

Laboratory Diagnostic Testing for *Treponema pallidum*



Expert Consultation Meeting Summary Report

January 13-15, 2009

Atlanta, GA



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In the last decade there have been major changes and improvements in STD testing technologies. While these changes have created great opportunities for more rapid and accurate STD diagnosis, they may also create confusion when laboratories attempt to incorporate new technologies into the existing structure of their laboratory. With this in mind, the Centers for Disease Control and Prevention (CDC) and the Association of Public Health Laboratories (APHL) convened an expert panel to evaluate available information and produce recommendations for inclusion in the Guidelines for the Laboratory Diagnosis of *Treponema pallidum* in the United States. An in-person meeting to formulate these recommendations was held on January 13-15, 2009 on the CDC Roybal campus. The panel included public health laboratorians, STD researchers, STD clinicians, STD Program Directors and other STD program staff. Representatives from the Food and Drug Administration (FDA) and Centers for Medicare & Medicaid Services (CMS) were also in attendance. The target audience for these recommendations includes laboratory directors, laboratory staff, microbiologists, clinicians, epidemiologists, and disease control personnel.

For several months prior to the in-person consultation, these workgroups developed key questions and researched the current literature to ensure that any recommendations made were relevant and evidence based. Published studies compiled in Tables of Evidence provided a framework for group discussion addressing several key questions.

The major recommendations of the consultation are summarized in the box below. The remainder of the following report summarizes the major discussions pertaining to chlamydia/gonorrhea diagnosis that were held over the course of the three day meeting and does not represent the final recommendations of the workgroup. The ensuing months will involve further expert discussion and literature review before the final development and publication of an STD Testing Guidelines document.

Summary of Major Conclusions:

- There is still a role for Dark Field Microscopy in the diagnosis of syphilis. Measures need to be taken to maintain quality DF testing, and to expand testing in sites which see a high prevalence of primary and secondary syphilis.
- Proper serologic diagnosis of syphilis in adults requires both a treponemal test and a non-treponemal test result. A single serologic test is not useful.
- The traditional algorithm of screening with a non-treponemal test followed by a treponemal test continues to have value. However, this algorithm is labor intensive. A syphilis testing algorithm using a high throughput treponemal test as the initial screen was proposed by the expert consultation group (Figure 1).

Laboratory Diagnosis of *Treponema pallidum*

Direct Detection of *Treponema pallidum*

Background:

Methods for the direct detection of syphilis include rabbit infectivity testing (RIT), dark field (DF) or microscopy following immunostaining (direct fluorescent antibody/silver staining), and most recently polymerase chain reaction (PCR).

RIT can be used for blood, cerebrospinal fluid (CSF,) amniotic fluid, primary and secondary lesion exudate, and lymph node (LN) aspirate. The technique is very sensitive and very specific, capable of detecting as few as 1 to 2 organisms¹. However, it is only available in a limited number of research settings and, although in these settings it has proven to be valuable, it is not practical as a diagnostic tool.

DF microscopy detects *Treponema pallidum* (Tp) based upon characteristic morphology and motility. It can be used for primary and secondary lesions (except oral lesions), exudate, LN aspirate, CSF, amniotic fluid, and other fluids. DF microscopy is a very valuable tool as it is sensitive, inexpensive, and can be performed at the point of care. The sensitivity of DF microscopy depends on the state of lesion development, but can reasonably be expected to detect approximately 10^5 Tp/mL². Specificity is highly dependent on the skill of the microscopist, making training and the maintenance of quality assurance programs very important.

Immunostaining identifies Tp based on antigen detection and morphology. DFA-TP (direct fluorescent antibody staining for Tp), is an immunofluorescence enzyme-based microscopy method that can be used for lesions smears, concentrated fluids, tissue brushings, and fixed or unfixed tissues. The specificity of the technique depends on the type of primary antibody used (polyclonal, monospecific, monoclonal). Sensitivity depends upon the concentration of Tp in the sample. Monoclonal antibodies are better for touch preps while polyclonal antibodies are optimal for formalin-fixed tissues. Although there are several specific antibodies commercially available for use in research, there is no FDA approved DFA-TP diagnostic test.

PCR identifies Tp by amplifying organism-specific DNA or RNA sequences. PCR can be performed on lesion swabs, LN aspirates, CSF, blood, amniotic fluid, and fixed or unfixed tissue samples. However, the sensitivity varies greatly by specimen type. PCR can theoretically detect 1 gene copy and the specificity, while intrinsically very high, depends on primer selection, skill of the laboratorian, and sample type, quality, and handling^{3,4}. There is no commercially available PCR diagnostic test for syphilis available in the United States.

The Syphilis workgroup was tasked with forming recommendations for the following questions regarding direct detection:

- How can the quality assurance of DF microscopy be ensured?

- Should serological testing be used to confirm DF?
- What is the role of immunostaining in the US?
- What is the role of PCR for the diagnosis of primary and secondary syphilis in the US?

General Conclusions/ Recommendations:

- The use of DF microscopy should be maintained in existing laboratories and expanded in sites with a high prevalence of primary and secondary syphilis.
- Quality assurance of DF microscopy is essential. This may be accomplished by:
 - Sponsoring training courses through regional STD Prevention Training Centers.
 - Production of video or web-based training packages both in the areas of microscopy and specimen collection.
 - Provision (potentially by CDC) of live Tp to testing sites. Different live TP species should be included to obtain experience in differentiation. Motile organisms are essential for distinguishing Tp from other oral spirochetes or commensal treponemes. However, this option is questionable as the shipment of viable treponemes is problematic.
- Serologic testing should always be used in conjunction with direct detection methods as a diagnostic tool in the evaluation of a person with suspected syphilis. However, a DF positive specimen that does not have a corresponding reactive serology test should still be treated as a positive result.
- In cases where there is a high degree of suspicion for syphilis, and lesions test DF negative and serology non-reactive, alternative tests such as DFA-TP and PCR should be made available.
- There is a role for immunostaining in the identification of Tp positive primary lesions as data show a high sensitivity and specificity for the identification of Tp in touch preps of chancres or mucosal lesions and biopsy samples. However, the sensitivity for secondary rash specimens is unknown. The greatest obstacle in the use of this test is the lack of an FDA approved diagnostic reagent. Currently, all commercially available reagents are only approved for research use.
- PCR can be a very useful test in the diagnosis of primary syphilis in lesions as it is very sensitive and specific. It would be especially useful in situations where DF is unavailable and serologic tests non-reactive. However, the usefulness of PCR in the identification of secondary rash lesions is unclear.
- PCR is not a useful tool for the identification of Tp in blood, serum, plasma, or CSF samples owing to low sensitivity.
- While RIT is more sensitive in detecting Tp in CSF in neonates, PCR for amniotic fluid appears to have an equivalent sensitivity to RIT.
- An FDA approved PCR test for syphilis diagnosis is needed. An alternative is for laboratories to develop and verify their own Laboratory Developed Tests (LDTs).
- CDC should consider establishing a mechanism to provide proficiency testing of in-house Tp PCRs (similar to the CDC Model Performance Evaluation Program for TB drug susceptibility and HIV testing).

Research & Development Needs:

- An FDA approved rapid point-of-care immunostaining test for detection of Tp in lesions.
- An FDA approved Tp PCR for lesions and other tissue specimens.
- Evaluation of Tp in secondary lesions (numbers, duration, best specimens) using real-time PCR.
- Investigation of the persistence of both live Tp and Tp DNA in tissues and fluids after treatment.
- Evaluation of the quality and specificity of commercially-available antisera for Tp.

Testing & Training Needs:

- Expansion of DF microscopy capacity and capability in the US.
- Development of training programs for DF microscopy.
- Development of training programs for proper specimen collection techniques for direct testing.

Serologic Testing for the Diagnosis of Syphilis in Adults

Background:

Syphilis serologic diagnosis relies on testing for nontreponemal and treponemal antibodies. These antibodies differ markedly with respect to antigenic reactivities and kinetics during the disease process. Treponemal tests detect antibodies to specific antigenic components of *T. pallidum*. Traditionally, non-treponemal tests detected antibodies to putative nonspecific antigens (primarily cardiolipin) produced by the host in response to syphilis infection. Recent studies suggest that cardiolipin is also a component of *T. pallidum* cells and that the formation of “non-treponemal” antibodies is also an immune response to specific antigens, but that the antigen is lipidoidal in nature⁵. A list of available serology tests for syphilis is shown in Table 1.

Table 1

Serological Tests For Syphilis	
Non-treponemal Tests	Treponemal Tests
Complement fixation tests - Wasserman reaction	<i>Treponema pallidum</i> immobilization assay (TPI) Fluorescent treponemal antibody absorption (FTA-ABS)
Flocculation Tests - Rapid Plasma Reagin (RPR)	<i>Treponema pallidum</i> hemagglutination assay (TPHA)
- VDRL	<i>Treponema pallidum</i> passive particle agglutination assay (TPPA)
- TRUST	Enzyme Immunoassay (EIA) Western Blot (WB) and Pseudoblots Automated Chemiluminescence platforms Chromatographic Point of Care (POC) tests Microsphere Immunoassay

The traditional syphilis testing algorithm employs a non-treponemal test such as the RPR test to screen patients and a treponemal test to confirm reactive serological tests. In recent years, laboratories have overwhelmingly switched to screening populations with low prevalence of the disease with treponemal tests which can be automated in EIA or similar formats⁶. The logic behind this approach is to reduce high labor costs, and with a low prevalence of the disease, few cases of syphilis would require confirmation using a labor intensive non-treponemal test⁷. Treponemal tests will continue to be used more widely and this workgroup has been tasked with providing guidance as to how they can be used more effectively. This task is especially difficult owing to the limited performance data available for syphilis serologic tests.

There are inherent flaws in the serologic diagnosis of syphilis. The appearance and disappearance of antibodies does not necessarily correlate precisely with the appearance and disappearance of the pathogen⁸. The diagnosis of early infection is dependent on the sensitivity of the methods being used. The specificity of immunological diagnosis is highly dependent on the specificity of the antibodies produced and the methods used to detect them.

Perhaps the biggest question to answer in terms of syphilis serology is how to interpret a positive treponemal, but negative non-treponemal result. Answering this question has implications for decisions regarding treatment, contact investigations, and reporting. There needs to be a systematic evaluation of the reproducibility of treponemal tests. Reactive EIA results usually indicate a truly positive result, but reflect lifetime exposure to treponemal disease rather than active infection⁹. However, there may be cross reactive antibodies in normal sera that cause a number of false positives, which are often repeated with other treponemal test methods¹⁰.

Most treponemal tests detect IgG antibodies although some tests detect IgM antibodies as well. IgM antibodies appear earlier in the course of syphilis infection. However, the benefit of testing for IgM to detect early syphilis in adults is not well understood. There are limited data regarding such issues as when IgM antibodies first appear, how long they last, what happens to IgM antibodies during treatment, and best methods for detecting IgM.

- The Syphilis workgroup was tasked with forming recommendations for many questions regarding adult syphilis serology. These questions include:
- Which serologic tests should be used for screening and diagnosis and in which order (non-treponemal versus treponemal)?
- Are there differences in the performances of treponemal tests?
- What are the implications of using treponemal tests for screening?
- Is there any value in performing a quantitative treponemal test?
- Are all syphilis EIAs created and performed equal?
- What is the value of testing for IgM to detect early syphilis?
- How should the performance of serologic tests be measured?
- What factors need to be considered in test selection?

- What tests can be recommended for patient management and possible reinfection?
- Is there a relationship between nontreponemal antibody titers and activity/stage of disease
- What is the role of POC tests in the US?
- Which diseases are responsible for biological false positives?

General Conclusions/ Recommendations:

- When selecting a specific test for screening, the setting, the population, and the individual patient should be considered. For this reason, multiple algorithms may be necessary.
- Normally, a single treponemal test cannot be solely relied upon for syphilis diagnosis unless characteristic lesions are apparent upon patient presentation or patient history provides additional information. Thus, a combination of treponemal and non-treponemal tests must be used^{7,11}. For resolving discrepant results, antibodies against different Tp antigens using different platforms may need to be tested using different assays. Clinicians must consider the clinical presentations and behavioral profiles of patients in the interpretation of syphilis serological test results; laboratory testing can only be a part of an overall assessment.
- There are both benefits and drawbacks to screening with treponemal tests.
 - Benefits include: the high sensitivity and specificity; the feasibility for automation and thus high throughput; the ability to interface the instrumentation with Laboratory Information Management Systems (LIMS) to reduce transcription errors; and the removal of operator bias since many treponemal tests are objective rather than subjective.
 - Drawbacks include: the inability of treponemal tests to distinguish between active and previously treated disease leading to over diagnosis and over treatment⁷; and the need for additional Epi/ DIS (Health departments) resources to conduct more frequent sexual contact investigations.
- Syphilis serology results provide indirect evidence for the presence of active disease. For this and other reasons, defining one specific testing algorithm will continue to be problematic. Other reasons for this difficulty include: high and low risk populations may have different testing needs; various stages of syphilis may have different testing needs; and collecting additional specimens may be necessary during potential periods of seroconversion in order to resolve testing discrepancies.
- It may be necessary to develop multiple algorithms to address scenarios where non-treponemal tests are used to screen and scenarios where treponemal tests are used to screen. Multiple algorithms would also be useful to account for differing needs (e.g. levels of risk) in various settings. The traditional algorithm of a non-treponemal screen, followed by confirmation with a treponemal test, continues to be effective in syphilis testing^{9,12}. The taskforce proposed an algorithm for initial treponemal screening Figure 1.
- When considering which tests need to be used the following factors should be considered: prevalence of disease within the population; performance of the test; purpose of the test (screening, confirmation, or disease management); subjectivity of

the test/experience of the technologist; need for capital equipment; automation; technical requirements; and cost.

- Algorithms for adult serology need to be based on the treponemal tests that are currently FDA-approved. However, limited data is available on the comparative performance of these tests at this point in time.
- Most EIAs designed to detect antibodies against treponemal antigens perform well when used for screening¹³, however ideally there should be more data available especially in early disease to make this determination. Theoretically, EIAs that detect both IgG and IgM should be more sensitive in early disease than those that detect only IgG^{14,15}. There appears to be a benefit of having multiple recombinant antigens rather than antigens obtained from whole cell lysates^{16,17}.
- There is insufficient data on the use of chemiluminescence-based and microsphere-based tests in the literature.
- Currently available EIAs are based on the use of recombinant antigens to avoid cross reactivity with antigens from other treponemal species.
- There is variability in the performance of different treponemal tests due to antigens, conjugates, and methods. The overall agreement between various treponemal tests appears to be >95%^{18,19}. Historically, the FTA-ABS test has been reported to detect antibodies earliest among the traditional treponemal tests (TPHA, TP-PA, WB)²⁰. However, the FTA-ABS has not been compared “head to head” with many of the newer generation EIAs and studies that use FTA-ABS as comparator can be problematic as the test is considered subjective.
- Currently, there is no suitable or consistent gold standard treponemal test available. The FTA-ABS test is too subjective since it depends on the skill level of the microscopist. A standardized Western blot or pseudo-blot would be the most likely candidate for this purpose. However, at this time, these assays are not commercially available in the US.
- Point-of care tests (POC) have contributed major advances to syphilis control in developing countries with a high prevalence of disease. POC testing has the potential to play a similar role in the US, but probably not using any of the existing tests. Although sensitivities ($\pm 95\%$) and specificities ($\pm 98\%$) of POC tests are comparable to other treponemal tests^{21,22}, existing tests have very low positive predictive values (<50%) in detecting active disease in low prevalence settings²³, thereby resulting in a high number of over treatments and unnecessary stress on patients and their contacts. In certain high prevalence settings, where immediate treatment is the overriding concern due to a likely lack of follow up care, the existing treponemal POCs may prove to be better than no test at all; however, at this time POC tests cannot be recommended for general use in the United States.
- There is a need for a comprehensive study to compare a range of treponemal tests in US patients. When comparing the performance of treponemal tests, a number of different tests should be used and tests should be evaluated against sera from various stages of disease including HIV-coinfected patients. It is difficult to obtain appropriate numbers of sera from different stages of disease within the United States; researchers may need to look outside the country. Other sera that should be assembled and included in studies should be from patients with diseases other than syphilis (DOS) as potential

biological false positives. On the other hand, specificity measurements should be made using sera from the United States and should include large numbers of sera from pregnant women and blood donors.

- The performance of non-treponemal tests should be compared using a varied group of positive low-titer sera.
- IgM detection has the potential to improve the diagnosis of primary syphilis. However, there are not enough existing data to make a definitive recommendation regarding its use at this time. The potential of IgM detection needs to be explored further and better IgM tests need to be brought to market. IgM may have role in a situation where a treponemal screening test is reactive and a non-treponemal test is non-reactive. In this situation, a second test that is known to detect syphilis IgM may be indicated^{5,24}. There is no apparent advantage to performing quantitative treponemal tests when compared to quantitative non-treponemal tests²⁵. In addition, quantitative treponemal tests are very expensive.
- The serologic response following successful treatment of syphilis infection remains unclear. It was noted that seroreversion of both non-treponemal and treponemal tests may occur more frequently than previously thought; especially following treatment of early disease²⁶. Patients with primary and secondary syphilis should demonstrate a 4 fold drop in non-treponemal test titers within 3-6 months. However, there is insufficient data to make a definitive statement on what increasing titers mean.
- There have been no studies on changes in EIA and chemiluminescence assay signals following treatment. Determination of what constitutes a significant change in signal should be explored.
- Most biological false-positive (BFP) reactions in both nontreponemal and treponemal tests are seen in the sera of healthy individuals^{27,28}. Several possible causes of BFPs include pregnancy, systematic lupus erythematosus (SLE) and other autoimmune diseases, EBV co-infection, Lyme, and periodontal disease. Data generally does not support that pregnant women have a higher rate of BFPs^{29,30,31}. The association could be biased by the large number of tests performed on pregnant women. High rates of BFPs in elderly individuals led the group to believe that periodontal disease is a possible cause of BFPs that requires further investigation³².

Research and Development Needs:

- A large bank of well characterized sera differentiated by clinical stage needs to be accumulated.
- There is a great need for a comprehensive study to compare a range of treponemal and non-treponemal tests that are currently FDA-approved in the United States using the bank of characterized sera.
- Studies should be designed to determine the cause of BFPs in both treponemal and non-treponemal tests.
- Data should be gathered to definitively determine if one treponemal test can be used to confirm another.
- Studies should be designed to answer the following questions:

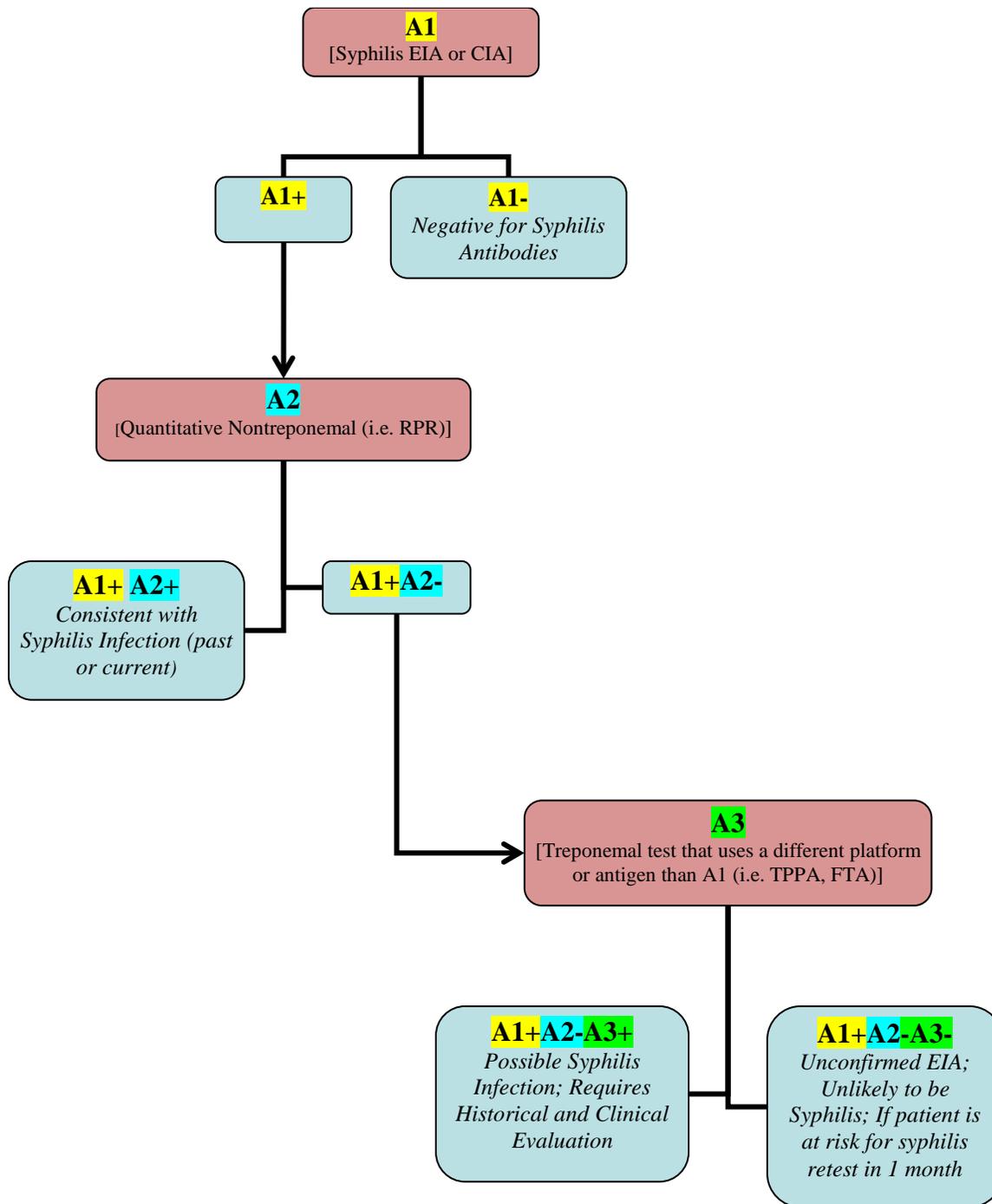
- How soon after exposure should a person be screened and which tests should be used?
- How soon should at-risk people with negative tests return to be rescreened?
- New and/or improved treponemal tests should be brought to market. The development of a Western or pseudo blot would be most helpful. Avidity testing and POC tests are other priorities.
- Research is needed regarding the usefulness of assays that detect IgM and the improvement of existing assays.
- Studies should be designed to determine if IgM persists beyond early latent syphilis in adults

Testing and Training Needs:

- Positive screening tests, whether non-treponemal or treponemal, should be confirmed with a test from the complementary category (either non-treponemal or treponemal). This may be difficult to implement, since most insurance companies do not necessarily reimburse for reflex testing.
- Alter current reporting requirements to call for laboratories to report reactive/ non-reactive results for all tests that were performed on a given patient to both the Health Department and the clinician. All results should be reported to the clinician and health department within 7 days.
- Education of physicians as to what constitutes a “high risk” (for syphilis infection) patient and to communicate this to the laboratory in order to ensure appropriate test utilization.

Figure 1: Suggested Syphilis Testing Algorithm with Treponemal EIA or CIA as Initial Assay

Key: A= Assay



Note: The laboratory should report the results of all assays conducted within 7 days

Serologic Testing for the Diagnosis of Congenital Syphilis

Background:

Congenital syphilis is caused by transplacental transmission of spirochetes³³. It is typically diagnosed based on maternal serological results and risk. Infants of mothers with positive serology may be tested using direct detection methods on any lesions or using non-treponemal serological tests. The use of IgM tests provides another possibility for the diagnosis of congenital syphilis; however, their utility is not well documented or understood.

General Conclusions/ Recommendations:

- A combination of IgM and direct Tp detection from multiple sites as well as the use of a treponemal test at >12 months will identify many or most infants with congenital syphilis.
- Testing for specific IgM antibodies is useful if the test is reactive, but in cases where the test is nonreactive it does not rule out congenital syphilis. IgM may be used in concert with Tp detection through immunostaining, PCR or DF microscopy.
- A four-fold or greater ratio of neonatal to maternal titers is rarely useful.
- The treatment and diagnosis of congenital syphilis should be separated. Treatment decisions should be made based on maternal infection or risk and not exclusively on laboratory results. Clinicians should always err on the side of over-treatment.

Research and Development Needs:

- Development of standardized immunoblot or line-blot assays for the detection of syphilis IgM
- Development of monoclonal antibodies for formalin-fixed tissue samples
- Development of assays for examining umbilical vein brushings for *T. pallidum*.

Testing and Training Needs:

- Evaluation of laboratory test performance on placentas.
- Use examination and laboratory testing of stillbirths as sentinels for congenital syphilis in the community and as a method of quality control in testing.
- Development of improved FDA approved IgM Assays.

Laboratory Diagnosis of Neurosyphilis

Background:

It is well recognized that CNS invasion by *T. pallidum* is very common and often happens very early in infection. Most people clear or control their CNS infection and have no late sequelae³³. However, this may not always be true, especially when individuals have not responded to treatment for primary, secondary, or latent syphilis, are co-infected with HIV, or have another condition that compromises their immune system³³.

Lumbar puncture is required for the diagnosis of neurosyphilis. Traditionally, CSF testing includes microscopic analysis for the presence of white blood cells, protein, and, VDRL analysis, although, a non-reactive VDRL does not rule out neurosyphilis. Treponemal tests are not typically used. Tests that were considered for inclusion in the groups recommendations for neurosyphilis diagnosis are shown in Table 2.

Questions analyzed for these guidelines include:

- What criteria should be used for the serologic diagnosis of neurosyphilis?
- What tests should be used for testing CSF specimens for syphilis?
- What are the indications for performing lumbar puncture in syphilis patients?

Table 2

<u>Potential Gold Standards for Syphilis Diagnosis in CSF Specimens</u>	
Clinical Abnormalities	CSF WBC levels
Nontreponemal Tests – CSF-VDRL - CSF - RPR	CSF Protein Levels
Direct Identification of <i>T. pallidum</i> in CSF	CSF B cells
Treponemal Tests – CSF-FTA-ABS - CSF TPHA Index - CSF TPPA	CSF CXCL13

General Conclusions/ Recommendations:

- Neurosyphilis cannot be diagnosed serologically.
- Serological tests can predict which asymptomatic individuals are most likely to have CSF findings consistent with neurosyphilis. A serum RPR > 1:32 may be used as an indicator of neurosyphilis, independent of stage of disease and recent treatment.
- Some agree that the use of VDRL in evaluating CSF may be worthwhile. Other promising tests include: WBC cut-offs, TP-PA (1:320), CXCL13.
- Protein levels and TPHA titer index are not useful tests for evaluating CSF.
- A combination of tests is likely the optimal choice for analyzing CSF.
- There was no consensus on what indications should be used for performing LP in suspected neurosyphilis cases. No data exists to resolve this issue. The two opposing views are:
 - To prevent risk of neurorelapse in asymptomatic HIV +, syphilis + individuals, early diagnosis and treatment of neurosyphilis is critical.
 - Early CNS abnormalities are not predictive of serious sequelae in HIV + individuals.

Research Needs:

- Further evaluation of optimal diagnostic test combinations for CSF examination to establish cut-offs and testing algorithms.

- Real longitudinal data on prognostic relevance of reactive CSF-VDRL tests in early syphilis infection in individuals who are also HIV infected.

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