CLINICAL MICROBIOLOGY REVIEWS, Jan. 1995, p. 1–21 Vol. 8, No. 1
0893-8512/95/$04.00+0

Laboratory Diagnosis and Interpretation of Tests for Syphilis

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HISTORICAL PERSPECTIVE

Test Development

Syphilis, caused by the spirochete *Treponema pallidum* subsp. *pallidum*, is a chronic infection with many diverse clinical manifestations that occur in distinct stages. Over the years, because *T. pallidum* cannot be readily cultured (51, 147) or stained with simple laboratory stains (197, 213), other laboratory methods to identify infection in the various stages of syphilis have been developed. These tests for syphilis fall into four categories: (i) direct microscopic examination, used when lesions are present; (ii) nontreponemal tests, used for screening; (iii) treponemal tests that are confirmatory; and (iv) direct antigen detection tests currently used in research settings and as gold standards for test evaluation.

Direct detection methods. The oldest, and also the newest, methods for the diagnosis of syphilis are direct antigen detection procedures. Before the etiologic agent for syphilis was isolated, nonsyphilitic humans were inoculated with specimens

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from infected persons, and their reactions were examined to differentiate chancroid from syphilis and to study the nature of the disease (123). The various sources of inoculum ranged from lesion exudate to blood. The uninfected recipient was inoculated by single or multiple injection or by implanting biopsied material into the forearm or thigh (123). Although lesions developed, precise interpretation of results was difficult because of the diverse clinical symptoms of syphilis and the lack of serologic or microscopic proof of infection (16). The first transfer of *T. pallidum* from a human to the testicle of a rabbit was reported in 1907 (150). Later, rabbit infectivity test (RIT) studies (122) determined that one or two treponemes were infectious for 47% of rabbits inoculated intratesticularly with *T. pallidum*. The oldest technique is still the most sensitive of the methods. The newest technology, PCR, has not reached the sensitivity level of the RIT (65) (see below).

The first association of *Spirochaeta pallida*, as *T. pallidum* was then known, with the disease syphilis was made in 1905 by Schaudinn and Hoffmann (176), who used a modified Giemsa stain to examine lesion material from individuals with chancres. Coles (25) in 1909 described the use of dark-field illumination for the examination of *S. pallida*, noting especially the motility of the organism. Today, dark-field examination is still a viable method for the diagnosis of syphilis. In the mid-1960s, the direct fluorescent-antibody (DFA) test for *T. pallidum* was developed (100, 101, 217); later, it was modified for use with monoclonal antibodies (74, 85) and tissue sections in the direct fluorescent-antibody tissue test for *T. pallidum* (DFAT-TP) (76, 80, 85, 86).

Nontreponemal tests. Coincidentally, around the same time that the etiologic agent of syphilis was being observed, the first nontreponemal test for syphilis was also being developed. In 1906, Wassermann et al. (209) adapted the complement fixation test, previously introduced by Bordet and Gengou in 1901 (7), to serologic testing for syphilis. The antigen used in the Wassermann test for syphilis was an extract of liver from newborns who had died of congenital syphilis. Initially, the Wassermann antigen was thought to be specific, but later Landsteiner demonstrated that other tissues, particularly beef heart extracted in alcohol, could be used equally well as antigens (44). Cholesterol and lecithin were added to increase the sensitivity of the antigens (44). Although the complement fixation tests contributed immensely to the diagnosis of syphilis, they were complicated to perform and required many reagents and 24 h to complete. Work in 1907 by Michaelis (136), using watery extracts of syphilitic liver, and of Meinicke (132) in 1917, using distilled water or sodium chloride extracts of liver, resulted in the first precipitation tests that did not require complement. In 1922, Kahn (96) introduced a flocculation test without complement that could be read macroscopically in a few hours. Many modifications of the Kahn test appeared, each identified by the name of its developer and each claiming greater degrees of sensitivity or specificity (97). However, the drawback of all these tests was that their antigens, prepared from crudely derived extracts of tissue, varied in quality. Standardization of the tests was difficult and relied on comparison with stored serum samples and antigen controls to determine the differences in the sensitivity and specificity of the test antigens. Then in 1941, Pangborn (149) successfully isolated from beef heart the active antigenic component, a phospholipid, cardiolipin. Cardiolipin, when combined with lecithin and cholesterol, forms a serologically active antigen for the detection of syphilitic antibody. In contrast to the crude tissue extract antigens, the pure cardiolipin-cholesterol-lecithin antigens could be standardized chemically as well as serologically, thus ensuring greater reproducibility of test results both within and between laboratories (172). With the advent of these new purified antigens, microflocculation tests, such as the Venereal Disease Research Laboratory (VDRL) test (68), were developed. These flocculation tests, in which standardized reagents were used, yielded reproducible results, could be rapidly performed, gave acceptable levels of sensitivity and specificity, and were soon converted to methods for mass screening. The addition of choline chloride and EDTA to the VDRL antigen enhanced the reactivity of the test and stabilized the antigen suspension (159). In the resulting unheated serum reagin (USR) test, as the name implies, the need for heating serum was eliminated and plasma was also found to be an acceptable sample source (159).

The original rapid plasma reagin (RPR) (161) test was developed to be a field screening procedure in which large numbers of specimens could be rapidly tested. The original RPR was a crude test performed on an unmeasured amount of plasma, but it had a high degree of reactivity. The test is no longer used because of lack of specificity and is of historic importance only. The plasmacrit test (3) was similar to the original RPR test but used the residual plasma portion of blood from a microhematocrit determination. Since venipuncture was not necessary to obtain a specimen, this test could be done easily on infants and children.

The RPR teardrop card test (160), a further refinement of the RPR test, was developed as a screening procedure to be used in the field (48). Laboratory equipment was not essential because all test materials were contained in a disposable kit. Plasma from blood obtained by finger stick was collected on a plasma collection card and then placed in a teardrop-shaped test area on a plastic-coated card. Next, a stabilized modified RPR antigen suspension, incorporating charcoal particles to aid in reading the reaction, was added to the plasma, and the test card was rocked by hand. The results were read macroscopically as reactive or nonreactive. The RPR teardrop card test is still employed in field work and as a screening procedure in some laboratories, but recent reports have found the test to be less sensitive than the currently used RPR 18-mm circle card test with serum as a sample source (205).

In contrast to the RPR teardrop card test, which was designed to be performed in the field, the RPR circle card test was designed for use in laboratories in which testing is performed on large numbers of specimens (48). In the 1960s, an automated version of the RPR card test was developed: the automated reagin test (129). In the automated reagin test, a measured portion of each specimen was automatically mixed with a continuous flow of the RPR card test antigen. A wash occurred between each sample to prevent carryover of the reactivity between samples. A portion of the antigen plus the sample mixture was then deposited on a continuously moving filter paper strip. The test was applicable for the daily processing of large numbers of samples in which the reactivity rate was low, as in blood banking, hospital admission testing, and premarital screening. In the late 1970s, when the rates of syphilis dropped dramatically, many states dropped premarital screening tests and hospitals no longer performed preadmission testing; thus, the need for the more expensive automated method disappeared. Later, additional modifications to the basic antigen used in the RPR card test were made. Like the RPR circle card test, the toluidine red unheated serum test (TRUST) (110, 155) and the reagin screen test (126) are macroscopic card tests for syphilis. The TRUST differs from the RPR circle card test in that sized particles of the red paint pigment toner, toluidine red, replace the charcoal as the visualization agent (155). The reagin screen test differs from

the RPR circle card test and TRUST, because Sudan Black B, a lipid-soluble diazo dye, is used to actually stain the reagin screen test antigen (126). The reagin screen test is no longer available in the U.S. market. The newest of the nontreponemal tests is an indirect enzyme-linked immunosorbent assay (ELISA) method based on the VDRL antigen (152, 211). (See below.)

Treponemal tests. Because nonspecific or false-positive reactions occur with the nontreponemal tests, usually as a result of damage to the host's tissue by infection, immunization, or autoimmune disease, the search for a specific serologic test for syphilis continues. Initial attempts to develop a test using an antigen derived from the treponeme itself were unsuccessful until 1949, when Nelson and Mayer developed the first treponemal antibody test, the *T. pallidum* immobilization (TPI) test (142). The TPI test uses *T. pallidum* (Nichols strain) grown in rabbit testes as the antigen and is based on the ability of patient's antibody and complement to immobilize living treponemes, as observed by dark-field microscopy. The TPI test was rapidly accepted as a specific test for syphilis. However, because the TPI test was complicated, technically difficult, time-consuming, and expensive to perform, a simpler procedure was sought. In addition, studies (71, 167) in the 1970s found that the TPI test was less sensitive and specific than the treponemal tests that appeared in the 1960s. Today, in the United States, the TPI test is used in research laboratories only.

As with the nontreponemal tests, an array of treponemal tests was later developed, some of which enjoyed short periods of popularity. In 1953, D'Allesandro and Dardanoni (34) prepared an antigen from *T. phagedenis*, the Reiter treponeme, a nonpathogenic organism which, unlike *T. pallidum* subsp. *pallidum*, can be easily cultivated in vitro. The Reiter antigen was thought to be treponeme specific not only to the Reiter treponeme but also to *T. pallidum* subsp. *pallidum*. When used in a complement fixation test for syphilis, the Reiter antigen detected an antibody different from that detected by nontreponemal tests. The most widely used tests employing the Reiter treponeme or an extract of the organism were the Reiter protein complement fixation test and the one-fifth-volume Kolmer test with Reiter protein antigen (26). However, a significant proportion of false-positive reactions occurred with these tests, and subsequent evaluation proved them to be less specific and sensitive than the TPI test (190).

In 1957, a major breakthrough in treponemal antigen tests occurred with the development of the fluorescent treponemal antibody (FTA) test (39). The original FTA procedure used a 1:5 dilution of the patient's serum in saline solution, reacted with a suspension of killed treponemes. A fluorescein-labeled anti-human immunoglobulin was used as the conjugate, and the test was read under a microscope with a UV light source. When an improved fluorescein compound (fluorescein isothiocyanate [FITC]) was used to prepare the labeled anti-human globulin conjugate, nonspecific reactions were encountered in approximately 25% of normal serum specimens (208). To eliminate these false-positive reactions, the test was modified by diluting the patient's serum 1:200, the FTA-200 (40). However, the FTA-200 test, although highly specific, was not very sensitive. The nonspecific reactions of the original FTA test were found to arise because of shared antigens common to *T. pallidum* and the nonpathogenic treponemes that occur as part of the normal bacterial flora of humans (41). Deacon and Hunter (41), by preparing a sonicate from cultures of the Reiter spirochete, removed the common antigens by absorption; their work led to the development of the more specific and sensitive FTA absorption (FTA-ABS) test (79). The FTA-ABS and its counterpart, the FTA-ABS double-staining test, used with incident light microscopes (81), remain the standard treponemal tests for syphilis today.

In 1965, Rathlev (163) reported the first reliable application of hemagglutination techniques to the serologic diagnosis of syphilis. The antigen used in her procedure was formalinized, tanned sheep erythrocytes sensitized with ultrasonicated material from *T. pallidum* (Nichols strain). The presence of treponemal antibody in the patient's serum was detected by the indirect agglutination of the sensitized erythrocytes and the subsequent formation of a mat of erythrocytes upon their settling. Rathlev published the results of her experience with this method on 300 serum samples in 1967 (164). Modifications of Rathlev's procedure followed, including the use of an improved reaction medium (199) and a sorbent much like the one used in the FTA-ABS test for removal of group treponemal antibody (200). The hemagglutination procedure was first a tube test known as the *T. pallidum* hemagglutination test. Subsequently, reagents for a microvolume (27) hemagglutination test, the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP), became commercially available. Another hemagglutination test for syphilis (HATTS) (210), based on the use of sensitized turkey cells and performed in microtiter plates, was considered a standard test for syphilis from the mid-1970s to late 1980s, when the product was withdrawn from the American market because of a lack of reproducible results between batches of reagents. The hemagglutination tests are simpler to perform than the fluorescent-antibody tests, and one hemagglutination method has recently been automated, the PK-*T. pallidum* (PK-TP) test (see below).

Test Standardization

Since the development of the first serologic tests for syphilis, the evaluation and standardization of these tests have been a major activity in syphilis serology. The major factors considered in test evaluation and standardization are the sensitivity, specificity, and reproducibility of the test. Sensitivity is defined as the ability of the test to detect the presence of syphilis in patients with syphilis, whereas specificity refers to the test's lack of reactivity with samples from individuals who do not have syphilis. Reproducibility refers to the ability of the test to give the same result when tested with the same sample a given number of times. Reproducibility in the quantitative nontreponemal tests is acceptable if the endpoint titer plus or minus one dilution is obtained each time the sample is tested.

In 1923, the Health Organization of the League of Nations attempted to judge the relative value of the serodiagnostic tests for syphilis that had been developed subsequent to the Wassermann test. In 1934, the first actual comparative evaluation of the serologic tests for syphilis was undertaken by the Public Health Service, and in 1938 the Assembly of Laboratory Directors and Serologists selected the most efficient tests. These standard procedures were published in the first manual of tests for syphilis in 1939 (203). The manual has been revised periodically to delete procedures that have become obsolete and to include new or improved and adequately evaluated procedures. The latest edition of the manual was published in 1990 (113). In the 1970s, the number of new tests being developed and the number of new companies undertaking the production of reagents for the diagnosis of syphilis increased. To maintain standardization of these reagents, the Centers for Disease Control and Prevention (CDC) responded by establishing an Advisory Panel on New Tests for Syphilis. The panel published guidelines for evaluation and acceptance of new serologic tests for syphilis in 1977 (17). An addendum was

placed in the guidelines in 1986 to address the use of a treponemal test as the initial test for syphilis. According to the guidelines, each new or improved test should pass through three stages of evaluation after the initial research stage. Each stage includes test evaluation and publication of the findings of these evaluations. In the investigational stage, the test should have a specific written technique based on substantial research, and the test should be in a form for the laboratory that developed the test and others to conduct studies of the new test in parallel with standard status tests to determine the clinical usefulness of the new test. By the time the test reaches provisional status, a revised technique resulting from the investigational and provisional studies has been written, the test has been found to be as sensitive and specific as the standard test with which it was compared in the earlier studies, and the test has survived large-scale independent studies. The provisional stage is the true evaluation of the test's worth, for during this stage large-scale production-size batches of reagents are prepared and the test protocols are used by investigators other than the test developer. Frequently, protocols are rewritten and production methods are changed during this stage. In addition to publications by independent test users, test usage and consistency between lots over a period of time are considered as the test progresses from the provisional stage to the standard stage and subsequently to standard status. Once standard status is reached, the test is included in the manual of tests for syphilis.

When medical management decisions are to be made, the laboratory should use only those procedures that have been thoroughly evaluated. Tests with standard or provisional status can be used in medical management, if the results obtained in the user laboratory are comparable to results previously reported (standard status) or if test results of the new tests are comparable to results obtained with the standard test currently being used when the two tests are performed in parallel (provisional status). Tests with investigational status should not be used as the sole criterion for diagnosis of syphilis.

In addition to the new tests for syphilis, there is another category of tests: the reagents made by manufacturers for a test that is already considered a standard status test for syphilis. These are the so-called me too tests. For example, around the mid-1980s, the number of reagent manufacturers or distributors in the United States selling antigen for macroscopic card tests, such as the RPR and TRUST, increased fourfold. To obtain a license to sell a "me too" product, new manufacturers must provide to the Food and Drug Administration proof that the results obtained with their tests are substantially equivalent to the results obtained in a standard status test already on the market. Only limited comparative testing is required of the manufacturer or distributor for a "me too" test, in contrast to the thorough clinical trials required when a new test is placed on the market.

STANDARD METHODS FOR THE DIAGNOSIS OF SYPHILIS

Direct Detection of *T. pallidum*

Animal inoculation. The oldest method for detecting infection with *T. pallidum* is animal infectivity testing. This technique probably offers the most sensitive method for detecting infectious treponemes and is used as the gold standard for measuring the sensitivity of methods such as the PCR (65, 175). Any source of specimen can be used for rabbit infectivity testing as long as the material is less than 1 h old or was flash-frozen immediately after collection and maintained in liquid nitrogen or at temperatures of −78°C or below (202).

Numerous animal species, from hamsters to chimpanzees, have been used to either maintain treponemes or determine infectivity (213). However, not all animals develop visible signs of infection or reactive serologic tests. The rabbit is the most practical animal because a local lesion can be produced at the site of inoculation, the tissues remain infective for the life of the animal, infection can be transferred from one animal to another using minced lymph nodes or testes, and serologic tests for syphilis become reactive (213). Inoculations of samples suspected of being infective are usually intratesticular or intradermal. However, the rabbits may be infected by ocular, intravenous, or scrotal inoculation as well. Detailed methods for maintenance and passage of *T. pallidum* in rabbits are available from the CDC (23). The incubation period in rabbits from inoculation to the formation of lesions is inversely proportional to the size of the inoculum (122). In our laboratory, rabbits infected intratesticularly with patient material are monitored at day 7 for the development of orchitis and then at 2-day intervals, if orchitis failed to develop at day 7. Serum samples are obtained from rabbits in which orchitis has not developed at days 18 and 30, and serologic tests for syphilis are performed. Rabbits with reactive serologic results are sacrificed, and the popliteal lymph nodes are removed, minced, and passed to a second rabbit. Infection in the second animal is required to confirm the presence of *T. pallidum*. Rabbits that have nonreactive serologic tests at days 18 and 30 are bled again at days 60 and 90. If reactive results are obtained, the preceding procedure is followed. If serologic test results remain nonreactive at day 90, the results are reported as "*Treponema pallidum* undetected in lesion material or cerebrospinal fluid [CSF]." The sensitivity of the RIT approaches 100% if the number of organisms exceeds 23 (123) and the patient has not received antibiotic treatment.

Dark-field microscopy. When lesions are present, the most specific and easiest means of diagnosing syphilis is by direct detection of the organism. Currently, dark-field microscopy (29) and DFA tests are commonly used to detect *T. pallidum*. A positive result on microscopic examination is definitive evidence of syphilis if infection with other pathogenic treponemes can be excluded. (See below.)

When collecting, preparing, and examining specimens, observe universal safety precautions (18, 19, 21). Because viability of the treponeme is necessary to distinguish *T. pallidum* from morphologically similar saprophytic spirochetes within and near the genitalia (Table 1), dark-field examination must be accomplished immediately after the specimen is obtained. Equipment and personnel for dark-field examination must be readily available, or the patient must be sent to a facility where the procedure can be carried out.

Dark-field examination is most productive during primary, secondary, infectious relapsing, and early congenital syphilis when moist lesions containing large numbers of treponemes (e.g., chancres, condylomata latum, or mucous patches) are present. Enlarged regional lymph nodes can also serve as a specimen source if the involved node is aspirated and the material obtained is examined. Dark-field examination of lesions of the cervix and vagina are possible if special techniques are used for collection of the specimen. Briefly, a specimen for dark-field microscopy consists of serous fluid that contains *T. pallidum*, but it should be free of erythrocytes, other organisms, and tissue debris. The lesion should be cleansed only if encrusted or obviously contaminated, and only tap water or physiologic saline (without antibacterial additives) should be used. A minimum amount of liquid should be used

for cleaning because large amounts may dilute organisms and hinder the ability to recover treponemes. Antiseptics or soaps should not be used because they may kill the treponemes and invalidate interpretation of the dark-field examination. After cleansing the lesion, gently abrade it and apply gentle pressure until only clear serum exudes. Place a drop of serum on the surface of a coverslip or slide. Then place the coverslip on a microscope slide, and examine the specimen while the organism is still motile. For cervical and vaginal lesions, after the lesion is identified by speculum examination, it should be cleansed with physiologic saline and then abraded by being rubbed with a gauze pad held in suitable forceps. When serous fluid appears, the specimen should be collected with a bacteriologic loop or Pasteur pipette and transferred to a glass slide for examination. Even the experienced observer may find it difficult or impossible to differentiate *T. pallidum* from saprophytic spirochetes in the mouth; thus, dark-field microscopy should not be used for the examination of samples from oral lesions.

Because of their narrow width, treponemes cannot be observed with the ordinary light microscope. Microscopes equipped with a double-reflecting or single-reflecting dark-field condenser, a ×40 to ×45 objective, and a ×90 to ×100 objective with a funnel stop are needed to perform the dark-field examination. Illumination for dark-field microscopy is obtained when light rays strike the object in the field at an oblique angle so that no direct light rays enter the microscope, but only the rays that are reflected from the object. Therefore, the object itself appears to be illuminated against a dark background (29). Details for the test are found in *A Manual of Tests for Syphilis* (29). Because the specimen must be read immediately, the microscope adjustment should always be completed, and the microscope should be in satisfactory working condition before the specimen is collected. Usually a stock strain of a nonpathogenic treponeme or gingival scrapings in a drop of saline are used for adjustment.

T. pallidum is distinguished from other spiral organisms by the tightness of the spirals and characteristic corkscrew movement (Table 1). However, *T. pallidum* subsp. *pallidum* cannot be distinguished from the other pathogenic organisms, *T. pallidum* subsp. *pertenue*, *T. pallidum* subsp. *endemicum*, and *T. carateum. T. pallidum* is a delicate, corkscrew-shaped organism with rigid, uniform, tightly wound, deep spirals. The length of the organism is 6 to 20 μm; the width is 0.10 to 0.18 μm. The length of the spiral wave is 1.0 to 1.5 μm, and the spiral depth is 0.5 to 0.7 μm. The characteristic motion of *T. pallidum* is a deliberate forward and backward movement with rotation about the longitudinal axis. Rotation may be accompanied by a soft bending, twisting, or undulation of the organism from side to side. When attached to or obstructed by heavier particles, the organism may contort, convolute, or bend and thereby distort the coils, but the organism will snap back to its original form in a coil-like manner. Organisms easily confused with *T. pallidum* are *T. refringens* and *T. denticola* (29).

Positive findings on dark-field examination permit a specific and immediate diagnosis of syphilis. Also, primary syphilis can be diagnosed by dark-field examination several days to several weeks before the appearance of reactive serologic tests. However, a negative dark-field finding does not exclude the diagnosis of syphilis. Too few organisms may be present to be observed, because the lesion may be in the healing stage, or the spirochete may have been altered by systemic or topical treatment. In addition, the dark-field sample may be reported as unsatisfactory as a result of the presence of too many blood cells, air bubbles, or tissue fragments for an accurate reading.

The sensitivity of the dark-field examination approaches

TABLE 1. Morphology and motility of *T. pallidum* subsp. *pallidum* and related nonpathogenic species

Organism	Location	Coils	Length (μm)	Width (μm)	Wave Length (μm)	Wave depth (μm)	Translation	Rotation	Flexion
T. pallidum subsp. *pallidum*	Skin and mucosal lesions	Spiral shape, 10–13 coils (6–20)[a]	Medium, 10 (6–20)[b]	Very thin, 0.13–0.15 (0.10–0.18)	Tight, 1.1 (1.0–1.5)	Deep, 0.5–0.7	Slow, deliberate	Slow to rapid; like a cork-screw, may rotate without changing place	Soft bending in middle; pops back into place with a spring
T. refringens	Normal genital flora	Spiral shape, 2–3 coils (2–5)	Short, 5–8 (5–8)	Thick, 0.20–0.30 (0.20–0.50)	Loose, 1.8 (1.5–2.5)	Shallow, 0.4–0.6	Rapid	Very rapid; active serpentine-like, rotates sometimes so rapidly that it looks straight	Marked bending, relaxed coils
T. phagedenis, Reiter treponeme	Normal genital flora	Spiral shape, 10–12 coils (10–30)	Medium long, 10–12 (10–30)	Thick, 0.20–0.25 (0.20–0.40)	Loose, 1.4–1.6 (1.5–2.0)	Shallow, 0.4–0.6	Slow, jerky; deliberate	Slow to rapid; rotates without changing place	Jerky; twists or undulates from side to side
T. denticola	Normal oral flora[c]	Spiral shape, 6–8 coils (2–8)	Medium, 8 (6–16)[d]	Very thin, 0.15–0.20	Tight, 0.9 (0.8–1.2)	Deep, 0.4–0.6 (approximately)	Slow, deliberate	Slow to rapid, often jerky	Soft bending; bends, twists, or undulates

[a] Values in parentheses are ranges.
[b] Slightly longer than the diameter of an erythrocyte, 8 μm.
[c] Note: avoid collecting specimens from the mouth because *T. denticola* and *T. pallidum* look very similar. If that is the only collection site, use a fluorescent-antibody test because *T. pallidum* fluoresces, but *T. denticola* does not.
[d] Slightly shorter than *T. pallidum*.

80% (35, 170). Adequate training and experience are necessary to make an accurate diagnosis by dark-field microscopy. The untrained observer may be deceived by artifacts such as cotton fibers and Brownian motion. Because other sexually transmitted diseases are also characterized by lesions, when the direct microscopic results are negative, other diseases, such as herpes and chancroid, should be considered.

DFA-TP. Lesion samples for the DFA for *T. pallidum* (DFA-TP) examination (75) are collected in the manner described for dark-field examination. The test detects and differentiates pathogenic treponemes from nonpathogenic treponemes by an antigen-antibody reaction; thus, the organism is not required to be motile in the DFA-TP. Because the conjugates used are specific for pathogenic strains of *Treponema* spp., the DFA-TP is applicable to samples collected from oral or rectal or intestinal lesions. However the test cannot distinguish between the pathogenic strains of *Treponema* spp. The DFA-TP method has been used to detect the presence of *T. pallidum* subspecies in tissues (80, 85, 86, 215), body fluids (188, 214), secretions, and lesion exudates (85, 86, 101) as well.

A fluorescence microscope equipped with a dark-field condenser and the standard set of filters is required. Initially, samples are air dried, and immediately before staining smears are fixed with either acetone for 10 min or 100% methanol for 10 s, or slides are gently heat fixed. Smears are stained with FITC-labeled anti-*T. pallidum* globulins prepared from the sera of humans or rabbits with syphilis and that have been absorbed with Reiter treponemes. More recently, mouse monoclonal antibody to *T. pallidum* has been used in the DFA-TP (71, 85, 170). When fluorescing treponemes displaying typical morphology are seen by microscopic examination, the results are reported as follows: "treponemes, immunologically specific for *T. pallidum*, were observed by direct immunofluorescence." Negative results are reported as follows: "treponemes were not observed by direct immunofluorescence." Like the dark-field examination, a negative test result does not exclude the diagnosis of syphilis since the ability to demonstrate pathogenic *Treponema* spp. can be affected by the condition of the lesion or sample, plus various technical factors. However, the sensitivity of the DFA-TP approaches 100% when fresh lesions are examined (35, 85). As with all tests for syphilis, proper controls of the technique and reagents used in these methods are mandatory if meaningful results are to be obtained.

DFAT-TP test. The use of the DFA-TP test has been extended to include the staining of tissue sections (DFAT-TP) (76, 80, 85, 86). A combination of the DFAT-TP test and histologic stains may be used to examine biopsy and autopsy material for the presence of pathogenic *Treponema* spp. Any tissue can be used, but most frequently tissue for paraffin-embedded sections are collected from the brain, gastrointestinal tract, placenta, umbilical cord, or skin. Often DFAT-TP is used to diagnose late-stage or congenital syphilis or to distinguish skin lesions of secondary or late syphilis from those of Lyme disease (85). A fluorescent microscope equipped with a dark-field condenser and the standard set of filters is required. To perform the DFAT-TP test, tissues from punch biopsy or surgical excision are initially fixed for 24 h in 10% neutral buffered formalin and then embedded in paraffin blocks. Sections from the blocks are cut 2 μm thick and attached to microscope slides. Slides are deparaffinized and then pretreated with either NH_4OH or trypsin. Next, slides are stained with an FITC-labeled monoclonal conjugate as for the DFA-TP (85) or by an indirect method by using, first, either an antibody to *T. pallidum* prepared from the serum of a human with syphilis or from an immunized rabbit or a mouse monoclonal antibody, followed by an FITC-labeled anti-human, anti-rabbit, or anti-mouse globulin. A testicular tissue section from a *T. pallidum*-infected rabbit is used as the control. The condition of the initial biopsy specimen or autopsy sample and the thickness of the tissue sections affect the outcome of the test. Results are reported as for the DFA-TP. When the DFAT-TP test and the Steiner stain (197), a silver stain, were used to examine umbilical cords, we found the agreement between the two tests to be 100% (184).

Nontreponemal Tests

Unlike most bacteria, *T. pallidum* subspecies cannot be readily isolated or sustained in cell culture for numerous generations (51, 147). In addition, a specimen source is not available in the latent and late stages, since lesions are absent. Therefore, serologic tests for syphilis are performed. The serologic tests used fall into two categories, nontreponemal or treponemal. Four nontreponemal tests are currently considered standard tests: the VDRL slide, USR, RPR 18-mm circle card, and the TRUST. These standard status nontreponemal tests can be used as qualitative tests for initial screening or as quantitative tests to follow treatment (9, 53–56, 171). All four tests are based on an antigen composed of an alcoholic solution containing measured amounts of cardiolipin, cholesterol, and sufficient purified lecithin to produce standard reactivity (172). The nontreponemal (reagin) tests measure immunoglobulin M (IgM) and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material (127) and possibly by cardiolipin released from the treponemes. The antilipoidal antibodies are antibodies that not only are produced as a consequence of syphilis and the other treponemal diseases, but also may be produced in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs (16). Without some other evidence for the diagnosis of syphilis, a reactive nontreponemal test does not confirm *T. pallidum* infection.

When collecting, preparing, and examining specimens, observe universal safety precautions (18, 19, 21). Detailed methods for specimen collection are described in the 1990 edition of *A Manual of Tests for Syphilis* (105). Serum is the specimen of choice for both nontreponemal and treponemal tests. However, plasma samples may also be used in the RPR card test and TRUST. The technician must check the product insert to be sure that the plasma sample has not exceeded the recommended storage time and that the blood was collected in the specified anticoagulant. Plasma cannot be used in the VDRL, since the sample must be heated before testing, and plasma cannot be used in the treponemal tests for syphilis. The VDRL is the only test that can be used for testing CSF (112). The CSF is not heated before the test is performed. In screening for congenital syphilis, the CDC recommends the testing of the mother's serum rather than cord blood (204). Recent studies (24, 165) compared the reactivities of the mother's serum, cord blood, and infant's serum and found that the maternal sample is the best indicator of infection, followed by neonatal serum, with cord blood being the least reactive. Infant's serum is the specimen of choice for the IgM-specific tests, as well.

In the qualitative nontreponemal tests, undiluted patient's serum is used simply to measure the presence or absence of antibody. In the quantitative nontreponemal tests, serial twofold dilutions are made, and the serum is diluted until an endpoint is reached. Quantitative reactions are reported in terms of the highest (last) dilution in which the specimen is fully reactive. Quantitative tests are more informative than qualitative tests alone. Quantitative tests establish a baseline of

TABLE 2. Sensitivity and specificity of nontreponemal tests

Test	% Sensitivity at given stage of infection				% Specificity (nonsyphilis)
	Primary	Secondary	Latent	Late	
VDRL	78 (74–87)[a]	100	95 (88–100)	71 (37–94)	98 (96–99)
RPR	86 (77–100)	100	98 (95–100)	73	98 (93–99)
USR	80 (72–88)	100	95 (88–100)		99
RST[b]	82 (77–86)	100	95 (88–100)		97
TRUST	85 (77–86)	100	98 (95–100)		99 (98–99)

[a] Range of sensitivity or specificity in CDC studies.
[b] RST, reagin screen test.

reactivity from which change can be measured; recent infection can be demonstrated by a fourfold rise in titer, and reinfection or relapse can be detected among persons with a persistently reactive (serofast) test for syphilis. All of the nontreponemal tests have approximately the same sensitivity and specificity (Table 2), but their reactivity levels may differ because of the variation in antigen preparation. The different levels of reactivity are reflected in the different endpoint titers obtained when the same serum specimen is tested in the four tests (155, 156). Because success or failure of treatment is based on just a two-tube decrease in titer, the serum sample used as the baseline should be drawn the day treatment is begun (124). Also, because reactivity levels vary among the tests, the same test used in the initial testing should be used to monitor treatment (Fig. 1).

Nontreponemal tests used for screening have the advantage of being widely available, inexpensive, convenient to perform on large numbers of specimens, and necessary for determining the efficacy of treatment. Limitations of the nontreponemal serologic tests include their lack of sensitivity in early dark-field-positive primary cases and in late syphilis and the possibility of a prozone reaction or false-positive results.

Serum samples containing large amounts of nontreponemal antibody occasionally demonstrate a prozone reaction in the nontreponemal serologic tests. Prozone reactions occur in 1 to 2% of patients with secondary syphilis (95, 189). A prozone reaction occurs when antibody is in excess, is incomplete, or blocks the normal antigen-antibody reaction. Initially, these strongly reactive serum samples may show a weakly reactive, atypical, or on rare occasions, a negative "rough" reaction or grainy appearance in undiluted serum. Upon dilution of a serum sample exhibiting the prozone reaction, the reactivity will increase and then decrease as the endpoint titer is approached. Dilution of the antibody to 1:16 is usually adequate to obtain the proper optimal concentration and a readily detectable reaction. All tests with a rough appearance should be quantitated. A serologist may not detect a prozone reaction because of a lack of reading experience. When fresh serum samples are tested, some exhibit an innate roughness. Many serologists compensate for this roughness in their reading and fail to quantitate all serum samples exhibiting a grainy appearance (negative roughs). A second reason serologists may not detect the prozone reaction is that they incorrectly perform the test by adding antigen to the serum and then spread the sample to fill the circle. The physical spreading of the serum before first adding antigen dilutes the antibodies slightly, thus frequently preventing the prozone reaction.

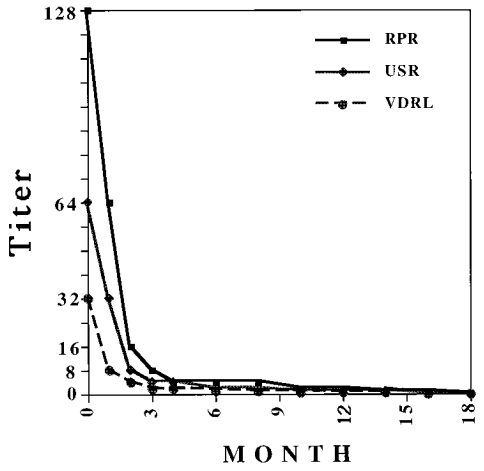

FIG. 1. Antibody levels of sera of patients monitored for 18 months after treatment by three nontreponemal tests. Reactivity levels vary among the nontreponemal tests: therefore, the same test used in the initial testing should be used to monitor treatment. Usually the titers are higher in the macroscopic test than in the microscopic tests. Following adequate therapy for primary and secondary syphilis, the titer should decline by at least fourfold by the third or fourth month and by eightfold by the sixth to eighth month (9). For most patients treated in early syphilis, the titers decline until little or no reaction is detected after the first year (53, 56).

The incidence of false-positive reactions depends on the test used and the population studied (172). False-positive reactions occurring with the nontreponemal tests can be divided into two groups: those that are acute false-positive reactions of <6 months in duration and those that are chronic false-positive reactions that persist for >6 months (89). Acute false-positive nontreponemal reactions have been associated with hepatitis, infectious mononucleosis, viral pneumonia, chicken pox, measles, other viral infections, malaria, immunizations, pregnancy, and laboratory or technical error (66, 89, 172, 173, 190). Chronic false-positive reactions have been associated with connective tissue diseases such as systemic lupus erythematosus or diseases associated with immunoglobulin abnormalities, which are more common in women; thus, chronic false-positive reactions are more common in women than in men. Other conditions associated with chronic false-positive reactions are narcotic addiction, aging (201), leprosy, and malignancy (89). At one time, titer was used to distinguish between false- and true-positive results, with titers of >8 considered true positives and those of <8 considered possible false-positives. The titer of false-positive reactions is usually low, but on rare occasions it can be extremely high; therefore, the quantitative titer cannot be used to differentiate between a false-positive reaction and syphilis. This is especially true for persons who inject illegal drugs. Over 10% of intravenous drug users have false-positive test results with titers of >8 (109).

The interpretation of the results of the nontreponemal tests depends on the population being tested. The predictive value of the nontreponemal tests is increased when combined with a reactive treponemal test. Therefore, when the nontreponemal tests are used as screening tests in a low-risk population, all reactive results should be confirmed with a treponemal test. In some low-risk populations, every reactive result may be a false-positive result (113, 114). Nontreponemal test results also must be interpreted according to the stage of syphilis suspected (see below). On the basis of the number of participants in the 1993 College of American Pathologists Syphilis Serology Survey that use the RPR card test rather than the VDRL or other nontreponemal test techniques, the RPR card test is currently the most widely used nontreponemal test in the United States.

Microscopic nontreponemal tests. The VDRL slide test and USR are classified as microscopic tests because the antigen-antibody reaction must be viewed with a light microscope

equipped with a ×10 ocular and a ×10 objective. In the microscopic tests (31, 32), the liposomes formed in the cardiolipin-cholesterol-lecithin emulsion are barely visible. In agglutination, precipitin, and flocculation reactions, antigen-antibody recognition occurs first, followed by the aggregation and antigen-antibody lattice formation. Because the reaction in the nontreponemal tests stays suspended and the two reactants themselves are not readily visible, the term flocculation is used rather than agglutination to describe this type of reaction. To perform the VDRL test, a fresh antigen suspension is prepared daily by adding VDRL antigen to a VDRL-buffered saline. Before patient samples are tested, the antigen suspension is tested with control serum samples of various reactivity levels to determine whether the suspension reproduces the established reactivity pattern of the controls. Initially, a qualitative test is performed by placing 50 μl of heated serum into one 14-mm ring of a paraffin- or ceramic-ringed slide and then adding one 17-μl drop of antigen, using either a syringe and needle or a safety pipetting device calibrated to deliver this amount. Next, the slide is placed on a mechanical rotator and rotated for 4 min at 180 ± 2 rpm. Immediately after rotation, the slide is read. Results of the VDRL are reported as reactive (if large clumps are seen), weakly reactive (if small clumps are seen), and nonreactive (if no flocculation is noted). A reactive or weakly reactive result usually indicates an active infection; however, a reactive or weakly reactive result can also indicate a person who is serofast or could represent a false-positive reaction. A nonreactive result indicates no active infection but cannot be used to rule out an incubating infection.

All serum samples exhibiting any degree of reactivity or roughness should be quantitated. The quantitative test is performed in a manner comparable to the qualitative test, except that serial twofold dilutions of the serum samples are prepared in 0.9% saline. Quantitative results are reported as the highest dilution giving a reactive (not weakly reactive) result.

When neurosyphilis is suspected, a VDRL test on the CSF is usually requested. The VDRL-CSF test should be performed only if the patient's serum treponemal test is reactive. To perform the VDRL-CSF test (32), the VDRL antigen suspension is prepared as previously described; then a 1:2 dilution of the suspension is prepared in 10% saline. This sensitized antigen must be used within 2 h after preparation. The VDRL-CSF test is performed in a manner similar to that of the test with serum, except the antigen drop size for the CSF test is 10 μl and the slide is rotated for 8 min. The results of the VDRL-CSF test are reported as either reactive (definite clumping from slight to marked) or nonreactive, as defined above. A quantitative test is performed to determine the titer of the antibodies in the CSF by preparing twofold serial dilutions in saline. The endpoint titer is the last dilution in which a reactive result is observed. False-positive VDRL-CSF test results have been reported infrequently (33, 172).

As mentioned earlier, the USR test antigen is the VDRL antigen stabilized by the addition of EDTA (159). Thus, the need for daily preparation of an antigen suspension is eliminated. Choline chloride is added to eliminate the need to heat inactivate the serum. Apparently, the addition of choline chloride also enhances the reactivity of the antigen. The USR test is performed and reported in a manner similar to the VDRL slide test on serum.

Macroscopic nontreponemal tests. The macroscopic test antigens, the RPR 18-mm circle card test and the TRUST, are based on the USR antigen (30, 110). The TRUST and RPR card test antigens differ only in the visualization agent added to the antigen. For the RPR card test, sized charcoal particles are added to the antigen; for the TRUST, paint pigment particles are added. The particles of both tests become entrapped in the antigen-antibody lattice formed with a reactive serum.

Before patient samples are tested, the antigen suspension is tested with control serum samples of various reactivity levels to determine whether the suspension reproduces the established reactivity pattern of the controls. The RPR card test and TRUST are performed in a similar manner: first, 50 μl of the control or patient's serum is placed on a plastic-coated card; next, the serum is spread within the area of the circle, and antigen is added with a 20-gauge dispensing needle calibrated to deliver 17 μl/drop or 60 ± 2 drops/ml of antigen. A mechanical rotator is used to promote the union of the antigen and antibody. The mixture is rotated for 8 min at 100 ± 2 rpm. Immediately after rotation, the slides are read macroscopically to determine the presence of clumping (flocculation) of the antigen-antibody complex. Results of the card tests are reported as either reactive, regardless of the size of the clumps, or nonreactive. All serum samples exhibiting any degree of reactivity or roughness should be quantitated to an endpoint titer. The quantitative test is performed in a manner comparable to the qualitative test, except that serial twofold dilutions of the serum samples are prepared in 0.9% saline or 0.9% saline containing 2% normal human serum for dilutions of ≥1:32. Because of a lack of sensitivity and specificity, the card tests cannot be used to test CSF (112). A major source of error in the nontreponemal tests is improper temperature of the laboratory, specimens, or reagents. If the temperature of any one of these is less than 23°C, test reactivity is decreased; if the temperature is greater than 29°C, test reactivity is increased. Other sources of errors include the testing of hemolyzed specimens (especially in the VDRL-CSF) or contaminated or lipoidal specimens, improper rotation time or speed, and incorrect antigen delivery.

Treponemal Tests

Three tests are currently considered standard treponemal tests: FTA-ABS, FTA-ABS double staining, and MHA-TP. All of these tests use *T. pallidum* as the antigen and are based on the detection of antibodies directed against treponemal components. Treponemal tests are used primarily to verify reactivity in the nontreponemal tests. The treponemal tests also may be used to confirm a clinical impression of syphilis in which the nontreponemal test is nonreactive but there is evidence of syphilis, such as might occur in late syphilis. Unfortunately, treponemal tests are technically more difficult and costly to perform than nontreponemal tests and cannot be used to monitor treatment. For 85% of persons successfully treated, test results remain reactive for years, if not a lifetime (182). The treponemal tests are not intended for routine use or to be used as screening procedures. Problems arise when these tests are used as screening procedures because about 1% (62) of the general population will have false-positive results. However, a reactive treponemal test result on a sample that is also reactive in a nontreponemal test is highly specific.

Although false-positive results in the treponemal tests are often transient and their cause is unknown, a definite association has been made between false-positive FTA-ABS test results and the diagnosis of systemic (2, 187), discoid, and drug-induced varieties of lupus erythematosus (103, 104, 130, 137). Patients with systemic lupus erythematosus can have false-positive FTA-ABS tests that exhibit an "atypical beading" fluorescence pattern. To resolve these types of false-positive reactions, absorption with calf thymus DNA can be used to remove the anti-DNA antibodies in the serum (104).

TABLE 3. Sensitivity and specificity of treponemal tests

Test	% Sensitivity at given stage of infection				% Specificity (nonsyphilis)
	Primary	Secondary	Latent	Late	
FTA-ABS	84 (70–100)[a]	100	100	96	97 (94–100)
MHA-TP	76 (69–90)	100	97 (97–100)	94	99 (98–100)
FTA-ABS double staining	80 (69–90)	100	100		98 (97–100)

[a] Range of sensitivity or specificity in CDC studies.

Unexplained reactive serologic results also may occur, particularly in elderly patients. Some false-positive reactions may be due to the failure of the sorbent used in the tests to remove all cross-reacting group, genus, or family antibodies, e.g., in Lyme disease (82, 121). In these instances, absorption with Reiter treponeme or the use of the hemagglutination test may be the only means of differentiating between syphilis and a false-positive reaction.

Of the two standard treponemal test systems (fluorescent antibody and hemagglutination) currently in use, the MHA-TP gives fewer false-positive test results (87, 111, 210). In general, the occurrence of false-positive hemagglutination tests is rare in "healthy" persons (<1%). Inconclusive hemagglutination tests have been reported for patients with infectious mononucleosis, especially in the presence of a high heterophil antibody level (93). Presumably, false-positive hemagglutination tests also occur in samples from drug addicts, patients with collagen disease, patients with leprosy, and patients with other miscellaneous conditions (111, 167, 210). In some cases, the results are difficult to assess because syphilis may actually coexist with these other infections. If both the FTA-ABS and MHA-TP tests are reactive, the sample is most likely (95%) from a person who has or has had syphilis (167). However, the final decision rests with clinical judgment.

The treponemal tests, if used appropriately as confirmatory tests, have few technical limitations, but they do have a cost limitation. Treponemal tests vary in their reactivity in early primary syphilis (Table 3); the varied sensitivities of the treponemal tests in primary syphilis are related to the time of serum collection after lesion development.

FTA-ABS tests. The FTA-ABS test is an indirect fluorescent-antibody technique (77, 78, 81). In this procedure, the antigen used is *T. pallidum* subsp. *pallidum* (Nichols strain). The patient's serum is first diluted 1:5 in sorbent (an extract from cultures of the nonpathogenic Reiter treponeme) to remove group treponemal antibodies that are produced in some persons in response to nonpathogenic treponemes. Next, the serum is layered on a microscope slide to which *T. pallidum* has been fixed. If the patient's serum contains antibody, it coats the treponeme. FITC-labeled anti-human immunoglobulin is added and combines with the patient's antibodies adhering to *T. pallidum*, resulting in FITC-stained spirochetes that are visible when examined by a fluorescence microscope. A modification of the standard FTA-ABS test is the FTA-ABS double-staining test (78). The FTA-ABS double-staining technique employs a tetramethylrhodamine isothiocyanate-labeled, anti-human IgG and a counterstain with FITC-labeled anti-*T. pallidum* conjugate. The counterstain was developed for use with microscopes with incident illumination to eliminate the need to locate the treponemes by dark-field examination when the patient's serum did not contain antibodies to *T. pallidum*. Therefore, counterstaining the organism ensures that the nonreactive result is due to the absence of antibodies and not to the absence of treponemes on the slide when it is read with incident illumination. Results of both FTA-ABS tests are reported as reactive, reactive minimal, nonreactive, or atypical fluorescence observed (77, 78). Specimens initially read as 1+ (reactive minimal) should always be retested. If the results are again read as 1+, they should be reported as such with the statement that "in the absence of historical or clinical evidence of treponemal infection, this test result should be considered equivocal." A second specimen should be obtained 1 to 2 weeks after the initial specimen and submitted to the laboratory for serologic testing. Because beaded fluorescence (atypical staining) has been observed in serum from patients with active systemic lupus erythematosus and from patients with other autoimmune diseases, this observation should be reported as well.

The sources for errors are numerous with the FTA-ABS tests because they are multicomponent tests, and each component must be matched with another. Conjugates must be properly titrated, and controls for reactive, reactive minimal, nonreactive, and nonspecific staining and sorbent must be included. Slides must be evaluated to ensure that the antigen is adhering. In addition, the microscope must be in proper operating condition with the appropriate filters in place.

MHA-TP. Passive hemagglutination of erythrocytes sensitized with antigen is an extremely simple method for the detection of antibody (102). The antigen used in the procedure is formalinized, tanned sheep erythrocytes sensitized with ultrasonicated material from *T. pallidum* (Nichols strain) (164, 199). The patient's serum is first mixed with absorbing diluent made from nonpathogenic Reiter treponemes and other absorbents and stabilizers. The serum is then placed in a microtiter plate, and sensitized sheep erythrocytes are added. Serum containing antibodies reacts with these cells to form a smooth mat of agglutinated cells in the microtiter plate. Unsensitized cells are used as a control for nonspecific reactivity.

Results are reported as reactive, nonreactive, or inconclusive. Reactive results are reported over a range of agglutination patterns: from a smooth mat of agglutinated cells surrounded by a smaller red circle of unagglutinated cells with hemagglutination outside the circle, to a smooth mat of agglutinated cells covering the entire bottom of the well. With highly reactive serum samples, the edges of the mat are sometimes folded. Nonreactive results are reported when a definite compact button forms in the center of well, with or without a very small "hole" in its center. Results that show a button of unagglutinated cells with a small hole in center are initially read as ± and should be repeated. If the same pattern is again observed, then the report should state "nonreactive." If agglutination is observed with the unsensitized cells, the test is repeated as a quantitative test. Twofold dilutions of the absorbed serum are prepared in absorbing diluent in two rows of the microtiter plate wells. Sensitized cells are added to each well in one row, and unsensitized cells are added to each well in the other row. A reactive result is reported, without reference to titer, if (i) the hemagglutination with sensitized cells is at least two doubling dilutions (four times) greater than with unsensitized cells, and (ii) the first dilution showing no hem-

TABLE 4. Comparison of the Captia syphilis M enzyme immunoassay and FTA-ABS 19S IgM test for the diagnosis of congenital syphilis

Infant category[a]	No. reactive/no. tested (%)	
	Captia syphilis M	FTA-ABS 19S IgM
Symptomatic	17/19 (90)	14/18 (77)
Asymptomatic, high risk	3/63 (5)	1/58 (2)
Asymptomatic, low risk	0/83 (0)	1/72 (1)

[a] Symptomatic = babies born with multiple signs of congenital syphilis; asymptomatic, high risk = babies born to serologic reactive mothers with no history of treatment; asymptomatic, low risk = babies born to serologic reactive mothers with a history of treatment.

agglutination with unsensitized cells has a 3+ or 4+ reaction with sensitized cells. Otherwise, the test results are reported as inconclusive.

Because the test is based on agglutination, quantitation of treponemal antibody is possible but has not proven to be worthwhile. Most studies demonstrate no practical relationship between the titer and either the progression of the disease or the clinical stage of syphilis diagnosed, and unlike the quantitative nontreponemal tests, the quantitative MHA-TP test does not seem to be useful in posttreatment evaluation (11, 28, 93). The sources for error with the MHA-TP are usually associated with the use of dusty or improper plates, pipetting errors, and vibrations in the laboratory.

PROVISIONAL TESTS FOR THE DIAGNOSIS OF SYPHILIS

Nontreponemal Tests

VISUWELL Reagin test. A new nontreponemal test in the provisional status is the VISUWELL Reagin test, an indirect ELISA (152, 211). In the indirect ELISA procedure, VDRL antigen coats the wells of a microtiter plate. The patient's serum sample is added, and nontreponemal antibody attaches to the VDRL antigen. These antibodies are then detected with an anti-human immunoglobulin conjugate labeled with the enzyme urease. Finally, at a specified time, a stop solution is added, and results are read spectrophotometrically. Studies (152, 211) have shown the test to have a sensitivity in untreated syphilis of 97% and a specificity of 97%. Like the other nontreponemal tests, the reactivity of the VISUWELL Reagin test usually disappears with treatment of the patient. The major disadvantages of VISUWELL Reagin tests are the time required for test performance, the need for equipment, and, most importantly, the inability to quantitate the patient's serum to an endpoint titer in order to assess the efficacy of treatment. With the VISUWELL Reagin test, several hundred tests can be performed in a day. This test, with a 10-min substrate incubation time or total test time of 60 min, is acceptable for both clinical laboratory and blood bank testing, if serum is used. The test does not have provisional status with plasma. When large numbers of serum samples are to be screened in a day, as in a state health department, with the addition of an automated ELISA processor, the VISUWELL Reagin becomes a viable alternative to the standard nontreponemal tests.

Treponemal Tests

Captia syphilis M test. Another test with provisional status is the Captia syphilis M test (83, 117, 195) for the detection of congenital syphilis in the newborn. Because infected infants can produce IgM in utero after 3 months of gestation and the fetus can be infected with *T. pallidum* at any time during gestation, an IgM ELISA has been developed. The test is based on using anti-human IgM antibody to capture IgM in the patient's serum, followed by the addition of a purified *T. pallidum* antigen to detect those IgM antibodies in the patient's serum directed toward *T. pallidum* (83). Next, a monoclonal antibody to *T. pallidum*, conjugated with the enzyme horseradish peroxidase, is used to detect the antigen-patient antibody reaction through the enzyme's reaction with a substrate. One study (195) found that the IgM capture ELISA was more sensitive than the FTA-ABS 19S IgM test in detecting probable cases of congenital syphilis (Table 4 [195]); however, another study (8) found the test to be equal in sensitivity to the IgM Western immunoblot in neonatal congenital syphilis but less sensitive than the Western blot in detecting delayed-onset congenital syphilis. Current interpretations of the IgM capture test results are given in Table 5. The interpretation of the test result is linked to the treatment status of the mother and her stage of syphilis.

Olympus PK-TP. A new hemagglutination test has also achieved provisional status, the Olympus PK-TP. This test was developed mainly for use in blood banking. The instrument used is a PK7100, an automated pretransfusion blood testing system, which is the basic equipment for blood grouping and typing used by the American Red Cross. The instrument is capable of running 240 samples per hour. The PK-TP reagent is composed of chicken erythrocytes which have been fixed and then sensitized with components of sonicated *T. pallidum*. Plasma is tested first; if it is reactive, a serum sample is then tested. If the serum is reactive, then the serum is tested in the RPR, and if reactive in the RPR, it is confirmed by the FTA-ABS test. This sequence of testing is done because the PK-TP identifies all individuals who currently have syphilis or who have ever had syphilis. Because this testing sequence is contrary to the epidemiologic procedures in the United States that are based on identifying active cases of syphilis, the use of the test as a screening test is restricted to blood bank populations at this time. Early experience with the PK-TP in blood banks (58) indicates that the PK-TP is at least twice as specific as the RPR card test.

TABLE 5. Interpretation of Captia syphilis M results for the diagnosis of congenital syphilis

Infant's Captia syphilis M result	Clinical signs/ symptoms in infant	Mother's treatment history	Serodiagnosis
Nonreactive	Present	Untreated or inadequately treated	Congenital syphilis
Nonreactive	Absent	Untreated or inadequately treated early syphilis	Possible incubating congenital syphilis
Nonreactive	Absent	Untreated or inadequately treated latent syphilis	Unknown risk of congenital syphilis
Reactive	Present	Untreated or inadequately treated	Congenital syphilis
Reactive	Absent	Untreated or inadequately treated	Suggestive of congenital syphilis

INVESTIGATIONAL TESTS FOR THE DIAGNOSIS OF SYPHILIS

Treponemal Tests

ELISA IgG antibody tests. Several tests using the ELISA format have been developed and evaluated as tests for syphilis (12, 49, 83, 117, 138, 141, 152, 153, 157, 206, 218). Of the commercial ELISAs designed to replace the FTA-ABS tests and MHA-TP as confirmatory tests for syphilis (117, 138, 157), initial evaluations have found all to have sensitivities and specificities similar to those of the other treponemal tests, but more extensive evaluation is necessary (146). Limitations of the ELISAs are time and costs when small numbers of samples are to be processed. The main advantages are the capacity to process large numbers of samples and the automated readout. No longer is the reading of the test subjective, as for the FTA-ABS tests and MHA-TP, but a spectrophotometric determination is made and an objective reading is provided as a printout.

FTA-ABS 19S IgM test. An older technique for the diagnosis of congenital syphilis that remains in the investigational status is the FTA-ABS 19S IgM test (1, 42, 92, 125, 139, 185, 186). The FTA-ABS 19S IgM test is not available in kit form, and matching the reagents to be used in the test is extremely difficult. The predecessors of the FTA-ABS 19S IgM test, the FTA-IgM and the FTA-ABS IgM, were reported to be both nonspecific and lacking in sensitivity (1, 92, 99, 116, 125, 139). The specificity of the FTA-ABS IgM test for neonatal congenital syphilis, as well as other indirect immunofluorescent IgM tests for prenatal infections, has been questioned by the observation that newborns may produce IgM antibodies in response to passively transferred maternal IgG antibody rather than in response to the infectious agent itself (116, 139, 166, 172). The fractionation of the infant's serum appears to eliminate the majority of the problems with the specificity of the test. False-positive reactions in the current version of the FTA-ABS IgM test with the 19S fraction of IgM have been reported in normal infants, but they are infrequent and the test may be a useful confirmatory procedure to differentiate passive transfer of maternal antibody from active infection (139, 195). Still, the major drawback of the FTA-ABS IgM 19S test for neonatal congenital syphilis lies in its insensitivity (166, 195). Therefore, while the FTA-ABS 19S IgM test for congenital syphilis may be useful as a confirmatory test, it should not be used as a screening procedure and, at this time, cannot replace careful repeated clinical assessment combined with serial quantitative nontreponemal tests in the evaluation of the newborn with possible congenital syphilis.

FTA-ABS CSF. Another older test technique that is still in investigational status is the FTA-ABS CSF test for the diagnosis of neurosyphilis. FTA tests used for CSF examination have included techniques that used undiluted CSF (the CSF-FTA) (43, 47, 59, 69, 88, 112, 214) and tests that used CSF diluted 1:5 with sorbent (the CSF-FTA-ABS test) (43, 47, 59, 69, 88, 112, 214). FTA tests on CSF are more sensitive than the CSF-VDRL slide test. However, the clinical significance of this greater sensitivity remains to be established, because the test may be reactive as a result of antibodies remaining in the CSF of patients who had been adequately treated for early or latent syphilis rather than as a result of neurosyphilis (88). False-positive results have been reported with the CSF-FTA tests (47, 112) that appear to be eliminated by diluting the CSF in sorbent (112). The results of three studies appear to support the use of a nonreactive result with the FTA-ABS CSF test to rule out neurosyphilis (88, 112, 119).

Western blotting. The last of the investigational tests is the Western blot to detect either IgG (14, 146) or IgM (135, 174). Performance of the Western blot techniques for syphilis is similar to the techniques used for confirmation of antibodies to human immunodeficiency virus (HIV). To prepare the strips for *T. pallidum* immunoblotting, initially a boiled sodium dodecyl sulfate extract of the organism is electrophoresed through a gradient gel. After electrophoresis, a sheet of nitrocellulose is placed on top of the gel, and the protein immunodeterminants are electrophoretically transferred to the blot. The blot is cut into strips and incubated with the patient's serum sample. If a commercial test kit is used, the starting point is the nitrocellulose strip. After incubation of the strips with the patient's serum, antibodies are detected using a second antibody as in an indirect test, labeled with either an enzyme and substrate or a radioactive material. To date, many investigators (148), and the manufacturer of the one commercial product that is under evaluation, agree that antibodies detected to the immunodeterminants with molecular masses of 15.5, 17, 44.5, and 47 kDa appear to be diagnostic for acquired syphilis. The test using IgG conjugate appears to be at least as sensitive and specific as the FTA-ABS tests (14), and efforts have been made to standardize the procedure (60). Several studies (8, 42, 118, 135, 174) have found the Western blot for *T. pallidum* to have its greatest value as a diagnostic test for congenital syphilis when an IgM-specific conjugate is used. The IgM Western blot for congenital syphilis appears to have a specificity of at least 90% (135) and a sensitivity of $\geq$83% (135, 179), which is greater than the sensitivity of 73% recently reported for the FTA-ABS 19S IgM test (195).

DIAGNOSIS OF TREPONEMAL INFECTIONS USING STANDARD TESTS

Sexually Acquired Syphilis

For the laboratory diagnosis of syphilis, each stage has a particular testing requirement. In the United States, the routine testing scheme is direct microscopic examination of lesion exudates followed by a nontreponemal test which, if reactive, is confirmed with a treponemal test (Fig. 2). The relationship of serologic test reactivity to disease stage is shown in Fig. 3. Criteria for the diagnosis of syphilis are divided into three categories: definitive, presumptive, and suggestive (22).

Primary syphilis. Sexual transmission of syphilis requires direct contact with infectious lesions. Approximately 30% of persons who have sex with an infected partner will develop syphilis (187). Within hours after initial contact with the organism, treponemes disseminate throughout the body: however, preferential multiplication occurs at the site of entry (52). The primary-stage chancre usually appears within 3 weeks after infection, although the incubation period between the time of infection and formation of a lesion is 10 to 90 days (191). The lesions, once present, may spontaneously resolve in 1 to 5 weeks. During the primary stage, the inguinal lymph nodes may be slightly enlarged but are rarely tender. Serous fluids from the lesion contain numerous treponemes, detectable by either dark-field microscopy (29) or the DFA-TP (75). Humoral antibodies, as detected by the standard nontreponemal and treponemal serologic tests for syphilis, usually do not appear until 1 to 4 weeks after the chancre has formed. Approximately 30% of those with early primary syphilis will have nonreactive nontreponemal test results on initial visit. A definitive diagnosis of primary syphilis requires the direct microscopic identification of *T. pallidum* in lesion material, lymph node aspirate, or biopsy section. A presumptive diag-

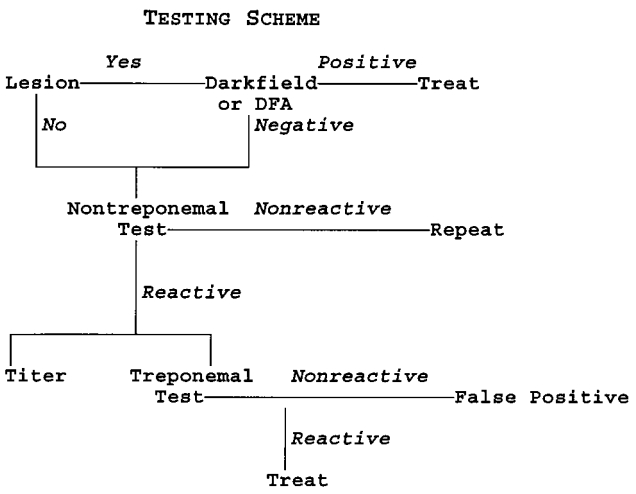

FIG. 2. In the United States, the routine testing scheme is direct microscopic examination of lesion exudates in early syphilis (primary and secondary stages), followed by a nontreponemal test which, if reactive, is confirmed with a treponemal test. In latent syphilis, reactivity in serologic tests is the only means of diagnosis.

nosis is based on the presence of a typical lesion and either a reactive nontreponemal test and no history of syphilis or (for persons with a history of syphilis) a fourfold increase in titer on a quantitative nontreponemal test when results of past tests are compared with the most recent test results. In primary syphilis, the MHA-TP is less sensitive than the FTA-ABS test, and probably is less sensitive than the nontreponemal tests, when used to confirm reactivity in the nontreponemal test (87, 111, 114). A suggestive diagnosis of primary disease is based on the presence of a lesion and sexual contact within the preceding 90 days with a person in whom syphilis has been diagnosed.

Secondary syphilis. By the secondary stage of syphilis, the organism has invaded every organ of the body and virtually all body fluids. Nonspecific symptoms develop (usually within 6 weeks to 6 months after infection or 1 to 5 weeks after the primary chancre has healed) and may include fever, headache, sore throat, arthalgias, and anorexia (191). However, a generalized rash (with diverse manifestations often resembling other

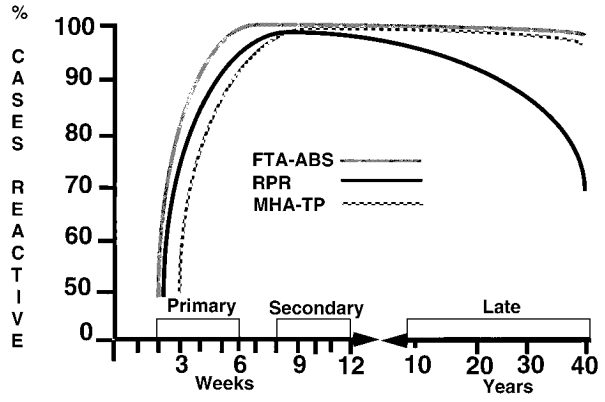

FIG. 3. For the majority of patients, the RPR card test and FTA-ABS test become reactive within 1 week after the primary chancre appears. The MHA-TP test becomes reactive somewhat later. Although shown here as distinct stages, primary and secondary symptoms may overlap, with secondary rash appearing before the primary chancre has resolved.

dermatoses), mucous patches, and condylomata lata are the most characteristic symptoms of secondary syphilis. These manifestations spontaneously resolve, usually within 2 to 6 weeks, but may recur during the first year of infection if the patient is not treated. As the disease reaches the secondary stage, with few if any exceptions, all serologic tests for syphilis are reactive, and treponemes may be found in lesions by direct microscopic examination. As with primary syphilis, the definitive diagnosis of secondary syphilis is based on the observation of *T. pallidum* by direct microscopic examination. A presumptive diagnosis is based on the presence of typical lesions and a reactive nontreponemal test titer of ≥ 8 and no previous history of syphilis or, for persons with a history of syphilis, a fourfold increase in the most recent titer compared with past test results. For patients with atypical lesions and/or nontreponemal test titers of <8, the nontreponemal tests should be repeated and a confirmatory treponemal test should be performed before a presumptive diagnosis is made. In the secondary stage of syphilis, the sensitivity of the MHA-TP is equal to the 100% sensitivity of the FTA-ABS tests. A suggestive diagnosis is made only when serologic tests are not available and is based on both the presence of clinical manifestations and sexual exposure within the past 6 months to a person with syphilis.

Latent syphilis. A period of latency may occur between primary and secondary stages; however, the term latent syphilis usually refers to the period following the disappearance of symptoms of secondary syphilis. In the early latent stage, the disease progresses from an acute to a chronic infection. During the latent period following the secondary stage, a relapse to the secondary stage may occur. Infection of less than 1 year in duration that is asymptomatic is arbitrarily defined for epidemiologic purposes as "early latent stage" (191). Serologic tests are reactive in the early latent stage, but the reactivity in the nontreponemal tests decreases with increasing latency. Since lesions are not present, a definitive diagnosis of latent syphilis based on the observation of treponemes does not exist. The presumptive diagnosis of latent syphilis is based on a combination of serologic results and a history of a nonreactive nontreponemal test the prior year, a fourfold increase in titer compared with the most recent test for persons with a history of syphilis, or a history of symptoms compatible with those of the earlier stages of syphilis. A suggestive diagnosis of latent syphilis is based on a reactive nontreponemal test result and a history of sexual exposure within the preceding year.

Patients with late latent syphilis, i.e., with reactive nontreponemal and treponemal tests, no clinical and historical findings, and an unknown history of treatment or nonreactive serologic results, should be evaluated for potential asymptomatic neurosyphilis. Even when the patient has not received treatment, the nontreponemal tests may be only weakly reactive.

Tertiary syphilis. Syphilis will progress to the late stage in approximately one-third of persons who fail to receive treatment (204). The pathogenesis of the various forms of the late or tertiary stage of syphilis is not completely understood. Often, symptoms of late-stage syphilis occur 10 to 20 years after the initial infection. Because results for approximately 30% of patients with late syphilis will be nonreactive in the nontreponemal tests, treponemal test results should be obtained if syphilis in these stages is suspected and the nontreponemal tests are nonreactive. The laboratory should be informed that late syphilis is suspected; otherwise, according to laboratory policy, a treponemal test may not be performed in the absence of a reactive nontreponemal test. Treponemal tests are almost always reactive and may be the only basis for diagnosis (Table 2), even though the sensitivity of the trepo-

nemal tests also declines somewhat in the late stages of syphilis (Table 3). In the future, PCR techniques may play a major role in establishing the diagnosis of late syphilis.

In the lesions (gummas) of benign late syphilis, few treponemes are found by direct microscopic examination. However, if gummas are present, then a definitive diagnosis is based on the observation of *T. pallidum* in biopsy samples by DFAT-TP. Presumptive diagnosis is based on a reactive treponemal test and no known history of treatment for syphilis.

The lesions in the aorta in cardiovascular syphilis are related to the multiplication of treponemes (52). Diagnosis of cardiovascular syphilis is made on the bases of symptoms indicative of aortic insufficiency or aneurysm, reactive treponemal test results, and no known history of treatment for syphilis.

The lesions in the central nervous system in neurosyphilis are also related to the multiplication of treponemes (52). Although neurologic forms of syphilis (e.g., syphilitic meningitis) may develop during the secondary stage, neurosyphilis is usually a complication of late syphilis; however, it may occur as early as 2 years after initial infection. Neurosyphilis can take many forms, yet symptoms consistent with neurosyphilis may not always be present (133). CSF examinations, such as the VDRL-CSF slide test, total protein, and leukocyte counts should be performed on the spinal fluid of persons with late latent syphilis as well as on the CSF of those patients with clinical symptoms and signs consistent with neurosyphilis (36, 37, 133). Diagnosis of neurosyphilis requires a reactive treponemal test result with a serum sample, a CSF cell count of more than five mononuclear cells per cubic centimeter, and CSF total protein in excess of 40 mg/dl. A definitive diagnosis is made on the bases of a reactive serum treponemal test, a reactive VDRL-CSF on a spinal fluid sample, and identification of *T. pallidum* in tissue by microscopic examination of tissue stained with silver stain or fluorescent antibody. When a patient is treated for neurosyphilis, the CSF cell count should return to normal first, followed by the CSF protein concentration and, finally, the results of the VDRL-CSF test (36, 37, 133, 204). The results of serial VDRL slide quantitative tests on CSF can be used to monitor response to treatment, similar to the use of quantitative serum serologic tests for syphilis. However, the response of the CSF quantitative VDRL to treatment is less predictable; it may take many years for the CSF nontreponemal test to become nonreactive, although the titer should drop progressively.

Evaluation after treatment. To follow the efficacy of therapy, patients should be monitored to ensure that signs and symptoms have resolved and that titer has declined. To monitor the efficacy of treatment, quantitative nontreponemal tests should be performed on the patient's serum samples, which are drawn at 3-month intervals for at least 1 year. Following adequate therapy for primary and secondary syphilis, there should be at least a fourfold decline in titer by the third or fourth month and an eightfold decline in titer by the sixth to eight month (9) (Fig. 1). For most patients treated in early syphilis, the titers decline until little or no reaction is detected after the first year (53, 56). Patients treated in the latent or late stages, or who have had multiple episodes of syphilis, may show a more gradual decline in titer (54, 55). Low titers will persist in approximately 50% of these patients after 2 years. As far as can be determined, this persistent seropositivity does not signify treatment failure or reinfection, and these patients are likely to remain serofast even if they are retreated.

Diagnosis of syphilis among HIV-infected persons. In the 1980s, studies showed that 70% of serum specimens from homosexual men with AIDS reacted in treponemal tests for syphilis (168). More recent studies in the heterosexual population indicate that approximately 60% of HIV-infected females had reactive syphilis serologic test results (15) and that 15% of the adolescents that were reactive in the tests for syphilis also tested positive for HIV antibodies (128). Although most HIV-infected persons who also are infected with *T. pallidum* appear to respond normally in the serologic tests for syphilis and have typical clinical signs (63, 169), several exceptions have been published (38, 108, 162) or reported to the CDC. The problems in the diagnosis of syphilis are (i) confusing clinical signs and symptoms (38, 162); (ii) lack of serologic response in a patient with a clinically confirmed case of active syphilis (64, 72); (iii) failure of nontreponemal test titers to decline after treatment with standard regimens; (iv) unusually high titers in nontreponemal tests (140), perhaps as the result of B-cell activation (107); (v) rapid progression to late stages of syphilis and neurologic involvement even after treatment of primary or secondary syphilis (6, 50, 91, 98, 120, 140); and (vi) the disappearance of treponemal test reactivity over time (67). Whether these problems in the diagnosis of syphilis occur more frequently in HIV-seropositive persons than in HIV-seronegative persons with syphilis is unclear (169, 171).

Aberrant results in the serologic tests for syphilis appear to be related to abnormally low absolute CD4 cell counts and are relatively rare (64, 72). The diagnosis of syphilis in these cases was supported either by an observation of *T. pallidum* in material from typical lesions or by the appearance of serologic reactivity after treatment. The delay in development of a response to syphilis theoretically should be expected in persons with abnormal lymphocyte counts; however, the frequency of this occurrence is unknown. In studies conducted by the CDC (15), the serologic test results for syphilis in heterosexual populations indicated that HIV-seropositive persons were twice as likely as HIV-seronegative persons to have reactive serologic tests for syphilis. Another study (183) found that HIV-seropositive homosexual men were five times more likely than their HIV-seronegative counterparts to have a reactive treponemal test. Thus, the HIV-seropositive individuals in these two studies were assumed to have had a previous history of syphilis. In addition, recent studies (63, 95, 169) found that the serologic response in the tests for syphilis, as indicated by endpoint titers, was greater among HIV-infected persons than among HIV-nonreactive individuals.

The failure of nontreponemal test titers to decline after treatment with standard therapy has been documented for HIV-seronegative persons treated during latent-stage or late-stage syphilis and in persons treated for reinfection (54, 55, 171). Therefore, the failure of titers to decline with treatment for syphilis in HIV-infected person is probably related to the stage of syphilis rather than to HIV status.

Previously, infection with *T. pallidum* has been reported to be immunosuppressive (90, 151). Recently, we reported that, although the percentages of CD4 and CD8 lymphocyte subsets for persons with syphilis were within the range of those percentages found in uninfected persons, the percentages of CD4 cells were significantly lower ($P = <0.001$) and those of CD8 cells were higher ($P = 0.03$) among patients with syphilis than in the uninfected population (158). Early findings in our most recent study (169) indicated that infection with *T. pallidum* in the HIV-positive person exacerbates the depletion of CD4 receptor cells.

The disappearance of reactivity in the treponemal tests after treatment and over time has been reported in the past, before the identification of HIV type 1. Schroeter et al. (182) reported that 14% of patients with early syphilis lost their reactivity in the FTA-ABS test within 2 years after treatment. Further studies (67) found that specimens from 36% of men with

AIDS, 43% of men with AIDS-related complex, and 7% of asymptomatic HIV-seropositive men with histories of treatment for syphilis became nonreactive in the treponemal tests, whereas the treponemal tests results of the HIV-seronegative men remained reactive. Loss of reactivity in the treponemal tests was related to decreased total CD4 lymphocytes and CD4/CD8 ratios and a nontreponemal test titer of <32 at the time of treatment. In this study (67), none of the persons had active syphilis. On the other hand, when persons being treated for active syphilis (with and without HIV infection) were followed up over time (63), 16% of the patients lost treponemal test reactivity after treatment. The loss of reactivity could not be related to HIV, CD4 count, or stage of syphilis. However, initial low nontreponemal test titer was weakly associated with the loss of treponemal test reactivity.

The literature abounds with descriptions of syphilis in so-called late-stage forms in relatively young persons who are HIV seropositive (38, 50, 91, 98, 108, 162), but neurologic symptoms may be present among patients with secondary syphilis who are HIV seronegative as well (133). One study (120) found indications of *T. pallidum* in the central nervous system of 40% of patients with early syphilis. However, no correlation was found between HIV infection and the invasion of the central nervous system by *T. pallidum*. *T. pallidum* was isolated from 27% of HIV-infected persons and 32% of HIV-negative persons in the study (120).

Even though most HIV-seropositive persons treated with standard regimens for syphilis appear to respond to treatment (63, 169), one cannot totally disregard the role of cell-mediated immunity in protecting the person against the progression of syphilis (134) or the interaction of an intact immune system with therapeutic agents (50). Recommendations for diagnosing and treating syphilis among HIV-infected persons have been made by the CDC (20). The recommendations include the increased use of direct microscopic examinations of lesion or biopsy material when clinical findings suggest syphilis but serologic test results are nonreactive; careful serologic and physical examination follow-up to ensure adequacy of treatment; a CSF examination of patients in whom syphilis is latent and in whom the duration, if known, is >1 year; the treatment of neurosyphilis among HIV-infected patients with at least 10 days of aqueous crystalline penicillin G or aqueous procaine penicillin G plus probenecid; and the reporting of unusual manifestations of syphilis to state epidemiologists.

Congenital Syphilis

The control of congenital syphilis (219) can be accomplished by treating pregnant women. In pregnancy, although false-positive nontreponemal test results have been reported in samples from pregnant women (10), reactive results should be confirmed with a treponemal test, and if it is reactive, the patient should be treated. Also in pregnancy, nontreponemal titers remaining from previous treatment of syphilis tend to increase nonspecifically. This increase in titer may be confused with the diagnosis of reinfection or relapse. An increase in titer may be considered nonspecific if previous treatment can be documented and if positive lesions, a fourfold increase in titer, and a history of recent sexual exposure to a person with infectious syphilis are absent. Previously, the difference in the mother's nontreponemal test titer and the infant's titer at delivery was thought to be a means of distinguishing infected from uninfected infants. If the infant's titer was higher than that of the mother's, then the infant had congenital syphilis (194). However, the converse has not been found to be true. A lower titer in the infant's serum than in the mother's does not rule out congenital syphilis (195). Examination of serum sample pairs from mothers and infants in cases of congenital syphilis indicated that only in 22% of the cases did the infant have a titer higher than the mother (195).

A primary stage does not occur in congenital syphilis because the organisms directly infect the fetal circulation. Treponemes or the effects thereof are detectable in almost every tissue of the infant (204). The standard serologic tests for syphilis, based on the measurement of IgG, reflect passively transferred antibodies from the mother to the infant, rather than IgM antibodies produced during gestation. The passively transferred antibodies should be catabolized and undetectable among noninfected infants between the ages of 12 to 18 months (84). Currently, the diagnosis of neonatal congenital syphilis depends on a combination of results from physical, radiographic, serologic, and direct microscopic examinations. Clinical signs of congenital syphilis include hepatosplenomegaly, cutaneous lesions, osteochondritis, and snuffles (99, 204). Although some clinical manifestations may be present at birth, they are more often seen at 3 weeks to 6 months. At birth, up to 50% of the infants with congenital syphilis are asymptomatic (99, 204); other stigmata that may develop later include teeth and bone malformation, deafness, blindness, and learning disabilities (204). The diagnosis of neonatal congenital syphilis is definitive if *T. pallidum* is demonstrated by direct examination of umbilical cord, placenta, nasal discharge, or skin lesion material. A presumptive case of congenital syphilis is defined as an infant born to a mother who had untreated or inadequately treated syphilis at delivery, regardless of findings in the infant, or any infant who has a reactive treponemal test result and any clinical sign or symptoms of congenital syphilis on physical examination, or an abnormal CSF finding without other cause, or a reactive VDRL-CSF test result, or a reactive IgM test specific for syphilis.

Other Treponematoses

To date, no laboratory method has been devised to distinguish the other pathogenic treponemes from each other or from *T. pallidum* subsp. *pallidum* (45, 46). The standard serologic tests for syphilis are uniformly reactive in the diseases caused by *T. pallidum* subsp. *pertenue* (yaws), *T. carateum* (pinta), and *T. pallidum* subsp. *endemicum* (nonvenereal endemic syphilis or bejel) (5). Interestingly, results from animal model studies indicate differences in susceptibility of the hamster and the rabbit to infection with *T. pallidum* subsp. *pallidum*, *pertenue*, or *endemicum*. The Syrian hamster is not highly susceptible to *T. pallidum* subsp. *pallidum*, yet the LSH hamster develops extensive skin lesions when infected with *T. pallidum* subsp. *endemicum* (178). When rabbits are infected with *T. pallidum* subsp. *pertenue*, tissue alteration and infectivity are not observed to the extent seen with *T. pallidum* subsp. *pallidum*; on the other hand, *T. pallidum* subsp. *pertenue* is considerably more virulent for the LSH hamster than *T. pallidum* subsp. *pallidum* (177). In the hamster, lesions are consistently seen in infections with $>10^4$ *T. pallidum* subsp. *pertenue* organisms and local infection in the lymph nodes can be demonstrated with even lower doses. Infection of the hamster is performed by taking the material suspected of containing *T. pallidum* subsp. *pertenue* and injecting 0.1 ml of the sample intradermally in the inguinal area of the skin. The treponemes colonize the skin and especially the inguinal lymph nodes from the draining lymphatics. Although hamsters are far more sensitive to infection with *T. pallidum* subsp. *pertenue* than to that with *T. pallidum* subsp. *pallidum*, they can be infected by the latter. Therefore, any infection shown by

hamster injection must be confirmed by clinical evaluation to differentiate syphilis and yaws.

Newer test techniques such as Western blotting assays do not differentiate the antibodies formed in response to syphilis from those formed in response to yaws or pinta (57, 143). Most molecular approaches, such as DNA sequencing, DNA probes, and PCR techniques, have also failed to individualize the pathogenic treponemes (143). A single nucleotide substitution has been reported in the gene for the 4D antigen (an oligomeric protein of unknown function or location in the cell) in *T. pallidum* subsp. *pallidum* compared with *T. pallidum* subsp. *pertenue* (144). The data were not convincing, though, since up to 30% of the isolates tested had the nucleotide in the position expected for the other subspecies (144). Until a great deal more is known about the biology of *T. pallidum* and the function of the various cloned proteins, it is doubtful that probes can be used to differentiate the different subspecies. Much greater sensitivity could be expected if actual differences were known in the presence of various enzymes or structural proteins in the two subspecies, but this is not a viable possibility at the present time.

Current diagnosis of these diseases as separate entities is based on the clinical appearance of the lesions formed by *T. pallidum* subsp. *pertenue*, *pallidum*, or *endemicum* and *T. carateum*, the anatomical location of the lesion, the mode of transmission, the age of the individual, and the geographical location of the infected individual. Because the nonsexually acquired treponemal infections are often childhood diseases, a combination of the age of the individual and the nontreponemal test titer is used to distinguish test reactivity due to active sexually acquired syphilis from test reactivity due to earlier nonsexually acquired infections. For example, the nontreponemal test titers of adults is expected to be ≤ 8 for those from a geographic region in which endemic treponematoses were virtually eliminated by mass campaigns in the 1950s and 1960s (4, 131). Therefore, any titer of >8 is indicative of sexually acquired syphilis for adults from these regions (61, 115). Likewise, a titer of ≥ 8 in a child suggests a resurgence of yaws in these particular areas (154).

MOLECULAR BIOLOGY-BASED METHODS FOR THE DIAGNOSIS OF SYPHILIS

The shortcomings of the standard tests for syphilis for the diagnosis of early primary, congenital, and neurosyphilis, as discussed above, have made techniques based on the detection of treponemal DNA or antigens very appealing. The greatest difficulty with the DNA approach is the lack of understanding of the biology of the organism, which complicates the selection of target antigens or DNA sequences. Despite these difficulties, some progress has been made in treponemal diagnosis using techniques based on molecular biology. All of these techniques are presently experimental, but they offer the possibility of great sensitivity and in some cases increased potential for automation of syphilis testing.

DNA Probes

DNA probes have already found clinical application for the identification of a number of clinically important pathogens (207, 216). Experience with other spirochetes indicates the possible applicability of this methodology to the identification of *T. pallidum*. Random probes labeled with ^{32}P by nick translation of bulk DNA from various strains of pathogenic leptospires has been used successfully in a dot blot assay for identification of this spirochete (198). The assay gave a positive result when approximately 2,500 leptospires were present in the sample. Only in certain specimens would this be sufficiently sensitive to serve as a clinical test, but no attempt was made to optimize the assay by determining what sequences would be most suitable; thus, the potential of this method is still undetermined. In many of the problem areas in syphilis diagnosis (neurosyphilis and congenital syphilis), only rarely are large numbers of treponemes seen in clinical samples (184); thus, the sensitivity of the dot blot would have to be considerably enhanced to make this a viable method for diagnosis of syphilis.

PCR

Because of the low sensitivity of DNA probes as cited above, PCR-based methods might offer better possibilities for clinical applications. PCR-based tests have been developed by several laboratories either as potential diagnostic tests or to identify *T. pallidum*-infected animals in experimental animal systems (13, 65, 145, 175, 212). Most of the tests have been based on membrane lipoproteins, of which a large number have been cloned and sequenced. This work has been significantly aided by the fact that *T. pallidum* is extremely genetically conserved. Whereas variation in membrane and surface-associated proteins is common among many species of bacteria, this variation has never been shown with *T. pallidum*; even the different subspecies show essentially identical patterns by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (196). This extreme genetic stability is probably related to the apparent lack of significant DNA repair capacity in *T. pallidum* (193) and the probable absence of the *recA* gene product (192), which would indicate little or no recombinational capacity in this spirochete.

Two PCR-based techniques were described almost simultaneously in 1991 (65, 145). Since sensitive and inexpensive serologic tests are available for most stages of syphilis in adults, the investigators in these two papers concentrated on problem areas in which definitive diagnosis is beyond the abilities of most clinical laboratories. The first of these was based on the amplification of a 658-bp segment of the gene for the 47-kDa surface antigen; this is a lipoprotein that is antigenically dominant in the human immune response to *T. pallidum* (94). The test was performed on clinical specimens (amniotic fluid, neonatal sera, and neonatal CSF) for use in the diagnosis of congenital syphilis in neonates (65, 146, 175). The test results were verified with the RIT. In the various tissue fluids examined (65, 175), the overall sensitivity was 78% compared with results obtained in the RIT. The major difficulty with the test was one that has hindered the application of PCR in other clinical settings: lack of sensitivity in detecting positive specimens. False-positive results, caused by the improper handling of the specimens, and resulting in contamination with extraneous treponemal DNA, did not seem to be a problem (106). None of the samples negative by RIT were positive by PCR; rather, the sensitivity problems revolved around false-negative results. Only in the amniotic fluid samples was the PCR sufficiently sensitive to be considered a diagnostic test; CSF and serum results correlated approximately 60 to 67% with positive results by the RIT. The lack of sensitivity has generally been found to be related to nonspecific inhibitors of the PCR reaction. To eliminate inhibition, Grimprel et al. (65) used four different methods to isolate DNA from the samples. The four methods were a boiling method, a low-spin separation, an alkaline lysis, and a spin separation method. The low-spin and alkaline lysis methods seemed to give the best correlations with rabbit infectivity. Interestingly, the alkaline lysis methods

showed better results with small volumes of sample from the clinical specimen. More effective and simpler methods for sample preparation are clearly needed for this and most other PCR-based methods if this technique is to be more than a curiosity in clinical laboratories.

Noordhoek et al. (145) reported on the use of PCR for the diagnosis of neurosyphilis almost simultaneously with Grimprel et al. (65). Several studies have indicated that *T. pallidum* can persist for long periods in the central nervous system, even apparently in patients who have received what was considered adequate antibiotic treatment (6, 70). Serologic tests have generally not proved adequate to diagnose these patients since their results commonly remain positive in the treponemal tests whether they are still infected or not (88, 112). Thus, a method that could detect the presence of *T. pallidum* in small samples of CSF could prove extremely valuable, especially in determining whether treatment was sufficient in cases where neurosyphilis is suspected or known to have occurred.

Noordhoek et al. (145) used as their target the gene coding for a 39-kDa basic membrane protein. Primers derived from this gene showed a serious lack of specificity, but the authors were able to largely overcome this problem by using a second pair of nested primers within the sequence used in the first round of amplification. A very serious difficulty developed in this series of tests, analogous to problems found in serologic diagnosis of neurosyphilis, namely, the inability to differentiate between patients with active infections and patients who no longer appeared to harbor living treponemes. All patients who had been previously infected with *T. pallidum* were positive by PCR regardless of previous treatment status. Because there is considerable controversy over whether the present treatment regimens successfully eliminate *T. pallidum* from the central nervous system (6, 18, 91, 120, 140), it was not possible to evaluate these results with certainty. At present, it is not possible to differentiate between the persistence of small numbers of viable treponemes and that of dead organisms containing DNA which could be amplified.

At least two other groups have used PCR to detect *T. pallidum* in either clinical samples or experimental animals (14, 15, 70, 212). The first group (14, 70) used primers derived from the gene sequence of TmpA (a 45-kDa membrane protein) and the 4D antigen (an oligomeric protein with multiple forms) as primers. They found a sensitivity of detection equivalent to 65 organisms in nonclinical specimens but did not determine the sensitivity in CSF. They indicated that PCR could be used for determination of treatment success, but this was before the report by Noordhoek et al. (145); also, they did not evaluate the PCR against known serologic tests, so the reported sensitivity could not be compared with that of standard clinical methods. The second group (212) used a primer sequence derived from basic membrane protein, a cloned membrane protein with a basic pH. The specimens tested were from a rabbit model of congenital syphilis. An interesting result of these experiments was that only whole blood proved to be positive by the PCR; serum samples were uniformly negative, with the exception of a single sample that was partially hemolyzed. No inhibitory effect was seen with heparin, although this polysaccharide is known to cause inhibition of PCR in other systems (16, 73). The system as described would seem to have fewer difficulties in most cases; e.g., specimens can be frozen and there are fewer problems with inhibitory materials in the sample. The reason for the inconsistencies with results of other systems is difficult to identify because the animals and samples involved were different, as was the PCR target sequence. Because of the controversy over sample source (i.e., whole blood, lesion material [212], CSF, serum, or amniotic fluid [175]), the appropriate sample source is still under consideration (146).

PCR could be extremely valuable in diagnosing infection in congenital syphilis (passively transferred antibodies now confuse the diagnosis), in diagnosing neurosyphilis (the only serologic test is only 50% sensitive), in diagnosing early primary syphilis (the only tests available currently are microscopic), and finally, in distinguishing new infections from old infections (now only a rise in titer can be used).

USE OF CLONED ANTIGENS IN SEROLOGIC TESTS

Although the data are incomplete, it appears that at least some of the cross-reactive antigens are components of the endoflagellar complex whose protein components are highly conserved. The problems of nonspecificity or the necessity for absorbance steps could theoretically be eliminated by using antigens that are specific to *T. pallidum* for the serologic diagnosis of syphilis.

The majority of cloned antigens appear to be membrane-associated proteins; most of them have been shown to be lipoproteins (20, 180). Many of these proteins are highly antigenic. The lipid component has been suggested to function as a hapten that increases the antigenicity of the proteins involved. The relationship of this putative reactivity to the almost invariant response to lipid antigens seen in primary syphilis and the autoimmune response seen in tertiary syphilis is presently unknown. In serodiagnosis, the most important principle to be determined is whether the response is mainly to the protein or the lipid hapten; if to the former, the potential for cross-reactivity to the native protein could be a potential problem. One way around this problem is to use nonacylated recombinant proteins. In this way, any reactivity to the lipid component is eliminated, although there is the potential for some loss in sensitivity also.

The tests being developed that utilize cloned antigens specific for *T. pallidum* are mainly ELISA or Western blotting tests. Two major problems have plagued the development of tests against specific antigens in the past. The first has been the lack of sufficient quantities of treponemal proteins to use in a test. An attempt was made to circumvent this problem by using purified flagella from *T. phagedenis* (18, 153), but high levels of antibody against these shared antigens in normal sera have hampered this ELISA from becoming commercially viable. Recombinant proteins can be produced in large quantities in *Escherichia coli*; however, the major problem is purification, since high levels of antibody against *E. coli* are found in all normal human sera. Thus, either the recombinant proteins need to be purified or the sera must be adsorbed with *E. coli* before use to prevent unacceptable levels of background response. Both of these methods have potential drawbacks: (i) absorbance of anti-*E. coli* antibodies is not always consistently reproducible, leading to variations in background; and (ii) the purification of the recombinant protein to eliminate reactions to the cloning host *E. coli* can be expensive.

The second major problem has been cross-reactivity with commensal spirochetes. This cross-reactivity can be seen even with some of the specific treponemal proteins because of reactivity to the lipid component of the *T. pallidum* membrane lipoproteins (26). An ingenious method to overcome this problem is the use of nonacylated recombinant fusion proteins (20, 175). Preliminary work has begun on this method, but results of tests of clinical efficacy of the tests so developed have not been reported.

The first cloned antigen to be used in the development of a serologic test for the diagnosis of syphilis was the TmpA

protein (19, 181). This protein has since been shown to be a membrane-localized lipoprotein. It is found closely associated with another membrane protein (TmpB), and the two genes may be transcriptionally coupled since the start ATG of one overlaps the termination codon of the other (181). The gene yields a mature protein of approximately 42 kDa, which has been used in the development of an ELISA for syphilis (181). When used for an ELISA, the protein proved to be both sensitive and specific; results were almost exactly the same as those seen with the MHA-TP and FTA-ABS tests (181). The authors found a significant correlation between the antibody titer against this antigen and efficacy of treatment of patients. Therefore, they suggested that the test could be of significance in monitoring treatment for syphilis with antibiotics. The test is not available on the U.S. market, to date.

Several other recombinant proteins have since been used to develop ELISAs, most of them by the same research group. The most prominent are the TmpB and TmpC proteins. A number of other groups have reported the cloning of proteins that are secreted from the cytoplasm (148, 196). Many of these proteins have been shown to be reactive with antitreponemal serum samples by one of several different tests, most commonly, protein (Western) blotting.

The protein on which the greatest amount of work has been done is the 47-kDa protein (94), which is now thought to be a penicillin-binding protein. This protein has been shown to be immunodominant and produced in large quantities by the treponemes and does not cross-react to any extent with similar proteins from the commensal treponemes. The ELISA system that uses this protein is currently under evaluation by a number of laboratories, but the product is not yet on the U.S. market.

CONCLUSION

The diagnosis of syphilis and the related treponematoses offers challenges for the clinical laboratory not associated with most other bacterial infections. The lack of a culture system that allows for the growth of the organism from clinical specimens has necessitated the use of alternative methods for detection of the treponemes or antibody against *T. pallidum*. Many of these techniques were developed 20 or more years ago and have been refined and standardized over the years. These tests have proved to be useful but suffer from a lack of sensitivity in some stages of syphilis and a lack of specificity in some populations. With the development of molecular techniques, new and potentially more sensitive techniques may soon be available to aid in the diagnosis of syphilis. The clinical relevance of the results obtained in a test to diagnose syphilis, the simplicity with which the test can be performed, and the test's sensitivity and specificity must be considered before a new test is placed into routine use in the clinical laboratory.

ACKNOWLEDGMENT

We thank Kathy Perry for clerical support.

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