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Comparison of the fluorescent treponemal antibody test with the standard tests for syphilis

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A COMPARISON OF THE FLUORESCENT TREPONEMAL ANTIBODY TEST WITH THE STANDARD TESTS FOR SYPHILIS

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This evaluation deals primarily with the Fluorescent Treponemal Antibody Test (FTA) in comparison with the Standard Tests for Syphilis (STS). However, in order to appreciate their differences and the advantages and uses of each, a brief summary of what the STS consist of is in order.

In the course of syphilis as well as less frequently in other disease processes an antibody-like substance termed reagin appears in the affected patient's serum. It has the ability to combine with colloidal suspensions of lipids extracted from animal tissue and thereby form visible clumped masses. In addition, such combinations of reagin and lipid particles are able to fix or absorb complement. These properties of reagin are the basis for the two types of STS:

(1) flocculation tests, and

(2) complement fixation tests.[16]

The development of complement fixation test begins with Pfeiffer who in 1894 showed that serum of guinea pigs previously infected with Vibrio comma possessed bacteriolytic activity. The bacteriolytic property of this serum was due to two substances. One, termed "amboceptor," is a heat resistant substance found only in the blood of an immune animal; and it is a specific antibody formed against bacteria. The other, termed

"complement," is a heat sensitive substance; and it is present in the serum of nearly all warm-blooded animals. The "antigen" is the bacteria or material that induced the immune response. Whenever the union of these three bodies takes place, bacteriolysis results if the antigen is capable of being lysed.[5]

Subsequently, Bordet and Gengou demonstrated that hemolysis is controlled by the same conditions that govern bacteriolysis. That is, red blood cells sensitized by specific antibody are lysed or destroyed in the presence of complement. Since hemolysis is much easier to recognize than bacteriolysis, the former has served as the basis for the indicator system in the complement fixation test.[15]

With the discovery of Treponema pallidum as the causative microorganism of syphilis in 1905, the complement fixation test was soon applied to syphilis. Two workers proposed the use of the complement fixation test at about the same time: Wasserman in Berlin, and Detra in Budapest.[15]

The antigen used in Wasserman's original studies was a saline extract of various syphilitic organs. The use of non-syphilitic organs to fix complement was accomplished at a later date by Marie and Levaditi. Other important milestones include the initial attempt of

using cholesterol in tissue extract antigen, first carried out by Browning, Cruickshonk, and Mckenzie. However, Hans Sacks was the one who discovered that normal heart extract antigen to which cholesterol has been added gave comparable results to the other Wasserman antigens in use at that time, including cholesterolized alcoholic extracts of syphilitic liver.[15] This finding, of course, greatly simplified the preparation of antigen for use in the Wasserman test.

The antigen used in the present day STS have been further purified by various workers. However, since Panghorn in 1941 showed that the active component of beef heart extracts is a chemically defineable substance, termed "cardiolipin," most of the complement fixation tests contain the latter as well as lecithin and cholesterol in the proper proportion.[22]

The original Wasserman technique has been altered in other ways, but all these modifications depend on a hemolytic system to detect the presence or absence of reagin. If the latter is present, their combination with the antigen binds the complement and no hemolysis will take place when the sensitized sheep cells are added. On the other hand, if no reagin is present, complement remains unbound and is free to complete the indicator system with the resultant hemolysis taking

20
place.

The development of a practical precipitation test for syphilis came later than the complement fixation tests. Rudolph Kraus in 1897 was first to observe the phenomenon of precipitation on mixing soluble elements of bacteria with specific antiserum. Later on, Michalis carried out precipitation studies on syphilitic serums using a saline extract of syphilitic liver. However, none of the early precipitation tests were as sensitive as the Wasserman test even though the latter was not nearly as refined as the present-day Wasserman techniques.

It remained for Kahn to demonstrate that by changing the following four variables, the sensitivity of the precipitation tests could be altered:

(1) Concentration of reagents. The basic effect of concentration, whether of the antigen or the serum, is to increase the sensitivity.

(2) Physical state of the antigen. As the turbidity of the antigen suspension increases, the sensitivity of the test will increase. The most important limitation being that the antigen suspension remains opalescent in non-syphilitic serums.

(3) The quantitative relations between antigen suspension and serum dilutions. For optimal precipitation results, it is essential to mix serum with antigen suspension in a proper ratio.

(4) Agitation of serum-antigen mixture. Sensitivity increases with agitation.[15]

The current flocculation tests including the Hinton, Kahn, Kline, Mazzini and VDRL differ from one another by variations in the above four factors and in the sources and preparations of this antigen. However, they are similar in that they all represent a colloidal reaction with the end-product being macroscopically visible particles resulting from the combination of the reagin in the patient's serum and particles of lipid-coated cholesterol with or without cardiolipin.[5]

These reagin tests have been further refined with the development of the rapid screening tests for syphilis such as the Rapid Plasma Reagin Test (RPR), the RPR card test, the Plasmacrit Test, and the Unheated Serum Reagin Test (USR). In these tests, time lost in allowing for clotting of blood and inactivation of serum has been eliminated by the use of such chemicals as choline chloride which inactivates substances in unheated plasma or serum which interfere with the clump-

ing of antigen and antibody.[11]

The antigen used in the standard serodiagnosis of syphilis is unique in that it is not derived from the particular etiological organism but rather from tissue extractives that are not susceptible to infection with Treponema pallidum. So the STS is merely an empirical reaction between a nonspecific antigen and associated antibody reagin.[22]

A serological test making use of spirochetal antigen should theoretically possess a greater degree of immunological specificity than the tests using a lipid antigen.[2] For this reason, some workers prefer the cultivable Reiter strain rather than cardiolipin as their antigenic source. However, the origin of these organisms has never been resolved, and it is still unclear whether they were originally virulent pathogens that have lost their virulence or whether in the beginning they were merely indigenous saprophytes. At any rate, the Reiter strain that has been maintained in laboratories through the years has now lost nearly all similarity it might have once had with saprophytic or pathogenic spirochetes. But these organisms do have the one advantage of being easily cultivable under anaerobic conditions in such media as standard thioglycollate broth containing 10% serum; and because of the

ease by which they can be handled in the laboratory, they have been extensively studied and used.[27]

However, a more precise antigen than the Reiter strain is the causative organism itself, Treponema pallidum. Although the latter has not been cultivated, it does grow readily in the testicles of the experimentally innoculated rabbit, and extracts thereof serve as a source of supply for this antigen.[2]

It is now recognized that reagin is formed in response to a large number of acute and chronic conditions other than syphilis which can produce a "biological false positive" (BFP) test. Hence, a positive reaction only means that the test procedure detected the presence of reagin or a reacting antibody of some type and does not necessarily indicate syphilis. Because of the nonspecificity of the antigens used in STS, the substance giving the positive reaction may be due to a number of conditions. One classification for such causes for non-syphilitic reactions to STS include the following.[16]

(1) Technical False-Positive Reactions. These result from human errors, such as mistakes in the collection of specimens, the use of faulty reagents, and errors in the performance of the tests or in recording the results. Laboratory errors also occur when the specimens are contam-

inated by bacteria or have been previously hemolysed.

(2) Variations in the Normal. A few normal patients produce an excess of reagin. This has been estimated to occur in one in 3,000 to one in 5,000.

(3) Diseases Allied to Syphilis. These diseases include those caused by treponemata, such as yaws, begel, and penta. They give positive results with those serological reactions relying on specific antibodies. These are not false-positive reactions in the true sense, in that they give firm evidence of the presence of treponemal disease.

(4) Biological False-Positive Reactions. These result from the presence of non-syphilitic diseases. Some of these are acute reactions occurring during or shortly after an acute infectious process such as malaria, vaccinia, virus pneumonia, glandular fever, typhus, Weil's disease, filariasis, and active pulmonary tuberculosis. These usually disappear spontaneously after a few weeks. Others are chronic reactions in that they occur in presence of certain chronic diseases such as leprosy and disseminated lupus erythematosus.

In these cases, the STS may remain positive for many years.[16]

The exact incidence of biologically false positive reactions is difficult to estimate. However, one group in 1952 expressed the opinion that in certain population groups in the United States, especially in white persons of relatively high socioeconomic status, at least 50% of seropositive reactors discovered in mass blood testing represented biologically false positive reactions.[22] In a more recent survey involving 30,000 serums which had been evaluated with the Treponema pallidum immobilization (TPI) test, the percentage of biologic false positive reactors had increased from 54.2 to 70.7% during the decade 1953-1962.[4]

Because there is no ideal test for syphilis, a number of laboratories customarily use multiple non-treponemal tests in their investigation of those serums found to be reactive in the initial screening procedure. With this approach some have attempted to distinguish the BFP serums from true syphilitic reactors. But, unfortunately, the use of a battery of tests results in a decrease in specificity although there is a proportional increase in sensitivity. "Sensitivity" in this sense refers to the ability of a test to be reactive in the presence of syphilis, while the "speci-

ficity" of a test refers to its ability to be nonreactive in the absence of syphilis.[25]

The loss of specificity with the use of multiple tests follows from the fact that no standard test for syphilis is designed to be specific. Therefore each one produces a certain number of BFP, the latter not necessarily occurring in the same serum with all tests. Hence the resultant total of BFP reactions is the algebraic sum of BFP occurring from the individual tests. In summary, the use of multiple nontreponemal tests decreases specificity although sensitivity is proportionally increased.

This limitation of the reagin tests for syphilis has served as an impetus for the development of an antigen derived from the etiological agent of the disease for the purpose of employing it in a specific test for syphilis. Such a test is needed for the following diagnostic problems:

(1) to distinguish between biologic false positive and true positive reagin tests for syphilis;

(2) to establish a correct diagnosis in patients who have clinical evidence of syphilis, but who have nonreactive blood and spinal fluid reagin tests;

(3) to assist in the diagnosis of patients who epidemiologically should have the disease, but who have negative serological evidence.[11]

Towards this end a number of treponemal tests have been developed during the past fifteen years including the Treponema Pallidum Agglutination Test, the Treponema Pallidum Immune Adherence test, the Treponema Pallidum Complement Fixation test, the Reiter Complement Fixation test, the Treponemal Wasserman test, the Treponema Pallidum Immobilization test (TPI), and the Fluorescent Treponemal Antibody test (FTA).[11] Of these, the TPI and FTA are superior to the others in terms of specificity, and they will be considered in more detail.

The era of treponemal testing began in 1949 with the development of the Treponema Pallidum Immobilization Test (TPI) by Nelson and Mayer.[5] This test and the other treponemal tests detect specific antibodies against the etiological agent of syphilis. However, in the TPI test the treponemal antibodies are assumed to be responsible for the immobilization of treponema by syphilitic serum in the presence of complement. The test itself involves the use of Treponema pallidum, Nichols strain, extracted from rabbit testicles and placed in survival media. The serum to be tested and the complement are then added, and this is allowed to

incubate. The results are based on the percentage of treponema immobilized.[11]

Although in theory this is a very simple test, complex technical problems have limited the practicality of the test. Difficulties inherent in obtaining satisfactory treponemal suspensions from experimentally infected rabbits, maintenance of motile organisms during the test period, inability to obtain valid results with serums containing many of the commonly used antibiotics, as well as its high cost have restricted the general use of this procedure.[8] Only a few laboratories have the facilities or the personnel to perform the TPI test as a diagnostic service. Nevertheless, because of its great specificity, all subsequent treponemal tests have been compared to it.[11]

But from a practical standpoint there still remains a need for a less intricate but still specific treponemal test which can be performed in the average laboratory with a minimum of ease and materials. It is hoped that the FTA test will fulfill this need, and hence enable many serology laboratories to perform this test rather than transmitting specimens to one of the few laboratories established to perform the TPI.

Fluorescent antibody methods were first introduced by Coon and his associates in 1942 when he published a

procedure for antibody-fluorescein conjugation and a description of fluorescent reactions obtained by means of ultraviolet. The application of this technique was originally in the study of viruses and in the differentiation of protozoa and bacteria. Deacon in 1956 was the first to use fluorescent antibody techniques in the serologic diagnosis of syphilis.[12]

Immunofluorescent staining depends on certain dyestuffs known as fluorochromes. These compounds can be observed visually from the radiation which is emitted upon stimulation by the light of a shorter wavelength range. The nature of fluorescent compounds is such that upon absorption of a certain quantum of radiation, it attains an excited state with a different electronic distribution. This extra energy is quickly lost to surrounding molecules, and the excited fluorescent compound returns to its original state by emitting radiation. Emission of this type is termed fluorescence and is almost always of longer wavelength in the visible range, than the exciting radiation which is in the ultraviolet range.[23]

The selection of fluorochromes has been almost entirely empirical inasmuch as very few general rules or guides can be made about fluorochromes as a group. However, the one important structural characteristic that

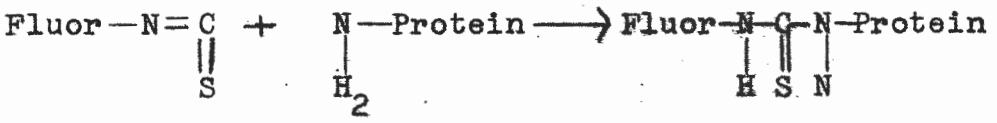
fluorochromes have in common is that they possess a conjugated system of bonds and have sufficiently stable excited states to permit fluorescent emission. In contrast, most other molecules which absorb radiation in the visible or near ultraviolet range are degraded from the excited state by collision with neighboring molecules before fluorescent emission can occur.[23]

The two fluorochromes that have been most widely used are fluorescein and rhodamine B. Fluorescein has a yellowish green fluorescence with a maximum at about 500 mu, and rhodamine B has a reddish-orange fluorescence with a maximum at about 640 mu. In most immunofluorescent techniques fluorescein has been preferred because the eye is more sensitive to the apple green of fluorescein than to the reddish range of rhodamine B, and secondly, because red autofluorescence is more common in nature than green autofluorescence.[1]

These fluorochromes are used to prepare conjugates or fluorochromes labeled proteins. In this process the fluorescent dye is coupled or labeled to the protein, usually an antibody, by means of a chemical attachment or bonding. Fluorescein is a very suitable fluorescent label inasmuch as this chemical union with the protein forms a stable unit which does not dissociate under experimental conditions. The efficiency of the fluor-

escence remains high, and no significant alteration in the antigenic character of the protein takes place.[23]

In the early studies with fluorescent protein tracers, an isocyanate derivative of fluorescein was commonly used in labeling the protein. The latter was prepared by first reacting the amino group of the fluor with phosgene. The isocyanate then reacts with free amino groups of the protein to form a carbamido linkage. Later, Riggs introduced a procedure for conjugating isothiocyanate derivatives with protein as follows:[1]



The isothiocyanate form offers the advantage of being prepared from thiophosgene rather than the more toxic phosgene gas.[26] In addition, the isothiocyanate is superior to the isocyanate in terms of degree of fluorescence as well as stability.[18] At present, fluorescein isothiocyanate is much more widely used.

All immunofluorescent staining methods used in syphilitic serology are similar to the point that they conjugate antibody containing serum globulin with a fluorescent dye, allow the labeled antibodies to react with specific antigen and observe the reaction product under the fluorescent microscope, that is, an ultraviolet light source.[1]

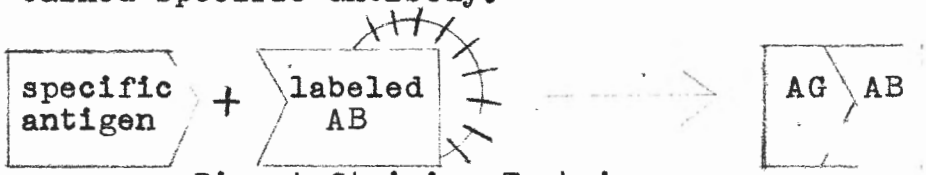
The serum globulins for conjugation may be prepared by fractionating serum with 50% saturated ammonium sulfate or 23% sodium sulfate, or by the cold alcohol method of Cohn.[18] Various methods have been devised for the coupling of the fluorescein dye to the globulin, including impregnating filter paper with fluorescein isothiocyanate and then simply adding the serum globulins and shaking; or by adding acetone, dioxone, and carbonate buffer to the serum globulin before the dye is mixed.[1] However, Marshall and his associates have shown that isothiocyanate powder poured directly into a cooled, dilute, buffered antiserum yielded a product which was as good as, if not superior, to that prepared by any other method.[18]

Once the fluorescent labeled gamma globulin has been prepared, it may be used in the following types of reactions:

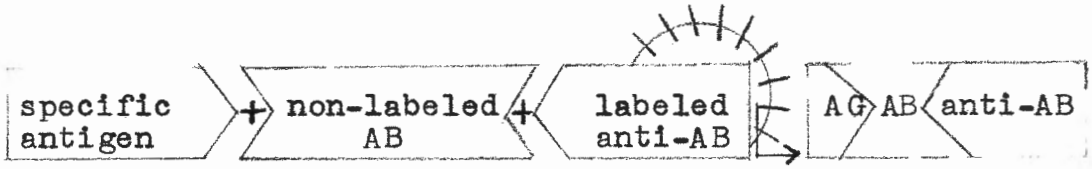
(1) The Direct Reaction. The direct reaction is the coupling of a specific labeled antibody to a microorganism to prove its identity. The antiserum is prepared in an appropriate animal with the resultant gamma globulin being conjugated with fluorescein.[6]

(2) The Indirect Reaction. The indirect reaction is obtained when a known microorganism is

treated with an unknown, unlabeled serum. The antibody coupling is then demonstrated by exposing the microorganism to fluorescein-labeled anti-globulin.[12] The FTA test is an example of the indirect reaction and is used to detect human treponemal antibody. The pathogen (Nichols strain of Treponema pallidum) is a known quantity. This quantity is first treated with test serum (human treponemal antibody in the case of syphilis.) Upon the addition of fluorescein-labeled goat antihuman globulin, the resultant immune complex will fluoresce if the test serum contained specific antibody.[6]



Direct Staining Technique



Indirect Staining Technique

Schematic Representation of Direct and Indirect Staining Technique[1]

(3) The Blocking Reaction. With the blocking technique the coupling of a specific labeled anti-

serum to a microorganism is prevented or blocked by a preceding exposure of the microorganism to the nonspecific antibody in unlabeled serum. Thus, common antigen receptor sites are inactivated or bound by nonspecific antibody, and the later attachment of specific, labeled globulin is prevented.[12] This technique has recently been applied in a modification of the FTA test. With this method the antigen (T. pallidum) is first exposed to rabbit Reiter treponeme antibody for the purpose of binding or inactivating T. pallidum common antigen receptor sites prior to contact with the patient's serum.[13]

(4) The Absorption Reaction. The FTA absorptive procedure, a new variation of the FTA test, is an attempt to remove the nonspecific antibodies involved in syphilitic serum. In this procedure a sonicate of the Reiter treponeme antigen is added to the test serum to absorb out or eliminate the group nonspecific antibody. The test serums are then exposed to the specific antigen (T. pallidum) in essentially the same manner as in the standard FTA test.[14]

Materials

The serum bank at the Nebraska University Hospital Laboratory provided the test specimens. Specimens evaluated were from patients with reactive or weakly reactive serologic tests for syphilis (STS). In most cases there were no histories or clinical manifestations directly related to the disease.

Before testing, each serum was heated in a 56°C. water bath for 30 minutes and then allowed to cool to room temperature before a 1:200 dilution was prepared.

Treponema pallidum, Nichols strain, extracted from rabbit testicular tissue served as antigen. This suspension contained 50-75 organisms per high power field.

Fluorescent goat anti-human globulin was used throughout the test as the indicator system. Each lot of conjugate was titered by the manufacturer and was prepared with buffer containing 2% Tween prior to use in the test.

Phosphate buffered saline, pH 7.2, was used in preparing the serum dilutions and in rinsing slides in the staining procedure.

A Leitz fluorescent microscope which allowed visualization of treponemes by regular darkfield before applying ultraviolet light was used throughout this study.

The light source was a 200 amp Osram high pressure mercury vapor lamp. Optimal fluorescence was obtained with a BG-12 4mm excitor filter which removed the visible spectrum of light, and a UG barrier filter which excluded any residual wave lengths under 500Å.

Procedure

The experimental procedure was conducted in the following manner in accordance with the recommendations of the Communicable Disease Center as outlined in the manual, Laboratory Procedure for Modern Syphilis Serology, 1961 edition.

1. Slides were placed in 75% ethanol overnight to remove any grease and then wiped dry with gauze. On the grease-free slides a circle approximately one cm. in diameter was cut with a diamond stylus.
2. Using a disposable pipet, 0.01 ml. of antigen was placed in the circle, spread with a small, footed glass rod and allowed to air dry.
3. Slides were fixed in a precooled (-78°C.) acetone-dry ice bath for two to three minutes after which the slides were drained and air dried.
4. 0.03 ml. of the 1:200 dilution of inactivated patient's serum was placed over each smear and spread to cover the entire area enclosed by the etched circle.

5. To prevent evaporation of serum dilutions, the slides were covered with petri dishes which contained moistened filter paper. The slides were then rotated at 100 rpm for 30 minutes in an incubator set at 37°C.

6. The slides were then rinsed in buffered saline and soaked in two changes of buffered saline for a total of 10 minutes and then blotted gently with filter paper.

7. 0.03 ml. of optimally diluted fluorescein conjugate was spread over each slide.

8. Steps (5) and (6) were repeated.

9. Slides were mounted with glycerol and either read immediately or placed in a refrigerator at 6-10°C. for examination at a more convenient time.

A number of variations from the above procedure have been tried in the past. Some workers eliminate the rotation of the slides altogether; others prefer to use room temperature instead of 37°C. during the incubation period. However, both the use of an incubator at 37°C. and the rotation of the slides during this step seem to increase the sensitivity of the test to a slight degree, as has been shown in studies in which these factors were the only variables.[24]

The most critical factor is the dilution of the patient's serum. In the past, numerous studies have been

conducted with 1:5 or 1:100 serum dilutions. Because these serum concentrations resulted in an oversensitive response, they have for the most part been discarded for the more specific and less sensitive 1:200 dilution.[24]

Interpretation of Test Results

Serums yielding maximal fluorescence (4+) to moderate fluorescence (2+) were designated as reactive; serums yielding minimal fluorescence (1+) or rendering the organisms barely visible (± or -) were designated as non-reactive.

Results and Discussion

The purpose of the following survey was to investigate the value of the FTA test in the serological diagnosis of syphilis. The results from the following procedures were compared on 205 serums:

1) Mazzini Flocculation test;
2) VDRL Flocculation test;
3) Kolmer Complement Fixation test (KCF);
4) Reiter Protein Complement Fixation test (RPCF);
5) Fluorescent Treponemal Antibody test.

TABLE I

A Comparison of 205 Positive Mazzini Serums to the VDRL, KCF, RPCF, and FTA Tests

Test	Number Positive	Number Negative	%Agreement with Mazzini
Mazzini	205	--	--
VDRL	167	38	82%
KCF	117	89	57%
RPCF	98	107	48%
FTA	95	110	46%

From the preceding table it can be seen that there is a progressive decrease in sensitivity from the Mazzini to the FTA. However, the loss in sensitivity is accompanied by an increase in specificity.

This survey demonstrates the value of using a battery of tests for syphilis inasmuch as it reveals the many false positive reactions resulting from a simple screening technique as is the Mazzini. Hence, the use of the more specific tests such as the Reiter and FTA to confirm a positive Mazzini gives one a better basis for making a serologic diagnosis of syphilis.

Furthermore, one cannot be assured that individual positive tests are not due to technical error. Duplication of procedures is desirable for this reason alone, but it is also a safeguard against the inherent error of the different tests and the technical variation which might occur in any laboratory.[6]

From Table I it might appear that the FTA test adds

little to what information can be obtained from the RPCF method in that they are nearly equal in terms of percentage agreement with the Mazzini. However Table II shows that the percentage agreement between the RPCF and the FTA is somewhat less than might be expected from the figures in Table I. In 22% of the serums there were diverse findings, indicating a difference in reactivity between the two procedures.

TABLE II

Comparison of FTA and RPCF Test Results	
Number of Serums Evaluated	286
Reactive in Both Tests	76 (26.6%)
Nonreactive in Both Tests	148 (51.4%)
FTA-Reactive RPCF-Nonreactive	32 (11.5%)
FTA-Nonreactive RPCF-Reactive	30 (10.5%)

A similar comparison between the FTA test and RPCF has been carried out by Montgomery on a mixed population group including normals, and patients with syphilis both before and after treatment.[21]

TABLE III

A Comparison of FTA and RPCF Test Results

Number of Serums Evaluated	223
Reactive in Both Groups	104 (46.6%)
Nonreactive in Both Groups	84 (37.7%)
FTA-Reactive RPCF-Nonreactive	29 (13.0%)
FTA-Nonreactive RPCF-Reactive	6 (2.7%)

In 32 cases (11.5%) in our series and in 29 cases (13.0%) of the Montgomery series the FTA was positive while the RPCF was negative. This discrepancy between the two test procedures is probably based on a lack of sensitivity of the RPCF or a technical error.

Originally it was felt that the RPCF was a relatively sensitive test and useful as an exclusion procedure. However, in one recent series of 125 patients, 94 serums were reactive to the TPI while only 54 were positive with respect to the RPCF (see Figure A). Only 5 of the RPCF reactive patients were TPI negative. This finding would indicate that the Reiter Protein Complement Fixation Test currently available is significant only when reactive.[28]

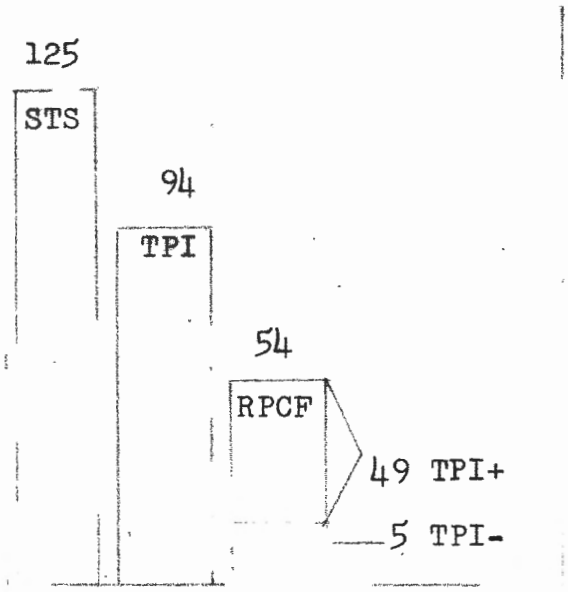


Figure A. Comparative Test Results

Furthermore, the Reiter test has had a low correlation with the TPI test in the group of patients in which serologic confirmation is most needed; that is, in cases characterized by no clinical or historical evidence of syphilis and a reactive reagin test. In another study of syphilis in the male Negro, the RPCF was again much less reactive than the TPI; the difference was even greater in those patients having inadequate treatment than in those having had no treatment. On the basis of these and other studies, it was found that in syphilis of long standing, whether treated or untreated, the Reiter test will be nonreactive in no more than 50% of the cases.[11]

In our series the patients were not categorized

with respect to the various stages of syphilis. However, certainly a significant number of the positive syphilitic serums represent either late or latent syphilis, treated or non-treated. Because the Reiter is not a sensitive test in the later phases of syphilis, it can be assumed that the disagreement in at least some of those 32 cases where the FTA was positive and the Reiter negative was on the basis of the inherent lack of sensitivity in the RPCF test.

Laboratory error, no doubt, also contributed to the lack of correlation between the FTA test and RPCF. The complexities associated with the antigen and the labeled globulin necessitates rigid laboratory control with the introduction of each new lot of reagent into the test procedure. Other problems include adherence of sufficient numbers of T. pallidum to the slide, nonspecific fluorescence, and the differentiation of T. pallidum from detached fluorescent coils in the tails of spermatozoa.[3]

Perhaps the greatest source of technical error occurs in the reading of the results. The distinction between a 2+ (reactive) and a 1+ (nonreactive) is an important one; but unfortunately this is often a difficult judgment as it is an entirely subjective impression.

Moreover, the use of an unfamiliar instrument, that is, the ultraviolet microscope, tends to complicate the procedure further.[19] However, like other test methods with the employment of properly standardized reagents and controls, results usually become quite satisfactory with experience.

Another pitfall associated with the FTA is the occurrence of BFP. This has been well documented in those cases of rheumatoid arthritis and other macroglobulinemias with a positive FTA and negative TPI. Most of the serums falling into this classification have betonite titers greater than 512.

Since the most striking serological feature of rheumatoid arthritis is the appearance of the so-called " rheumatoid factor," which is a high molecular weight component of gamma globulin with a sediment coefficient of 22S, this macroglobulin and conceivably other macroglobulins have been implicated in the pre-disposition of patients with rheumatoid arthritis to false FTA reactions.

One hypothesis suggests that BFP occur with the FTA as a result of sensitization of the spirochete in rabbit testes with 7S gamma globulin being bound to the surface of the organism. The majority of 19S or greater sediment coefficients are noted for their ability to

react with 7S gamma globulin. Therefore, the high molecular weight component in serums from patients with macroglobulinemias such as rheumatoid arthritis could combine nonspecifically with the specifically bound 7S gamma globulin. The fluorescein-labeled antihuman gamma globulin would then react with the macroglobulins that were bridged to the spirochetes by rabbit antibody and thereby produce a false FTA reaction.

Others feel that sensitization of the spirochetes does not occur in the rabbit, but rather macroglobulins are absorbed directly onto the spirochetes and in turn react with the fluorescein-conjugated antihuman gamma globulin.

In either case, serums containing macroglobulins are rare and do not constitute a problem that seriously limits the usefulness of the FTA as a diagnostic procedure. Furthermore, valid FTA results can be obtained even with macroglobulinemia serums simply by centrifuging these specimens for one hour at 100,000 rcf and testing the supernatant.[10]

Sensitization of the antigen can result in a false negative as well as a BFP. In these cases <u>in vivo</u> sensitization of the treponemes with rabbit antibody occurs. Syphilitic antibody produced by the experi-

mentally infected animal combines with the antigenic constituent on the surface of the treponemes. Since human antibody is prevented access to the antigen, a false negative is the result in the presence of a positive serum. A negative reaction with a known positive control would be indicative of *in vivo* sensitization with rabbit antibody.[9]

Other false negatives can occur with extended use of the same mercury arc lamp, for its light output gradually diminishes with age, this reduction being sufficient to influence test results. However, diminution of ultraviolet light intensity can be detected by noting the concomitant reduction of reaction with control serum containing a threshold amount of antibody.[9]

From Table II it is seen that in 30 cases (10.5%) from our study the Reiter was reactive while the FTA was nonreactive. Most of these cases probably represent a BFP, a laboratory error, or a falsely negative FTA.

The diagnosis of a BFP obviously depends on what criteria are used in the diagnosis of syphilis. For this purpose comprehensive clinical histories provide the best data. But, unfortunately, they are often difficult to obtain, and their validity is dependent upon the skills and thoroughness of the examining physician. In view of these difficulties the TPI test,

which has been extensively evaluated with carefully documented serums and found to exhibit an exceptionally high degree of specificity, has become a standard by which the distinction between a BFP and true syphilitic serum is made.[9] Hence, the laboratory diagnosis of a BFP is made on the basis of a negative TPI.

In those 30 cases of a reactive Reiter in the presence of a nonreactive FTA, a TPI would have been very helpful in the identification of the BFP. Unfortunately, our laboratory is not equipped for the TPI. However, in other laboratories parallel studies between the FTA and TPI have been carried out on problem serum. The results of these surveys are in Table IV.[17, 19, 29]

These studies revealed a high degree of correlation (88%) between the FTA and TPI. Because of the significant degree of agreement between the FTA and TPI, it is reasonable to state that a negative FTA in association with a positive RPCF is indicative of a BFP in most cases if the possibility of a laboratory error has been ruled out.

However, the FTA is not a completely satisfactory substitute for the TPI inasmuch as 8% of the cases were nonreactive with respect to the FTA but reactive to the TPI. In other words, in approximately one out of ten cases of problem serum, if one relied solely on the FTA

TABLE IV

Comparison of the TPI and FTA

Worker	Number of Serums	Reactive in Both Tests	Nonreactive in Both Tests	FTA-Reactive TPI-Nonreactive	FTA-Nonreactive TPI-Reactive	% Agreement
Leibovitz	261	125 (48%)	107 (41%)	11 (4%)	18 (17%)	89%
Wilkinson	144	41 (29%)	84 (58%)	2 (1%)	17 (12%)	87%
Miller	104	29 (28%)	65 (62%)	4 (4%)	6 (6%)	90%
Total	509	195 (38%)	256 (50%)	17 (3%)	41 (8%)	88%

and not both of these procedures, he would have the mistaken impression of a nonsyphilitic serum--at least on the basis of the laboratory evidence.

One might wonder if a test which is designed for specificity, as the FTA is, would lack sensitivity since in the STS there is an inverse relationship between specificity and sensitivity--the greater the specificity the less the sensitivity. However, this is not the case in the treponemal tests for syphilis as is apparent from recent surveys and an understanding of the underlying immunology involved.

Treponemal antigens may be separated into two major components: group antigen and specific antigen. This is true whether the microorganism is a pathogen or a saprophyte. Furthermore, crossreaction occurs between the various treponemes as is seen from the upper block (Block A) in Table V. However, Block B shows that antiserums absorbed with the Reiter treponeme lose their common or nonspecific component, revealing only the specific fraction peculiar to each treponeme.

The RPCF test then detects only the common or group antibody although the FTA may also indicate the presence of this antibody because of crossreaction. This is clear from Table VI where a clear-cut antibody difference is noted between the normal and syphilitic

TABLE V

Differentiation of Treponemes by Means of Absorbed Fluorescein-labeled Antiserums[7]

Fluorescein-labeled Treponemal Antiserums	Antigens			
	T. pallidum	Reiter treponeme	T. zuelzerae	T. microdentium
A. Unabsorbed Serums:				
Treponema pallidum	R	R	R	N
Reiter treponeme	R	R	R	N
Treponema zuelzerae	R	R	R	N
Treponema microdentium	R	R	R	R
B. Absorbed with Reiter treponeme:				
Treponema pallidum	R	N	N	N
Reiter treponeme	N	N	N	N
Treponema zuelzerae	N	N	R	N
Treponema microdentium	N	N	N	R

serum after the absorption with the Reiter.[7]

TABLE VI

Fluorescent Treponemal Antibody Quantitations Before and After Reiter Treponeme Absorption

Serums	Unabsorbed FTA dilutions	Absorbed FTA dilutions
Normal	100	5
Primary syphilis	100	50

From these findings it is apparent that the greater specificity of the FTA rests with its non-Reiter component. In other words, since the FTA is carried out with the T. pallidum species, it would appear that the major cause of nonspecificity in the FTA is the occurrence of common or group antigens shared by pathogenic, saprophytic, and Reiter treponeme.[14]

The FTA test can be made to operate without interference from group antibodies by means of Reiter treponeme absorption. This type of manipulation has resulted in a new modification of the FTA procedure known as the FTA-ABS (Fluorescent Treponemal Antibody-Absorption Procedure).[13] At least one series has already been carried out showing the FTA-ABS to have specificity equal to, if not greater than, the FTA and TPI (see Table VII).

TABLE VII

A Comparison of the FTA, FTA-ABS, and TPI

FTA		FTA-ABS		TPI	
R	NR	R	NR	R	NR
77	123	111	89	99	92

Summary

In order to appreciate the uses of the FTA test, one must have an understanding of what information is provided by the STS.

Both the flocculation tests and complement fixation tests merely detect the presence or absence of reagin. In the case of the complement fixation tests if reagin is absent, then complement is available to combine with the sensitized sheep cells and hemolysis results. On the other hand, if reagin is present, its union with the antigen fixes the available complement and no hemolysis occurs. In the case of the flocculation tests, reagin combines with particles of cholesterol-coated cardiolipin, the result being macroscopically visible particles. The sensitivity of this test will depend upon the concentration of reagents, the physical state of the antigen, the quantitative relations between antigen suspension and serum dilutions as well as the degree of agitation of the serum-antigen mixture.

Because the antigens used in the STS are non-specific in the immunological sense, a positive reaction does not necessarily indicate syphilis. In fact, a host of acute and chronic disease processes can give a BFP. In some series over one-half of the positive reactors to the STS proved to be nonsyphilitic serum on further evaluation with the treponemal tests for syphilis. To distinguish the BFP from the true syphilitic serum, a simple and specific test is needed. The FTA is designed to fulfill this need.

The test itself involves the use of fluorescein isothiocyanate which is a fluorescent dye in that it has the ability to absorb light energy of one wave length and emit light of another wave length. Anti-human globulin labeled with this dye retains its immunological specificity. This specificity allows the conjugate to combine with its homologous antigen, human globulin, and the resultant fluorescence is viewed through a fluorescent microscope. The FTA procedure is then not dissimilar from the "sandwich" technique employed by the indirect Coombs. However, in this case a Treponema pallidum species serves as antigen, the test serum or reagin as the non-labeled antibody, and goat antihuman globulin as the labeled antibody.

In this study the FTA was compared to two standard

flocculation tests, the Mazzini and VDRL; and two complement fixation tests, the KCF and RPCF. The survey consisted of 205 serums which were positive with respect to the Mazzini in the original screening procedure. Of these serums 82% were positive with the VDRL, 57% with the KCF, 48% with the RPCF, and 46% with the FTA.

Although the RPCF and FTA are nearly equal in terms of percentage agreement with the Mazzini, in 22% of the serums there were diverse findings between the two. In nearly one-half of these cases the FTA was reactive while the RPCF was nonreactive. The divergence of results between these two tests could be on the basis of either laboratory error or lack of sensitivity with the Reiter inasmuch as the latter has been shown to be nonreactive in at least 50% of cases of latent or late syphilis. Another much less likely explanation for the discrepancy in results is those rare instances of rheumatoid arthritis and other macroglobulinemias which have led to BFP with the FTA.

In those serums in which the FTA was nonreactive while the RPCF was reactive, one must consider the possibility of a laboratory error, a BFP, or a falsely negative FTA. False negatives with the FTA are not

uncommon. In several series comparing the FTA and TPI about 8% of the serums were nonreactive to the FTA but reactive to the TPI. However, for the most part, the specificity of the FTA procedure is restricted only by the common antibody component in the patient's serum; and this limitation can be eliminated by the new FTA-Absorption procedure.

Conclusions

1. In a parallel study of 205 serums which were positive with respect to the Mazzini, 82% were positive with the VDRL, 57% with the KCF, 48% with the RPCF and 46% with the FTA.
2. In 11.5% of the cases the FTA was reactive while the RPCF was nonreactive. In 10.5% of the cases the FTA was nonreactive while the RPCF was reactive.
3. Other studies have shown the Reiter to be a relatively insensitive test in the late and latent phases of syphilis.
4. The degree to which laboratory error contributed to the lack of correlation between the FTA and RPCF is not known exactly, but no doubt was significant.
5. BFP have been known to occur in cases of rheumatoid arthritis and other macroglobulinemias.

6. False negatives can result from *in vivo* sensitization of the antigen with rabbit antibody.

7. The degree of correlation between the TPI and FTA in several studies has consistently been in the range of 88%.

8. The specificity of the FTA test procedure is limited only by the common antibody component of the test serum.

9. The FTA-Absorption procedure may offer a greater degree of specificity than the FTA or TPI by eliminating the nonspecific or group antibodies.

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