Biomarkers for Infectious Disease Diagnostics in the Developing World:

Diagnosis of Syphilis in Asymptomatic Pregnant Women

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In this review of syphilis diagnosis for pregnant women, Section 1 discusses the current need for diagnostic products in resource-limited settings, given the clinical information and the user specifications that are required. In Section 2 we present the biomarkers, sample types, and technologies that would be most appropriate for diagnosis of syphilis in pregnant women. Sections 4 through 7 outline the steps that need to be taken, if any, to develop these products. Finally, in Section 8, the recommended course of action is presented.

1. Needs for Syphilis Diagnosis in Resource-Limited Settings

Syphilis is caused by the spirochete *Treponema pallidum* which is transmitted sexually through contact with an open sore, or chancre, which appears during the primary stage of the disease. It is also transmitted sexually during later stages of disease through direct contact with mucous membranes. Congenital syphilis is caused by transplacental passage of the bacterium from mother to fetus, or direct contact with a lesion during delivery. Congenital infection often results in fetal death or significant medical complications to the newborn, including growth retardation, premature delivery, as well as congenital syphilis.[1] If detected, syphilis can be cured with common inexpensive antibiotics; furthermore if treated during the second trimester, the negative impact on the fetus during pregnancy can be significantly reduced.[2]

The clinical and biological features of syphilis have been extensively reviewed.[3] Syphilis is clinically described in terms of its four stages. During the primary stage, a chancre, 0.3 to 3.0 cm in size, usually develops at the site where the bacteria entered the body.[3] This commonly occurs three weeks after infection, but can occur anywhere from three to 90 days after infection.[3] Although the treponemes rapidly disseminate to all organs in the body, the level of reproduction is highest at site of entry. In women, chancres can develop on outer genitals, or on the inner part of the vagina. The chancre is usually painless and often difficult to notice if located vaginally near the cervix. Thus, primary infection can go unnoticed in women. The chancre usually heals in 30 to 40 days without treatment.

During primary syphilis infection, a person is highly contagious and even after the chancre has healed, the infected person can pass the treponeme on to others, including an unborn fetus. The rate of vertical transmission during primary syphilis (mother to infant) ranges from 70% to 100%.

During the secondary stage of syphilis, which may occur before or after chancre healing, treponemes will have infected virtually every organ and can be found in almost any body fluid. Clinically, a generalized skin rash often develops, indicating that the infection has spread throughout the body. Small open sores may be present on mucous membranes, especially in women's genital regions. The skin rash usually heals without scarring within two to seven weeks. A person is highly contagious during the secondary stage, including vertical transmission from mother to infant.

The third stage of disease, latent syphilis, generally lasts two to twenty years. During this stage of syphilis, the infected individual is generally asymptomatic, although 30% of those infected may relapse to secondary syphilis one or more times.

An accurate diagnosis during latent syphilis can only be made through blood tests. Diagnosis of latent syphilis in a woman can also be made if her newborn infant is infected with syphilis. A person with asymptomatic latent disease is not considered contagious through contact; however a pregnant woman can transmit treponemes vertically to her infant with infectivity rates of 40% (for early latent) and 10% (for late latent disease).[3] In the final, fourth stage of disease (late stage syphilis), severe clinical symptoms develop, including neurologies and cardiomyopathies.

The infected mother can transmit infection transplacentally to the fetus or during birth by direct contact of the infant with a genital lesion. If identified and treated before or during the second trimester, fetal morbidity has been shown to be significantly reduced in both developed and developing countries. In developed countries, or in urban areas of developing countries, the identification of infected pregnant women using existing testing approaches and subsequent treatment are often readily available. In outlying areas of developing countries, however, often prenatal care is not available. Furthermore, existing testing approaches are not suitable for deployment in resource limited settings.

In the developing world, syphilis remains a significant health concern during pregnancy. The prevalence of syphilis (based upon seroreactivity) among pregnant women attending antenatal centers in Africa ranges from 3% to 18%.[1] Prevalence in antenatal centers varies from country to country (1999: 2.5% Burkina Faso to 17.4% Cameroon) as well as from province to province, for instance ranges from 1.5% to 6% have been reported in South Africa (WHO; www.who.int/hiv/pub/sti/en/who_hiv_aids_2001.02.pdf). Of important note, increased focus on disease diagnosis and aggressive treatment programs have been shown to have a positive impact on reducing the prevalence of syphilis in pregnant women. For example, in over 300 antenatal care sites in 9 provinces in South Africa, the average prevalence rate in pregnant women has steadily decreased from 11% in 1997 to 1.6 % in 2004 (National HIV & Syphilis Antenatal Sero-prevalence Survey in S Africa: 2004).

The goal of congenital syphilis eradication programs is to identify pregnant women at risk, to test for the presence of disease, and then to treat the individuals who are infected. In an area of high disease prevalence and incidence all pregnant women should be considered at risk. Due to the sporadic availability of antenatal care in resource-limited environments, it is of utmost importance to identify infected women and to provide treatment during the same healthcare encounter.

The Sexually Transmitted Diagnostic Disease Initiative (SDI) of Unicef/UNDP/World Bank/WHO has established the criteria that a diagnostic test must meet for acceptance in resource-limited environments within developing countries (http://www.who.int/std_diagnostics/). These criteria are symbolized by the acronym, ASSURED: Affordable, Sensitive, Specific, User Friendly, Rapid and Robust, Equipment Free, and Deliverable to Developing Countries. In the broad context of ASSURED criteria, this manuscript reviews the currently available biomarker-based approaches for syphilis diagnosis, identifies their strengths and weaknesses for use in resource-limited environments, and identifies newer approaches that have the potential to provide an impact in resource-limited settings.

2. Status of Current Diagnostic Approaches and Available Biomarkers

The currently used methods to diagnose syphilis are based upon clinical findings, examination of a chancre lesion (if present) for treponemes, and serology tests (treponemal and non-treponemal). In routine clinical practice in developed countries, serological tests are usually used to make the diagnosis of syphilis because the prevalence of disease is low and most patients have no signs or symptoms of disease. However, if a chancre or other suspect lesion is present, exudate could be collected and examined using microscopy to determine the presence of treponemes.[4]

Diagnosis of syphilis in the highly infectious primary stage, before the development of a lesion at the site of infection, is difficult. Since there is no lesion to sample for treponemes, the microscopy based approaches cannot be used. Furthermore, the appearance of host antibodies to the infectious agent does not occur until a few days to one week after the appearance of a chancre lesion, thus rendering serology based methods inadequate. Regardless of these shortcomings, the time interval before the appearance of the chancre lesion is relatively short (approximately three weeks) thus making this period of difficult diagnosis rather short compared to the time span of the four stages of disease.[1] Once a lesion appears during primary syphilis, regardless of whether it is observed or not, syphilis can be readily diagnosed in a standard laboratory setting using serology approaches with a high degree of sensitivity and specificity. Although such diagnostic procedures are routine in established clinical laboratories, they are difficult to implement in resource-limited antenatal care clinics in developing nations due to testing complexity, stability, cost, sampling and other issues.

Historically, the approaches used to diagnose syphilis have been two-pronged. Syphilis diagnosis relies on initial "screening" tests that detect antibodies directed against non-specific lipid membrane components of the infective bacterium (often called "non-treponemal" tests) and later confirmation with a test that detects antibodies that react to protein antigens produced by the bacteria (specific "treponemal" tests). Recently, new assay formats have been introduced that rely on using the treponemal tests for both screening and diagnosis. A variety of biomarkers are employed. Such advances are based upon ELISA, Western blot, recombinant antigens, and lateral flow device immunoassay technologies. In addition, nucleic acid based technologies, such as PCR, have been used to determine the presence of treponeme genomes in serum/plasma as well as other specimen types. All of these approaches are reviewed in the following sections.

2A. Direct Microscopic Detection of Treponemes

A chancre observed during primary disease, or a lesion observed during secondary syphilis, or even swollen lymph nodes, can serve as source material for direct observation of bacteria using microscopy. Material, such as lesion exudate, is collected onto a microscope slide. Safety concerns arise here because the exudate may contain infectious treponemes. The oldest and most difficult method of analysis of exudate involves wet-mount and dark field microscopy. The material must be examined within minutes of collection, using specialized equipment for the presence of live treponemes. Sensitivity approaches 80% for highly trained microscopists.[4] An alternative approach is to dry the slide and then examine it at a later date using fluorescently tagged antibodies and fluorescence microscopy (DFA-TP test). This approach is more sensitive than dark field microscopy. However, specialized expensive equipment, a

sophisticated laboratory, and highly trained microscopists are required. The advantage of these direct tests is their specificity and ability to provide a conclusive diagnosis of active syphilis.

In summary, the available data indicates that direct detection of treponemes via microscopy in lesions is highly specific; however the absence of lesions that could be examined over the vast duration of this disease makes this approach, in general, an inadequate method for the diagnosis of syphilis during the course of disease when, for the most part, lesions are absent.

2B. Non-treponemal tests: Detection of Anti-Lipid Membrane Antibodies Using Immunodiagnostic Approaches

The non-treponemal tests are best represented by the venereal disease research laboratory (VDRL) and rapid plasma regain (RPR) tests. These tests use a lipid antigen mixture of lecithin, cholesterol and cardiolipin which is prepared with well defined ratios of the three components. Each of these components is found in mammals; however, in *T. pallidum* membranes these lipids exist in a specific ratio that is immunogenic in humans. Thus infected humans elicit immune responses against the *T. pallidum* lipid membrane. Since these lipid components are found individually outside of treponemes, the tests are termed non-treponemal. Freshly collected or stored (frozen and thawed or refrigerated) serum (VDRL) or serum/plasma (RPR test) are used in a format that allows antibodies in the specimen to cross link the lipid vesicles and cause these vesicles to flocculate together. As an example, the RPR test is performed in a card format using one drop of serum is mixed with one drop of lipid reagent inside an 18 mm circle. The card is placed onto an orbital shaker for eight minutes and then read in available light.

Tests that detect anti-*T. pallidum* antibodies are used in either a qualitative or quantitative format, with the latter being based upon 2-fold sample dilution endpoint titer approaches.[4] Using the tests in a quantitative manner provides a baseline end-point titer value. Recent infection can be indicated by a minimum four-fold increase in the quantity of anti-*T. pallidum* antibodies in subsequent sampling (e.g., the endpoint dilution for detect ability increasing from 1:4 to 1:32). Conversely, effectiveness in treatment can be assesses by a four-fold decrease in the quantity of anti-*T. pallidum* antibodies over two to 12 month period post treatment. Thus eradication of disease can result in diminished reactivity in either a quantitative or qualitative format, and furthermore, treatment success often results in test seronegativity 1-2 years post infection. This feature allows the identification of active or recurrent infections using tests that detect the levels of anti-*T. pallidum* antibodies (i.e., non-treponemal-based serologies).

Tests that detect anti-*T. pallidum* antibodies become positive four to eight weeks after infection is acquired.[5,6] Both tests show similar sensitivities (44% to 85% primary disease, 100% for secondary disease, 95% for latent disease, and 70% for late disease), and specificities of 98 to 99% when thoroughly analyzed in controlled laboratory settings.[4, 16] False negative reactivity occurs in less than 1% of infected individuals who have circulating antibodies. This is attributed to a “prozone effect” and can be overcome by diluting sera prior to testing.[4,7] Reports suggest that this prozone effect may be exaggerated in HIV/syphilis co-infection.[8-11] Thus, although not a general concern, this may become significant in regions of high HIV prevalence. It is important to note that false positive reactivity occurs in individuals that have never had treponeme infection and the false positive rates can be influenced by the population being tested (pregnancy, elderly, sick, liver disease, autoimmune diseases, viral infections, etc.).[4] False

positivity in pregnant women is of particular concern in the context of screening large numbers of pregnant women for syphilis.

It is also very important to note that interpretation of these tests is subjective and open to differences in training and environmental conditions. West et al. compared the RPR test results when identical specimens were obtained from pregnant women in Gambia and tested in the field at the site of collection and in a centralized laboratory.[12] The environmental conditions in the field testing included a dusty atmosphere, excessive heat (35 to 42°C), and poor available light. Reagents were stored and testing was performed under manufacturer specified conditions by using ice boxes and orbital rotators powered by generators. Out of 1,295 venous serum samples, the positive reaction rates were 5.9% in the field and 3.5% in the laboratory. One percent of the samples were called negative in the field and positive in the laboratory. These discrepancies were attributed to temperature sensitivity[13] of the reagents during testing (not during storage), the tendency to dry while mixing and rotating, and the dusty environment used for test interpretation. Similar poor performance of RPR card tests in the field have been reported by others.[14]

In summary, the available data indicates that the RPR card tests are highly accurate for use in well established laboratories; however these tests are subject to false positivity when testing serum from pregnant women and furthermore are highly sensitive to extreme environmental conditions during testing. These non-treponemal tests offer advantages in resource-limited settings. First, they are inexpensive (<$0.20 USD), widely available, and do not require specialized equipment except for a rotator which could be battery operated. Furthermore, they are generally easy to interpret using appropriate lighting conditions and provide reasonable sensitivities and specificities in the field setting. The limitations are due to subjective interpretation of results, susceptibility to environmental conditions (especially temperature), and the need for a serum or plasma specimen and therefore venipuncture. The specimen must not contain red blood cells. It is possible that a finger lancet, combined with a capillary microhematocrit centrifuge device could be used to prepare a specimen. However, this adds an additional level of complexity to specimen collection and processing. It is conceivable that an alternative specimen, that requires minimal processing, such as saliva or urine, might prove to be adequate for syphilis testing, however such studies have not been performed. Taken together, the limitations raise significant concerns for implementing such tests in resource-limited environments.

2C. Detection of Anti-Treponemal Protein Antibodies Using Immunodiagnostic Approaches

Tests that detect antibodies directed against protein antigens encoded by *T. pallidum* (i.e. “treponemal-based tests”) fall into two categories: those that use proteins obtained from whole treponeme preparations and those that use recombinant *T. pallidum* antigens. The main difference between these tests and the non-treponemal test is that treponemal tests react despite successful treatment and therefore should not be used in measuring success to therapeutic interventions.[4,15] That is, antibodies against the *T. pallidum* organism do not wane, as opposed to antibodies raised against the lipid mixture used in non-treponemal tests.

The first non-microscopy test that utilized whole treponemes was the *T. pallidum*- FTA Abs test.[4] Although this test has evolved over time the principles remain the same. Sera is allowed to react with *T.*

pallidum organisms dried to a microscope slide (usually the sera is first pre-treated to absorb out non-specific anti-spirochete antibodies). The presence of anti-*T. pallidum* antibodies is determined with a secondary, fluorescently labeled anti-human IgG and fluorescent microscopy. The next test based upon the use of whole treponemes was the treponemal hemagglutination assay (TPHA) test. This test was later adapted to a more sensitive format using micro-well technology and is termed the MHA-TP (micro hemagglutination treponemal) test. In this test, sheep red blood cells, coated with *T. pallidum* whole organism sonicate, are allowed to react with test serum. If anti-*T. pallidum* antibodies are present in the test serum, they will agglutinate the sheep red blood cells. Reactive results are observed visually as a mat of agglutinated cells, compared to a button of non-agglutinated cells in the bottom of the testing well. Both of these tests have been used primarily as confirmatory test in the context of non-treponemal test reactivity. In the mid 1990s, numerous ELISA tests were developed using whole treponemes. These tests generally gave sensitivities similar to the MHA-TP and FTA-Abs tests;[4] however, a more recent side by side comparison of nine different ELISA methods, using highly characterized sera from primary syphilis patients that were all negative for the MHA-TP test, demonstrated superior sensitivity compared VDRL tests (49-77% vs. 44% for the VDRL).[16] These results were attributed to assay design (ease of use, sample dilution issues, and non-visual interpretation) as well as the ability of the ELISAs to detect IgM antibodies. Although these assays showed promise in a research setting, they were never broadly adopted for a variety of reasons, including cost, throughput, challenges in scaling-up the treponeme preparations, difficulty of use, and equipment requirements. Furthermore, since the assays were based upon proteins obtained from whole-organism sonicates, cross reaction to other spirochete species was obtained. The latter point is significant to consider in the context of developing nations where non-*T. pallidum* spirochete infections, such as *T. pertune* the causative agent of Yaws, can be of high prevalence (5%). Considering the limitations, neither of these tests is suitable for a rapid testing, resource-limited environment.

The sequencing of the *T. pallidum* genome in conjunction with the emergence of new technology platforms has significantly impacted the potential to develop new treponeme based tests. The first influence emerged with the utilization of Western blotting to identify serologic reactivity to specific antigens.[4] This converged with the introduction of recombinant immunoblots (such as the INNO-LIA from Innogenetics, Ghent, Belgium) where specific recombinant antigens were placed on membrane strips. Visual reactivity to specific antigens was used to confirm the presence of *T. pallidum* antibodies. Sensitivities and specificities compared to TPHA tests were in excellent agreement and usually ranged from 99 to 100%,[17] however, the test was limited by test complexity, requiring overnight incubations and multiple reagent additions and wash steps. Finally, the emergence of lateral flow point of care immunochromatographic devices and cartridge technology, combined with recombinant *T. pallidum* antigens as binders, have provided a new and alternative approach for rapid syphilis testing.

Until the early 1980s, *T. pallidum* proteins were prepared by growing the organisms in rabbit testicles, followed by harvest, and sonication of resultant treponemes. Although useful in research settings, this approach could not provide a sufficient quantity of materials for the large-scale production of commercial tests. In the late 1980s, the production of recombinant *T. pallidum* proteins was first reported which were used as binders in an ELISA format.[4,18] This assay showed excellent concordance with the FTA-ABS test. Furthermore, the cloning and expression of antigens in *E. coli* overcame the issues of source

materials. Sequencing and preparation of clones[19] covering the entire genome of *T. pallidum* has provided a bonanza of candidate assay proteins, as well as the ability to identify immunodominant proteins that do not cross react with other treponeme species. The *T. pallidum* genome is a circular chromosome of approximately 1.1 million nucleotide base pairs. It contains 1,041 open reading frames of which approximately 55% have been assigned a biological role.

Highly specific recombinant *T. pallidum* antigens have been adapted to three assay formats, the ELISA, line-tests, and lateral flow immunochromatic devices. In the ELISA format, individual antigens or combinations of antigens are adsorbed to the microplate and then stored under refrigeration prior to use. Sample and control sera or plasma are added to the plate and after one to two hour incubation at room temperature, the plate is washed and the presence of human IgG/IgM bound to the antigen is detected with an enzyme labeled secondary antibody. Enzymatic readout is usually based upon a colorimetric substrate and can be measured either an optical instrument or visually with the naked eye. Although excellent performance has been reported compared to gold standard methods, the test is limited by a need to refrigerate reagents, the time to result (which can be hours), the requirement for skilled personnel, power and instrument requirements, and finally a format which requires all 96 wells of the plate to be tested simultaneously.[16,20] This latter limitation is somewhat alleviated by the use of a strip format that utilizes only eight wells at a time.

Recombinant antigens have also been applied to the line immunoassay (LIA) format. As an example, the INNO-LIA™ brand test (Innogenetics, Netherlands) has been developed and commercialized as a confirmatory test for *T. pallidum* antibodies and has been evaluated in a controlled clinical setting.[17] The test contains four recombinant antigens (TpN15, 17, 47 and TmpA) placed in specific locations on a membrane strip. Sera is incubated with the strip overnight, the strip is rinsed and then developed using a secondary antibody. Although excellent concordance with a reference test was obtained (100% sensitivity, 99% specificity compared to TPHA & FTA-ABS), the test has significant limitations such as the overnight incubations, lability of reagents, and estimated cost of $30 per strip.[17]

Lateral flow membrane technology, in combination with recombinant *T. pallidum* antigens (e.g. TpN 47, 15, 17, and 15-17) have been used to create immunochromatographic strip (ICS) tests also known as rapid syphilis tests (RSTs). These tests are intended to provide rapid serological testing for syphilis in non-laboratory settings to guide decision making. Over 20 different versions of the test have been commercialized (see Table 1) and can cost less than $0.5 USD per test. The specimen type may be either serum, plasma, or whole blood; depending upon test manufacturer validation. The sample is placed (either manually or via a dipstick approach) on one end of the strip or device and is allowed to wick through the strip. In some formats, a few drops of a buffer reagent are added to the strip to enhance capillary flow. As the specimen moves through the membrane, antibodies react with *T. pallidum* antigens and detection reagents. The tests usually are visualized within 10 to 20 minutes as reactive “stripes” in an interpretation zone, and are validated with internal negative and positive controls. It is usually recommended that the tests be stored at temperatures ranging from refrigerated up to 30°C, and be kept out of direct light and heat.

The performance of the RSTs has been evaluated in a number of studies. In one study, an ICS test was found to correlate well with a FTA-ABS test but was also reactive with RPR negative sera from previously treated individuals.[21] In another study, a RST was evaluated in portable field laboratories in 20 Gambian villages. Reagents were kept on cool boxes with ice packs. Venous blood was collected, allowed to clot and 100 µL of the resultant serum was placed in a tube along with the RST. Compared to RPR/THPA testing performed on the identical specimens in a controlled laboratory setting, the RST provided 77% sensitivity with a 41% PPV and 99% NPV.[12] The authors concluded that the RST test was easy to use and interpret in the field compared to RPR testing, but did not provide adequate positive predictive power for the presence of active syphilis.

The World Health Organization (WHO) invited RST manufacturers to participate in a comparative evaluation of the various tests (see http://www.who.int/std_diagnostics/publications/meetings/SDIReport.pdf). Testing was performed in eight proficient laboratories using serum that was also evaluated with TPHA. A high degree of operational ease was noted for the tests. Sensitivities and specificities compared to TPHA ranged from 85 to 97% and 85 to 98% respectively. The results were considered satisfactory, and the evaluation program is being advanced to field testing and the use of whole blood specimens. It was pointed out in the published report that it is ideal to distinguish an active vs. a treatment-resolved infection. The RST tests that detect the presence of host antibodies are not capable of making this distinction; and thus it was proposed that initial screening be performed with RST followed by RPR to confirm a successful treatment (RPR would be negative).

In summary, the available data indicates that detection of anti-*T. pallidum* antibodies coupled with an easy to use, rapid, robust platform that is not subject to extreme environmental conditions, including reagent/device storage might provide a reasonable approach for the diagnosis of syphilis. The main concerns are robustness of the assay system in difficult testing environments, and the inability of such tests to distinguish active vs. previously treated infection.

2D. Detection of T. pallidum DNA Using Nucleic Acid Amplification Methods

The shortcomings of strategies that detect host-derived antibodies for the diagnosis of early primary syphilis, as well as a desire to identify an active infection, have driven the development of approaches that detect treponemal genomic DNA or protein antigens of interest. Direct detection of treponeme DNA or protein antigens in specimens is limited by the relative insensitivity of the method combined with the observation that only rarely are large numbers of treponemes found in clinical specimens.[4] This low sensitivity of direct tests has led to the exploration of genomic amplification using a variety of methods including PCR.[4,22] Using a PCR primer pair located in the TmpA gene (the same gene product targeted with recombinant antigen serology tests) species specificity was obtained and a sensitivity of 65 organisms per sample was achieved. Higher levels of sensitivity have been reported with the use of different primer pairs as well as adaptation to the TaqMan platform.[23]

PCR has been used to measure the presence of organisms in specimens such as serum and provides potential utility for very early (pre-chancre appearance) diagnosis of *T. pallidum* infection,[24] diagnosis of congenital syphilis and assessment of therapy effectiveness. The widespread adoption of this method in

resource-limited environments nevertheless presents significant challenges, including relatively high cost, technical difficulty of use, time to results, power requirements, reagent storage needs, temperature influences and specimen cross contamination concerns. Thus although this approach provides utility in a clinical laboratory setting, it is not currently useful in resource-limited environments. The picture could change with improved technical approaches.

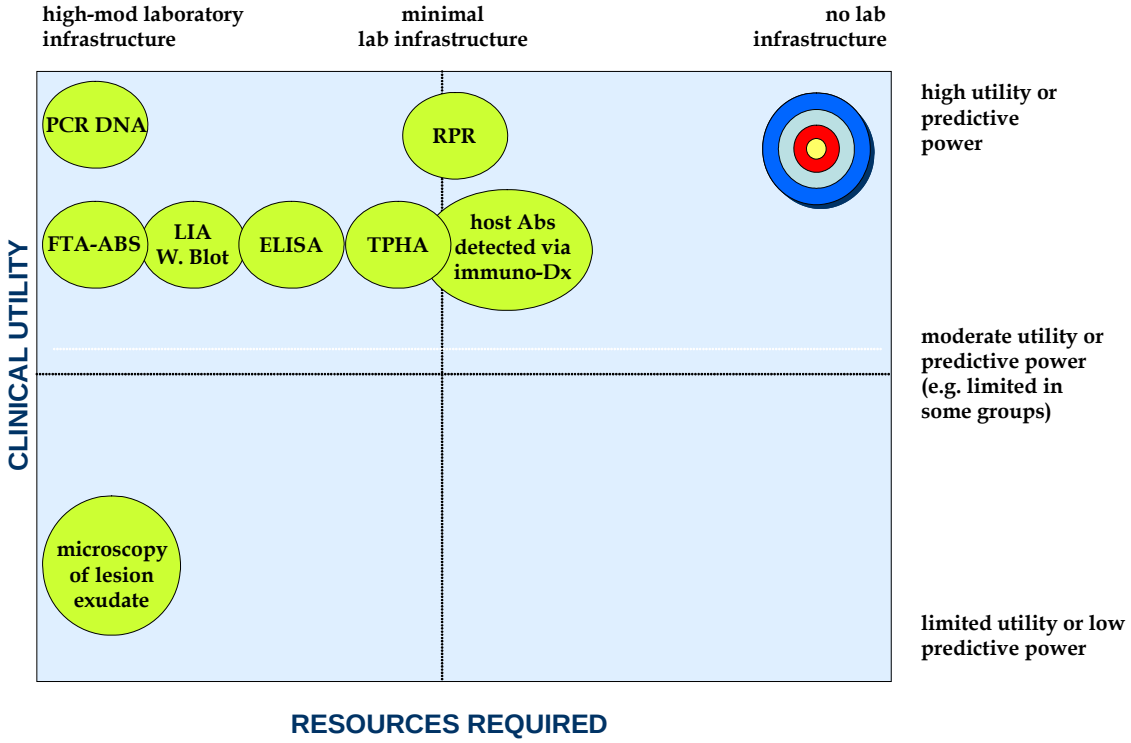
A comparison of the currently available biomarkers, diagnostic approaches, and methods for diagnosing syphilis is presented in Table 1 (next page). The ideal test represents most of the attributes encompassed within the UNICEF/UNDP/World Bank/WHO ASSURED test criteria.

Table 1. Comparison of the Currently Available Biomarkers and Diagnostic Approaches for Syphilis

Test Class	Name	Dx of active vs. previous infection?	Specimen	Equipment & power	Reagent storage	Time required	Ease of use & interpret-tation	Success in field trials*
The Ideal Test		Yes	Easily obtain-ed, non-invasive	Disposable, no power required	Room temp. for storage, 18-44°C for field use	<10-15 minutes	Easy, visual, controls included	
Non Trepo-neme based	RPR, VDRL	Yes	Serum or plasma	Rotator, electricity or battery	2-8°C	10 minutes	Easy in lab; poor in field	Poor
Trepo-neme based	FTA-ABS	No	Lesion	Microscope, electricity	2-8°C	1-2 hrs	Moderate, Technical skills in lab	Not tested
	TPHA	No	Serum or Plasma	Laboratory supplies	2-8°C	1-2 hrs	Moderate	Not tested
	ELISA	No	Serum or plasma	Many lab supplies, power for plate reader & computer	2-8°C	2-3 hrs	Easy, especially with computer data analysis	Not tested
	LIA W Blot	No	Serum or plasma	Many supplies, rockers, electricity required	2-8°C	overnight	Easy with training, visual interpretatio n	Not tested
	RST	No	Serum, plasma, whole blood	Few, self contained system; no electricity	2-30°C	15-30 minutes	Easy; controls provided for interpretatio n	Good, using serum
	PCR-DNA	Yes	Many specimens including serum, plasma, CSF, etc.	Complex, many reagents, equipment and power required	Frozen and 2-8°C	1-3 hrs	Easy with computer analyses; difficult visually	Not tested

The relative merits of the major diagnostic approaches discussed above are presented diagrammatically in Figure 1. In this figure, the ideal approach would be in the upper right quadrant, because of its ability to diagnose an active syphilis infection across all stages of disease and the low level of resources required for successful implementation. In addition, approaches with potential for broad diagnosis of active syphilis, but that currently require significant resources, are seen in the upper left quadrants.

Figure 1. Map of Currently Available Biomarkers and Diagnostic Approaches for Syphilis



3. Current Deficiencies in the Diagnosis of Syphilis in Resource-Limited Settings

Although syphilis can be easily diagnosed in a cost effective manner in clinical settings in the developed world, the tests used in the diagnostic process are generally inappropriate for resource-limited settings. Features that are desired for a test to diagnose syphilis in pregnant women in a resource-limited setting are described below. In this context of features, the applicability of currently available testing methods is briefly discussed.

1. Easy to perform test with two to three manipulations over a five to 15 minute time period. This is a critical issue and must be strongly weighted because technical expertise is often limited in field antenatal settings and furthermore, diagnosis must be made during the antenatal visit so that the woman can be treated and enabled with partner notification at that time. Without same day results, the woman could fail to return to the clinic for test results and treatment. The only tests that meet these criteria are the RSTs based upon lateral flow (dipstick) technology.

2. An easy to interpret test that can be done with the naked eye under variable lighting conditions. The hemagglutination, VDRL, RPR, and RST methods fall into this category. Out of these tests, the hemagglutination tests are the most difficult to interpret with the naked eye in the laboratory and hence in a resource-limited setting such interpretation would undoubtedly become even more difficult. The RPR test has been field tested[12, 14] and has been shown to have interpretation issues, especially in low light, dusty settings. The RST methods have shown superior interpretation, including internal positive and negative controls, in resource-limited field tests.

3. Insensitive to environmental challenges, including temperature extremes both during reagent storage and testing. All of the currently used tests must be stored in controlled conditions that range from refrigeration to a maximum of 30°C. It is conceivable that two types of reagent storage could be established. Test kits could be manufactured then shipped to and stored at centralized laboratories where environmental temperatures can be managed. These kits could then be deployed to the field in "batch-mode" where they would only receive short term (two to four weeks) exposure to elevated temperatures. It may be possible that existing kits could be stable using this approach, either as they exist today or with minor modifications. High temperatures, such as > 37°C, during testing can cause reagents to evaporate and hence either invalidate the result or provide the wrong result. This is an issue that all of the currently-used tests face. None of these tests have been validated for use in extreme environmental conditions, including high humidity, elevated temperatures, and dusty atmospheres.

4. Works on a readily available specimen in the priority of saliva> urine> finger prick whole blood>serum. The direct microscopy based tests require exudate from a lesion and hence are inappropriate. The serology tests are based upon serum or plasma, with the exception of some of the RSTs where claims have been made for whole blood. In the case of almost all the RSTs a finger prick drop of blood is collected into a microcapillary and either allowed to clot or applied directly to the RST membrane. If clotted, serum could be obtained with a battery or generator powered microhematocrit centrifuge; however this requires specialized skills and time both of which are addressed in #1 above. It is of interest to note that HIV serology has evolved from serum to saliva and urine as a specimen.[25] In fact, numerous manufacturers of rapid lateral flow HIV serology tests have demonstrated excellent concordance with serum serology. A recent report using oral fluid specimens in a recombinant *T. pallidum* antigen-based fluorescence immunoassay demonstrated 100% sensitivity and 98% specificity, in primary syphilis compared to standard serology.[26] Although the number of study subjects was relatively small, these results are very encouraging towards the potential utility of using oral fluid specimens in syphilis serology. In 2000, OraSure was awarded a $1 million USD grant by the NIH to develop a 20 minute RST using saliva. This test is yet to be made available.

5. Has reasonable sensitivity and specificity compared to standardized laboratory testing methods. In resource-limited environments, one has to be practical and assume that test performance will not exactly match that obtained with the same specimens tested in an established laboratory setting. Nevertheless, one needs to consider sensitivity, specificity, and positive and negative predictive values in the context of the population being tested. Although health economic

outcome studies have yet top be published, it is fair to assume that a test that could be readily deployable and adopted in areas of high syphilis disease prevalence, such as Africa, could provide positive health outcomes, because they are rapid and the patient that reacts positive in the test would not be lost to follow-up.

6. Can differentiate between active infection and previously treated infection. Aside from the direct microscopy based approaches that investigate lesions for the presence of treponemes, there are few tests that differentiate active from previous treated infection soon after treatment. *T. pallidum* genome detection by PCR is one test that can meet this characteristic, using a blood based specimen; however, for reasons previously discussed, PCR is not amenable to use in field antenatal clinics. The RPR and VDRL tests do differentiate between active and past treated infection, but it can take six months to two years to observe a decrease in antibody titer. Furthermore, these tests must be performed in semi-quantitative end-point dilution mode, which is difficult and expensive. Finally, interpretation of tests results requires comparison to endpoint titers obtained months to years earlier, and in field antenatal settings such information can not be easily managed or retained.

7. Can identify active infection in both early and late stages of disease. The good news is that the course of primary disease is relatively quick and that serology methods become effective within four to eight weeks after infection. Prior to positive serology, *T. pallidum* can only be detected by the direct microscopy methods or PCR-based approaches, both of which are currently unsuitable in resource-limited environments. The ideal test would be one that measures treponemes directly in a readily available specimen such as blood. Unfortunately, the treponemes are at relatively low concentrations and test methods such as lateral flow devices adapted to protein antigen detection most probably would not provide the requisite sensitivity. A breakthrough advance in rapid immunoassay detection of antigens would be required.

There are at least three clinical decision points for diagnosing syphilis in pregnant women: 1) Diagnosis in early primary syphilis prior to seroconversion, 2) Diagnosis once a chancre appears (if noticed) which is concomitant with the onset of seroconversion, and 3) Differentiation of an active syphilis infection from a previous one that had been successfully treated. The deficiencies of the current biomarkers and diagnostic approaches in their ability to address these clinical decision points are summarized in Table 2. Cells that are filled in light blue are the characteristics that limit the utility of the test in resource-limited settings.

Table 2. Summary of Current Deficiencies of Diagnostic Tests for Syphilis in Resource-limited Settings

Clinical Decision	Approach	Test	Specimen types	Sensitivity/Specificity (Limit Of Detection)	Resources & Infrastructure
Early primary Syphilis: Diagnosis prior to IgG seroconversion	*T. pallidum* in chancre	Microscopy	Lesion exudate	High	Established lab, not appropriate for field use
	T. pallidum in body fluids	Genomic DNA (e.g., PCR) or circulating antigen	Serum/Plasma*	High for DNA, low for antigen detection	Established lab, not appropriate for field use
	IgM detection	ELISA	Serum/plasma	Not established	Established lab, not appropriate for field use
Diagnosis post seroconversion	Serology (detection of anti-T. pallidum antibodies)	RST	Whole blood, saliva	High, 84-97% compared to other lab methods; varies by manufacturer. Specificity, 93-98%, varies by manufacturer	Good for serum; whole blood testing study in progress
	T. pallidum in body fluids	Genomic DNA (e.g., PCR) or circulating antigen	Serum/Plasma*	High	Established lab, not appropriate for field use
Differentiate active from previously treated infection	Waning antibodies	RPR/VDRL	Serum/plasma	Variable, endpoint titer wanes 6-24 months post-Rx	Established lab, possible but challenges exits for field testing
	T. pallidum in body fluids	Genomic DNA (e.g., PCR) or circulating antigen	Serum/plasma*	High	Established lab

*If a specimen other than serum or plasma is required, such as urine or oral fluids, then specimen type would no longer be considered a deficiency

In summary, there are three major categories of problems with syphilis diagnostics when it comes to implementing them in resource-limited sites. First, specimen type is an issue. Few tests have been validated to work with whole blood that could be obtained from a finger prick or other non-invasively obtained specimens such as saliva or urine. Second, all tests except for the lateral flow RSTs require technical expertise, controlled environments for reagent storing and testing, and/or are subject to variability in interpretation, including the relatively easy to perform RPR card tests. Third, there are no simple tests to differentiate active from previously treated infection.

A final point to take into consideration in the diagnosis and treatment of all infectious diseases is the identification of resistance to therapeutic options. Of note, as of the year 2000, resistance to penicillin, the primary therapeutic choice for syphilis, especially in pregnant women, had not been reported among any isolates.[1] In the years 2002 and 2003, the CDC reported eight cases where the drug azithromycin had no effect on syphilis in HIV co-infected patients. The patients were eventually cured with penicillin or doxycycline. Whether the treponeme had become resistant to azithromycin or whether the drug was an inappropriate first line choice is not clear. Nevertheless, it appears that drug resistance is not an issue in *T. pallidum*.

4. Opportunities to Improve the Clinical Performance of Existing Biomarkers

Many improvements could be made to existing biomarkers and test technologies that would allow the currently available biomarkers to deliver adequate performance in resource-limited settings. The key is to fill in the gaps that prevent tests, such as *T. pallidum* recombinant antigen immunoassays in a lateral flow devise (RSTs), from being deployed and implemented in resource-limited environments.

Specimen type is one area where improvements could be made. Blood collected via phlebotomy creates a technical challenge for specimen collection and biohazard management in resource-limited settings. In some locations, such collection is culturally unacceptable. Preparation of serum or plasma from such specimen creates another technical challenge. Many of the syphilis serology RST tests have been adapted to use whole blood, collected via finger prick and microcapillary, and then directly applied to the test device. Although certainly an easier approach, the field personnel are still confronted with biohazard issues, especially in regions with a high prevalence of blood borne pathogens such as HIV and HBV. More importantly, this specimen/test combination has not been rigorously tested in resource-limited field sites.

It is important to note though, that in a multi-center international laboratory based study, the WHO evaluated six different RSTs using archived sera and found excellent overall performance compared to the reference standard TPHA or TPPA tests (http://www.who.int/std_diagnostics/publications/meetings/SDI_Report.pdf). They are advancing four of the RSTs that are claimed to perform well with whole blood and do not require refrigeration for field trials. The stability of kits under storage and the robustness of testing have not been evaluated under extreme environmental conditions, or even under controlled conditions that mimic those found in resource-limited field settings. This is a gap that needs to be filled. Of further interest, Baguley has recently demonstrated that syphilis serology potentially may be performed on oral fluids.[26] This specimen type obviates many of the biohazard concerns and simplifies collection. Combining the simplicity of RST methodology with an oral fluid specimen may significantly simplify syphilis serology in resource-limited settings.

A test that may seem easy to perform in a routine laboratory setting is not necessarily transportable to field settings. An example of this was reported by West for the simple RPR card tests using sera collected by standard phlebotomy.[12] The only way to ensure that a test is robust in such settings is to design and develop it with very specific user requirements in mind and then test it in the appropriate field settings. Unfortunately, none of the currently available tests were developed with hot, dusty, resource-limited

environments in mind. In the context of an "ideal test", one would want to detect the presence of treponemes or treponeme products (e.g., genomic DNA) in a readily available body fluid such as saliva, urine or whole blood. The presence of the organism would indicate active infection while the absence of the organism would indicate an absence of infection (for instance, a previously treated patient). A rapid simple PCR-based method, combined with a readily available specimen type, would meet the criteria. Unfortunately, even the most portable, user friendly cartridge system marketed by Cepheid Corporation (www.cepheid.com) would not meet the ease of use specification. Many others are in development.

In summary, the RSTs provide a simple and straight-forward approach for syphilis diagnosis. The gap is that they need to be further developed and/or validated for storage and use in remote field settings. In addition they need to be validated (or further developed) for use on an easily obtainable specimen such as finger-prick whole blood. A further advance would be if such tests used an oral fluid or urine specimen. The problem with *T. pallidum* immunoassay RSTs is that they do not distinguish current vs. previous syphilis infection. This limitation could result in over-treatment of a population; however, over-treatment with a relatively safe and affordable therapeutic might outweigh the health outcome of under-treatment. Approaches to distinguish active vs. previous infection are discussed in the following sections.

5. Known Biomarkers That Have Not Yet Been Clinically Validated

Opportunities exist in syphilis diagnosis for known biomarkers that remain to be validated in the clinic. One example of this is the detection of genomic DNA sequences in readily-available specimens other than blood. Numerous studies using both human and animal specimens have shown that the *T. pallidum* genomic sequences can be detected in blood, CSF, as well as in tissue specimens.[4, 27, 28] Furthermore it has been shown that the DNA of dead organisms is readily cleared from the host within 15 to 30 days, whereas a persistent infection results in ongoing detectability of DNA.[28] In another related example, PCR has been used to identify the presence of spirochete DNA (*B. burgdorferi*) in urine and CSF obtained from patients with both early and late Lyme neuroborreliosis.[29] Taken together, these findings suggest that urine may be a potential specimen for diagnosing persistent active *T. pallidum* infection compared to a previously treated infection. The potential of this is significant in that urine is a readily available specimen type and is relatively void of inhibitors that might impact DNA amplification methods such as PCR. Furthermore it equally plausible that oral fluids might provide a suitable specimen for *T. pallidum* DNA based diagnosis. Although these specimen types are ideal in resource-limited settings because of ease of collection, lack of need for sample preparation or purification, and a smaller biohazard risk, easy to use methods for DNA amplification and detection are not currently available.

There is a fundamental need to conduct clinical studies to validate these sample/marker combinations. There is no need to conduct the studies at the remote sites of eventual test use; the clinical work can take place in a standard clinical laboratory with sophisticated capabilities anywhere. This would allow for an immediate thorough validation of the approach, including identification of strengths and weaknesses, as well as the determination of key performance parameters such as sensitivity and specificity. Such an approach could be performed in parallel with a technology development program aimed at creating easy to use, environmentally robust, and inexpensive DNA amplification and/or analysis platforms.

6. Approaches for the Discovery of Novel Biomarkers for Syphilis Diagnosis

It would be useful to differentiate pregnant women who have been successfully treated for a previous *T. pallidum* infection from those whom are actively infected (i.e., the syphilis test should not be reactive with samples obtained from someone whom is not actively infected). This would help to minimize "over treatment" Treatment regimes based upon single doses of benzathin penicillin[1] have been shown to be 98% effective in preventing fetal infection and are safe, excluding penicillin allergy. Thus, the issue of treating individuals who are not actually infected is a cost-driven concern more than a safety concern. As discussed previously, diagnostic approaches that detect anti-*T. pallidum* antibodies cannot distinguish between active and previously treated infections. Since these tests, especially the RSTs, show the most promise for an immediate impact in syphilis diagnosis in resource-limited settings, an opportunity exists for a companion test that could be used to rule out a previously treated infection. It has been proposed that the RPR card test could fit this need (WHO); however, this approach has two potential problems: 1) a single RPR test is not informative in this context unless it is negative, and 2) RPR tests are not robust in environmentally challenging environments.[12] Thus, there remains the need for a simple robust companion test that would confirm active syphilis.

One approach for a companion test might be a method that measures the presence of a active *T. pallidum* infection but as a stand alone test is not specific (i.e., has a high false positive rate). The concept is that a positive result with this test alone is non-specific, but in combination with a positive RST would provide a significant indicator of active infection. A potential solution for such a test could be a lateral flow device (similar to those used in RST formats) that measured a general inflammatory response. For example, molecules such as inflammatory cytokines fall into this category. The biology behind cytokine production during disease processes, including viral and bacterial infection, and the measurement of such markers in blood has been reviewed by others.[30] Of particular interest, there has been an interest in studying cytokine responses to syphilis infection in both animal models and humans. Podwinsky et al. demonstrated that the pattern and level of cytokines secreted by Th1 and Th2 lymphocytes of syphilitic patients correlate to the progression of disease.[31] Others have followed T- and B-cell responses (including cytokine secretion) during the course of experimental syphilis in rabbits.[32, 33] Thus, the potential appears to exist for identifying specific cytokines or cytokine patterns that would indicate an active infection. The key to understanding the utility of such an approach would be to perform cross-sectional and longitudinal studies to identify the best cytokine candidates and then validate their utility in study populations representative of the intended treatment group.

Immunoassays are commercially available from many vendors in an ELISA format for the quantification of cytokines in serum or plasma. These assays could be used in discovery programs using either the rabbit testis model or retrospectively collected human specimens. Since human specimens are readily available in areas where syphilis is highly endemic, it may be reasonable to perform discovery work directly on human specimens. The process used for such discovery might be as follows: 1) Literature search to identify candidate cytokines. 2) Assembly of specimens from individuals representing all stages of syphilis (including specimens from syphilis subjects that have underlying diseases prevalent in the population of interest, such as HIV), those successfully cured without underlying diseases, those successfully cured but also have prevalent underlying infections, and normal healthy individuals. 3)

Perform complete syphilis serology work-up and correlate with clinical information available for the specimens. 4) Using the specimens and candidate cytokines, perform the appropriate assays. 5) Perform bioinformatic analysis of the resultant data. It will be important to note the quantitative levels of the individual cytokines in the context of the sensitivity expected for the field testing device (i.e., make sure that quantitative cytokine levels can be readily measured in the test device). In the data analysis it will be important to identify potential cytokine biomarkers that are not only significantly different in the study populations but also generalizable to a large number of subjects in the study population. Any biomarkers identified will require additional validation in prospective studies.

Another approach to identify active infections involves the detection of *T. pallidum* protein antigens. Since the *T. pallidum* genome has been sequenced, the open reading frames identified, and the genes encoding these protein antigens cloned, this approach is intriguing. However, the sensitivity of existing immunodiagnostic technology for detecting protein antigens (such as those expressed by *T. pallidum*) may make this approach unfeasible. For example, today's most sensitive ELISA antigen assays can detect1 to 10 picograms of an antigen per ml of sample. Considering a protein with a molecular weight of 50 kDa, this translates to an abundance of 10^7 to 10^8 proteins/mL. If 100 copies of a protein were produced per treponeme present, then 10^5 to 10^6 organisms/mL of sample would be required to be detected. Compounding the matter further is the relative insensitivity of lateral flow antigen detection devices compared to the most sensitive ELISA technology. Taking these assumptions together, at least 10^7 to 10^8 organisms/ml would be required in an accessible biological specimen such as blood or saliva to make an immunochromatographic antigen detection approach viable. Considering the biology and natural history of *T. pallidum* infection, namely that it is usually found in low levels in most if not all tissues during a large course of infection, existing immunoassay technology may not have sufficient sensitivity to detect *T. pallidum* in blood or oral fluids. Two situations could make the detection of treponeme antigens via immunochromatographic formats a viable option. First, researchers might discover a protein produced by treponemes that is present at a very high abundance. This protein would need to have epitopes that are unique to *T. pallidum*. Alternatively, improvements could be made to point-of-care immunoassay technology (e.g., lateral flow technology) that enhance the sensitivity by orders of magnitude.

7. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

As in any study, it is of utmost importance to collect serum and oral fluid specimens from the correct study populations and ensure that integrity is maintained. In Africa for instance, the prevalence of syphilis in patients attending non-field referral centers can range from 2 to 12%; thus, there appears to be an ample number of subjects for specimen collection in an environment where serum and oral fluid specimens would be appropriately tested with existing syphilis testing procedures, categorized and frozen. To perform the cytokine-based discovery described above, the numbers of subjects in each category would not need to be large (approximately 50) because the analysis will look for strong trends, not small trends that are slightly statistically significant. Considering the number of categories, the number of specimens required may be in the range of 500. If a study was to evaluate 15 different candidate cytokine markers, and specimens were to be tested in duplicate, and assuming that 96 well ELISA plates were used

according to SOPs, approximately 200 ELISA plates would be required. Such a study is doable within a one year timeframe or so.

8. Discussion and Recommendations for the Improvement of Diagnostics for Syphilis in Pregnant Women

The following recommendations are presented for consideration, given the deficiencies of current diagnostic test methods for deployment in resource-limited settings and the opportunities for improving the deployment of existing biomarkers.

8A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for Syphilis?

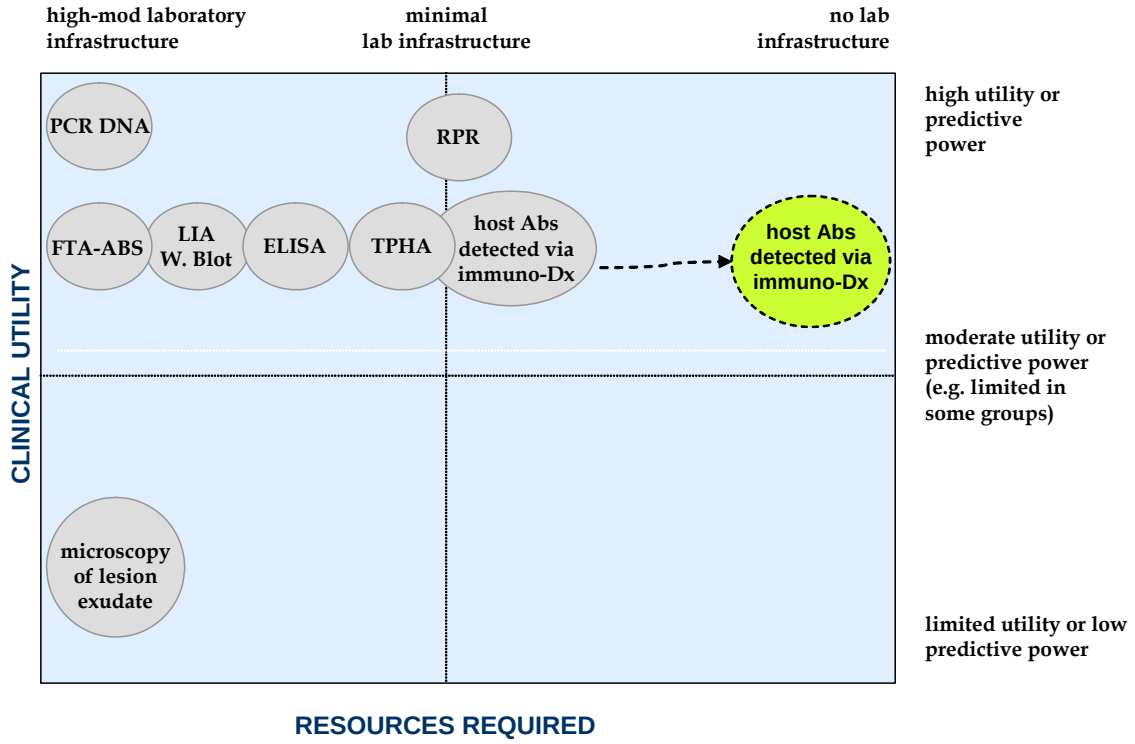
The assay should be able to identify the presence of *T. pallidum* infection and hence syphilis with a reasonable level of sensitivity (approximately 85%, or greater) and specificity (approximately 90%, or greater). The test needs to use a format that is easy to perform in resource limited settings.

8B. What biomarkers, sample types, and technologies are most appropriate for Syphilis?

The most likely candidates for practical specimen types are oral fluids, urine, and whole blood. These specimens have been shown to be appropriate for immunoassays that detect the presence of host antibodies against *T. pallidum.* In addition they have been shown to be appropriate for nucleic acid (pathogen DNA) based tests. Serum or plasma from whole blood is the only specimen that has been extensively validated for the detection of host antibodies against the pathogen and these specimens are used in routine syphilis testing in developed countries; however, we do not recommend using serum or plasma in resource limited settings.

The future diagnostic approaches for syphilis in resource-limited settings are depicted in Figure 3. In this Figure, the current approaches are shown as light gray spheres and the future approaches are shown in color. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource-limited setting (y-axis). In these Figures, therefore, an ideal biomarker and test method will be in the upper right quadrant, with a high predictive power and low resource requirements.

Figure 2. Future Approaches for Diagnosing Syphilis and Their Utility in Resource-Limited Settings



8C. Recommended Course of Action and Resources Required

There are several sets of potential activities that could lead to the development of future diagnostic tests that are depicted in Figures 3 for syphilis, which are discussed in the subsections below.

We recommend a health economic study comparing correctly diagnosed active and current infections versus the misdiagnosis of current infection in persons that were previously infected and cured. The models would compare treating women with a test that can not differentiate an active infection from a previous treated infection, a test that could differentiate current from previous infections and no test and treatment (which is often the case in resource limited settings). This modeling needs to be performed using various levels of disease prevalence (e.g., 0 to 20%) along with various levels of previous treatment (e.g., 0 to 20% previous treatment).

If this modeling study demonstrates that no diagnosis and no treatment provides a worse health outcome compared to over treatment, even at high prevalence levels, then the treponemal RSTs should be advanced. If the study demonstrates that there are levels of disease prevalence and/or previous treatment where over treatment is a major issue (e.g., the outcome of over treating a population where the level of previous infection is 20% is worse than no treatment) then the degree of previous treatment and disease prevalence in the target population would need to be determined. This can potentially be estimated via patient interviews but might also require testing sample populations with both non-treponemal tests (e.g. RPR tests) and treponemal tests (e.g. ELISA). If actual testing is required, serum samples will need to be collected from sample target populations and transported to a well equipped laboratory for testing. From

a practical perspective, and considering the low cost and minor toxicity associated with over treatment vs. the serious health outcomes associated with no diagnosis and treatment in regions of moderate to high disease prevalence, we expect that the health economic modeling will indicate that current RSTs should be advanced. If the modeling reveals that over treatment has dire consequences, the development of a test that can differentiate active from past infection would be necessary. Another outcome of the modeling would be a set of sensitivity and specificity goals that are based upon the economic and health impact at specific prevalence levels.

Potential Path Forward 1 Detection of Syphilis antigen using RSTs

Before moving RSTs forward into clinical field evaluation, they must be evaluated for robustness and stability under conditions that mimic those encountered in resource limited settings. This can be done in an established laboratory using repository serum specimens. It is essential that user specifications for testing and test kit storage be rigorously established and accurately reflect those conditions that would be encountered in the field. This approach will minimize the risk of failure in field trials which can be costly and time consuming.

If the RSTs pass this evaluation then the existing kits would be considered ready for field trials. If they fail, then the root cause for failure would need to be determined and the RSTs need to be re-developed. Re-development could be a lengthy and difficult task depending upon the source of instability (e.g. reagent instability, evaporation).

In parallel with testing the robustness of RSTs, the appropriateness of using finger prick whole blood specimens needs to be evaluated. Although some RSTs have been validated for such specimens, they have not been tested and validated under field conditions encountered in resource-limited settings. Such testing should again be performed in a controlled laboratory setting using conditions that mimic and even surpass the extremes that one might encounter. Some form of manipulation of the finger prick specimen, such as adding it to a buffer solution to minimize drying, may be required. This needs to be evaluated if testing a neat (non-treated) whole blood specimen fails. If finger prick whole blood specimens pass testing, then finger prick blood would be ready to be tested in field trials with RSTs that pass robustness testing. If finger prick whole blood fails, then alternative specimens such as saliva or urine should be evaluated for sensitivity, specificity and robustness. Once again, re-development of the RSTs could be warranted.

Once the appropriate specimen is identified and the appropriate RST validated for robustness, they should then be advanced to field trials. We believe that the technological risk for identifying/developing a stable RST combined with validating an easily obtainable specimen is medium.

If health economic modeling indicates that over treatment is a major issue or if an appropriate specimen/RST combination can not be identified or developed, then the need will arise to develop a test that measures the level of *T. pallidum* in a easily obtainable biological specimen as a measure of current infection. We believe that these scenarios are unlikely for the reasons stated above, but in case that this situation is encountered, potential path forward #2 is presented below.

Potential Path Forward 2: Adaptation of DNA Testing to a Rapid, Robust Platform

A global impact would be made with a test that could easily detect genomic *T. pallidum* DNA in urine, saliva, or finger prick blood using a simple, inexpensive approach. Significant effort would be required to develop a DNA amplification and/or detection device that parallels the convenience, robustness, and costs of lateral flow RSTs. However, if developed, such a device would prove valuable, not only to address limitations of syphilis diagnosis, but on a grander scale by providing a means for the diagnosis of many other pathogens using urine or oral fluid specimens. Designing and developing such a DNA analysis device would be an expensive and multi-year, project, involving multi-disciplinary teams with broad skill sets. Several groups world wide are already engaged in these activities, but in most cases they do not appear to understand the user requirements necessary.

If such a system were available, it would be possible to adapt the nucleic acid amplification tests which have been developed in many laboratories world wide. This would involve validating the pathogen genome sequences being amplified and detected, and demonstrating the stability of testing reagents under conditions that are appropriate for resource limited settings.

9. Summary of Recommendations

In summary, we recommend that a health economic analysis be performed to understand the impact of over treatment of syphilis to understand the need to differentiate active infections from previous and treated infection. If this analysis indicates that over treatment is not an important issue, then existing RSTs should be tested for robustness and alternative specimen types need to be evaluated for use in resource limited settings. This should be done in controlled laboratory settings, followed by field testing. Extensive improvements to RST kits could be needed. These activities should be performed in a parallel manner to understand the probability of existing RSTs meeting the performance criteria required in a resource limited environments.

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