Diagnosis of syphilis*

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The demonstration of Treponema pallidum in early specimens is still the most important procedure for definite diagnosis of the disease. Nonspecific lipoidal antigen tests, as well as assays using T. pallidum antigen, are used for the detection of antibodies in sera. The techniques, the interpretation of the results, the onset and limits of reactivity, as well as the sources of error of the VDRL (RPR), FTA-ABS, TPHA (MHA-TP, AMHA-TP), IgM FTA-ABS, 19S IgM-FTA-ABS, and IgM-SPHA tests are described. The presence of 19S IgM antibodies against T. pallidum indicates activity of the disease and their disappearance is evidence of cure. Positive results in the VDRL test are also strongly suggestive of active disease but are less precise. A TPHA index for CSF of more than 100 and a positive result in the IgM-SPHA test on CSF are indicative for neurosyphilis.

A haemagglutination assay is suggested for screening, if possible combined with the VDRL test. The FTA-ABS test is recommended for confirmation of the diagnosis and the response to treatment can be assessed by the IgM-SPHA test or by changes in the VDRL titre.

The incidence of syphilis increased during the 1960s, decreased slowly during the early 1970s, but has remained at a fairly high level for the last 5–10 years in most countries for which statistics are available. The increased prevalence of latent infections indicates, in particular, the need for the application of reliable diagnostic methods. 2

Although the most important basis for a proper diagnosis of syphilis is a careful history and physical examination, the clinical diagnosis must always be confirmed by laboratory techniques, when facilities are available.

IDENTIFICATION OF THE CAUSATIVE ORGANISM

The most important laboratory procedure for definite diagnosis is still the demonstration of the causative organism, Treponema pallidum, by dark-field examination of early specimens. Immunofluorescent staining is somewhat less reliable owing to several sources of error that may give rise to nonspecific results. Burry's indian ink method is outdated.

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Ultramicroscopic identification by the negative contrast staining technique is very accurate but its application remains limited to research purposes.

Histopathological changes are typical in early and late lesions but demonstration of *T. pallidum* is required to confirm the syphilitic origin of the changes. Silver-staining does not permit a clear differentiation between the causative organism and treponema-like shaped tissue fibres and is therefore not as reliable as the direct immunofluorescence technique.

**IDENTIFICATION OF THE CIRCULATING ANTIBODIES IN SERUM**

**Lipoidal antigen tests**

The original antigen used by Wassermann in the complement-fixation test to demonstrate the presence of antibodies in the serum of syphilis patients, was eventually shown to contain not *T. pallidum* antigen but a lipoidal substance, cardiolipin; and the immunoglobulins that react in lipoidal antigen assays were later called reagins or Wassermann antibodies. Research during the last decade has revealed that these reagins are actually autoantibodies against substances of the mitochondrial membranes.\(^b\)

The best standardized method using lipoidal antigens, and the method that is recommended by WHO, was originally developed by Harris et al. in the Venereal Disease Research Laboratories (VDRL), Atlanta, Georgia, USA.

**The VDRL test**

The antigen for the VDRL test consists of 0.03% of cardiolipin + 0.9% of cholesterol + 0.21% of lecithin. The antigen is added to the patient's inactivated serum, usually on a microscope slide. The slide is then rotated for 4–5 min at room temperature and the result can be read immediately. The presence of reagins in the serum causes a macroscopically visible flocculation.

Reactivity in the VDRL test can usually be observed about 4 weeks after infection and a quantitative evaluation of the circulating antibodies can be obtained by diluting the serum before the test in a geometrical progression, i.e., 1:2, 1:4, 1:8, 1:16, etc. The greatest dilution that can be classed as reactive is reported as the titre.

A positive reaction in the VDRL test strongly suggests infection with *T. pallidum*. Titres are high in active disease, but normally fall gradually after effective treatment and are usually classed as non-reactive within 1–2 years in cases of early syphilis. After adequate therapy of late disease, serum may remain reactive at a low value (e.g., 1:8 or less) for many years. However, the reactivity may cease spontaneously in about 20–30% of untreated patients during latency and even more frequently during the late phase of the disease.

False positive results (biological aspecific reactive) may be caused by autoantibodies (rheumatoid factors) that occur in immune disorders (e.g., forms of lupus erythematosus, collagenoses, rheumatism, Sjögren’s syndrome, dysgammaglobulinaemia) as well as in diseases that are connected with an increased decomposition of cell nuclei, such as malaria, psittacosis, bacterial infections, virus pneumonia, and carcinoma.\(^c\)

The main advantages of the VDRL test are that it is inexpensive, easy to perform, gives a quick result, and is quite sensitive as well as specific. The antigen and the test procedures are well standardized. The main disadvantages are that the sensitivity and specificity of the results are lower than those of tests using *T. pallidum* antigen.

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**Treponema pallidum antigen tests**

For 25 years, the *T. pallidum* immobilization test (TPI or Nelson-Mayer test), based on the ability of syphilitic serum to immobilize *T. pallidum*, dominated syphilis serology. However, the method is complicated, expensive, needs highly qualified personnel, laboratory animals, etc., and is no longer used.

**Fluorescence Treponema pallidum absorption test (FTA-ABS)**

The antigen used consists of *T. pallidum*, from specific rabbit orchitis, which are fixed on a slide with acetone. Lyophilized *T. pallidum* can also be used after reconstitution in saline solution.

Inactivated serum is incubated with a sorbent consisting of Reiter treponemes for absorption of non-specific group antibodies. The serum is then dropped onto the antigen on the slide. Specific antibodies (globulins) bind to the surface of *T. pallidum*. After rinsing, a conjugate of antihuman globulin with a fluorescent stain (fluorescein isothiocyanate, FITC) is then added to the treponemes on the slide. The conjugate links to the human globulin on the walls of *T. pallidum* and can be recognized by fluorescence microscopy. After at least 2 hours the degree of fluorescence can be graded as non-reactive, borderline or reactive (++, ++, ++++, +++++). A reaction of ++ or greater is regarded as indicating infection.

Reactivity can be expected at about the beginning of the third week of infection and is permanent in untreated patients. It may still be observed several years after successful treatment of early infection but usually lasts for decades, or is permanent, in patients who receive adequate therapy of late disease.

A positive reaction in the FTA-ABS test indicates an extremely high probability of syphilitic infection. False negative results are exceptionally rare and may be due to poor quality antigen, particularly with lyophilized treponemes from rabbits that were killed more than 6–8 days after infection. False positive results may be caused by group-antibodies that were not eliminated during the absorption procedure or by unsatisfactory reagents. False positive findings also occur sometimes in patients with hepatic cirrhosis, balanitis, herpes gestationis, collagenosis, lupus erythematosus, and very rarely in pregnant women, or even in healthy persons, for reasons unknown.

In fact, false positive results occur in about 0.18–0.26% of all sera from hospitalized patients and may represent about 7–10% of all reactive samples from selected groups of donors.

The main advantages of the FTA-ABS are its high specificity and sensitivity as well as the early onset of reactivity. The results are most reliable and may be decisive in doubtful cases. However, the FTA-ABS test requires highly trained personnel, is time consuming, the reading of results is tiresome, and the whole procedure is rather expensive. It should therefore be applied only for the confirmation of a serological diagnosis.

**Treponema pallidum haemagglutination assay (TPHA)**

For this test the antigen consists of sheep or turkey erythrocytes coated with particles of *T. pallidum* obtained from infected rabbits and then fragmented by ultrasonication.

Absorbing diluent (consisting of parts of sheep or turkey and cow erythrocyte membranes, Reiter treponemes, rabbit testis-tissue, as well as rabbit serum) is dropped into the wells of the plates before inactivated serum is added. The absorbing diluent binds non-specific group antibodies. Specific immunoglobulins against *T. pallidum* cause agglutination which prevents the complexes from sedimenting to the bottom of the well.

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*d LUGER, A. ET AL. Recent observations on the serology of syphilis. British journal of venereal diseases, 56: 12-16 (1980).*
A preliminary result can be read after incubation for 3–4 hours and the final result is obtained after 18 hours at room temperature. A uniform pink colour indicates a positive reaction whereas a dark-red precipitate in the form of a small spot or ring indicates sedimentation of the red blood cells.

The specificity and sensitivity of the TPHA are higher than those of all other methods for the detection of antibodies against *T. pallidum* provided satisfactory reagents are available.

The time before the onset of reactivity depends on the IgM-binding capacity of the antigen and is usually 3–4 weeks after infection if Fujizoki products are used. Preparations with improved IgM sensitivity may be available in the near future. Reactivity occasionally fades many years after effective treatment of early syphilis but is usually permanent regardless of therapy.

Reactivity in the TPHA test is almost always indicative of syphilitic infection.

False negative results may occur at the beginning of an infection as mentioned above. On the other hand, false positive findings are very rarely observed and are usually due to autoantibodies in the serum. If Fujizoki reagents are used, not more than 0.07% of false positive results are obtained and in the order of 0.008% of false negative results.

The *T. pallidum* haemagglutination assay is easy to perform and does not require highly trained personnel and the results are the most reliable in syphilis serology. However, the reagents are rather expensive, the quality differs even from kit to kit from the same producer, the IgM-binding capacity varies, and standardization cannot be expected within the near future.

**Micro haemagglutination assay with T. pallidum antigen (MHA-TP)**

This test is a variant of the TPHA. It is performed on microtitration plates, it needs less serum, 1/16 of the absorbing diluent, and less than 1/6 of the antigen. The reagents and results are same as in the TPHA, but it is less expensive than the macro method and the final result is available after only 4 hours of incubation.

**Automated micro haemagglutination assay with T. pallidum antigen (AMHA-TP)**

Automation of the filling of the test plates as well as of the dilution steps makes this assay still less expensive than the MHA-TP, and it is thus particularly suitable for mass examinations. Otherwise, there is no difference from the MHA-TP.

**SYPHILIS IMMUNOLOGY**

**Types of antibody**

After infection, the first humoral immune response is the production of antibodies of the IgM type, but these immunoglobulins disappear soon after elimination of the antigen. *T. pallidum*-specific IgM is detectable during the second week of infection and disappears within three months of the beginning of treatment in cases of early syphilis or within one year of the beginning of therapy of late disease.

The detection of *T. pallidum*-specific IgM in the serum of an untreated patient indicates the need for appropriate therapy, as does the persistence of reactivity at unchanged titres for a period of more than 3–4 months following the administration of penicillin in adequate schedules. Reinfection can be distinguished from a relapse following ineffective treatment

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by the reappearance of IgM antibodies.

The production of IgG immunoglobulins normally begins during the fourth week after infection and the serum level usually reaches much higher titres than those for IgM antibodies. IgG secretion may be continued by memory cell clones long after elimination of the antigen and this mechanism is responsible for the persistence of reactivity in sensitive tests.

The VDRL, FTA-ABS, and TPHA tests described above all show reactivity with IgM as well as with IgG antibodies.

Detection of IgM antibodies against *T. pallidum*

*IgM-FTA-ABS test*

The first attempt to identify IgM antibodies against *T. pallidum* was by the FTA-ABS method, using an anti-IgM conjugate instead of the anti-human globulin reagent. However, this method produces many erroneous results; false negative results occur as a result of competitive inhibition of IgM by IgG. The receptors on the surface of *T. pallidum* become occupied by IgG before the big IgM molecules can react, this situation usually occurring when an enormous surplus of IgG is found in the serum, particularly at the beginning of the secondary stage and after reinfection. False positivity is caused mainly by autoantibodies—IgM immunoglobulins against antitreponemal IgG, rheumatoid factors, and others.

**19S IgM-FTA-ABS test**

The shortcomings of the FTA-ABS test for IgM stimulated the search for a more precise method. First, attempts were made to separate the big 19S IgM antibodies from the smaller 7S IgG ones. Several different methods were tried, including ultracentrifugation, adsorption to protein A, and gel-filtration. Precise separation was eventually achieved by gel-filtration using Ultragel AcA 34 with a tris buffer at pH 8.0 in a K 26/100 column with a gel volume of 380 ml, a flow rate of 27 ml/hour, and a temperature of 24 °C. In this system the first peak of eluate contains the pure 19S IgM fraction. This process of filtration separates the loose immune complexes and use of the 19S IgM fraction in the FTA-ABS test eliminates all known sources of error.

However, the 19S IgM-FTA-ABS test can be performed only by highly trained and experienced persons in specialized laboratories with expensive equipment. The procedure is time-consuming and must thus be limited to a few "problem" sera.

**Solid-phase haemadsorption (IgM-SPHA) test**

The need for a simple, cheaper method, which could be applied on a larger scale, led to the development of the IgM-SPHA test. The 96 cavities of a polystyrene microtitration plate are coated with antihuman IgM (μ-chain specific) and are then filled with 50 μl of serum + 50 μl of absorbing diluent. Gentle rotation and incubation for 2 hours is followed by removal of the sera and careful rinsing of the wells. Then 50 μl of a 1:16 dilution of TPHA antigen are added, and the plate is rotated and incubated again. A preliminary reading is possible after 3–4 hours, and the final result is obtained after 18 hours. The reagents are the same as those used for the AMHA-TP and the colour reaction is the same. IgM antibodies are bound to the coated walls of the wells. Sera from untreated patients contain

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specific IgM antibodies which become linked to the antigen and prevent the erythrocytes from sinking to the bottom.

A quantitative evaluation is performed as in the AMHA-TP. Reactions at dilutions of 1:4 and above are considered positive, 1:2 is classified as borderline. The margin of error due to false positive results caused by autoantibodies, rheumatoid factor, and other unknown factors is 0.3% of all sera examined and about 3.7% of all samples positive in the IgM-SPHA test. The rate of agreement with the 19S IgM-FTA-ABS test is 96.3%. Reactivity is first seen during the second week of infection and declines as indicated for IgM on page 650.

The IgM-SPHA technique seems to meet all requirements, the results are highly specific, the method is easy to perform, even less expensive than the AMHA-TP, it does not require specialized laboratory personnel or facilities, and it is applicable for routine and mass examinations. However, the IgM-SPHA test has been used in syphilis serology for only eighteen months. Other laboratories are about to start using it, but some have encountered problems, mostly due to difficulties in following the recommended procedure.

**DIAGNOSIS OF NEUROSYPHILIS BY EXAMINATION OF CEREBROSPINAL FLUID**

The occurrence of total protein values above 400 mg/litre and cell counts exceeding 5000/litre in cerebrospinal fluid (CSF) indicate inflammation of the central nervous system (CNS) according to the Dattner-Thomas formula. A negative result in either the TPHA or the FTA-ABS test on CSF excludes neurosyphilis. A positive result in the IgM-SPHA on CSF and a TPHA index above 100 strongly suggest a syphilitic process in the CNS. The TPHA index is calculated by the formula:

$$\frac{\text{CSF-TPHA titre}}{\text{albumin quotient}}$$

—and the albumin quotient, which defines the blood-brain barrier function, by the relation:

$$\text{CSF-albumin (μmol/litre)} \times 10^8 / \text{serum albumin (μmol/litre)}.$$

**SEROLOGICAL TESTS IN PRACTICE**

**Diagnosis of cases**

The serological diagnosis of an infection with *T. pallidum* can be established beyond doubt in cases where the serum gives a positive result in the TPHA (AMHA-TP) and in the FTA-ABS test (Table 1). In order to exclude various sources of error, these results should be confirmed by the examination of a second sample taken on a different occasion from the same patient.

**Screening**

The need to intensify screening for syphilis was emphasized in the introduction of this paper.

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The preferred method for this purpose is the AMHA-TP, if possible combined with the VDRL test (Table 1). In areas with limited laboratory facilities, the VDRL test or the RPR may be used. However, use of the VDRL test alone would fail to detect reactivity in 60% of sera from patients who have had an infection with *T. pallidum*. The importance of the AMHA-TP for screening is illustrated by reference to one study in which use of this test detected 6 patients with late syphilis (4 with mesoartitis, 2 with active neurosyphilis) among a selected group of 53 patients who showed no reaction in the VDRL test.

The cost/benefit aspects of serological screening for syphilis were examined in 1977, and at that time, one examination by the VDRL test cost approximately US$ 0.66, including all the expenses for personnel, equipment, reagents, wear and tear, buildings, electricity, water, etc. Estimated on a similar basis, one examination by AMHA-TP costs about US$ 1.16. On the other hand, it was estimated in 1971 that institutional care of persons with syphilitic psychoses cost the American government US$ 41 million annually. This figure seems to justify the use of AMHA-TP for screening, particularly in view of expected improvements in the IgM-binding capacity of the reagents, which should improve recognition of cases of early infection. Sera that give a positive result in the TPHA (AMHA-TP) and/or the VDRL test should be checked by the FTA-ABS in order to confirm or exclude a diagnosis of syphilis as mentioned above.

**Problem sera**

Sera for which the results in the TPHA (AMHA-TP) and the FTA-ABS do not agree, require further investigation. However, such disagreement occurs in only about 0.5% of all samples examined.

The TPI test is not of any use in such cases, but the SPHA test, or if necessary the 19S IgM-FTA-ABS test, can be valuable in deciding whether treatment is necessary. A positive reaction in either of these tests indicates the presence of *T. pallidum* in untreated patients.

In one study, isolated positivity in the TPHA (AMHA-TP) test occurred in about 0.45% of all samples from hospital patients (i.e., 17.07% of all specimens that were positive at screening). About 86% of these sera were from patients who were, or had been, infected with *T. pallidum*. Therefore, provided that reliable reagents have been used in the test, isolated reactivity in one of the haemagglutination assays strongly suggests a previous syphilitic infection which has either cured itself spontaneously or been cured by antibiotic treatment.

Isolated positivity in the FTA-ABS test may occur for the reasons explained on page 649. It may also occur during the third or fourth week of infection (see page 649 and page 650) or as a result of the presence of a factor in the serum that inhibits haemagglutination in spite of all precautions. However, this factor has been found in only one patient during the past 4 years among about 400 000 sera examined.

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Criteria of cure

Decline of the IgM-SPHA titre and of reactivity in the 19S IgM-FTA-ABS within the time intervals mentioned on page 650 can be considered to indicate cure. A decrease in the VDRL titre (1:8 or below), according to the parameters described on page 648 also indicates the effectiveness of therapy.