td>
<td>NT</td>
<td>Agglutination</td>
<td>Qualitative/Quantitative</td>
</tr>
<tr>
<td>VDRL</td>
<td>NT</td>
<td>Agglutination</td>
<td>Qualitative/Quantitative</td>
</tr>
<tr>
<td>TPPA</td>
<td>T</td>
<td>ELISA</td>
<td>Qualitative/Quantitative</td>
</tr>
<tr>
<td>TS-EIA</td>
<td>T</td>
<td>Agglutination</td>
<td>Qualitative</td>
</tr>
<tr>
<td>HC RST</td>
<td>T</td>
<td>Lateral Flow</td>
<td>Qualitative</td>
</tr>
<tr>
<td>CAPTIA IgM</td>
<td>T</td>
<td>ELISA</td>
<td>Qualitative/Quantitative</td>
</tr>
</tbody>
</table>

Note: NT = nontreponemal; T = treponemal
1.4 APHL/CDC Expert Consultation Meeting

Advances in STD testing technologies in the past decade have brought a number of new methodologies onto the market. These technologies have provided laboratories with the opportunity for more rapid and accurate diagnosis of STDs [32]. The Association of Public Health Laboratories (APHL) in conjunction with CDC convened an expert consultation January 13-15, 2009 to assess the current state of syphilis diagnostics and provide recommendations for the forthcoming CDC Guidelines for Laboratory Diagnosis of Treponema pallidum in the United States. One of the major conclusions of the workgroup included a proposed high throughput treponemal test as the initial screen followed by a nontreponemal test to confirm a syphilis diagnosis [32]. Other research recommendations that require further study include the role of syphilis POC tests in the United States and the prevailing thought that a single test cannot be used to diagnose syphilis. With many unanswered questions, CDC testing guidelines for syphilis remain in limbo. This dissertation will provide APHL, CDC and other entities with important data to help inform these decisions regarding guidance.

1.5 Research Study Papers

This dissertation follows the three-paper format with an overall unifying introduction and conclusion describing a novel algorithm for the diagnosis of syphilis in a high prevalence setting, San Francisco, CA. The first paper is a direct comparison between the traditional serological algorithm and the reverse sequence algorithm. The second paper outlines a strategy to minimize the amount of second supplemental treponemal tests that need to be performed to decrease the bulkiness of the reverse sequence algorithm. The third paper describes how turnaround time to report positive results can be reduced by utilizing a rapid syphilis test as part of an algorithm.

1.6 Institutional Review Board Approval and Human Subjects

The project received institutional review board (IRB) approval from the University of California, San Francisco (UCSF) Committee on Human Research (CHR) on March 14, 2012. The IRB approval allows for researchers on the project to access the San Francisco Department of Public Health (SFDPH) informatics database, mLab, to lookup follow-up information to resolve discordant test results. This information helped to identify any history of previous syphilis infections, clinical stage of infection (i.e., primary, secondary, latent, tertiary) and whether the client was on antibiotic treatment at time the specimen submitted for testing was drawn.

The University of California, Berkeley (UCB) Committee for Protection of Human Subjects will rely on UCSF CHR’s IRB approval for the project. The Notice of Intent to Rely (NOITR) paperwork was submitted to UCB on April 12, 2012 and approved on August 8, 2012.
1.7 Funding Source

This project was supported in part by Cooperative Agreement # U60HM000803 from CDC. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC. Student support was provided by the California Department of Public Health (CDPH), through the LabAspire program, which was administered by the University of California, Berkeley.
Chapter 2

Evaluation of an Enzyme Immunoassay in an Alternative Syphilis Diagnostic Algorithm: Implications for a High Prevalence Setting

2.1 INTRODUCTION

There has been a large resurgence in syphilis cases in the past decade with the Centers for Disease Control and Prevention (CDC) reporting 13,970 cases of primary and secondary (P&S) syphilis cases in 2011. This is more than twice the number of reported cases in 2001 [8, 55]. Men who have sex with men (MSM) are disproportionately affected, accounting for 72% of new P&S cases [8]. California has the greatest number of P&S syphilis cases (n=2,443) in the nation and has the sixth highest rate of 6.6 cases per 100,000 persons in 2011 [12]. San Francisco County, California has one of the highest rates of P&S syphilis in the country, accounting for the third most cases annually (n=388) and the second highest rate (48.2 cases per 100,000 persons) in the United States in 2011 [15, 56]. These data indicate that San Francisco would be an excellent setting in which to examine how an alternative syphilis serological testing algorithm would perform in a high prevalence setting.

Traditional serological syphilis testing in the US currently includes screening with a nontreponemal assay (e.g., rapid plasma reagin [RPR] or Venereal Disease Research Laboratory [VDRL]) that detects antibodies to lipoidal material released from host cells damaged by *T. pallidum* in the blood as well as lipids on the surface of the treponemal organism itself [3, 6, 19]. Specimens with reactive nontreponemal tests are subsequently confirmed through the use of a test to detect antibody specific for *Treponema pallidum* (termed “treponemal”). Popular examples of treponemal tests include, the *Treponema pallidum* passive particle agglutination [TPPA] or fluorescent treponemal antibody-absorption test [FT-ABS]) [19]. Figure 1.2 illustrates the traditional serological testing algorithm. It has been suggested that this traditional serological algorithm could be reversed so that screening begins with a treponemal test, such as an enzyme immunoassay (EIA), and if reactive, infection would be confirmed with a nontreponemal test [17, 33, 38, 58]. This suggestion was stimulated by the automation of some screening methods, which opened up the possibility of increasing laboratory workflow, alleviating manual labor and potentially decreasing costs [33]. In cases of discordance between the screening treponemal test and the confirmatory non-treponemal test, the CDC suggests that a second treponemal test (such as a TPPA) could be utilized to serve as a supplemental test [17].

A reverse sequence testing algorithm (Figure 2.1) presents several advantages over the traditional algorithm for laboratories. Screening with an immunoassay (IA): (1) allows for the use of automation, thus potentially increasing laboratory efficiency;
(2) provides objective instead of subjective results; (3) reduces the amount of manual manipulation that is involved with nontreponemal assays. An algorithm that includes treponemal immunoassay based screening however may present certain dilemmas. Perhaps greatest amongst them is that such tests will be reactive in cases where the patient has previously been treated for syphilis. This may be particularly problematic in a setting with a high prevalence of syphilis where many patients being tested may have had a previous case of the disease.

The objective of this study was to directly compare the performance of the reverse sequence syphilis algorithm to the traditional algorithm in order to assess how screening with a treponemal EIA would function in the high prevalence of San Francisco, CA.

Figure 2.1 Potential Reverse Sequence Syphilis Testing Algorithm
2.2 MATERIAL AND METHODS

2.2.1 Specimen Collection

Serum specimens were prospectively collected from July 3, 2012 through August 15, 2012 from two STD clinics in San Francisco. These clinics were selected because they provide the most complete clinical follow-up information of all the sites that submit for STD testing in San Francisco. In total, 2,350 specimens were included in the study. A pilot study consisting of 330 specimens was conducted to provide proof of concept that a reverse sequence syphilis algorithm was feasible to conduct. The specimens from the pilot were included in the final evaluation of 2,350 specimens.

2.2.2 Traditional Syphilis Algorithm

The traditional serological algorithm performed for the study consisted of a VDRL (BD, Franklin Lakes, NJ) nontreponemal screen of all 2,350 serum specimens. A follow-up TPPA (Fujirebio Diagnostics, Inc., Malvern, PA) treponemal test was conducted to confirm all reactive VDRL results. Titers for all positive syphilis results were also obtained by VDRL and results were reported electronically to the corresponding clinician according to standard operating procedures of the San Francisco Department of Public Health Laboratory (SFDPHL). A state certified Public Health Microbiologist at the SFDPHL performed all testing for the traditional algorithm.

2.2.3 Reverse Sequence Syphilis Algorithm

Once syphilis results according to the traditional algorithm were reported out, remnant serum from all 2,350 specimens were aliquoted into new storage vials and stripped of any identifying information. A study number was assigned to each sample and each specimen then received a TREP-SURE™ EIA (TS-EIA) (Trinity Biotech, Jamestown, NY) and TPPA test. The TS-EIA is an antibody sandwich assay that detects both IgM and IgG antibodies to \textit{T. pallidum} in human serum or plasma [26]. Results are provided in the form of a qualitative output (i.e., positive, negative, or equivocal) and quantitative output (i.e., optical density [OD] value and signal-to-cutoff [S/CO] ratios). The S/CO ratio (or index value) of each result is calculated by dividing the OD value by the mean of the cutoff calibrator controls. These index values determine the qualitative output of each specimen tested and are proportional to the amount of antibody to \textit{T. pallidum} found in each specimen. For the TREP-SURE™ assay, an index value of <0.80 is considered ‘negative’, >1.20 is considered ‘positive’ and values from 0.80 to 1.20 are considered ‘equivocal’ [26].

2.2.4 Follow-up Data Collection

The CDC published a suggestion that a second treponemal test could be performed in cases where the initial treponemal EIA screen is not confirmed by a
nontreponemal test (e.g. RPR or VDRL) [17,32,57]. However, in such an algorithm, the initial screen and third supplemental test are both treponemal antibody tests that detect both IgG and IgM and therefore would be unable to differentiate between current and past-tREATED syphilis infection. For that reason, we performed an additional treponemal test that specifically detects only IgM antibodies to *T. pallidum* to help resolve discordant TS-EIA and VDRL results.

Assays that are designed to specifically identify *T. pallidum* IgM antibodies may be useful tools to detect early and active (i.e., not previously treated or not latent) syphilis infection [37,58,59]. The CAPTIA Syphilis-IgM Capture EIA (Trinity Biotech, Jamestown, NY) is a Food and Drug Administration (FDA) approved test for detection of syphilis IgM antibodies. It is an antibody capture assay specifically designed to detect IgM antibodies to *T. pallidum* as an aid to identify active syphilis infection. Similarly to the TREP-SURE™ EIA, the CAPTIA assay also provides a qualitative and quantitative result. A S/CO ratio of <0.90 is considered ‘negative’ and >1.10 is considered ‘positive’. Index values from 0.90 to 1.10 are ‘equivocal’ [52].

Institutional review board approval allowed researchers to review the SFDPH laboratory information system (LIS) for pertinent information related to a patient’s previous syphilis infection history and stage of disease. A patient was considered to have had prior syphilis infection if there was evidence of a previous TPPA reactive result in the LIS. Information about a patient’s clinical stage of syphilis infection (i.e., primary, secondary, early latent, late latent, tertiary) was also examined. This information was utilized to gain a better understanding of each specimen’s clinical profile.

### 2.2.5 Comparison of Algorithms Performance

Once results for all three tests (VDRL, EIA and TPPA) were obtained, comparisons between the traditional and reverse sequence syphilis algorithms were conducted. The total number of syphilis infections identified along with sensitivity, specificity and positive predictive value (PPV) for the reverse sequence algorithm were calculated. For the purposes of this study, the traditional algorithm of VDRL and TPPA was considered the “gold standard.” The traditional serological algorithm has been utilized and accepted by laboratories across the globe as the main strategy for the diagnosis of syphilis for many years and provides an accurate diagnosis of active syphilis infection [17,46,58,60]. Therefore, all comparisons of the reverse sequence algorithm were directly calculated against the traditional algorithm. Statistical analyses were conducted using STATA version 12.1 (StataCorp, College Station, TX).

### 2.2.6 Institutional Review Board Approval and Human Subjects

The project received institutional review board (IRB) approval from the University of California, San Francisco (UCSF) Committee on Human Research (CHR). The University of California, Berkeley (UCB) Committee for Protection of Human Subjects relied on UCSF CHR’s IRB approval for the project.
2.3. RESULTS

2.3.1 Results from Traditional Syphilis Algorithm

For the purposes of this study, it was necessary to define a “gold standard” to which the reverse sequence algorithm will be compared. Direct detection methods such as dark-field microscopy or direct fluorescent antibody (DFA) tests are not routinely performed in the United States, nor are there any molecular (DNA-based) methods for the direct detection of syphilis that are FDA-approved [61,62,63]. Direct methods also require timely specimen collection from patients since scrapings from syphilitic lesions are required for testing. All of these factors make direct detection methods difficult. For these reasons, serology is routinely performed for the diagnosis of syphilis. Due to its high sensitivity and specificity at various stages of syphilis infection, the TPPA treponemal test was selected as the basis for comparison to other treponemal tests [21]. The traditional syphilis algorithm of VDRL screen followed by TPPA confirmation was selected as the “gold standard” algorithm since it has been widely accepted as the standard algorithm [57,58,64].

The results of the traditional two-test algorithm consisting of 2,350 nontreponemal screens by VDRL followed by a treponemal confirmation by TPPA are illustrated in Figure 2.2. Overall, 198 (8.4%) of VDRL tests screened were reactive or weakly reactive, thus requiring a confirmatory test. Of those, 189/198 (95.5%) were TPPA reactive and 9/198 (4.5%) were TPPA nonreactive. The nine TPPA nonreactive specimens were considered VDRL biological false positives. Thus the traditional algorithm was able to identify 189 current and active syphilis infections.

![Diagram](image)

**Figure 2.2** Results from the traditional syphilis serological algorithm, beginning with a VDRL screen and concluding with a TPPA confirmation.
2.3.2 Results from Reverse Sequence Syphilis Algorithm

The overall results of the three-test reverse sequence algorithm consisting of treponemal screens by TREP-SURE™ EIA and followed by a nontreponemal confirmation by VDRL are illustrated in Figure 2.3. When the algorithm is reversed to begin with the TREP-SURE™ EIA, there was a large increase of positive screening test results, with 478 (20.3%) TS-EIA specimens testing positive on the initial screen. Of the specimens that tested positive by EIA, 186/478 (38.9%) were reactive upon subsequent VDRL analysis. Therefore, the reverse sequence algorithm, beginning with a treponemal EIA was able to detect 186 current and active syphilis infections. All 186 specimens were also TPPA reactive, thus confirming our conclusion of syphilis infection. The remaining 292 specimens that tested VDRL negative were subject to a supplemental test, which was another treponemal test, the TPPA. Of those specimens, 246/292 (84.3%) were TPPA reactive and 44/292 (15.1%) were TPPA nonreactive. There were an additional two specimens that were repeatedly inconclusive on TPPA and therefore excluded from the study. In clinical practice, these specimens would require a redraw for repeat testing.

![Diagram](image)

**Figure 2.3** Results from the reverse syphilis serological algorithm, beginning with an EIA screen utilizing the TREP-SURE™ and confirming with a VDRL. Discordant EIA/VDRL results were followed-up with a third test, the TPPA.
2.3.3 Follow-up Analysis of Discordant Results

An issue that required investigation was a determination of the actual status of the 246 TS-EIA positive, VDRL nonreactive, TPPA reactive specimens. Such specimens could represent patients who were previously infected by *T. pallidum*, and either cleared or were treated for the infection. These specimens could also represent patients with early, primary infection and are perhaps positive on the TS-EIA, while nonreactive by VDRL. In order to determine this all 292 TS-EIA positive, VDRL nonreactive discordant specimens were tested by the CAPTIA Syphilis-IgM Capture EIA (Trinity Biotech, Jamestown, NY). This FDA-approved assay is designed to detect human IgM antibodies directed towards *T. pallidum* and aid in the identification of early and active syphilis [37,52,58,59,65].

Results from the follow-up testing performed on the CAPTIA Syphilis-IgM Capture EIA are shown in Table 2.1. Of the discordant specimens tested, 0/292 (0%) demonstrated reactivity with the CAPTIA assay, indicating that they lacked detectable IgM antibodies to *T. pallidum* and therefore may not be associated with active syphilis infections.

Further examination into the SFDPH LIS database indicated that of the 246 TS-EIA positive, VDRL nonreactive, TPPA reactive specimens, 112 had a previous TPPA reactive result, which indicates a past-treated syphilis infection. The remaining 134 specimens had no locatable history of past treated syphilis infection and all tested negative for IgM antibodies on the CAPTIA assay. Unfortunately, no further clinical examination or observation notes were available for analysis.

<table>
<thead>
<tr>
<th></th>
<th>TREP-SURE™ EIA</th>
<th>VDRL</th>
<th>TPPA</th>
<th>CAPTIA Syphilis-IgM Capture EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive</td>
<td>292</td>
<td>0</td>
<td>246 (84.2%)</td>
<td>0</td>
</tr>
<tr>
<td>Nonreactive</td>
<td>0</td>
<td>0</td>
<td>44 (15.1%)</td>
<td>289 (99.0%)</td>
</tr>
<tr>
<td>Equivocal/Inconclusive</td>
<td>0</td>
<td>N/A</td>
<td>2 (0.7%)</td>
<td>3 (1.0%)</td>
</tr>
</tbody>
</table>

2.3.4 Analysis of Algorithms

Tabulations of how each specimen performed on all three tests were calculated and shown in Table 2.2. There were 186 specimens that were identified as having syphilis infection by all three tests (VDRL, TPPA and TS-EIA). An additional three specimens were VDRL reactive and TPPA reactive, thus considered syphilis infected by the traditional algorithm. Therefore the traditional algorithm identified a total of 189 syphilis infections. The reverse sequence algorithm, beginning with the TS-EIA, identified 186 syphilis infections.
Table 2.2 Summary of overall testing outcomes for VDRL, TPPA and TS-EIA

<table>
<thead>
<tr>
<th>VDRL</th>
<th>TPPA</th>
<th>TS-EIA</th>
<th># of Specimens</th>
<th># Detected Traditional</th>
<th># Detected Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1845</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>44</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>246</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>186</td>
<td>186</td>
<td>186</td>
</tr>
</tbody>
</table>

Total 2341\textsuperscript{a} 189 186

\textsuperscript{a}Four TPPA inconclusive and five TS-EIA equivocal results were excluded from final tally

The sensitivity, specificity and positive predictive value (PPV) of the reverse sequence algorithm were calculated as two distinct scenarios to demonstrate the drastic differences of infections identified if all of the supplemental treponemal TPPA results were considered positive. The first calculation (a) accounts for only the 186 specimens identified as TS-EIA positive and VDRL reactive. It assumes that all of the 246 reactive TPPA supplemental tests were past-treated infections and not active infections. The second calculation (b) accounts for the 186 TS-EIA positive, VDRL reactive plus 246 TPPA reactive supplemental tests as being syphilis infected. Both calculations are compared to the “gold standard” traditional algorithm. The comparisons are illustrated in Table 2.3.

Table 2.3 Sensitivity, specificity and PPV of two reverse sequence algorithm scenarios compared to “gold standard” traditional algorithm

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Infections Detected</th>
<th>Sensitivity 95% CI</th>
<th>Specificity 95% CI</th>
<th>PPV 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional (VDRL + /TPPA +)</td>
<td>189</td>
<td>100.0%</td>
<td>98.1%-100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>(a) Reverse (TS-EIA+ /VDRL +)</td>
<td>186</td>
<td>98.4%</td>
<td>95.4%-99.6%</td>
<td>100.0%</td>
</tr>
<tr>
<td>(b) Reverse (TS EIA + /TPPA +)</td>
<td>432</td>
<td>98.4%</td>
<td>95.4%-99.6%</td>
<td>88.6%</td>
</tr>
</tbody>
</table>

The (a) reverse algorithm (TS-EIA positive/VDRL reactive) yielding 186 infections identified is the more likely scenario for this study and does not significantly differ from the traditional algorithm that identified 189 syphilis infections. The 246 TPPA reactive results most likely are detecting previously treated and past infection. The
low PPV in the (b) reverse sequence algorithm is due to the scenario where the 246 TPPA reactive results are considered falsely identified infections.

2.4 DISCUSSION

To our knowledge, this is the first comprehensive laboratory study directly comparing the reverse sequence syphilis algorithm to the traditional serologic algorithm in a high prevalence setting. Two treponemal and one nontreponemal test were performed on all specimens in the study. Other studies have prospectively investigated the performance of a reverse sequence algorithm; however, such studies did not have complete and full testing data on each specimen [36-38,57,64]. Additionally, the study described herein included all specimens with discordant treponemal screening and nontreponemal results were tested by an IgM treponemal antibody capture assay. Clinical information, including information such as past-treated syphilis infection and stage of disease was also evaluated.

The proportion of specimens that were found positive by EIA (20.3%) in this population was higher than that of other published reports from high prevalence settings in New York (15.3%) and Chicago (13.3%) [36,37]. In the case where a third test is utilized to resolve discordant screening and confirmatory results, having a high prevalence of previous syphilis cases in the testing population may trigger a large amount of unnecessary testing. This appeared to be the case in San Francisco. The data herein indicate that the vast majority of EIA-positive, VDRL-nonreactive specimens may represent previous and not current infection. It is therefore unclear to what degree a second treponemal test can aid in the resolution of discordant specimens. Currently, no national guidelines exist to help direct laboratories, health departments and clinicians on how to resolve such discrepancies in test results. The data described herein would indicate that the use of a second treponemal test to resolve discordant specimens might have questionable value.

Results from the CAPTIA Syphilis-IgM Capture indicated no active syphilis infections for any of the 292 discordant screening and confirmatory results. This finding likely indicates that the 44 specimens that were solely reactive by TREP-SURE™ EIA results were falsely positive. These data also suggest that 246 supplemental TPPA reactive specimens are most likely past treated infection. Although unlikely, untreated late latent or tertiary disease cannot be completely ruled out and these patients should receive clinical follow-up to investigate their previous syphilis exposure and treatment history. The VDRL and CAPTIA EIA may have missed these cases because IgM titers and lipoidal antibodies diminish during those stages of disease [57,65,66].

Despite its aforementioned advantages of automation and providing subjective results, a three-test reverse sequence syphilis serologic algorithm in a high prevalence setting such as San Francisco may result in additional work and cost.
With such a high number of previously infected individuals in the screening population, (as also demonstrated by the low concordance between the TREP-SURE™ EIA and VDRL), an algorithm with a treponemal assay as the primary screening test would necessitate a large number of third supplemental treponemal tests that would have to be performed. In addition, the laboratory results provided to clinicians would be unclear. In our study, an additional 246 patients in a seven-week time period would require clinical follow-up including an evaluation of previous syphilis exposure, risk and treatment status. The discordant results would also require additional investigation by the health department to discern how many of the 246 additional patients identified were primary and secondary syphilis cases for the purposes of surveillance.

A three-test reverse sequence algorithm in a high prevalence setting may be especially cumbersome for the laboratory. There would be additional cost, quality assurance and increased turnaround time that accompanies maintaining a third test. In the SFDPHL, the reverse sequence algorithm required 47% more TPPAs (n=292) versus the traditional algorithm (n=198), which adds to the amount of manual labor that must be performed. The effect that manual pipetting has on repetitive motion injury to laboratory staff must also be strongly considered.

Syphilis diagnostics poses a difficult challenge. Direct detection methods are not commonly accessible, T. pallidum cannot be cultured, and true active syphilis infection is difficult to identify serologically, especially in high prevalence settings where many individuals become infected, receive treatment, are cured, and then become re-infected again. The traditional two-test algorithm, although imperfect, utilizes a combination of nontreponemal and treponemal tests so that past-treated infections can be weeded out. This study has highlighted one of those potential flaws to the “gold standard” traditional algorithm as untreated late latent and tertiary infected individuals may be missed on a nontreponemal screen. The number of true untreated syphilis infections probably lies somewhere between 189 and 432. However, without the proper diagnostic tests and mindful clinical follow-up, it will be difficult and sometimes impossible to discern a patient’s true syphilis disease status.

There are several limitations to this study. It should be noted that the history of past syphilis infection is incomplete since it only includes patients tested in the SFDPH system. It is conceivable that some patients may have contracted syphilis in the past in a different jurisdiction, prior to being tested in San Francisco. These patients would not be identified as having previously treated syphilis infection in the SFDPH database since it was diagnosed and treated in a different location. Also, follow-up information was limited to testing history and clinical stage of disease. Clinical observation notes were sporadic and incomplete. Finally, since this was a retrospective study conducted on remnant sera, we did not have the ability to monitor and track or request pertinent follow-up specimens on individuals that may be potential seroconverters.
Although seemingly challenging for a high volume laboratory in a high prevalence setting to implement, there are some modifications to the reverse sequence algorithm that perhaps could be conducted to make it more functional in this context. The next chapter explains a possible solution that can reduce the amount of third supplemental TPPA tests that would need to be performed as part of a reverse sequence algorithm, thus potentially facilitating its use.
Chapter 3

The Role of Enzyme Immunoassay Signal-to-Cutoff Ratios in Predicting Other Treponemal Assay Results

3.1 INTRODUCTION

Most commonly, syphilis testing in the US has included screening with a nontreponemal assay (e.g., rapid plasma reagin [RPR] or Venereal Disease Research Laboratory [VDRL]) that detects antibodies to lipoidal material released from damaged host cells caused by T. pallidum in the blood as well as lipids on the surface of nonspecific treponemes [3,6,19]. Reactive nontreponemal tests are then confirmed by a secondary treponemal assay (e.g., Treponema pallidum passive particle agglutination [TPPA] or fluorescent treponemal antibody-absorption test [FT-ABS]) that detect antibodies specific to T. pallidum [19]. This algorithm is illustrated in Figure 1.2. It has been suggested that this traditional serological algorithm be reversed so that screening begins with a treponemal test, such as an enzyme immunoassay (EIA), and if reactive, the EIA would be confirmed with a nontreponemal test [17,33,38,57]. This suggestion has been inspired by the advent of automated screening methodologies, which increase laboratory workflow, alleviate manual labor and potentially decreasing costs [33]. The CDC proposes that discordant screening and confirmation results receive a third test, in this case another treponemal assay, to serve as a supplemental test [17]. Direct detection methods, such as dark field microscopy and direct fluorescent antibody (DFA) tests are no longer routinely performed by laboratories in the US, thus making syphilis serology the common tool for diagnosis [57].

This reverse sequence serologic algorithm, illustrated in Figure 2.1, presents several advantages over the traditional algorithm for laboratories: screening with an EIA or chemiluminescence immunoassay (CIA) allows for automation, thus potentially increasing laboratory efficiency; the EIA provides objective instead of subjective results; and reduces the amount of manual manipulation that is involved with nontreponemal assays. A reverse algorithm however may present some new dilemmas, the most significant of which is that past-treated syphilis infection would screen positive in such an algorithm, triggering subsequent, perhaps unnecessary testing.

This reverse sequence algorithm could be particularly cumbersome for laboratories that serve high prevalence settings. This is because there will be a large number of discordant EIA positive screening and nontreponemal (i.e., RPR or VDRL) nonreactive confirmation results. Depending upon what algorithm is utilized, such discordance may trigger a third test, such as the TPPA [17,38]. Most treponemal tests detect IgM and IgG antibodies to T. pallidum and are unable to distinguish between current and past-treated infection with syphilis [57]. This leads to
additional cost and quality assurance issues related to carrying a third syphilis test in the laboratory. Studies conducted in the areas of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) diagnostics have demonstrated clinical usefulness of signal-to-cutoff (S/CO) ratios of screening EIAs in predicting positivity of confirmation tests and in doing so reducing the number of confirmation tests that need to be performed [34,35,44,45]. The objective of this study was to evaluate how well the S/CO ratios (or index values) provided by a screening treponemal EIA can predict positivity on another treponemal assay, the TPPA, thus potentially reducing the number of supplement treponemal tests that need to be performed to resolve discordant testing results.

3.2 MATERIAL AND METHODS

3.2.1 Specimen Collection

Serum specimens were prospectively collected from July 3, 2012 through August 15, 2012 from two clinical sites with high syphilis prevalence in San Francisco, CA. These clinics were selected because they provide the most complete clinical follow-up information of all the sites that submit for STD testing to the San Francisco Department of Public Health Laboratory (SFDPHL). In total 2,350 specimens were included in the study. A pilot study consisting of 330 specimens was conducted to provide proof of concept that a reverse sequence syphilis algorithm was feasible to conduct. The specimens from the pilot were included in the final evaluation of 2,350 specimens.

3.2.2 Reverse Sequence Algorithm

Remnant serum from all 2,350 specimens was aliquoted into secondary vials and stripped of any identifying information. A study number was assigned to each sample. One nontreponemal, VDRL (BD, Franklin Lakes, NJ), and two treponemal tests, TREP-SURE™ EIA (TS-EIA) (Trinity Biotech, Jamestown, NY) and TPPA (Fujirebio Diagnostics, Inc., Malvern, PA), were performed on all 2,350 specimens. The reverse sequence syphilis algorithm was calculated beginning with the TS-EIA as the initial screen. Any positive TREP-SURE™ screening results would reflex to a VDRL nontreponemal test for confirmation. Discordant screening and confirmation results would reflex to a second treponemal test, in this case the TPPA, to model the possible resolution of the difference in initial two test results.

The TS-EIA is an antibody sandwich assay that detects both IgM and IgG antibodies to T. pallidum in human serum or plasma [26]. Results are provided in the form of qualitative (i.e., positive, negative, or equivocal) and quantitative outputs (i.e., optical density [OD] value and signal-to-cutoff [S/CO] ratios). The S/CO ratio, also referred to as the index value, of each result is calculated by dividing the OD value by the mean of the cutoff calibrator controls. These index values determine the qualitative output of each specimen tested and are proportional to the amount of
antibody to *T. pallidum* found in each specimen. For the TREP-SURE™ assay, an index value of <0.80 is considered ‘negative’, >1.20 is considered ‘positive’ and values from 0.80 to 1.20 are considered ‘equivocal’ [26].

The TPPA assay is a qualitative assay used for the detection of *T. pallidum* antibodies in human serum and plasma to aid in the diagnosis of syphilis [25]. The principle of the test is based on agglutination of antibodies to colored gelatin particles sensitized with the Nichols strain of *T. pallidum* antigen. If specific *T. pallidum* antibodies are present in the serum or plasma specimen, a smooth mat of agglutinated particles will form in the microplate tray indicating a reactive result. A compact button formed by the settling of non-agglutinated particles is indicative of a nonreactive reaction [25].

### 3.2.3 Statistical Analysis for Comparison of EIA S/CO ratio to TPPA Positivity

Receiver operating characteristic (ROC) analyses were conducted to examine the relationship between the TS-EIA S/CO ratios and TPPA test results. With this analysis, sensitivity was plotted against the false-positive rate (1 - specificity), which was used to evaluate the optimal threshold cut-off point for the assay. Each point on the ROC curve represents a different cut-off for identifying a test as positive [67]. The area under the ROC curve (AUROC) was calculated to quantify the discrimination of the EIA in predicting TPPA results. An AUROC of 1.0 signifies perfect test while 0.5 signifies that the results are no better than chance.

A kappa statistic was also calculated to further examine the level of agreement between the two tests. The kappa statistic is a measure of agreement that takes into account chance [67]. The calculation is based on the difference between how much agreement is present compared to how much agreement is present from chance alone [73]. Kappa is a commonly utilized statistic when examining overall agreement [71]. A kappa of 1 equates to perfect agreement while 0 indicates agreement equal to chance [67,73]. Statistical analyses were conducted using STATA version 12.1 (StataCorp, College Station, TX).

### 3.2.5 Institutional Review Board Approval and Human Subjects

The project received institutional review board (IRB) approval from the University of California, San Francisco (UCSF) Committee on Human Research (CHR). The University of California, Berkeley (UCB) Committee for Protection of Human Subjects relied on UCSF CHR’s IRB approval for the project.
3.3 RESULTS

3.3.1 Results of Reverse Sequence Syphilis Algorithm

The results of the three-test reverse sequence algorithm consisting of treponemal screens by TS-EIA and followed by nontreponemal confirmations by VDRL are illustrated in Figure 2.3. When screening with the TS-EIA, 478/2,350 (20.3%), were positive. Of those 478 specimens, 186/478 (38.9%) were found reactive upon subsequent VDRL analysis. All 186 specimens were also TPPA reactive, consistent with a conclusion of syphilis infection. TPPA results were surveyed for the remaining 292 specimens that tested VDRL nonreactive. Of the 292 specimens, 246/292 (84.3%) were TPPA reactive and 44/292 (15.1%) were TPPA nonreactive. Two specimens of 292 were repeatedly inconclusive on TPPA and therefore excluded from the study. In clinical practice, these inconclusive specimens would require a redraw for repeat testing.

3.3.2 Relationship between EIA S/CO ratios and TPPA test results

Through the use of ROC analyses, the relationship between TS-EIA index values and TPPA test results was calculated, along with sensitivity and specificity of the TS-EIA predicting TPPA positivity. Based on this analysis, the threshold S/CO ratio that correlated best with a positive TPPA test result was determined as ≥3.464. For this S/CO ratio, the sensitivity and specificity were determined to be 96.8% and 99.5%, respectively. Figure 3.1 illustrates the relationship between TPPA results and TS-EIA S/CO ratios. The closer the curve is to the upper left hand corner of the graph, the better the test characteristics [67]. The area under the ROC curve (AUROC) was calculated as 0.9875, which indicates that the TS-EIA has an excellent ability to predict TPPA positivity. An AUROC of 1.0 signifies a perfect test.

The kappa statistic was also calculated to examine the level of agreement between the TS-EIA and TPPA assays. Kappa was determined as 0.926, 95% CI (0.906 – 0.945). Generally, kappa classifications of 0.8-1.0 are considered as almost perfect agreement. Therefore, the TREP-SURE™ EIA seems to provide nearly perfect concordance with the TPPA. Table 3.1 describes generally accepted kappa classifications according to published literature [67,68].
Figure 3.1 Receiver Operating Characteristic (ROC) curve demonstrating the discrimination of the TS-EIA in predicting TPPA positivity. Each point on the ROC curve represents a different cut-off for identifying the TPPA as positive. Area under the ROC curve (AUROC) is also illustrated. An AUROC of 1.0 is considered perfect.

Table 3.1 Kappa classifications

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.2</td>
<td>Slight</td>
<td>Poor</td>
</tr>
<tr>
<td>0.2-0.4</td>
<td>Fair</td>
<td>Fair</td>
</tr>
<tr>
<td>0.4-0.6</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>0.6-0.8</td>
<td>Substantial</td>
<td>Good</td>
</tr>
<tr>
<td>0.8-1.0</td>
<td>Almost Perfect</td>
<td>Very Good</td>
</tr>
</tbody>
</table>

Note: A kappa of 1 equates to perfect agreement while 0 indicates agreement equal to chance [67,73]. Modifiers describe qualitative agreement between the two tests.

3.3.3 Utilizing EIA S/CO Ratios to predict the results of a TPPA within the context of a three-test, reverse sequence algorithm

With a strong agreement between TS-EIA S/CO ratios and TPPA results established, we examined the number of secondary treponemal tests that could be eliminated in the reverse sequence algorithm. In this algorithm, the SFDPHL would have needed to run an additional 292 follow-up secondary treponemal tests to help resolve the discordant TS-EIA positive, VDRL nonreactive specimens. Among these discordant specimens, 254/292 (87%) TPPA results were above the threshold index value of
≥3.464. Had the necessity to perform all of these TPPA assays been eliminated, the laboratory would only need to have performed 38 follow-up TPPA tests instead of 292 (87% fewer tests).

The ROC analysis provides the threshold values for various sensitivity and specificity levels. While the cut-off of ≥3.464 maximizes sensitivity and specificity of the relationship between TS-EIA and TPPA, it was not in perfect agreement. There were ten TPPA specimens that were either nonreactive (n=9) or inconclusive (n=1) from the 439 TS-EIA specimens with S/CO ratios ≥3.464. Table 3.2 illustrates the relationship of TS-EIA index values and TPPA results. However, with a more conservative approach to maximize specificity and ensure that all TPPA results are concordant, a higher TS-EIA index value of ≥14.354 could be used. This would eliminate 154 of the 292 (58.8%) TPPA tests required, leaving 138 specimens for follow-up.

<table>
<thead>
<tr>
<th>S/CO</th>
<th>Positive</th>
<th>Reactive</th>
<th>Nonreactive</th>
<th>Inconclusive</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.80</td>
<td>0</td>
<td>11 (0.6%)</td>
<td>1854 (99.3%)</td>
<td>2 (0.1%)</td>
</tr>
<tr>
<td>0.80-1.20</td>
<td>0</td>
<td>3 (7.7%)</td>
<td>35 (89.7%)</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>1.21-3.463</td>
<td>39</td>
<td>429 (97.7%)</td>
<td>9 (2.1%)</td>
<td>1 (0.2%)</td>
</tr>
</tbody>
</table>

Note: S/CO ratios <0.80 are considered Negative, 0.80-1.20 is Equivocal and ≥1.21 are Positive.

Figures 3.2 and 3.3 further illustrate the relationship between TREP-SURE™ EIA S/CO ratios and corresponding positive TPPA results. Figure 3.5 examines this association for all positive TS-EIA specimens (n=478) in the study and Figure 3.6 shows the subset of discordant TS-EIA positive, VDRL nonreactive specimens (n=292) from the reverse sequence algorithm performed at the SFDPHL. These graphs demonstrate the high concordance between the two assays at varying ranges of TS-EIA S/CO ratios. For example, in Figure 3.3, for index values in the range of 6.001 to 10.000, 23 of 26 (88.46%) TPPA results were reactive, thus agreeing with the TS-EIA result. Moving up to the range of 10.001 to 14.000, 27 of 29 (93.1%) TPPA results were reactive and concordant with TS-EIA results.
Figure 3.2 Relationship between TREP-SURE™ EIA S/CO ratios and corresponding positive TPPA results for all positive TS-EIA specimens (n=478) from the reverse sequence algorithm performed at the SFDPHL. The graph shows high agreement between the two tests within various threshold TS-EIA S/CO ratio ranges. Index values from 6.001-10.000 have 88.89% concordance between TS-EIA and TPPA results.
3.4 DISCUSSION

There are numerous advantages to implementing a reverse sequence algorithm in a high volume laboratory. These include automation of syphilis screening, objective results provided by an EIA or CIA, reduction of manual labor and improved workflow. Unfortunately, for high volume laboratories in high prevalence settings, this three-test reverse sequence algorithm may be cumbersome. Complexity is added to the diagnostic algorithm since there would be additional costs, quality assurance and turnaround time (TAT) with performing and maintaining an additional treponemal test.

Wong et al. at the SFDPHL previously demonstrated in a smaller study that the TS-EIA S/CO ratios showed strong correlation to TPPA [64]. This study expands on that concept and sought to further build the evidence base. We are aware of no other studies that have investigated the relationship between syphilis EIA S/CO ratios and other treponemal assays. This study has identified such an association between the TREP-SURE™ EIA and TPPA assays, more specifically, how EIA S/CO ratios could predict positivity of the TPPA.
A major drawback for a high volume laboratory performing syphilis testing in a high prevalence setting is the number of supplemental treponemal tests that need to be run in a three-test reverse sequence algorithm. For this study population, 292 TPPA tests would have been needed to help resolve discordant TS-EIA positive screening and VDRL nonreactive confirmation results. This equates to an increase in cost, quality assurance measures, labor requirements and TAT for the laboratory. However, if EIA S/CO ratios are utilized to infer TPPA results, a large proportion of supplemental TPPA tests could be eliminated. The results of this study indicate that when utilizing the optimal threshold cut-off of index value ≥3.464, 87% of supplemental third TPPA tests could be eliminated, thus only requiring 38 to be performed over the seven-week study period. At the SFDPHL, which analyzes approximately 2000 specimens per month, this equates to about five TPPA tests that would need to be performed every week instead of 42 if EIA S/CO ratios were not considered.

The TPPA assay is a labor-intensive test that requires multiple dilution and manual manipulation steps. It also requires a two-hour incubation for the antigen-antibody agglutination reaction to occur. Additionally, once the unsensitized and sensitized particles are reconstituted, they expire in seven days. This limitation requires laboratories to conduct batched testing and to perform the TPPA at specified intervals (e.g., weekly) to conserve reagent and control costs. This may cause delays in reporting positive results, thus increasing TAT.

This study demonstrates the ability of a laboratory serving a high prevalence setting to drastically reduce the amount of secondary treponemal tests performed. Given the potential need to batch specimens for cost-saving reasons, this study also highlights the possible increase in TAT.

Some limitations to this study exist. First, only one EIA was utilized, therefore generalizations about how other EIA or CIA platforms would compare to TPPA cannot be made. Also, the S/CO ratios discussed in the paper are specific to the TS-EIA; therefore threshold cut-off values will most likely differ in other EIAs.

The next chapter discusses the possibility of reducing TAT through the removal of the TPPA from a reverse sequence algorithm.
Chapter 4

The Role of a Rapid Treponemal Syphilis Test in Various Syphilis Serological Diagnostic Algorithms in a High Prevalence Setting

4.1 INTRODUCTION

The role of rapid point-of-contact (POC) tests in patient diagnostics has increased significantly in recent years. In some cases, such tests have become the most commonly used tests for diagnosis of infections such as influenza, respiratory syncytial virus (RSV) and human immunodeficiency virus (HIV) [41,49,50,69]. With the advent of HIV rapid tests in the US in 2003 and wide utilization by community-based organizations and publicly funded testing sites, access to HIV testing services is now easier to obtain than ever before [28]. A number of POC rapid syphilis tests (RSTs) are commercially available internationally and exist in either the lateral flow membrane immunochromatographic strip test or agglutination tests of latex particles coated with treponemal antigen [19]. The use of rapid treponemal tests for syphilis in a high prevalence setting may possess challenges, if it is assumed that a high number of the infected population will have antibodies from previous syphilis infections [29]. The World Health Organization (WHO) suggests that rapid POC syphilis tests could be useful as diagnostic tools in areas where access to a laboratory is difficult and patient return rates are low [30].

Rapid tests may also be useful in a laboratory setting. Rapid influenza and HIV tests are routinely utilized in the laboratory as a way to provide prompt diagnosis of infection [50,69]. The first rapid syphilis POC test was FDA-approved on August 1, 2011 [51]. The assay is considered by Clinical Laboratory Improvement Amendments (CLIA) to be a test of moderate complexity [Personal Communication, Jenks, April 2012]. Therefore, it can be performed in traditional and modified laboratory (e.g., physician’s office laboratory, STD clinic) settings that have a CLIA license for moderate complexity testing. Consequently, there is potential for a RST to replace the TPPA as either the treponemal confirmatory test in a traditional serological algorithm or as the second treponemal test in a reverse sequence serological algorithm.

Most commonly, syphilis testing in the US has involved screening with a nontreponemal assay (e.g., rapid plasma reagin [RPR] or Venereal Disease Research Laboratory [VDRL]) that detect antibodies to lipoidal material released from damaged host cells caused by T. pallidum in the blood as well as lipids on the surface of nonspecific treponemes itself [3,6,19]. Reactive nontreponemal tests are then confirmed by a secondary treponemal assay (e.g., Treponema pallidum passive particle agglutination [TPPA] or fluorescent treponemal antibody-absorption test [FT-ABS]) that detect antibodies specific to T. pallidum [19]. It has been suggested
that this traditional serological algorithm could be reversed so that screening begins with a treponemal test, such as an EIA, and if reactive the EIA would be confirmed with a nontreponemal test [17,33,38]. This suggestion is due to the ability to automate the screening methodology thus increasing laboratory workflow, alleviating manual labor and potentially decreasing costs [33]. The CDC further suggests that discordant screening and confirmation results receive a third test, another treponemal test such as the TPPA, to serve as a tiebreaker [17,46]. Figures 1.2 and 2.1 illustrate the traditional and reverse sequence serological testing algorithms. It is notable however that the addition of a third-test to a multi-test algorithm would increase turnaround time (TAT) for a laboratory to report a positive test result. This study explores the potential use of a treponemal RST in the laboratory as an aid in reducing the TAT, therefore providing a prompt diagnosis of syphilis.

4.2 MATERIALS AND METHODS

4.2.1 Specimen Collection

This study utilized frozen and stored specimens from a larger primary study conducted at the SFDHPL. Serum specimens were prospectively collected from July 3, 2012 through August 15, 2012 from two health clinics in San Francisco. These clinics were selected because they provide the most complete clinical follow-up information of all the sites that submit for STD testing in San Francisco. In total 2,350 specimens were included in the primary study.

4.2.2 Sample Selection

Specimens for this rapid syphilis test study were purposefully selected from the larger principal syphilis study, which compared the reverse sequence syphilis algorithm to the traditional serologic algorithm. Selected serum samples represented various categories of interest. These included 50 confirmed positive (reactive on VDRL, TPPA, TREP-SURE™ EIA) and 50 confirmed negative (nonreactive on VDRL, TPPA, TREP-SURE™ EIA) syphilis specimens. The remaining specimens consisted of eleven primary infections, 28 secondary infections, nine biological false positive (BFP) samples, 50 treponemal-only reactive (nonreactive on VDRL; reactive on TPPA and TREP-SURE™ EIA), 44 isolated TREP-SURE™ EIA (TS-EIA) positive (i.e., reactive on TS-EIA; nonreactive on VDRL and TPPA) and eleven TPPA reactive, TS-EIA negative specimens. The 50 confirmed positive, negative and treponemal-only reactive specimens were randomly selected from a larger pool of specimens with the same characteristics. There was no randomization performed for the other specimens in the study since they represented their entire respective category. In total, there were 249 specimens across all the categories listed.
4.2.3 Study Protocol

Once specimens were selected, they were again randomized, blinded and remnant sera were aliquoted into new test tubes and stripped of any identifying information. A new study number was assigned to each sample and each specimen was tested by the Syphilis Health Check rapid syphilis test (HC-RST). The HC-RST is a qualitative rapid membrane immunochromatographic assay that detects antibodies to *T. pallidum* in human whole blood, plasma and serum. It is FDA-approved for use as either an initial screening test or in conjunction with a non-treponemal laboratory test to aid in the diagnosis of syphilis infection [31]. The assay contains a combination of three recombinant treponemal antigens (Tp15, Tp17 and Tp44) that are bound to a membrane with anti-human immunoglobulin gold conjugate for visualization [31,70].

Two drops (50 uL) of whole blood or one drop (25 uL) of plasma or serum are added to the device along with four drops of wash buffer. As the samples flow through the device, the anti-human immunoglobulins/protein A dye conjugate binds to human immunoglobulins in the sample, forming an antigen-antibody complex. This complex binds to the recombinant protein in the positive reaction zone on the strip and produces a pink-rose colored band. If antibodies to *T. pallidum* are absent, there is no line in the positive reaction zone. The reaction continues flowing through the device and unbound conjugate binds to the reagents in the control zone producing a pink-rose color band. This demonstrates that the reagents are functioning correctly. Results are read as soon as ten minutes but no more than 15 minutes [31].

The TPPA assay is a qualitative assay used for the detection of *T. pallidum* antibodies in human serum and plasma to aid in the diagnosis of syphilis [26]. The principle of the test is based on agglutination of antibodies to colored gelatin particles sensitized with the Nichols strain of *T. pallidum* antigen. If specific *T. pallidum* antibodies are present in the serum or plasma specimen, a smooth mat of agglutinated particles will form in the microplate tray indicating a reactive result. A compact button formed by the settling of non-agglutinated particles is indicative of a nonreactive reaction [26].

Once the rapid tests were conducted on all 249 specimens, performance was analyzed and results were compared to results of the TPPA assay obtained previously from the larger primary study.

4.2.4 Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Syphilis Health Check RST were calculated, along with percent positive agreement (PPA), percent negative agreement (PNA) and percent overall agreement (POA) to the TPPA. Percent agreement is the main statistic suggested by the FDA for comparison of a new test to an established method when no “gold standard” or
A reference test is available [71]. A kappa statistic was also calculated to further examine the level of agreement between the two tests. The kappa statistic is a measure of agreement that takes chance into account [67]. The calculation is based on the difference between how much agreement is present compared to how much agreement is present from chance alone [73]. Like percent agreement, kappa is also a commonly utilized statistic when examining overall agreement [71]. Statistical analyses were conducted using STATA version 12.1 (StataCorp, College Station, TX).

4.2.5 Institutional Review Board Approval and Human Subjects

The project received institutional review board (IRB) approval from the University of California, San Francisco (UCSF) Committee on Human Research (CHR). The University of California, Berkeley (UCB) Committee for Protection of Human Subjects relied on UCSF CHR’s IRB approval for the project.

4.3 RESULTS

4.3.1 Performance of Syphilis Health Check Rapid Test

Sensitivity and specificity can only be calculated if the true status of infection is known. The analysis in this section assumes that test results from other serological assays and that the clinical classification from the SFDPH STD Prevention and Control office are correct. Therefore, specimens with concordant reactive VDRL, TPPA and TS-EIA results were considered positive. Specimens found non-reactive by VDRL, TPPA and TS-EIA nonreactive were considered negative. Additionally, specimens that were identified as primary and secondary infection by SFDPH STD Control were also considered truly positive. Conversely, any BFP (VDRL reactive, TPPA nonreactive) specimens and isolated TS-EIA positive samples were considered truly negative. Based on these classifications, there were 89 positive and 103 negative specimens.

With these assumptions, sensitivity of the HC-RST from this population was calculated to be 92.7% and specificity was 98.1%. Positive predictive value (PPV) and negative predictive value (NPV) were also calculated and determined as 97.8% and 93.6%, respectively. Table 4.1 illustrates these results.

| Table 4.1 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Syphilis Health Check rapid test |
|-----------------------------------------------|-----------------|
| Sensitivity                                 | 92.1%           |
| Specificity                                 | 98.1%           |
| Positive Predictive Value                    | 97.8%           |
| Negative Predictive Value                    | 93.6%           |

95% CI:
- Sensitivity: 85.55%-97.01%
- Specificity: 93.27%-99.71%
- Positive Predictive Value: 92.27%-99.67%
- Negative Predictive Value: 87.32%-97.39%
4.3.2 Performance of Syphilis Health Check Rapid Test on Selected “Challenge” Specimens

The HC-RST was also evaluated using a panel of specimens that represented a range of infectious states and varying characteristics. This group is referred to as the “challenge” specimens. The samples from this group include primary and secondary (P&S), BFP, falsely positive TS-EIA, discordant TS-EIA/TPPA and discordant nontreponemal (i.e., VDRL)/treponemal (i.e., TS-EIA and TPPA) specimens. Table 4.2 shows the results of HC-RST testing when performed on these “challenge” specimens.

The HC-RST was able to identify 81.8% and 96.4% of P&S specimens respectively. The sensitivity of the HC-RST was lower for discordant specimens. It was able to correctly identify 60.0% of VDRL nonreactive/TPPA reactive/TS-EIA positive specimens and only 36.4% of TPPA reactive/EIA negative specimens.

Table 4.2 Performance of Syphilis Health Check rapid test against specimens of varying categories

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Number of Specimens</th>
<th>Reactive</th>
<th>% Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>11</td>
<td>9</td>
<td>81.8%</td>
</tr>
<tr>
<td>Secondary</td>
<td>28</td>
<td>27</td>
<td>96.4%</td>
</tr>
<tr>
<td>Biological False Positive</td>
<td>9</td>
<td>1</td>
<td>88.9%</td>
</tr>
<tr>
<td>Isolated EIA Positive</td>
<td>44</td>
<td>1</td>
<td>97.7%</td>
</tr>
<tr>
<td>Discordant Nontreponemal/Treponemal</td>
<td>50</td>
<td>30</td>
<td>60.0%</td>
</tr>
<tr>
<td>(VDRL-/TPPA+/TS-EIA+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discordant Treponemal(^a)</td>
<td>11</td>
<td>4</td>
<td>36.4%</td>
</tr>
<tr>
<td>(TPPA+/TS-EIA-)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)This group includes three primary and one secondary syphilis infection. The HC-RST correctly identified one primary (1 of 3) and one secondary (1 of 1) infection.

4.3.3 Agreement Between Syphilis Health Check Rapid Test and Treponema pallidum Passive Particle Agglutination Assay

In addition to examining the performance of the HC-RST, one of the aims of this study was to assess whether the rapid test could be a potential replacement for the TPPA assay in the laboratory. To accomplish this, we examined the level of agreement between the two tests. Table 4.3 illustrates the results of this comparison. The PPA and PNA were calculated as 78.1% and 86.4%, respectively,
which were comparable to the figures in the package insert from one study site that prospectively compared the HC-RST to TPPA [31].

**Table 4.3 Comparison of Syphilis Health Check Rapid Test to TPPA Assay**

<table>
<thead>
<tr>
<th></th>
<th>TPPA</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactive</td>
<td>Non-Reactive</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Syphilis Health Check</td>
<td>Positive</td>
<td>114</td>
<td>2</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>32</td>
<td>101</td>
<td>133</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>146</td>
<td>103</td>
</tr>
</tbody>
</table>

Percent Positive Agreement: 114/146 = 78.1% (95% CI=70.6%-84.1%)
Percent Negative Agreement: 101/103 = 98.0% (95% CI=92.5%-99.5%)
Percent Overall Agreement: 215/249 = 86.4% (95% CI=81.5%-90.1%)

The kappa statistic was also calculated to examine the level of agreement between the HC-RST and TPPA assay. Kappa was determined as 0.730 (95% CI=0.648-0.812). Generally, kappa classifications of 0.6-0.8 are considered to have substantial agreement with each other. Table 3.1 describes generally accepted kappa classifications according to published literature for comparison [67,68]. A kappa of 1 equates to perfect agreement while 0 indicates agreement equal to chance [67,73].

Since the HC-RST is a treponemal antibody test, we sought to explore what effects past-treated syphilis infection might have on the agreement between the two treponemal tests. We know that treated historical syphilis affects other treponemal tests, such as the TPPA, because they cannot differentiate detection between IgM and IgG antibodies. Kappa was determined to be lower (0.421, 95% CI = 0.197-0.645) among identified past-treated infection (n=77) than specimens (0.759, 95% CI=0.662-0.856) with no history of past syphilis infection (n=172). Although agreement was higher between the two tests in specimens that had no history of past syphilis, the kappa statistic does not provide insights into accuracy of either test.

**4.4 DISCUSSION**

To our knowledge, this is the first study that examines the performance of the only FDA-approved rapid membrane immunochromatographic syphilis test available in the US. The Syphilis Health Check and other RSTs currently in development have many potentially practical applications for both the POC and laboratory venues. The ability to provide accurate and prompt results is critical to their performance. In a laboratory setting, a treponemal RST, such as the Syphilis Health Check, can be used as either a confirmation assay in the traditional two-test algorithm or as a
supplemental secondary treponemal test in the reverse sequence algorithm. Both scenarios would provide quicker TATs and less manual manipulation.

The TPPA assay has conventionally been performed as a confirmatory test in the traditional algorithm or the supplemental third test in the reverse sequence algorithm. The TPPA test is labor-intensive, requiring multiple dilution and manual manipulation steps. It also entails a two-hour incubation period for the antigen-antibody agglutination reaction to occur. Additionally, once the unsensitized and sensitized particles are reconstituted, they expire after seven days. This limitation requires laboratories to conduct batched testing and perform the TPPA at specified intervals (e.g., weekly) to conserve reagent and control costs. This may cause delays in reporting positive results, thus increasing TAT.

The overall performance of the HC-RST compares well to other syphilis RSTs that are available in international settings [72]. It also performed well on some “challenge” specimens (i.e., primary and secondary infections, BFPs, isolated EIA positive), but not others (i.e., discordant nontreponemal/treponemal and discordant treponemal). It is unclear why the test performed well with some groups, but not others. The HC-RST had good agreement with its treponemal counterpart, the TPPA, and had very similar performance against what was published in the Syphilis Health Check package insert [31].

The data from this study may indicate a role of the HC-RST in multi-test algorithms. If the test were used in lieu of a TPPA, the TAT required to report a positive syphilis infection could potentially be reduced since specimens would no longer need to be batched prior to testing on a TPPA. For the traditional algorithm, this means a nontreponemal test could be performed as a screening assay and reactive results would then reflex to an RST for confirmation. This algorithm could be performed in a matter of hours instead of days. In the case of a reverse sequence algorithm, any specimens found discordant between EIA/CIA treponemal screening and nontreponemal confirmation results could immediately be assessed by a RST in the same day.

Some limitations to the study exist. First, repeated freeze-thawed serum specimens were utilized for the study. This process may have degraded some of the specimens although the performance of the HC-RST in this study was similar to others listed in the package insert. Also, this was a retrospective study that utilized archived and remnant specimens, therefore clinical follow-up and retesting of individuals was not possible. Finally, the number of specimens this study was small due to financial constraints. A larger prospective study performed on fresh serum samples to further examine the performance of the Syphilis Health Check rapid test on various “challenge” specimens and historical treated syphilis infection would be of great benefit.
Chapter 5

Conclusion

Diagnostic tests are seldom perfect and should always be utilized in conjunction with proper clinical examination to provide an accurate and proper diagnosis of disease. Syphilis diagnosis provides a particularly challenging task for laboratories, clinicians and epidemiologists. Its complex clinical course provides many challenges to understanding how to correctly diagnose the infection. The fact that it can be treated and cured only further complicates matters. Traditional direct detection methods, like dark field microscopy and DFA, depend on precise timing of screening and are no longer routinely performed in the United States. Molecular (DNA-based) methods are available only as in-house assays that are not FDA-approved. Therefore, serology continues to be the “gold standard” identification methodology for syphilis.

In recent years, some laboratories have switched from the two-test traditional serologic algorithm to a three-test reverse sequence serological algorithm. This change has been partly due to the ability to automate the screening methodology thus increasing laboratory workflow, alleviating manual labor and potentially decreasing costs [33]. However, these advantages may not outweigh the limitations of a reverse sequence algorithm for all settings. As observed in our studies, the reverse sequence algorithm may prove to be problematic for laboratories serving high prevalence populations. When screening with a treponemal antibody assay, the test will not be able to differentiate between current and past-treated syphilis infection. This will cause expanded follow-up for the laboratory, cloud the clinical picture for health care providers and make surveillance more complicated.

The most recent CDC STD Treatment Guidelines were released in 2010, with updates and revisions to past recommendations on treatment regimens and testing protocols. Plans for an accompanying testing guidance are also in the works, however additional data are needed to help inform these guidelines. The efforts to help obtain these data are being facilitated to an extent by APHL, a national membership organization that represents public health laboratories in the United States. Through a Cooperative Agreement with the CDC, APHL helped to fund the studies conducted at the SFDPHL. The data and information provided by this dissertation will be presented to the APHL and CDC so that some of the data gaps around how a reverse sequence serologic algorithm functions in a high prevalence setting can be filled. These data, along with others from high and low prevalence public health and clinical laboratories, will provide an evidence base as syphilis testing guidelines are developed.

San Francisco County has one of the highest rates of syphilis in the country [15]. The SFDPHL provided over 23,000 syphilis VDRL screening tests in 2012, which ranks syphilis as the second highest volume test in the laboratory behind chlamydia and
gonorrhea [48]. With such a high volume, moving to an automated platform would be beneficial. Table 5.1 illustrates the estimated per test cost taking into account reagent and analyst time only. Other benefits of automation, such as the ability to perform other duties during incubation steps and the reduction of repetitive motion injuries due to manual manipulation of VDRL and TPPA assays are not included. These would require complicated cost analyses that are beyond the scope of this dissertation.

**Table 5.1 Estimated costs for each type of test performed at SFDPH**

<table>
<thead>
<tr>
<th>Test</th>
<th>Cost/test</th>
<th>Analyst Time Required/test (min)</th>
<th>Automatable</th>
<th>Batching Capacity</th>
<th>Total Cost/test</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDRL</td>
<td>$0.26</td>
<td>1.67</td>
<td>No</td>
<td>24</td>
<td>$1.23</td>
</tr>
<tr>
<td>TPPA</td>
<td>$2.33</td>
<td>6.25</td>
<td>No</td>
<td>24</td>
<td>$5.97</td>
</tr>
<tr>
<td>TS-EIA (manual)</td>
<td>$1.59</td>
<td>2.27</td>
<td>Yes</td>
<td>88</td>
<td>$2.91</td>
</tr>
<tr>
<td>TS-EIA (automated)</td>
<td>$1.59</td>
<td>0.23</td>
<td>Yes</td>
<td>88</td>
<td>$1.72</td>
</tr>
</tbody>
</table>

Note: Total Cost/test calculated from minimum hourly wage of SFDPH laboratorian of $34.91/hour

The information from this dissertation presents a novel alternative to the traditional and reverse sequence algorithms to diagnose syphilis. The data from chapter 2, which investigated the performance of a reverse sequence algorithm in San Francisco, showed that this algorithm would cause a large amount of additional work for the laboratory. The amount of third supplemental TPPA tests that would need to be performed would drastically outweigh the benefits of automation of the TS-EIA. However, the data presented in chapter 3 allowed for a large reduction in the number of TPPA tests that needed to be run if TS-EIA S/CO ratios were considered as part of the algorithm. This makes the reverse algorithm more appealing, however, would increase the TAT required to report positive results because not all TPPA tests could be eliminated and specimens would have to be batched prior to being run on the TPPA to keep reagent costs down. The data from chapter 4 illustrated how a rapid syphilis test could potentially replace the TPPA as the second supplemental test in the reverse sequence algorithm, thus decreasing the TAT down to one to two days. This novel algorithm would allow for screening on a fully automated platform to improve workflow, reduce the amount of labor-intensive TPPA assays that would be required and reduce TAT for reporting of positive results to one to two days. These studies have shown the innovative utility of EIA S/CO ratios and rapid tests as modifications to the reverse sequence algorithm in order for it to function well in a high prevalence setting.

The scope of this dissertation was to provide key scientific findings and data to help inform new testing strategies. While solid scientific data may be the basis for implementing new tests and algorithms in the laboratory, many other factors weigh
into the equation as well. Issues such as cost, quality assurance, labor concerns, turnaround time and policy implications must also be considered. Cost implications include but are not limited to reagent acquisition, carrying a third assay, vendor contracts and Medicare/Medi-Cal reimbursement. Quality assurance requirements, such as additional proficiency testing and maintenance of equipment, would be increased if three tests were performed instead of two. One advantage of shifting to a reverse sequence algorithm is the potential reduction in manual labor required for screening with a nontreponemal test. The ability to fully automate screening on an EIA or CIA platform could reduce labor costs as well as repetitive motion injuries caused by the high volume of RPR or VDRL tests that would need to be performed with the traditional algorithm. Turnaround time is another factor that needs to be examined and will vary for each laboratory. There has been one study conducted by the CDC to compare the cost-effectiveness of the traditional two-test algorithm to the reverse sequence three-test algorithm in low prevalence settings [33].

This dissertation has contributed to the scientific foundation for a new testing strategy for the diagnosis of syphilis infection in high prevalence settings. In San Francisco, a high number of past-treated syphilis cases complicated the utility of the reverse sequence algorithm. However, given the potential benefits, key stakeholders, including laboratories, clinicians and STD control must decide which algorithm and testing strategy will perform best in their jurisdiction. Further research on how the reverse sequence algorithm performs in settings with different syphilis prevalence is needed to build the evidence base. Additionally, more in depth research and exploration into the cost-benefit and overall economic impact are needed to address the many other issues that accompany such decisions.


47. Centers for Disease Control and Prevention. Table 35b. Primary and Secondary Syphilis—Rates per 100,000 Population by Race/Ethnicity, Age Group, and Sex,


55. Centers for Disease Control and Prevention. Table 1. Cases of Sexually Transmitted Diseases Reported by State Health Departments and Rates per 100,000 Population, United States, 1941-2011 [Internet]. 2012 [Updated December 13, 2012; cited March 10, 2013]. Available from: [link]


