Laboratory Examination of Syphilis

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A B S T R A C T

Syphilis, is sexually transmitted disease caused by spirochete Treponema pallidum subsp.pallidum. It has many diverse clinical manifestations that occur in distinct stages. Early diagnosis and management are the main things to prevent transmission and complication. Direct test or morphological observation is the definitive diagnosis of syphilis. This can be done through animal inoculation test, dark field microscopy, direct fluorescence antibody (DFA), and nucleid acid amplification test (NAAT). While the indirect test is a nontreponemal serologic test consist of Wasserman test, venereal disease research laboratory (VDRL), toluidine red unheated serum test (TRUST), unheated serum reagin (USR), rapid plasma reagin (RPR) and treponemal serologic test, such as T. pallidum passive particle agglutination (TPPA), T. pallidum haemagglutination assay (TPHA), fluorescent treponemal antibody absorption (FTA-Abs), enzyme immunoassay (EIA) and rapid test. The algorithm of serologic test can be divided into traditional or reverse.

1. Introduction

Syphilis is a sexually transmitted infection (STI) caused by spirochaete *Treponema pallidum subsp.pallidum*. Syphilis is classified into primary, secondary, latent and tertiary stages.[1]

Based on the Integrated Behavioral and Biological Survey report in 2011 the incidence of syphilis in 2011 is 13% higher than 2007.[2] Study by the South Sumatra Provincial Health Office in 2014 found that the incidence of syphilis in female sex workers was around 4.5%.[3] Based on data from the General Hospital Dr. Mohammad Hoesin (RSMH) Palembang, Dermatology Venereology (DV) sexual transmitted infection outpatient clinic in 2018-2020, recorded the number of visits by syphilis patients is 76 patients.

Syphilis have several types of clinical manifestations based on the stage level. Primary and secondary skin lesions can mimic other diseases. In the latent stage, asymptomatic conditions can more complicate to be diagnosed. Early diagnosis and management can prevent complications, and disease transmission. Direct test or morphological observation is used for the definitive diagnosis of syphilis. This can be done through animal inoculation, test using a dark field microscope, direct fluorescence antibody (DFA), and a nucleid acid amplification test (NAAT). Indirect test through serologic test consists of nontreponemal and treponemal. Nontreponemal serologic test are

Wasserman's test, venereal disease research laboratory (VDRL), toluidine red unheated serum test (TRUST), unheated serum reagin (USR), and rapid plasma reagin (RPR) are used for screening, evaluate disease progression and therapy. While the treponemal serologic test consists of *T. pallidum* passive particle agglutination (TPPA), *T. pallidum* haemagglutination assay (TPHA), fluorescent treponemal antibody absorption (FTA-Abs), enzyme immunoassay (EIA) and rapid test can detect *T. pallidum* specific antibodies.[4][5]

There are 2 types of syphilis serologic testing algorithms, such as traditional and reverse. The traditional algorithm begins with a nontreponemal serologic test confirmed by treponemal serologic test. The reverse algorithm begins with a treponemal serologic test confirmed by nontreponemal and treponemal serologic test.

The aim of this literature review to remind the clinicians about laboratory tests for syphilis. Hopefully it can be used to determine the diagnosis, assess the stage and evaluate therapy.

Morphology

Treponema pallidum (*T. pallidum*) *subspecies pallidum* is a bacteria that cause syphilis infection.[1] This infection is transmitted through sexual contact, from mother to baby through the placenta, or blood transfusions. This bacterium belongs to the *Spirochaetaceae* family and associated with another pathogenic treponema that causes endemic treponematosis, such as *Treponema pallidum subsp. endemicum* (bejel), *T. pallidum subsp. pertenue* (yaws, patek), and *T. pallidum subsp.carateum* (pinta).[6]

On dark field microscopy, organisms can be recognized by their characteristic movements of moving forward and backward slowly, rotating on a long axis like a corkscrew, elongating and curving. Such movements are possible because there is an endophlagellae. *T. pallidum* varies in size, 6-15 μm in length, 0.2 μm in diameter, and there are 8-20 coils with 1 μm spacing.[6]

Several immunogenic *T. pallidum* antigens in

syphilis that can cause specific antibody responses are Tp15, Tp 17 and Tp 47. Until now, the function of Tp15 in the treponema structure still unknown. Most of the outer membrane of *T. pallidum* is composed of 17kDa lipoprotein (Tp17) which can bind to surrounding proteins, maintain treponemal membrane function and trigger activation of intercellular adhesion molecule 1 (ICAM-1), e-selectin, and monocyte chemoattractant protein-1 gene (MCP-1) in endothelial cells. Another immunogenic 47 kDa lipoprotein (Tp47) is a carboxypeptidase that can bind to penicillin and increase immune responses in endothelial cells.[7]

Classification of syphilis

The clinical manifestations of syphilis vary from stage and called a 'great imitator' or 'great mimicker'. The course of syphilis varies and classified into early and advanced stages of syphilis. Early stage is more infectious than late. Early stage syphilis, namely primary, secondary and early latent syphilis. Late stages of syphilis includes tertiary (gumma, cardiovascular syphilis, neurosyphilis) and late latent syphilis.[6][8]

The course of syphilis begins with sexual contact with early syphilis patients. *T. pallidum* bacteria will penetrate through mucosal surface or exposed skin, then adhere to epithelial cells and host extracellular matrix components and begin multiplying. Replication occurs at the site of inoculation every 30-33 hours, giving rise to a local inflammatory response in the form of a painless wound (chancre) occurring 3-6 weeks after infection. Syphilis lesions form approximately 3 weeks (9-90 days) after *T. pallidum* infection. If syphilis lesions are left untreated, they may disappear spontaneously in 2-3 weeks. The secondary stage generally occurs 4-8 weeks after the primary lesion has disappeared, and lasts several weeks or months.[6]

Secondary syphilis is a systemic vasculitis due to high blood levels of *T. pallidum* and an immunologic response. The first lesions that appear in secondary syphilis are pale red macules with a diameter of 0.5-1.5 cm on the body and extremities, called secondary syphilitic macular rash or syphilitic roseola. These

lesions can be found on the palms of hands, and feet also forehead. The rash consists of reddish-brown, copper or slightly bluish (50-70%) maculopapular, but can also be in the form of papules (12%), macules (10%), annular papules (6-14%), pseudovesicles, lichenoid, papulosquamosa, corimbiform, or ulcerated. In the scalp area can be found irregular and diffuse papular lesions and alopecia patches called "mouth-eaten alopecia". In oral mucosa can be found erythematous spots on the palate mole and durum, as well as the cheek mucosa.[6]

In the early latent stage the patient has no clinical symptoms but serologically indicates active syphilis. Early latent stage can progress to late latency starting within 1 year after infection. In this late latent phase it is not transmitted through sexual contact, but can be transmitted through the placenta to the fetus. This can be due to a high number of treponemes in the lesions, making them more infectious. Around 3-10 years, latent syphilis will progress into tertiary syphilis. [6]

Laboratory test

The diagnosis of syphilis is based on clinical suspicion combined with laboratory tests for direct and indirect detection of *T. pallidum*.

Test of the board of directors
Animal inoculation test

Several animals, including hamsters, chimpanzees, and rabbits have been used to assess *T. pallidum* infectivity. Rabbit is most often used because the changes can be immediately seen with the naked eye and serologically reactive, so it is called the rabbit infectivity test. The test is carried out by injection of a sample such as cerebrospinal or serous fluid into a seronegative adult New Zealand rabbit. After 1 week of inoculation, an inspection and serological test of rapid plasma reagin (RPR) and *T. pallidum* particle agglutination (TPPA) were carried out every 2 days for 1 month and every 2 weeks for 2 months. Samples from the rabbit were transferred to other seronegative rabbit, and the incidence of orchitis was observed. Sensitivity and specificity 100%.[9] A positive result if the serologic

T. pallidum is reactive, but a negative result does not necessarily rule out a diagnosis of syphilis because many factors influence the work technique. Although this test is the gold standard, it is highly dependent on work technique, professional laboratory personnel, animal protection ethics and takes a long time, so animal inoculation test is only used for research purposes.[5][10]

Dark field microscopy

Examination using a dark field microcopy is a simple definitive diagnostic method which is still applicable today.[1] Through this, we can immediately see the shape of *T. pallidum*. Microscope is divided into light and dark field. The microscope components consist of a diaphragm, a condenser, a condenser focusing device and an objective lens (Figure 2a).[11]

The main difference between dark and light field microscopy is that the light directly to the specimen and the objective lens is limited to a darkfield stop at the condenser, then light is absorbed only from the edges and results in a dark field of view (Figure 2b). [11]

The objective lens size and numerical aperture (NA) in dark field microscopy are important in successful diagnostic. Numerical aperture is the angle of light that will be received by the specimen. If the NA is too large, a hollow cone of light will not form, which can complicate the test (Figure 2b). The recommended objective lens size and NA are low to medium size, in the range of 2-10 times (0.05-0.25 NA). The ideal type of specimen is serous fluid from active lesions that are not accompanied by red blood cells. This fluid can come from lesions in the primary stage, namely chancre, condyloma lata or mucosal lesions in the secondary stage, and rhinitis fluid from congenital syphilis.[12]

The active lesion is cleaned first using gauze and sterile saline. After drying with sterile gauze, the active lesion is pressed from the edge with both fingers to increase the amount of serous fluid. The slide can be attached directly to the lesion to collect the specimen or a stainless steel spatula can be used to move the specimen. Specimens are given 1-2 checks for sterile saline and covered with a glass cover. Examination of

the specimen should be carried out immediately within 30 minutes because the motility of *T. pallidum* is the main feature for diagnosis. *Treponema pallidum* will appear spiral in shape, and are bright white. Surface area between 0.10-0.18 μm, length 6-20 μm. Typical movements are flexion and relaxation in an irregular direction (Figure 2c). [11][12]

Positive result if organisms with similar morphology and movements are found. False positives can occur when other types of treponemes are present in the preparation. False negative results can be found if the number of organisms is insufficient, history of treatment with topical antibiotics of the lesion, the lesion has resolved or the lesion is not part of syphilis. The sensitivity of dark field microscopy is 74-79%, with a specificity of 100% .[1][11] Dark field microscopy can give a positive result before reactive serologic studies. However, this test is not recommended as a routine screening because it is impractical, only for lesions with serous, microscopic field of view is very sensitive to changes in light and the presence of other particles.[13]

Direct fluorescence antibody (DFA)

Direct fluorescence antibody is a test to seeantigen-antibody bonds using fluorescent isothiocyanate (FITC). This test can uses specimen samples from oral and rectal lesions.[4] The lesion was cleaned with saline, minimal abrasion was performed to obtain serum. The DFA test is an alternative to the dark field microscope. This test does not require a specimen with live bacteria. Monoclonal or polyclonal antibodies are used from rabbits in Reiter's treponemes in order to remove non-specific antigen bonds. Detection of antibody antigen bonds conjucated with FITC will give an apple green color (Figure 3). The specimens used may come from serous fluid from oral or rectal lesions, cerebrospinal fluid, or paraffin deep tissue fixation. The fluid from the lesions can be drained and sent to the laboratory in a temperature of 4-8°C. A specimens must be viewed with an objective lens 40 times. For long-term storage, specimens can be stored at -20°C. [10]

Both negative and positive control antigens (stained

T. pallidum specimen as a positive control, and Reiter's treponema as a negative control) should be included in each test. DFA test of the tissue in paraffin, the tissue should be deparaffinised, rehydrated and given 1% ammonium hydroxide or PBS containing 0.25% trypsin. Positive control was derived from examining rabbit ants containing *T. pallidum* and tissue containing non-pathogenic Treponema. [10]

Specimens were given fluorescent anti-*T. pallidum* immunoglobulin, a monoclonal antibody derived from rabbits or mice and viewed with an electron microscope. It is positive if you see an apple green color on a fluorescent microscope, but false positives can occur if other types of treponemes are present (Figure 3). [10] A negative result if the treponema is not visible on the microscope, but a false negative if there is an error in the sampling or processing of the sample.[12][14]

The sensitivity of DFA test with conjunctival monoclonal antibody in specimens from the lesion is 73-100%, with a specificity of 100%, and assays using polyclonal antibodies is 86-90%, with a specificity of 96-97%. The sensitivity of the test with tissue material was 86-88% and specificity 97-100%. [10] Direct fluorescence antibody testing does not require live organisms, but is more expensive and fluorescent fluids are easily damaged.[15]

Nucleic acid amplification examiner (NAAT) or polymerase chain reaction (PCR)

Polymerase chain reaction or "molecular phototyping" is a technique of amplification of DNA segments to be duplicated into certain amount. Tools needed in PCR tests are thermo cyclers, DNA templates, two primers, taq polymerase, nucleotides and buffers. Polymerase chain reaction test can detect the DNA of organisms in specimens less than 10, by amplification of specific gene segments derived from *T. pallidum* DNA at the primary or secondary stage.[6][12] This test is mainly used for oral lesions or lesions of other commensal treponemes. Specimens can be collected from tissue, cerebrospinal fluid, or blood, whether they are fresh, frozen tissue, or fixated in paraffin.[16]

Polymerase chain reaction test can be done quantitatively or qualitatively. The quantitative method, also known as real time PCR, is by counting the amount of DNA in the sample to assess the severity of the infection. The sensitivity is lower if the sample is not from a serous lesion. Qualitative PCR methods are used to detect specific genes for bacteria. PCR test in the conventional way is carried out by making a specific primary bond to the DNA strand so as to limit the amount of DNA replication that is not needed. Some of the things that are needed in conventional PCR are aluminum blocks, DNA polymerase, buffers, primers and target DNA. The entire process takes time around 35-40 minutes and assessed by a gel electrophoresis technique. Multiplex PCR is a technique that can distinguish different types of pathogens in each sample by detecting strands of introns and exons in DNA. This test can be used to determine the presence of co-infection with syphilis separat on test of a blood sample for donation. This test is quite easy, fast and inexpensive and can separate several amplification reactions based on the fluorophore reaction. Sensitivity is 50-83.3%. Nested PCR can limit the formation of contamination in amplification products or incompatible primary DNA bonds. The sensitivity is 70% with a specificity 95%. fast and cheap and can separate several amplification reactions based on the fluorophore reaction. Sensitivity is 50-83.3%. Nested PCR can limit the formation of contamination in amplification products or incompatible primary DNA bonds. The sensitivity is 70% with a specificity of 95%. Nested PCR can limit the formation of contamination in amplification products or incompatible primary DNA bonds. The sensitivity is 70% with a specificity 95%.[16][17]

Routine and multiplex PCR tests can be used as additional screening methods in screening for early stage syphilis, while nested or real-time PCR can be used for confirmatory tests. [17] Fluorosens and genetic analyzers ABI310 can detect part of the treponemal DNA replication to enhance the PCR reaction in each specimen. The result is positive if 1 base pair is detected in the tpp15 gene *T. pallidum subsp. pallidum*

which is different from other subspecies.[18] Meanwhile, false negatives can occur if there is a specimen taking error. Specivisity is 93-100% and PCR sensitivity reaches 95% .[13] Polymerase chain reaction test can detect treponemes in small amounts, but it is more expensive, and trained personnel.[15]

Indirect test
Serologic test

There are 2 types of serologic test, namely treponemal and nontreponemal. Nontreponemal tests can detect immunoglobulin (Ig) G and M antibodies against lipoidal antigens released by the body as a consequence of cell and bacterial destruction. These antibodies can be detected 6 weeks after infection. Meanwhile, treponemal tests are used to detect specific IgG and IgM antibodies for *T. pallidum.* These antibodies can be detected within 3 weeks after infection (Figure 4).[13]

This test is divided into 2 types, namely nontreponemal, including Wasserman's test, venereal disease research laboratory (VDRL), toluidine red unheated serum test (TRUST), unheated serum reagin (USR), and rapid plasma reagin (RPR). Treponemal tests consist of fluorescent treponema antibody absorption assay (FTA-Abs), T.pallidum particle agglutination (TPPA), T.pallidum haemagglutination assay (TPHA), various treponema enzyme immunoassays (EIAs) and rapid testing.[1][6]

Nontreponemal serologic test

This test is used to detect antigen agglutination with immunoglobulin (Ig) G and IgM antibodies that are formed by the body due to cell damage.[19] The test was used as an evaluation of the therapeutic response with a decrease in titer of up to 4 times, or the equivalent of 2 dilutions (from 1:16 to 1: 4). There are 5 types of nontreponemal tests, namely Wasserman, VDRL, TRUST, USR and RPR tests.

Wassermans Test

The Wasserman test is the first nontreponemal

serologic test that uses antigens from bovine liver tissue. The results of Wasserman's test were strong positive (+4), positive (+3, +2), doubtful (+1) or negative based on the occurrence of hemolysis in the form of a reddish color in the patient's serum (Figure 5) .[19]

Wasserman test will be positive in 82-92% on primary stage, secondary 92-100%, and tertiary 75-95%. False positive results can be due to infection with other types of treponemes, malaria, Morbus Hansen or pregnancy. Meanwhile, false negatives can be caused by errors in working techniques, the patient received previous therapy or the test time was too early. The advantage of Wasserman's test is that it can see the antigen-antibody reaction in syphilis. Meanwhile, the antigen is less specific and cannot be used as a measuring tool for evaluation of improvement after therapy.[21]

Venereal disease research laboratory (VDRL)

The Wasserman test antigen was considered less specific, so a reagin modification was carried out for the VDRL test. This test uses an antigen consisting of a mixture of cardiolipin, lecithin and cholesterol. [12][22]

In treated primary syphilis patients, VDRL can be negative around 60-100% in 4-12 months. Meanwhile, secondary syphilis will be non-reactive at 12-24 months after therapy. In the latent stage of syphilis. will remain in low titers for 5 years or more (Figure 6) .[1][12]

The VDRL test can be done quantitatively or qualitatively. The quantitative way is to put the patient's serum into a centrifuge and heat it at 56°C. Then, the serum was mixed with the VDRL reagin into the cavity slide. After playing for 4 minutes, the mixture was viewed under a light microscope. Positive result if clumping is formed on the slide. While the qualitative method is done by giving 2 tests of NaCl 0.9% cavity slides 2 to 5, then followed by 3 tests to cavity slides 3 to 6. Then the patient's serum that has been diluted 8 times with 0.9% NaCl is examined. to the entire cavity slide. The reagin was examined on each cavity slide and placed in rotation for 4 minutes. The mixture is viewed under a light microscope to assess the clumping

reaction of each slide. This test is reactive at week 4 after infection for up to 1 year, and decreases slowly, so that late latent syphilis will appear low titers. Results are considered reactive if there is a quantitative titer of 1: 8. Non reactive when the titer is less than 1: 8.[12]

The sensitivity of the primary stage is 78% (74-87%), secondary is 100%, latent 96% (88-100%), and tertiary 71% (37-94%). Specificity is 98% (96-99%). [28] False positives can occur due to aging, pregnancy, other bacterial or viral infections, immunization or malignancy. False negatives can occur when there is a prozone reaction or the test is too early.[4] The advantages of VDRL are sensitive, more specific and stable reagins than the Wasserman test. The drawback is that false positives are common and the subspecies of treponemes cannot be distinguished. [1][4]

Rapid plasma reagin (RPR)

Rapid plasma reagin is a type of nontreponemal flocculation test, using a modified VDRL reagin. The reagin in RPR is added with choline chloride and ethylenediaminetetraacetic acid (EDTA) to stabilize the suspension and charcoal so as to make it easier to visualize the reading of the results.

This test can be carried out in a qualitative or quantitative way. The qualitative method is to check the reagin on the patient's blood serum on the circle of the RPR card. After turning it for 8 minutes, you will see a positive / reactive reaction in the form of blackness called clumping (Figure 7). [12][24]

Quantitative RPR was performed by mixed 0.9% NaCl with antigen and patient serum in each circle ranging from 1: 2 to 1: 512. The mixture will continue to be moved (1: 2 to 1: 4, 1: 8 to 1:16, 1:32 to 1:64, and 1: 128 to 1: 512) to be added again with 0.9% NaCl and antigen until dilution occurs, then the agglutination reaction that occurs is assessed. (Figure 8). [22][24]

The sensitivity of the RPR test depends on the duration of infection, the primary stage is 86% (77-99%), secondary is 100%, latency is 98% (95-100%), and tertiary is 73% .26 %). False positive can occur in the acute (<6 months) and chronic (>6 months) phase. In the acute phase, false positive occur in various

conditions, namely malaria, hepatitis, chickenpox, measles or vaccination. The chronic phase is often caused by connective tissue disease, malignancy, chronic infections, needle users, and aging. False negative results can be due to a prozone reaction that occurs when the count of nontreponemal antibodies is too high, leading to failure of the formation of the antigen-antibody reaction or if the timing of testing is too early. The advantages of RPR test are more sensitive, easier technique than VDRL and reagin can last longer.[22]

Toluidine red unheated serum test (TRUST)

This test uses a modified VDRL suspension antigen in the form of the addition of toluidine red pigment. This antigen can detect antilipoidal antibodies in the serum of syphilis patients. This test can be carried out in a qualitative or quantitative way. Qualitative is done by mixing the serum of syphilis patients with antigen suspension on the TRUST card. The card is placed on the rotator for 8 minutes. If reactive / positive, a clumping reaction will be found in the form of reddish particle agglutination (Figure 9). [27]

Toluidine red unheated serum test in quantitative by applied 0.9% NaCl to the TRUST card circle number 2 to 5. The patient's serum was examined and mixed in the second circle (1: 2). The mixture is moved to the 3rd circle (1: 4), then the mixture of the third circle is moved to the fourth (1: 8), the mixture of the 4th circle is moved to the fifth (1:16). The TRUST reagin was examined on all circles, then placed on the rotator for 8 minutes, and the clumping reaction was assessed. Positive result if a clumping reaction is found. The sensitivity of TRUST in the primary stage is 85% (77-86%), secondary is 100%, latent 98% (95-100%). While the specificity is 99% (98-99%). False positive results can be caused by aging, pregnancy and other acute or chronic diseases. False negatives can be caused by a prozone reaction, technical errors or inspection time too early. The advantage of TRUST test is that it is sensitive and does not require a microscope for analysis of the results. The drawback is that false positives are frequent and the subspecies of treponemes cannot be distinguished. [25][27]

Unheated serum reagin (USR)

Unheated serum reagin is a nontreponemal test using a modified VDRL reagin. In this test, the reagin is added with choline chloride so that it can react with the patient's unheated serum. This test can be carried out in a qualitative or quantitative way. The qualitative way is by mixing the patient's serum with the USR antigen. After being placed on the rotator for 4 minutes, the mixture was viewed under a light microscope to assess the clumping reaction. The quantitative method is by applying 0.9% NaCl to the entire cavity slide. The patient's serum is examined in the first (1: 1) and second (1: 2) circles and then mixed. The mixture is moved to the third circle, from the third circle it is moved to the fourth, from the fourth circle it is moved to the fifth. The cavity slide was placed on the rotator for 4 minutes and the results were read through a light microscope. The results are reactive if there is a clumping reaction.[29] The sensitivity of USR at the primary stage is in the 80% (72-88%), the secondary is 100% , and the latency is 95% (88-100%). False positive results can be caused by aging, pregnancy, autoimmune or other bacterial and viral infections. False negative results can be caused by a prozone reaction, technical errors or unstable reagins. The advantages of USR are that it does not require heated serum and the reagin can last longer. The disadvantage of USR is that it is very dependent on work tools and techniques. pregnancy, autoimmune or other bacterial and viral infections. False negative results can be caused by a prozone reaction, technical errors or unstable reagins. The advantages of USR are that it does not require heated serum and the reagin can last longer. The disadvantage of USR is that it is very dependent on work tools and techniques. pregnancy, autoimmune or other bacterial and viral infections. False negative results can be caused by a prozone reaction, technical errors or unstable reagins. The advantages of USR are that it does not require heated serum and the reagin can last longer. The disadvantage of USR is that it is very dependent on work tools and

techniques.[25][29]

Treponemal serologic test

This test uses an antigen derived from all or part of the T. pallidum fragment. This test is not used for evaluation of therapy because it can last a lifetime even if therapy is successful. [1][6]

Fluorescent treponemal antibody absorption (FTA-Abs)

This test uses *T. pallidum* suspension mixed with 1: 5 diluted serum. In this test, the patient's serum is given absorbent to remove non-specific antibodies. Monospesific antihuman fluorecein isothiocyanate (FITC) is a specific antisera against antitreponemal IgG, IgM and can be used to see other types of antibodies from patient serum, namely IgA, IgC and IgD. Anti-treponemal immunoglobulin M is an antibody that appears first during early infection and penetrates the placenta so that it can be used for the diagnosis of congenital syphilis using IgM-FTA-Abs. [12][28]

If there are treponemal antibodies, there will be antigen-antibody bonds that react with fluorescence. Fluorescence results in the form of a greenish yellow glow (Figure 3).[12] The level of sensitivity is 84% (70- 100%) at the primary stage, 100% at the secondary stage to latent, and tertiary 96% with a specificity of 97% (84-100%).[26]False positive results can occur in patient serum containing the antibody Borrelia burdorferi. The advantages of FTA-abs are higher sensitivity than TPPA or TPHA. The drawback is that the quality of the FTA-Abs test depends on the availability of tools, reagins and the ability of the investigator so that it cannot be used as a routine screening tool. [12][28]

T. pallidum haemagglutination assay (TPHA), T. pallidum passive particle agglutination (TPPA)

This test is a type of passive agglutination using ultrasonicated antigen or extract detergent (nichols strain) which is fixed in erythrocytes (TPHA), red gelatin (TPPA), bentonite or latex paricles. This reagin binds to the patient's serum IgG and IgM T. pallidum antibodies (Figure 8). This test detects treponemes in syphilis, yaws, pinta, bejel and endemic syphilis. This test can be used from the 3rd week after infection and will remain positive even after treatment. Positive results if an agglutination layer is formed on the microtiter tray well. If no antibodies are found, the particles will settle and form clots at the bottom. This test is commonly used to confirm a positive nontreponemal test.[31][32]This test can be carried out in a quantitative or qualitative way. The quantitative method is done by combining the dilution of the sample with the patient's serum into well 1 and mixing it. The mixture is transferred to the 4th well and dilution of the sample is added. The third well was given unsensitized particle (gelatin) as a control, and the 4th well was given sensitized particle (gelatin coated with T. pallidum) (Figure 10). A positive result will form a ring-like agglutination after 2 hours.[12][28][29]

The quantitative way, resembles the qualitative way. The mixture of diluent samples and patient serum from well 1 is transferred to the next well up to wells 11 to 12. The third well was given unsensitized particle (gelatin) as a control, and the 4th well (dilution 1:80) to twelveth (dilution 1: 20480) was given sensitized particle. The results were observed after 2 hours of test (Figure 11). [25][31]

The sensitivity of the primary stage is 88% (86-100%), secondary is 100%, latency is 97% (97-100%), and tertiary is 94%. Specificity is 99% (98-99%). The advantage of TPPA is that false positives are rare. While the drawbacks of this test are that it cannot be used to assess therapeutic response, is more expensive than nontreponemal tests and requires special tools.[28][31]

Enzyme immunoassay (EIA), chemiluminescence immunoassays (CIA)

Enzyme immunoassay is an indirect method for the detection of T. pallidum antibodies. This test is often used to screen for syphilis at blood banks. There are 2 types of reagins, namely for detection of IgG and IgM. IgM reagins are commonly used for detection of congenital syphilis. This test uses a reagin from the

sonicated T. pallidum antigen. The patient's serum was inserted into a well microtiter plate and then added with the antibody reagin. If there are specific antibodies against T. pallidum, an antigen-antibody bond is formed. After the excess antibody was removed, enzyme substrate was added and analyzed with a plate reader. Existing antibodies will cause color on the reading. Several other types of EIA, can use recombinant specific antigen treponema-15, 17,44.5, and 47kD antigens mixed with patient serum in a well microtiter plate. All proteins that are not bound to antigens will be lost during cleaning and the chromogenic substrate is added. Color changes can be seen by spectophotometrics.[23] Sensitivity at the primary stage is 77-100%, secondary 85-100%, latent 64-100% with a specificity 99-100%.[28] Chemiluminescent assay is an immunoassay technique using labels or reaction indicators in the form of luminescent molecules to determine the concentration of the analyzed sample. When administering antigen-isoluminol to the serum of syphilis patients, a positive result will be found a chemiluminescence color glow detected by a sophisticated photomultiplier system. This test has a sensitivity of 98% in the primary stage, 100% in the secondary, latent and tertiary stages with a specificity of 99-100%. will be lost during cleaning and chromogenic substrate is added. Color changes can be seen by spectophotometrics.[23] Sensitivity at the primary stage is 77-100%, secondary 85-100%, latent 64-100% with a specificity 99-100%.[28] Chemiluminescent assay is an immunoassay technique using labels or reaction indicators in the form of luminescent molecules to determine the concentration of the analyzed sample. When administering antigen-isoluminol to the serum of syphilis patients, a positive result will be found a chemiluminescence color glow detected by a sophisticated photomultiplier system. The sensitivity 98% in the primary stage, 100% in the secondary, latent and tertiary stages with a specificity 99-100%, will be lost during cleaning and chromogenic substrate is added. Color changes can be seen by spectophotometrics.[23] Sensitivity at the primary stage is 77-100%, secondary 85-100%, latent 64-100%

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99-100%.[26] Chemiluminescent assay is an immunoassay technique using a label or reaction indicator in the form of a luminescent molecule to determine the concentration of the analyzed sample. When administering antigen-isoluminol to the serum of syphilis patients, a positive result will be found a chemiluminescence color glow detected by a sophisticated photomultiplier system. This test has a sensitivity 98% in the primary stage, 100% in the secondary, latent and tertiary stages with a specificity of 99-100%.[4][10] This test can be done with a large amount of serum, but it cannot distinguish between active or past infections.[15][26]

Rapid test

Rapid test is a serologic test using a rapid membrane immune-chromatographic technique that provides qualitative results for the detection of *T. pallidum* antibodies in blood, serum or plasma within 10 minutes. The specimen can be collected via a fingertip blood sample. The result is positive if there is a line on C (control line) and T (test line) (Figure 12) .[31]

This screening is being developed in the United States for rapid screening in emergency departments, sexually transmitted infections-HIV outpatient clinic, health promotion, family clinics, antenatal programs, prisons, drug rehabilitation centers, and communities with risky behavior. This test is relatively inexpensive, fast, and does not require specialists. Rapid test has low sensitivity especially if the RPR titer is <1:16. Another drawback, generally only detects anti- treponemal antibodies. The sensitivity of the rapid test is 89.9% with a specificity of 99.3% for treponemal antibodies and sensitivity 94.2% with a specificity 62.2% for non-treponemal antibodies.[33]

Point of care is carried out by the nearest health worker with results in a short time. According to the World Health Organization (WHO) in 2017, the principle of POC test must meet the ASSURED criteria (Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment free, and Deliverable to users). Until now, POC tests have begun to be used in STI

clinics, pregnancy, and for detection among women sex workers (FSW) and MSM.[31]The disadvantage of a rapid test is that it cannot differentiate between new and old T. pallidum infections. The advantages of this test are its ease of use and early screening for syphilis. If the result is positive, it is necessary to check again with an RPR test. If the RPR is positive, treatment can be given, but if it is negative, a repeat test is necessary after 6 weeks from the last test. Health centers that do not have an RPR test facility can provide therapy to patients with a positive rapid test (Figure 13).[33]

Treponemal test can detect other spirochete subspecies such as yaws disease (*Treponema pallidum subsp. Pertenue*), pinta (*Treponema pallidum subsp. Carateum*), and bejel (*Treponema pallidum subsp. Endemicum*). False positives can be caused when multiple subspecies of T. pallidum are present. False negative results are more often due to laboratory errors or incorrect reading of results.[12]

Syphilis self-test is carried out by involving the patient to take a specimen, conduct and read the results of the test. This method is widely used by MSM who do not want to go to health centers because they are still afraid of the stigma of society. Patients can determine the location, condition and time of the test by themselves.[35]

Serologic test algorithm

Serologic testing is the standard to help diagnose syphilis. The serologic test algorithms are divided into traditional and reverse. Traditional serologic testing begins with a nontreponemal test (RPR). If the RPR is positive, it is necessary to confirm a treponemal test such as FTA-Abs or TPPA. The traditional algorithm is used more frequently because the RPR titer is more sensitive, decreases after therapy and is less expensive, but it is more manual and false-negative results are more common than the reverse algorithm (Figure 14).[13][38] The sensitivity of the traditional algorithm is 72.9%.[33]

In reverse algorithm (Figure 15), the test begins with a treponemal test (EIA or CIA). If positive, it is necessary to do a nontreponemal test (RPR). If the RPR is positive,

it needs to be confirmed again by treponemal test (TPPA). Although these algorithms are more expensive, less familiar and more complex, the results can be more sensitive and specific than traditional algorithm.

With the development of tools, easier procedures, fast test methods, and increase number of laboratories, the reverse algorithm is starting to be used more frequently by most laboratories in the United States.[35]

The discrepancy between syphilitic IgG and RPR results can be adjusted by a second treponemal test (TPPA) based on the CDC recommendation to choose a reverse algorithm. This is because more positive results are found with the reverse algorithm than traditional ones, so that the transmission of syphilis can be prevented. If the syphilis IgG is positive, the RPR is non-reactive and the second treponemal test is non-reactive it can be called a false positive. If the IgG results for syphilis and TPPA are positive but the RPR is negative then it is called a false positive or latent stage syphilis or a past history of syphilis with persistent syphilis IgG antibodies. The disadvantages of the reverse algorithm are that it has a higher number of false positives and a higher cost. The advantage of this algorithm is that it is good for screening high-risk patients and preventing transmission of congenital syphilis or neurosyphilis through early detection. The sensitivity of the reverse algorithm is 98.3%.[38-40]

Intepretation of serologic test

Diagnosis of syphilis by serologic test requires 2 tests are non-treponemal and treponemal. It is used to avoid false positives or negatives, to determine the stage and success of therapy.[33] If the serologic test found non-reactive treponemal (TPHA) and nontreponemal (RPR) tests, it can be concluded that the patient does not have syphilis or the test is too early. If the TPHA test is reactive and RPR is non-reactive, it can be concluded that the presence of syphilis in the past or a false positive for treponemal test. If the results of RPR and TPHA are reactive, but there is a history of treatment 3 months before and there are no new lesions, then the patient only needs to be observed and repeat the test 3 months later. If the RPR titer decreases or remains, then the treatment is not necessary and the test is repeated 3 months later. If the RPR titer rises, administer therapy as a new infection (Table 2).[2][36]

If the RPR result is ≤ 1: 4 (1: 2 or 1: 4), TPHA is reactive and there is no history of syphilis therapy in the last 3 months, then therapy is necessary as advanced latent syphilis. However, if the RPR titer is ≥ 1: 8 it can be treated as early (primary/secondary/early latent) syphilis. If the serologic test was performed 3 months after therapy and the RPR titer decreased by 2 stages or more, then the therapy was considered successful. Tests are evaluated every 3 months in the first year and 6 months in the second year. If the titer does not fall by 2 stages, re-evaluate for possible re-infection or latent syphilis.[2]
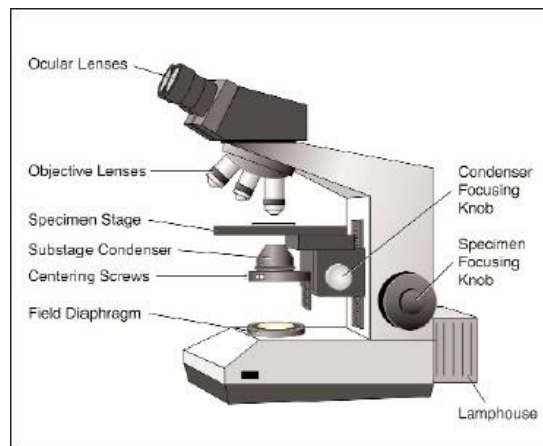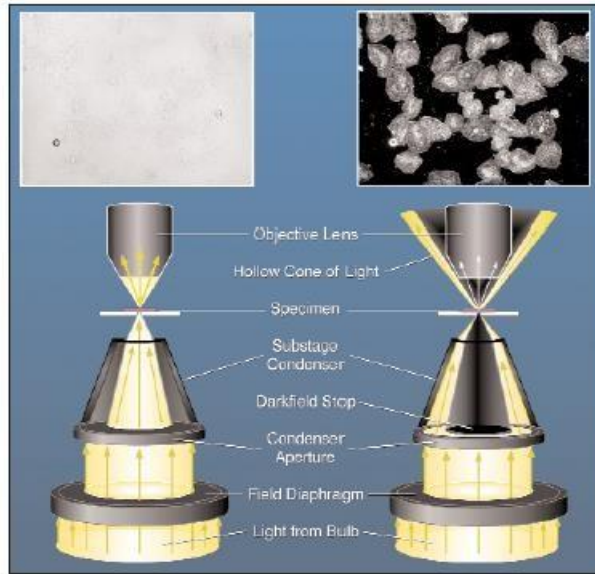

Figure 2a. Light microscope [11]

Figure 2b. Differences between light and dark field microscopy [11]

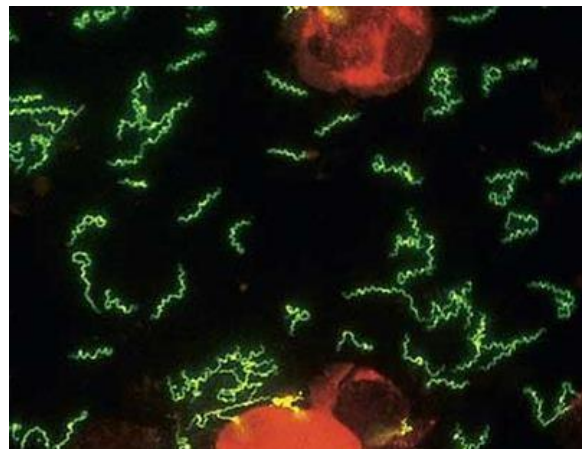Figure 2c. Treponema pallidum in dark field microscopy [1]

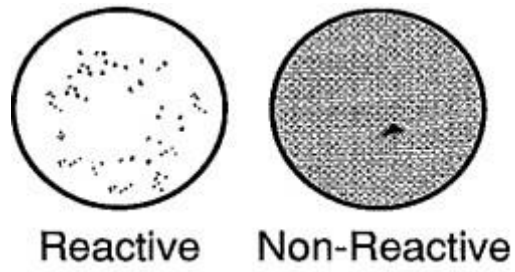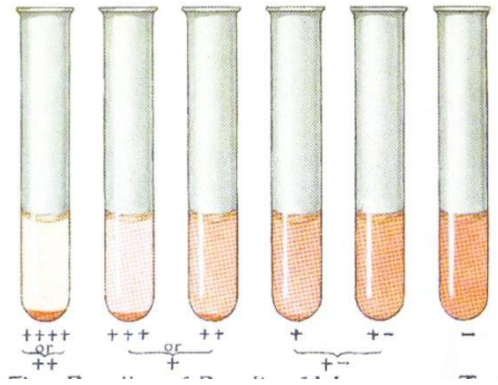Figure 3. Results of direct fluorescence tests [12]
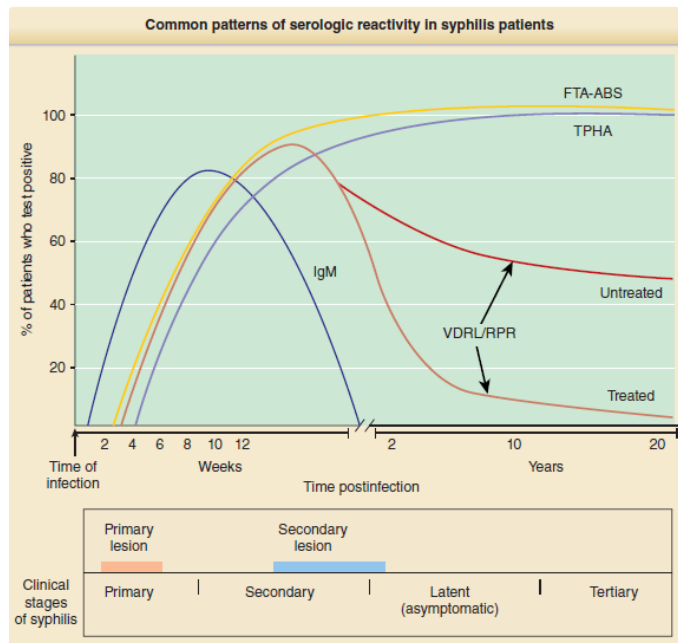
Figure 4. TRUST reaction [27]

Figure 5. Wasserman test [19]

Figure 6. Serologic pattern of syphilis [1]
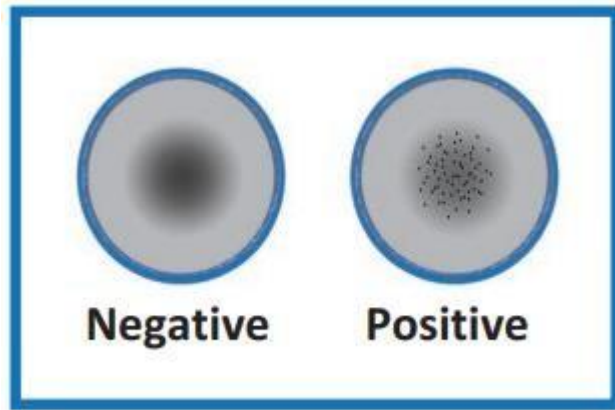
Figure 7. Reactive and non-reactive RPR [20]
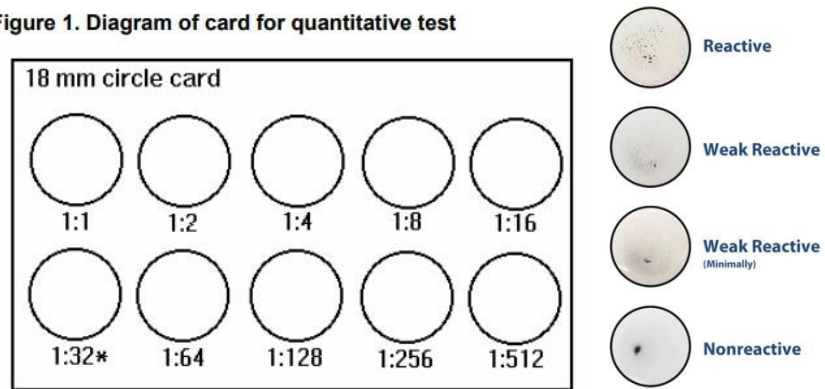

Figure 8. Quantitative RPR [22]
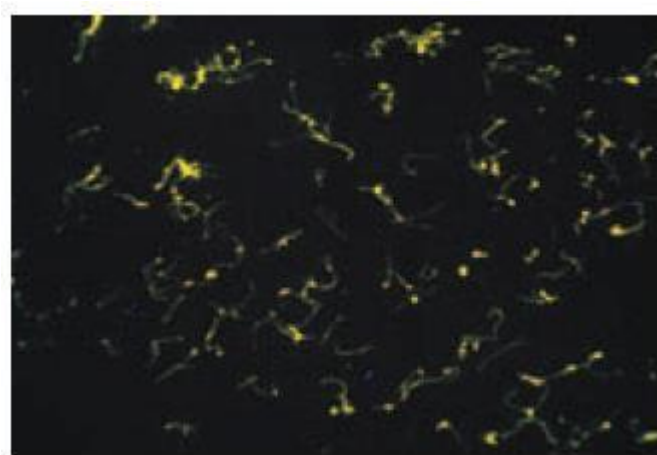
Figure 9. A positive FTA-Abs result indicates T. pallidum fluorescence [12]

WELL NO.	1	2	3	4	
Sample Diluent (μL)	100	25	25	25	(discard)
Test Specimen (μL)	25	25	25	25	25 μL
Test Specimen Dilution	1:5	1:10	1:20	1:40	
Unsensitized Particles (μL)			25		
Sensitized Particles (μL)				25	
Final Dilution			1:40	1:80	
Mix the content using a plate mixer, cover the plate and incubate for 2 hours					
Interpretation					
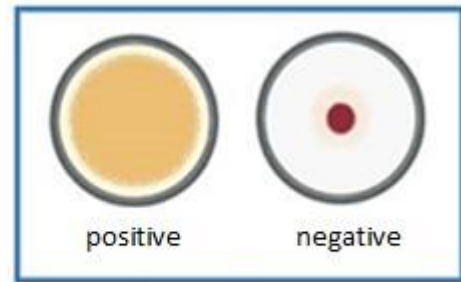
positive   negative

Figure 10. a. TPPA qualitative procedures, b. Positive-negative reactions of TPPA test [31]

Titer: 1/80   1/160   1/320   1/640   1/1280   1/2560   1/5120   1/10240   1/20480   1/40960
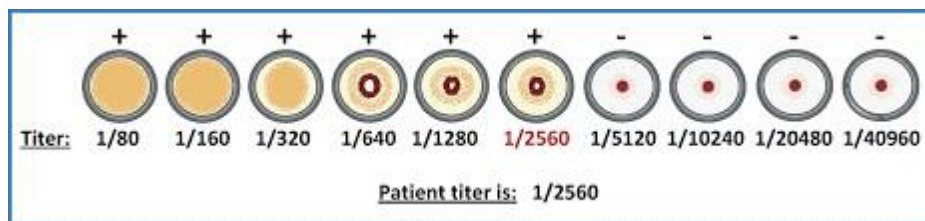
Patient titer is: 1/2560

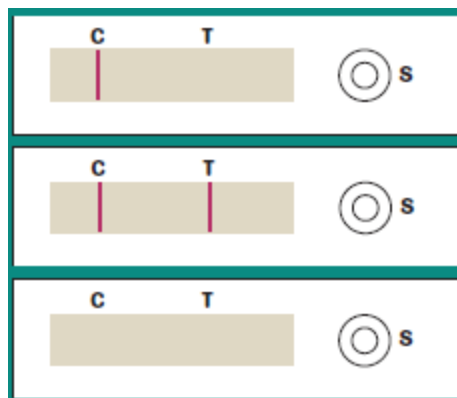Figure 11. a. TPPA quantitative procedure [31]

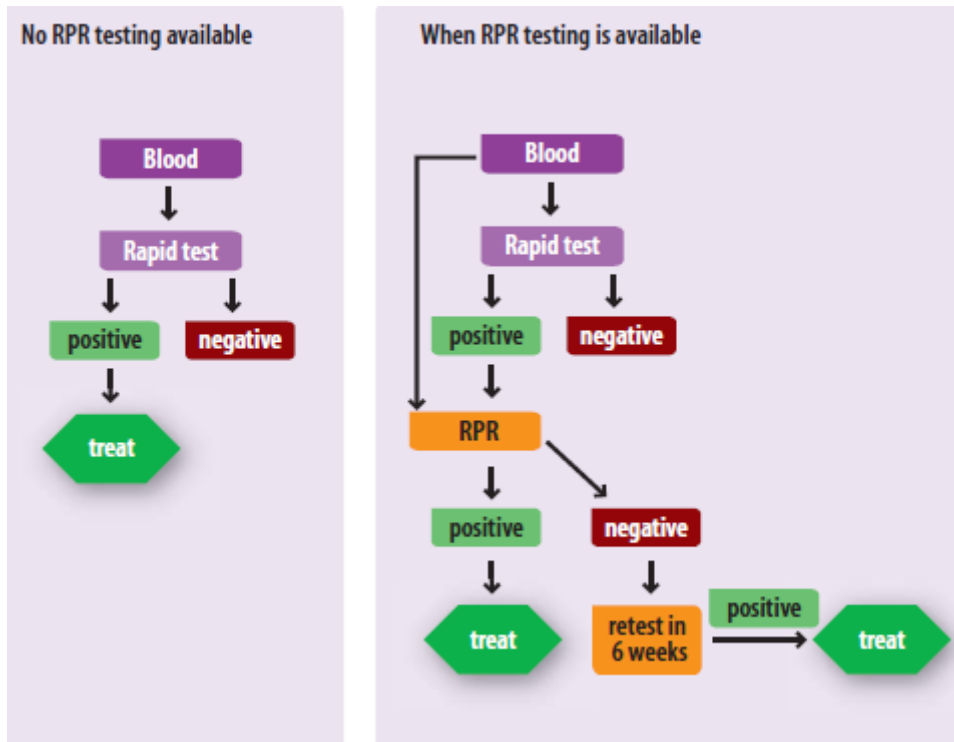Figure 12. Rapid test. Negative results, b. Positive results, c. Undetermined [31]
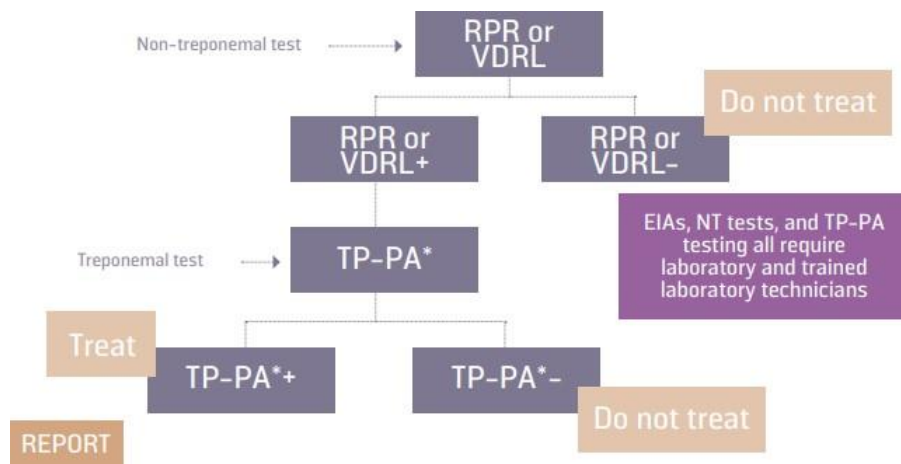
Figure 13. Rapid test [33]

Figure 14. Traditional algorithms [38]

EIA

EIA+ **EIA−** → Do not treat

EIAs, NT tests, and TP-PA testing all require laboratory and trained laboratory technicians

RPR or VDRL

Treat ← **RPR/VDRL+** **RPR/VDRL−**

REPORT

TP-PA*

Treat ← **TP-PA+** **TP-PA−** → Do not treat

REPORT

Syphilis unlikely. If at risk, repeat RPR in several weeks
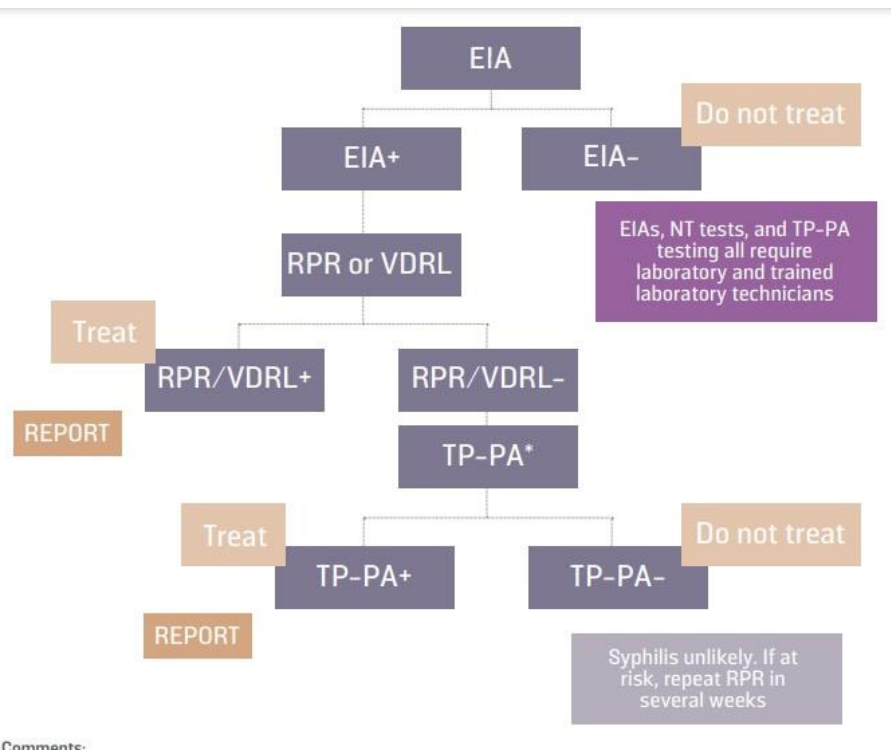
Comments:

Figure 15. Reverse algorithms [38]

Table 2. Syphilis serologic interpretation [2]

RPR	TPHA	RPR Titers and History		Interpretation	Action
Negative	No need	Not done		Negative	Re-test after 3 months
Positive	Negative	Not done		False positives	Re-test after 3 months
Positive	Positive	There is a history of syphilis therapy in the last 3 months, regardless of the titer		Therapy evaluation period	No need for therapy, re-test in 3 months
		There is no history of therapy in the last 3 months	1: 2 or 1: 4	Late latent syphilis	Treatment as advanced latent syphilis, evaluation 3 months later
			≥ 1: 8	Early syphilis: primary / secondary / early latent	Treatment as early siflis, evaluation 3 months later
Positive / negative	Positive	Compare with titer 3 months ago		If it goes down, therapy works	No need for therapy, observation and evaluation 6 months later

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Positive	Positive	Compare with titer 3 months ago	If it rises, new infections	Staged therapy

2. Conclusion

Detection of syphilis infection has been a challenge for clinicians to date. The definitive diagnosis of syphilis is by direct test. Nontreponemal or treponemal serologic test should be performed in patients with suspected syphilis. Nontreponemal serologic tests are available in most laboratories and can be used to screen a larger number of patients than treponemal tests. The combination of these two can be used to determine stages and evaluate syphilis therapy. Traditional or reverse serologic algorithms can be an option to further improve the diagnosis of syphilis.

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