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Serologic Testing for Syphilis: Benefits and Challenges of a Reverse Algorithm

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Abstract

Syphilis is a human infection of global importance. Its diagnosis can be challenging, requiring construction of a serologic profile based on the results of at least two types of antibody tests: treponemal and nontreponemal. The traditional approach to the serodiagnosis of syphilis has been the use of a nontreponemal screening assay followed by the performance of a treponemal confirmatory test if the initial nontreponemal screening test was reactive. With the increasing availability of automated, easier-to-perform, and rapid treponemal assays, an increasing number of laboratory testing sites are adopting reverse sequence screening for the serodiagnosis of syphilis: screening with a treponemal assay first, then confirmation with a nontreponemal assay and, when necessary, discrepant resolution using another treponemal test. In addition to offering automation and increased throughput, a reverse algorithm can increase disease detection, especially in late latent and early primary stages of infection when the nontreponemal antibody test may be nonreactive. However, a disadvantage to this approach is that there can be an increase in false-positive test results. This article reviews the clinical and workflow benefits and limitations of a reverse testing algorithm and discusses current guidance available from the Centers for Disease Control and Prevention.

Introduction

Treponema pallidum subsp. *pallidum* is a fastidious, microaerophilic spirochete that is the etiologic agent of syphilis. The diagnosis of syphilis, once called the “great imitator” because of its ability to produce a variety of clinical signs and symptoms of infection that can be easily confused with other diseases, can be a challenge.

Without treatment, syphilis is a chronic and progressive disease that can be associated with significant morbidity and mortality, especially when transmitted vertically from mother to child or in patients with advanced tertiary disease. Left untreated, infection is thought to proceed through a multistage process of primary, secondary, and tertiary stages (Fig. 1) that is often characterized by episodes of active disease between periods where signs or

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symptoms are absent (latency); latent disease is typically divided into early latent (less than 1 year from primary exposure) and late latent (greater than 1 year) (1). About 30% of untreated cases are thought to eventually progress to tertiary syphilis within 1 to 20 years of exposure (2). Primary and secondary disease affects skin, mucosal surfaces, and regional lymph nodes and can have misleadingly benign manifestations (e.g., painless chancre, rash, fever, malaise, muscle aches, and lymphadenopathy). In contrast, tertiary disease can occur in virtually any organ system following a spirochetemia that results in systemic dissemination of the organism. Tertiary syphilis is associated with serious complications, which can include cardiac or neurologic damage that can lead to death. Neurosyphilis, a serious complication, can occur at any stage if the spirochete invades the nervous system. Symptoms of neurosyphilis include headache, mental disturbance, and a Parkinson's disease-like movement disorder (3).

Syphilis is primarily acquired as a sexually transmitted disease (STD), so it is perhaps not surprising that data suggest an increased incidence of syphilis in certain high-risk populations, such as men who have sex with men and commercial sex workers (3). Beyond the greater generalized likelihood of contracting an STD through high-risk (e.g., unprotected) sexual behavior, having HIV does not appear to place an individual at higher risk of contracting syphilis (4,5). However, the likelihood of HIV transmission by an HIV-infected individual to an unprotected sexual partner may be increased if the partner has a syphilitic chancre that becomes exposed to HIV-bearing secretions (3,5). Thus, syphilis screening for at-risk and high-risk individuals plays an important role in public health.

Syphilis is usually transmitted sexually; however, it can also be passed vertically from mother to child either transplacentally (in utero) or perinatally as the newborn comes into contact with maternal blood and vaginal fluid during birth. Mother-to-child transmission of syphilis can have serious consequences. Approximately 69% of untreated infected pregnant women will experience an adverse pregnancy outcome, which can include serious birth defects, low birth weight, and prematurity. The consequences for a child infected with syphilis in utero can be lifelong. In addition, 25% of in utero infections result in late-term miscarriage or stillbirth, and 11% culminate in neonatal demise (3,6). Fortunately, routine screening and treatment of pregnant women have been shown to significantly reduce cases of congenital syphilis and can help to avoid serious consequences to the fetus or the newborn. Public health campaigns, such as the Syphilis Elimination Effort, and global initiatives by the World Health Organization (WHO) and the Pan American Health Organization in partnership with the United Nations Children's Fund seek to eliminate mother-to-child-transmission of syphilis by 2015, in part through targeted prenatal testing and educational efforts, as well as through improved availability of antenatal care (6). Despite such efforts, syphilis continues to present an important public health challenge in the United States and many other countries.

Regardless of the mode of transmission and despite the disappearance of symptoms in latency, *T. pallidum* infection persists until it has been treated. Antibiotic treatment can usually eliminate existing infection at any stage; however, there is no immunity associated with successful therapy, so reinfection can occur with subsequent exposure. The vast majority of infections are responsive to penicillin, which is still the drug of choice.

Fortunately, no penicillin-resistant strains of *T. pallidum* have been reported; however, penicillin crosses the blood-brain barrier only minimally and so may be of limited use for the treatment of neurosyphilis. For individuals allergic to penicillin, alternative antibiotics, such as tetracyclines (tetracycline and doxycycline) and macrolides (azithromycin and erythromycin), have been used for treatment. Unfortunately, the use of alternative regimens, in particular the macrolides, has produced antimicrobial resistance in some treponemal strains (7).

Laboratory Testing for Syphilis

Direct and molecular methods

Reliable testing is essential to establish the correct diagnosis and to institute the appropriate treatment—especially in latency, when there are no signs or symptoms of disease. In addition to a careful review of sexual history and physical presentation, several methods can be used to aid in the clinical diagnosis. In primary syphilis, if a patient presents with an open chancre, direct-visualization methods, such as dark-field or fluorescence microscopy, can be used to detect motile spirochetes in a fresh specimen collected by swab. Although microscopic methods can be used earlier in the course of disease than any other test (within 1 week of infection), they also have significant limitations. They are not useful for routine screening because they are labor-intensive, must be performed at the point of care, must be examined within minutes of specimen collection, and require the use of specialized and expensive equipment operated by personnel with appropriate experience and training. Additionally, a diagnosis can be missed if motile spirochetes are absent in the collection swab or if the primary chancre is absent.

The use of nucleic acid amplification tests is also currently being explored, and some success has been reported. However, existing methods consist of home brew tests that are unstandardized, as there are currently no FDA-cleared kits available for commercial use. Molecular assays, in general, are more expensive than performing standard serologic tests. Thus, molecular methods cannot be recommended for routine screening and diagnosis at this time, especially in resource-limited areas of the world (8).

Serologic testing

Serologic testing currently provides the best method for syphilis screening and diagnosis. In particular, serologic tests may provide the only evidence of infection during the latent period, and serologic profiles using a combination of test types can help determine the presence of untreated syphilis versus a patient with a treated past exposure (1). Because serologic testing for syphilis can be complex and often requires the results of at least two different serologic assays, clinicians need to understand the basis of these tests and the underlying immunological response to *T. pallidum* infection to accurately diagnose and manage disease.

Two types of serologic tests must be used to diagnose and to determine the stage of syphilis: nontreponemal tests and treponemal tests. Nontreponemal tests detect IgM and IgG antibodies directed against lipoidal antigens (such as cardiolipin and lecithins) released as a

consequence of cell damage from both the host and the bacterium (Fig. 2). Antibodies to these antigens are usually not detected until approximately 6 weeks after infection (Fig. 3) (9). Examples of nontreponemal tests are the rapid plasma reagin (RPR) test and the Venereal Disease Research Laboratory (VDRL) method. These tests can be run either qualitatively or quantitatively and are primarily based on either macroscopic or microscopic flocculation. Because nontreponemal tests are performed manually, they are labor-intensive and require a trained operator to conduct the test and interpret the results. While a reactive test can indicate syphilis infection, specificity is compromised by multiple factors, including other disease states that result in the production of anti-lipoidal antibodies, generating biological false-positive results (Table 1) (10).

False-negative results may also occur with nontreponemal tests because of a phenomenon known as the prozone reaction. This occurs when the nontreponemal antibody concentration is very high: the test antigen becomes saturated by antibodies, preventing formation of the antigen-antibody matrix required for agglutination (11). The prozone effect may be avoided by serially diluting the sample until the antibody titer is low enough to yield a positive response. This should be done if clinical suspicion of syphilis is high and the specimen yields a serologic result that is weakly reactive or atypical or has a rough, grainy appearance. Nontreponemal tests are valuable for measuring the efficacy of drug treatment because they are quantitative. A fourfold drop in titer is indicative of successful antibiotic treatment, and the nontreponemal antibody titer should be virtually undetectable following effective antibiotic treatment in most patients (11).

Because nontreponemal tests are not specific for syphilis, reactive sera are typically reflexed to a treponemal assay for confirmation. Treponemal assays detect the presence of IgM and IgG antibodies against proteins specific to *T. pallidum* (Fig. 2). In the native immune response, antibodies are generated against *T. pallidum* membrane lipoproteins (i.e., TpN15, TpN17, and TpN47) within ~3 weeks following infection, as shown in Fig. 3. Treponemal assays are qualitative and generally designed to detect one or more of the antibodies generated by these membrane antigens. Both laboratory-based and commercial tests have evolved over the years, and there are several types of formats in use. Manual assays include the fluorescent treponemal antibody absorption (FTA-ABS) test, which detects the whole organism, and the *Treponema pallidum* particle agglutination (TPPA) assay. Manual and automated versions include line blot assays, microbead immunoassays, enzyme immunoassays (EIA), and chemiluminescent immunoassays (CIA). Assays may use either naturally purified or recombinant antigens as the capture ligand. In many countries, rapid point-of-care testing for treponemal antibody is now also available (12,13). This format is particularly useful in areas with poor resource settings lacking routine access to laboratory-based methodologies. Some of these tests are being reviewed for WHO pre-qualification. However, none of the tests are FDA cleared as yet.

Despite the generally high sensitivity and specificity of treponemal assays, there are limitations inherent to the biology underlying their design. Treponemal assays will detect other related spirochete subspecies because they are antigenically indistinguishable. Although treatment is the same, this must be taken into consideration in regions with known endemic treponematosis that cause other non-venereal diseases, such as yaws (caused by

Treponema pallidum subsp. *pertenue)*, pinta (*Treponema pallidum* subsp. *carateum*), and bejel (*Treponema pallidum* subsp. *endemicum*) (14). As shown in Table 1, treponemal tests can also generate false-positive results in the presence of a variety of other diseases and conditions (8).

For establishing the serodiagnosis of patients with syphilis, it is important to understand the dynamic antibody profiles of both nontreponemal and treponemal assays, as outlined in Fig. 3, and to use the two tests in conjunction. Nontreponemal antibody typically declines in successfully treated patients as antigenic release from damaged cells is resolved. Quantitative nontreponemal testing using dilution titers can help identify initial infection; a reduction in the nontreponemal antibody titer relative to baseline usually signals successful therapy. Quantitative nontreponemal testing can also be used to determine if a patient who appears to have failed treatment has been re-infected or is “serofast.” Serofast patients fail to fully resolve serologically and perpetually exhibit low nontreponemal titers, whereas re-infected patients have persistently higher antibody titers (15). Conversely, treponemal antibody is generally detected in both infected and resolved cases. Approximately 85% of patients remain positive for life for treponemal antibody even with successful therapy (7). Evaluation of patients with reactive treponemal but negative nontreponemal results (discordant serologies) should be carefully considered for both current and previously treated infections.

Traditional versus reverse sequence syphilis screening algorithms

For many years, the traditional syphilis testing algorithm employed a nontreponemal assay for primary evaluation followed by a confirmatory treponemal assay on initially reactive samples (Fig. 4). Since both assay formats were originally manual, this approach made sense from both workflow and cost perspectives. However, the commercial availability of an increasing number and range of treponemal assays, including both EIA formats and fully automated CIA, has suggested an alternative approach to syphilis screening. Unlike nontreponemal assays, which with few exceptions are currently manual, automated assays require far less operator interaction, are easier and faster to perform, and are optimized for high volume and rapid turnaround. Naturally, the workflow advantages offered by automated treponemal assays suggested that reversing the order of the test types might make syphilis testing less labor-intensive while retaining diagnostic accuracy. Thus, it is not surprising that many laboratories have reversed the order in which treponemal and nontreponemal tests are performed, leading to the development of the reverse sequence syphilis screening (RSSS) algorithm.

In addition to the potential workflow improvement of RSSS, in the last several years, a growing body of evidence has suggested that the traditional testing approach could be missing some untreated cases, especially if the patient is in the late latent stage of disease where seroreactivity to nontreponemal tests declines (16). Use of the traditional screening approach could miss such cases, because an initial nonreactive nontreponemal test would not reflex to a treponemal assay. Across different studies and demographic populations (including HIV patients), up to 40% of untreated late latent cases were found to be

nonreactive using nontreponemal assays (17–19). This could create a diagnostic conundrum and could especially impact treatment decision making.

Other advantages offered by RSSS have helped drive the recommendation for its adoption in countries such as the United Kingdom (20). Treponemal assays detect primary infection at a slightly earlier stage than a nontreponemal assay (21). Also, published data support the value of using an RSSS approach for detecting syphilis in both low- and high-risk and low- and high-prevalence populations (22–24).

In the U.S., an increasing number of laboratories have either changed to or are considering adopting an RSSS algorithm. Changing paradigms in the order in which treponemal and nontreponemal tests are conducted is important to patient management, especially in patients with discordant results, where the current need to treat can be unclear. Laboratory professionals need to be fully aware of potential discrepancies in the serology to better support practitioner inquiries. Importantly, potential false-positive screening results can arise with significant frequency when using treponemal assays as the diagnostic starting point (24). Although the CDC currently continues to recommend a traditional testing approach, for laboratories or institutions wishing to adopt RSSS, the CDC has provided clear guidance and a recommended algorithm (Fig. 5) to resolve such discrepancies. In the CDC-recommended algorithm, initial testing is done with a treponemal assay (EIA or CIA), followed by a quantitative nontreponemal test for confirmation (quantitative RPR or other nontreponemal test). Discordant samples are resolved on the basis of a TPPA assay. The CDC specifically recommends the TPPA assay over the FTA-ABS test, citing concerns over specificity (24,25). All confirmed results should be reported concurrently to both the clinician and the appropriate public health agency.

However, some studies have suggested that while they have relatively high sensitivity and specificity, TPPA assays may provide different results in samples that are reactive by other treponemal assays (representing possible—but unconfirmed—infections) (26,27). In fact, most of the TPPA assay discrepancies were found in patients who were beyond the primary stage and who were co-infected with HIV (a population generally at higher risk for syphilis). Samples from this population have the potential to yield false-positive and false-negative results using both treponemal and nontreponemal tests (8,28,29). While concerns over use of the TPPA algorithm have led some laboratories to consider using alternative treponemal assays on discordant samples that are TPPA negative, the method’s relatively good performance is generally well accepted, and it is recommended by both the CDC and other authorities in countries outside the U.S. as an aid in resolving potential screening of false-positive results. Ongoing studies by the CDC involving a range of commercially available treponemal assays may provide further clarification, once published.

Analytic and clinical considerations with the RSSS

A 2008 study published by the CDC found that using a reverse screening approach (a treponemal assay followed by confirmation with a nontreponemal method) identified an additional 3% of patients who would have been missed with the traditional approach (17). However, the report noted that a relatively high rate of initially reactive treponemal results (17.2%) were nonreactive according to a second treponemal assay (suggesting the potential

for screening false-positive results). This finding was both reinforced and challenged by a 2011 study published by the CDC that supported the ability of the RSSS algorithm to identify samples that would have been missed with traditional screening but also found a presumptive high rate of treponemal false-positive results. As in the 2008 study, 56.7% of the specimens were initially treponemal test positive/RPR negative, and 31.6% of these were nonreactive using an alternate treponemal assay (either TPPA or FTA-Abs, depending on the laboratory) (24). Regardless of the second treponemal assay used, all laboratories reported a relatively high rate of false-positive results in both low- and high-prevalence populations examined. Importantly, out of the 140,176 specimens tested (both low and high risk), the overall treponemal reactivity was 3.4%, meaning the vast majority of samples were presumably correctly ruled out following the initial testing step. In the U.S., the probability that a nonreactive treponemal test represents a true negative is considered to be >98%, as the overall U.S. prevalence of syphilis is low (30,31).

The 2011 study noted that there were discrepancies between the initial and confirmatory treponemal test methods among the five participating laboratories. To arbitrate the results, the CDC announced plans for additional studies to compare multiple EIA, CIA, TPPA assays, and the FTA-ABS test in a head-to-head format using specimens from well-defined patient populations where clinical histories and risk factors for syphilis were known. Preliminary, but incomplete, results of this new study suggesting good performance by the treponemal assays evaluated have recently been published by CDC researchers. Despite some variability, overall there was good agreement for syphilis seroreactivity among the seven treponemal assays (three fully automated immunoassays [AIs] and four nonautomated assays, including the TPPA and FTA-ABS methods). Moreover, a minimum signal-to-cutoff ratio (which was different for each AI) could be associated with a positive TPPA assay result, suggesting that a second treponemal test may not be necessary to confirm AI-reactive, RPR-nonreactive sera (32). Additional data from this study are expected to be published and should further elucidate assay performance, as well as expand our understanding of how treponemal assays might be used in syphilis testing algorithms.

RSSS in the U.S

In many countries, treponemal screening using a reverse algorithm has become commonplace, though individual algorithms and discordant-specimen management vary. Both clinical and labor considerations contribute to the value of reverse screening for syphilis. In the U.S., the trend toward increased consolidation has meant that sample volume has often increased in laboratories offering syphilis testing. The current lack of a commercial, automated option for nontreponemal testing has driven interest in the growing availability of treponemal assays able to meet the demands of increased throughput. In addition to the nontreponemal assays being more labor-intensive, many clinicians, laboratorians, and public health agencies have cited concerns over the looming shortage of trained and experienced laboratory technologists who can accurately perform, interpret, and report the test results. While nontreponemal tests are clinically important for establishing the stage of disease and response to therapy, as with any test, both false-positive and false-negative results can be associated with them, stemming from time and temperature sensitivity, biologic false-positive results resulting from other diseases and physiological

conditions, and the subjective nature of the interpretation. However, the potential for a false-negative result in early primary syphilis or in late latent syphilis may be greater when using a nontreponemal assay for the initial screen (18,19,24,31,33).

Conclusion

Syphilis immunoassays remain important and commonly requested tests, both in low-risk populations, such as pregnant women, and in populations at higher risk or with clinical suspicion of infection. As traditional testing for syphilis (nontreponemal screening followed by treponemal testing for confirmation) is a long-held practice that is generally well known to clinicians, it is important for laboratories to be prepared to explain the interpretation of tests if moving to an RSSS algorithm. Because treponemal testing cannot distinguish between treated and untreated infections, patients with discordant results should be carefully considered for therapy, especially if the initial treponemal-test result is confirmed with an alternate treponemal assay. Currently, the CDC recommends the use of the TPPA test in resolving samples that fail to show reactivity with a nontreponemal test following a reactive treponemal result (24,25). Clinicians should be informed and educated on both the benefits and interpretive challenges of a reverse testing algorithm.

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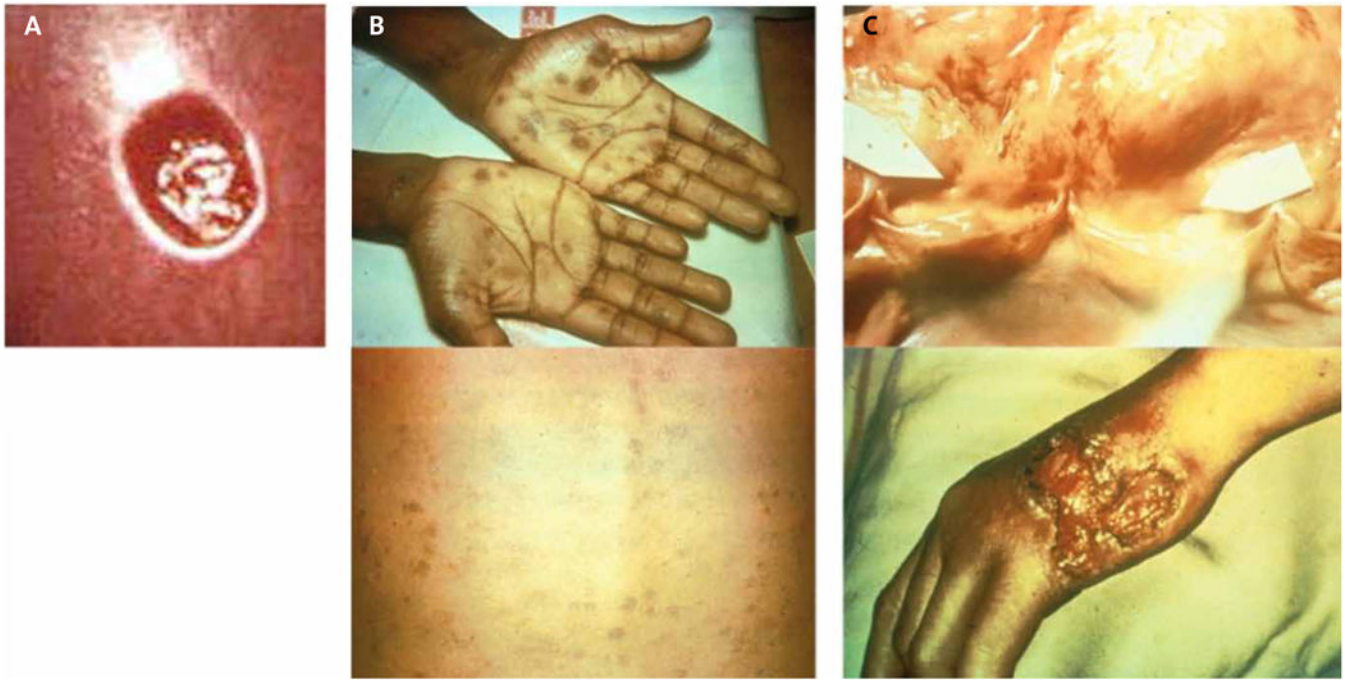

Figure 1.

Examples of the three clinical stages of syphilis (2)

a. Primary stage: chancre

b. Secondary stage: palmar rash, full body rash

c. Teritary stage: gummatous lesions in the heart and on the skin

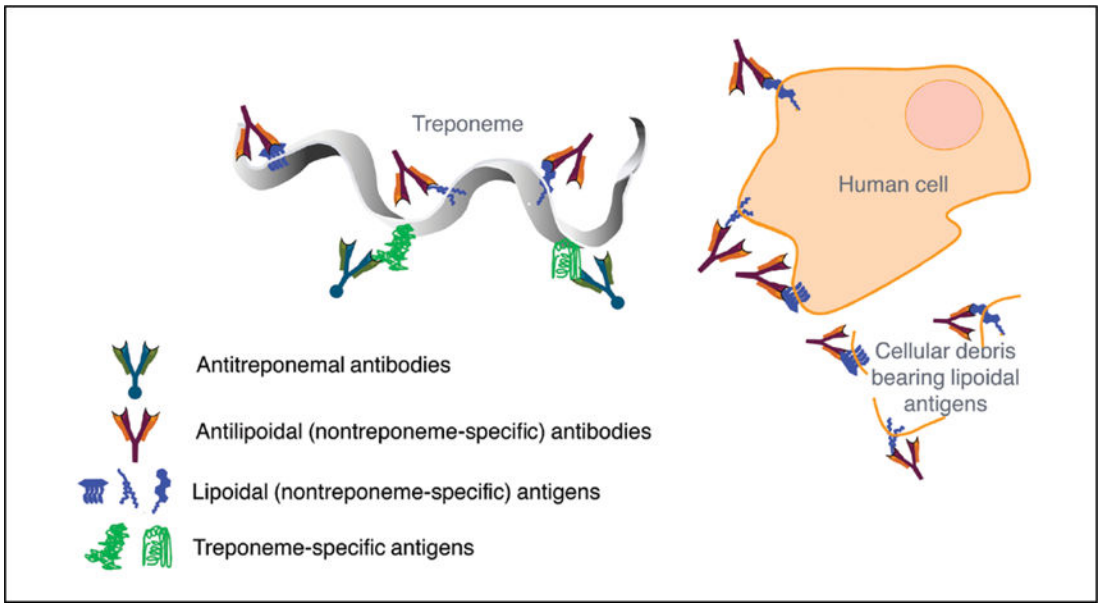

Figure 2.
Antibodies detected in treponemal and nontreponemal testing

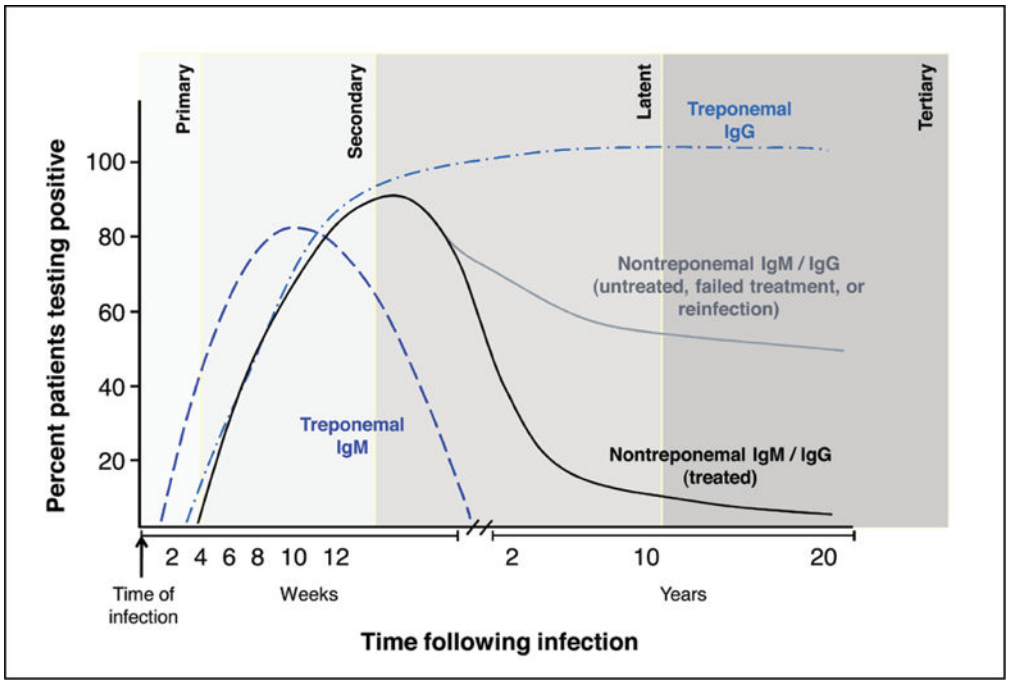

Figure 3.
Syphilis staging and serology (based on Peeling et al. [9]).

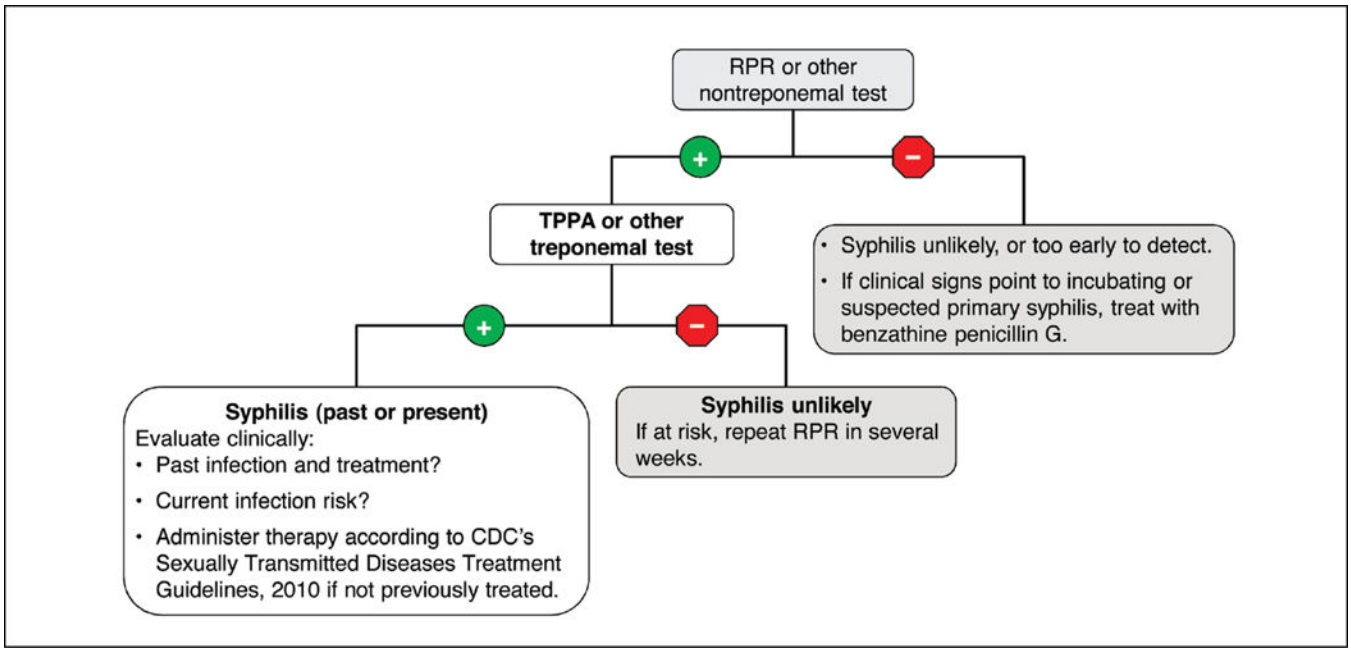

Figure 4.
The "traditional" syphilis testing algorithm (15,22)

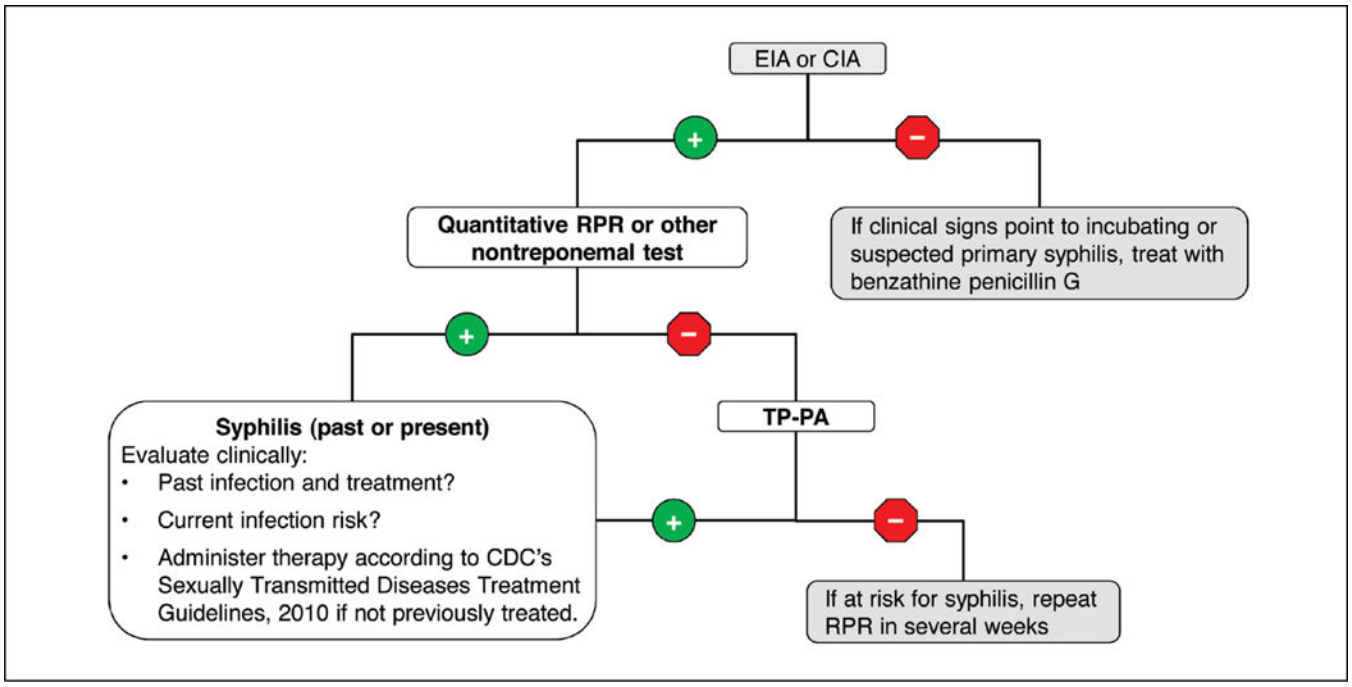

Figure 5.
CDC algorithm recommended for reverse screening

Table 1

Diseases and conditions that can cause false-positive results using nontreponemal and treponemal tests (8,10)

Test type	Disease or condition
Nontreponemal	Inflammation
	Autoimmune diseases (especially lupus erythematosus)
	Acute viral infections
	Hepatitis C
	Pregnancy
	Recent immunization
	Connective-tissue diseases
	HIV infection
	Injection drug use
	Malignancy
	Advancing age
Treponemal	Thyroiditis
	Systemic lupus erythematosus, scleroderma
	Infectious mononucleosis, genital herpes
	Cirrhosis
	Pregnancy
	Recent immunization
	Hyperglobulinemia
	Bacterial infections
	Brucellosis
	Leptospirosis
	Lyme disease
	Malaria
	Hansen’s disease
	Injection drug use
	Yaws, pinta, bejel
	Advancing age