EVALUATION OF THE REITER PROTEIN COMPLEMENT-FIXATION (RPCF) TEST FOR SYPHILIS*

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IN recent years many new serologic tests have been developed for the diagnosis of syphilis. Some of these tests are so complex as to be restricted to research institutions or large public health laboratories. The Reiter protein complement-fixation (RPCF) test, however, can be readily performed in a small hospital laboratory and, according to most published reports,[1-3] the results compare favorably with those of the more expensive and difficult *Treponema pallidum* immobilization (TPI) test for syphilis. For these reasons, it seemed desirable to evaluate the RPCF test along with the cardiolipin procedures that have been performed as standard procedures in our serology laboratory[4] (Kolmer complement-fixation test since 1921, and Kahn test since 1925).

Historical Background

The evolution of the various serologic tests for syphilis had a rather slow beginning, but has been progressing at great speed during the last decade. Schaudinn and Hoffmann[5] described the *Treponema pallidum* as the etiologic agent of syphilis in 1905, and in the following year a serologic test for syphilis was reported by Wassermann, Neisser, and Bruck,[6] who, using a complement-fixation procedure, employed saline extracts of organs containing many treponemata as antigens. In 1907, serviceable antigens were prepared from normal tissues, and by 1911 several antigens had been derived from alcoholic extracts of beef heart muscle plus cholesterol. The quality of lipoidal antigens has been improved by Pangborn's[7] isolation of cardiolipin (a phospholipid from beef heart), the development of methods for the purification of lecithin from beef heart and egg yolk, and the use of synthetic lecithin.

A great departure from the lipoidal antigens was the use of the living, virulent, Nichols strain of *Treponema pallidum* in the TPI test developed by Nelson and Mayer[8] and reported in 1949. Since that time, there has been a spate of diagnostic procedures employing various antigens derived from intact or chemically fractionated virulent (Nichols) or avirulent (Reiter) strains of *Treponema pallidum*. Garson[9] classified the various tests on the basis of derivation of their antigens. We have amplified his outline as follows.

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I. Those tests using whole-body virulent *Treponema pallidum* (Nichols strain) as antigen
   A. Tests using viable organisms from rabbit syphilomas
      (1) *Treponema pallidum* Immobilization (TPI): positive test consists in the demonstration under the dark-field microscope of immobilization of motile treponemata by syphilitic serum.
      (2) *Treponema pallidum* Methylene Blue (TPMB): performed as the TPI test except that the motile organisms take up the methylene blue dye and are more easily seen.
   B. Tests using, or usually using, nonviable organisms
      (1) *Treponema pallidum* Agglutination (TPA): poorly reproducible; detects reagin as well as treponemal antibody.
      (2) *Treponema pallidum* Immune Adherence (TPIA): in presence of syphilitic antibody and complement, killed organisms adhere to human erythrocytes and are absent from the supernatant following centrifugation.
      (3) Whole-Body *Treponema pallidum* Complement Fixation (WBTPCF): uses TPIA antigen in Kolmer-type complement fixation.
      (4) Fluorescent Treponemal Antibody (FTA): syphilitic antibody attaches to dried treponeme suspension, which in turn binds fluorescein-tagged human antiglobulin.

II. Those tests using a chemical fraction derived from whole-body virulent *Treponema pallidum* (Nichols strain) as antigen
   A. *Treponema pallidum* Complement Fixation (TPCF): employs aqueous desoxycholate extract of organisms; modification[10,11] as "tpcf 50" test increases speed and decreases cost of test.
   B. Treponemal Wassermann Reaction (TWR): antigen derived from mechanically disintegrated organisms and used in complement-fixation procedure.
   C. *Treponema pallidum* Cryolysis Protein Reaction (TPCPR): antigen derived by cycles of freezing and thawing.

III. Those tests using a chemical fraction derived from whole-body Reiter treponeme as antigen
   A. Reiter Protein Complement Fixation (RPCF): antigen prepared by ultrasonic or cryolytic disruption of organisms, precipitation with ammonium sulfate, followed by dialysis.
   B. Kolmer Test with Reiter Protein Antigen: as above.

The most recent advance in the serodiagnosis of syphilis is the rapid plasma reagin (RPR) test that employs unheated plasma[12] or serum,[13] and affords test results within 10 minutes from the time the blood specimen is drawn from the patient.

Most of the tests outlined above cannot be performed in routine serology

laboratories because of the complexity of the procedures, the danger of handling virulent organisms, the great amount of time and expense involved, and the difficulty in obtaining many of the antigens. None of these problems apply to the performance of the RPCF test.

The Reiter strain of *Treponema pallidum* was isolated in 1922 by Wassermann and Ficker[14] and, according to Sequeira,[15] subsequently studied by Reiter. This strain is distinctive in that it is avirulent, it can be cultured on laboratory media (Brewer's thioglycollate broth), and furthermore it can survive long periods without subculture. Antigens comprised of suspensions of intact organisms have been used in Germany since 1939, but were unsatisfactory because a lipid component in the treponemal antigen resulted in many nonspecific cross reactions with the standard Wassermann tests employing lipoidal antigens. In 1953, D'Alessandro and Dardanoni[16] isolated from the Reiter treponeme a protein, a carbohydrate that is relatively unimportant, and two lipid fractions, one of which is responsible for the nonspecific Wassermann cross reactions. The thermolabile protein antigen is prepared by the disruption of the treponemes by cryolysis or by ultrasonic waves and subsequent precipitation with ammonium sulfate, followed by dialysis. This protein antigen of the Reiter treponeme has been shown[17] to be immunologically identical with a protein fraction similarly derived from the virulent Nichols strain of *Treponema pallidum.* In addition to this common antigen, the virulent strain is also believed[18] to have a specific component that is lacking in the Reiter organism.

Material and Methods

In order to evaluate the RPCF test in parallel with the cardiolipin procedures, we conducted a comparative study in the following manner. Beginning in October, 1959, all serums received in the serology laboratory were tested by the qualitative Kolmer, Kahn, and RPCF tests. Each serum that showed a four-plus reaction was retested quantitatively with the antigen or antigens with which it reacted, in five-tube serial dilutions in the case of the RPCF and Kolmer tests, and in eight-tube serial dilutions for the Kahn test.

The Reiter protein complement-fixation test is performed according to standard complement-fixation technics, employing one-fifth quantities of reagents and using the Reiter protein antigen* according to the procedure outlined by the Venereal Disease Experimental Laboratory.[1] The Kolmer complement-fixation test is performed with Kolmer's cardiolipin antigen.† The three-tube Kahn test followed the accepted Kahn technic, using the standard Kahn antigen‡.

Results

During the four and one-half month period encompassed by this study, 10,292

**Commercially prepared by the Sylvana Chemical Company; and Organon, Inc.*

†Commercially prepared by the Sylvana Chemical Company.

‡Prepared in Doctor Kahn's laboratory at the University of Michigan.

serums were tested by all three procedures in the manner outlined. Of this group, 220 specimens reacted positively to at least one of the tests *(Table 1)*. The patients categorized as definitely having syphilis had well-documented clinical histories of this disease. The patients who had histories strongly suggestive of syphilis were

Table 1.—*Analysis of 220 specimens of serum having positive serologic reactions*

Tests	Category, number of patients				
	Syphilis		BFP reactors	Incomplete clinical data	Total
	Definite	Probable			
RPCF	3	13	8	27	51
RPCF, Kolmer, and Kahn	32	5	2	8	47
Kolmer	4	3	13	25	45
Kolmer and Kahn	12	2	9	9	32
Kolmer and RPCF	13	3	1	14	31
Kahn	0	1	7	5	13
Kahn and RPCF	0	0	0	1	1
Total	64	27	40	89	220

referred to as probably syphilitic. The patients classified as biologic false-positive (BFP) reactors had no clinical evidence of syphilis or history suggestive of the disease. Eighty-nine patients had no clinical evaluation of their syphilitic status at the Cleveland Clinic, either because of their return to their referring physicians, or the imperative nature of the underlying disease. These serums were not included in the final clinicoserologic evaluation of the tests for syphilis which we studied. The serums of 131 patients were retained for this evaluation. The 220 positive specimens reacted with the several serologic tests in the distribution outlined in *Table 1*. One-half of all patients who were classified as definitely having syphilis, reacted positively to all three of the serologic tests. Among the false-positive reactors the greatest number of reactions, 13, occurred with the Kolmer test; however, eight occurred with the RPCF test, and two patients reacted to all three of the serologic tests. A curious finding is the occurrence of only one positive reaction, among all clinical categories, to the combination of Kahn and RPCF tests. More serums reacted with the Kolmer antigen than with the Kahn or RPCF antigens. This high degree of sensitivity has long been known, and accounts for the many biologic false-positive reactions given by the Kolmer test.

Our study was conducted in a manner similar to that of Foster, Nicol, and Stone,[19] of St. Thomas' Hospital, London, and our two series are compared in *Table 2*. Seven times as many patients examined at the Cleveland Clinic had negative RPCF tests as those examined at St. Thomas' Hospital. The following hypotheses are suggested to explain this difference. The particular group of

Table 2.—*Comparison of patterns of reaction to standard serologic tests for syphilis and to the Reiter protein complement-fixation (RPCF) test*

Test combination	Series, per cent	
	Cleveland Clinic (220 cases)	St. Thomas' Hospital[18] (288 cases)
Positive STS* and positive RPCF	35.8	61.8
Positive STS* and negative RPCF	41.0	4.5
Negative STS† and positive RPCF	23.2	33.7

*Positive STS refers to either Kolmer or Kahn tests.

†Negative STS refers to both Kolmer and Kahn tests.

patients reported by the British authors might be comprised of more syphilitic persons. This possibility is further suggested by the greater number of these patients who had positive reactions to both the standard and Reiter tests for syphilis. In our experience, this pattern of reactivity to both the lipoidal and treponemal tests was most often encountered among patients who were considered clinically to be definitely syphilitic *(Table 3)*. Another suggestion to explain

Table 3.—*Clinicoserologic evaluation of 131 patients*

Test combination	Clinical category, number of patients		
	Definitely syphilitic	Probably syphilitic	Probably BFP
Positive STS* and positive RPCF	45	8	3
Positive STS* and negative RPCF	16	6	29
Negative STS† and positive RPCF	3	13	8
Total	64	27	40

*Positive STS refers to either Kolmer or Kahn tests.

†Negative STS refers to both Kolmer and Kahn tests.

the lesser numbers of RPCF reactors among the Cleveland Clinic patients could be that the widespread use of antibiotics by physicians in this country might have altered individuals' reactivity to the reagin and treponemal antigen tests.

Table 3 summarizes the clinicoserologic evaluation of the 131 patients in our series for whom there were adequate clinical data. It is apparent that most cases of syphilis were detected by all three serologic tests, and that the largest number of the BFP reactors were detected by the cardiolipin tests and not by the treponemal antigen. The 16 patients with syphilis in whom there were negative reactions to the RPCF antigen represent cases of long duration in which the

Reiter antibody became negative before the reagin did. Included in this group are seven cases in which spinal fluid serologic tests were positive, and three cases of congenital syphilis. The biologic false-positive RPCF reactions occurred in a group of 11 patients *(Table 4)* none of whom had a proved clinical history of syphilis, and all of whom had negative serologic tests for syphilis, either prior to the current study or upon repeated testing during the study. This group of false-positive reactors included six males and five females, whose ages ranged from 7 years to 77 years, and who had few clinical features in common. The major diagnoses of these patients were: demyelinization of the central nervous system, intervertebral disk, mesenteric lymphadenitis, traumatic paraplegia, benign enlargement of the prostate, renal hypertension, carpal tunnel syndrome, arteriosclerosis obliterans, pruritus ani, rheumatoid arthritis, and rheumatic heart disease. There were no clinical or therapeutic factors to account for the false-positive reactions in patients 1, 5, 8, 9, and 11. Patients 9 and 11 reacted positively to all three serologic tests. Patient 2 had malaria in 1941 and questionable syphilis in 1944. Patient 3 had positive serologic reactions during a period in which he was suspected of having an abdominal aneurysm; at laparotomy, intestinal adhesions and mesenteric lymphadenitis were found. Patient 4 had the false-positive RPCF reaction within a few days of a cerebral infarction. Patient 6 had hypertension associated with renal artery stenosis. Patients 7 and 10 had rheumatoid arthritis, which is reported to be often associated with false-positive serologic tests for syphilis. The occurrence of false-positive reactions to the treponemal antigen might suggest that perhaps all of the nonspecific lipoidal components had not been completely removed or that some of the patients actually had undiagnosed syphilis.

In order to obtain a rough comparison of the relative performance of these tests, we may calculate the specificity, sensitivity, and BFP rate of each individual test and each combination of tests. The specificity of a serologic test for syphilis is defined as the percentage of nonreactive results obtained in a nonsyphilitic population. For this group we selected the blood donors from the Cleveland Clinic blood bank. This particular group had been previously surveyed by King and Reich[4] who found that among 12,000 blood donor samples examined during a seven-year period, three cases of syphilis were discovered by the routine (Kolmer and Kahn) serologic survey. This gave an incidence of 0.025 per cent for this particular group. In our current study, among the 10,292 specimens of serum tested, 1058 were from professional donors in our blood bank. Of this group, three *previously negative* donors reacted positively as follows:

	Test, reaction		
Donor	Kolmer	Kahn	RPCF
A	+	—	—
B	—	—	+
C	—	—	+

We have assumed this to represent a lack of specificity of the particular tests, but have subtracted 0.025 per cent from the percentage of positive reactors (as representing the random chance that they might have actually had syphilis) to derive the specificity of each test.

The sensitivity of a serologic test for syphilis is defined as the percentage of

Table 4.—*Analysis of data of 11 patients who were biologic false-positive reactors to the RPCF test*

Patient, no.	Age	Sex	Test, reaction			Date
			Kolmer	Kahn	RPCF	
1	46	M	Neg.	Neg.	—	7/16/54
			Neg.	Neg.	—	10/13/58
			Neg.	Neg.	32200	11/19/59
			Neg.	Neg.	Neg.	1/19/60
			*Neg.	Neg.	Neg.	1/22/60
2	52	M	Neg.	Neg.	30000	11/24/59
			Neg.	Neg.	Neg.	12/8/59
3	56	M	Neg.	Neg.	Neg.	12/16/59
			Neg.	Neg.	44210	1/4/60
			Neg.	Neg.	44200	1/11/60
4	50	F	Neg.	Neg.	—	8/4/58
			*Neg.	Neg.	—	9/5/58
			Neg.	Neg.	44422	1/7/60
5	77	M	Neg.	Neg.	42200	1/11/60
6	42	M	Neg.	Neg.	Neg.	12/15/59
			Neg.	Neg.	44300	1/15/60
7	49	F	Neg.	Neg.	Neg.	12/1/59
			44443	Neg.	44200	1/15/60
			Neg.	Neg.	Neg.	2/16/60
8	65	M	Neg.	Neg.	31000	1/24/60
			Neg.	Neg.	Neg.	2/4/60
9	7	F	44321	2+	21000	1/25/60
			2+	Neg.	†AC	2/1/60
10	37	F	Neg.	Neg.	41000	1/25/60
			Neg.	Neg.	Neg.	2/10/60
11	57	F	Neg.	Neg.	—	7/21/59
			32210	2+	20000	2/24/60
			10000	2+	Neg.	3/1/60

*Cerebrospinal fluid specimen.
†Anticomplementary.

reactive results among a group of known syphilitic persons. The group of 64 patients classified as definitely syphilitic was used in this calculation.

A third expression of evaluation of these tests (suggested by Wheeler) is that of the BFP rate,[20] which is the percentage of reactive results among those patients whose previous serologic and clinical histories suggest a diagnosis of BFP sero-

Table 4.—*Concluded*

Patient, no.	Diagnosis	Remarks
1	Demyelinization of central nervous system; mechanical back pain.	
2	Protruded lumbar disk	Malaria in 1941; treated in 1944 for syphilis (?) without positive serologic test.
3	Mesenteric lymphadenitis	Laparotomy 1/7/60; hospital discharge 1/14/60.
4	Traumatic paraplegia; essential hypertension; cerebral infarction 1/4/60.	
5	Benign enlargement of prostate	
6	Renal hypertension; cerebral artery thrombosis	Ten negative Kolmer and Kahn tests since 1945 Splenoreal anastomosis 1/18/60; died 1/29/60.
7	Carpal tunnel syndrome; rheumatoid arthritis.	
8	Arteriosclerosis obliterans of femoral artery; dermatophytosis	
9	Pruritus ani	Mother's serologic test negative
10	Rheumatoid arthritis	Treated with ACTH, nitrogen mustard, and chloroquine phosphate 2/6/60.
11	Rheumatic heart disease; mitral insufficiency.	

logic reactions. The group of 40 patients classified as probable BFP reactors was used in this calculation.

The serologic tests listed in *Table 5* are in the order of their over-all performance. It is apparent that the performance value of a serodiagnostic test for syphilis

Table 5.—*Comparison of serologic tests (listed in order of over-all performance)*

Tests	Measurement of performance, per cent		
	Specificity	Sensitivity	BFP rate
Kolmer and RPCF	99.7	70.0	7.5
RPCF	99.8	75.0	27.5
RPCF and Kahn	99.8	50.0	5.0
Kolmer and Kahn	99.9	68.7	27.5
Kolmer	99.9	95.3	62.5
Kahn	100.0	68.7	45.0

is best reflected by a combination of the three parameters of specificity, sensitivity, and BFP rate. To consider any one of these parameters exclusively, will give a misleading impression of the value of the various test procedures. Furthermore, a different test or combination of tests excels in each of the categories. In our experience, the single most specific procedure was the Kahn test, the single most sensitive was the Kolmer test, and the combination resulting in the least number of BFP reactions was the RPCF and Kahn tests. In considering all three categories together, we have found that the best diagnostic procedure is to combine the Kolmer and the RPCF tests. This observation is consistent with the recommendations of other groups of investigators that a combination of a reagin and a treponemal test is better than either test alone, because each measures a different antibody and is a complementary procedure.[15]

Discussion

Since publication of the first evaluations of the RPCF test by DeBruijn[2] in Holland, and Cannefax and Garson[1] in the United States, many workers have reported the close correlation of the RPCF test with the TPI procedure. As regards specificity, these workers and the many institutions which participated in the Serology Evaluation and Research Assembly[20] (SERA) reported the RPCF to have a range of specificity from 94.4 to 97.7 per cent. This compares favorably with the TPI test, which has a reported specificity of 93.7 to 100.0 per cent. In DeBruijn's[2] series, a standard serologic test for syphilis, which uses a cardiolipin antigen, gave a specificity of 82 per cent; the various nontreponemal tests in the SERA[20] study gave specificities ranging from 83 to 99.5 per cent.

The sensitivity of the various serologic tests for syphilis is a function of the

particular stage of the disease *(Fig. 1).* In its earliest stages the disease is seldom detected with the TPI test, but is more readily diagnosed with the various nontreponemal and Reiter tests. However, in the late stages of syphilis, the TPI test remains positive long after the reagin and RPCF tests have become negative. In

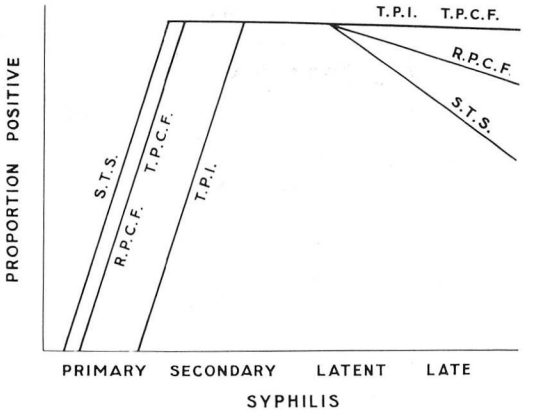

Fig. 1. Sensitivities of certain serologic tests at different stages of syphilis. (Courtesy of Sequeira, P. J. L.: Brit. J. Vener. Dis. 35: 141, 1959; and the Editor and Publishers of the *British Journal of Venereal Diseases,* London, England.)

its over-all sensitivity, the RPCF is reported[20] to surpass the TPI test and to be more sensitive than some, but not all, of the various nontreponemal tests.

In the SERA[20] report, Wheeler compares the BFP rate of the various types of tests. The RPCF and TPI procedures are reported to give ten times fewer BFP reactions than the various nontreponemal tests. The patients comprising this group were selected on the basis of previous clinical and serologic histories which along with a negative TPI suggested a diagnosis of BFP. In contrast to our 27.5 per cent BFP rate for the RPCF test, Wheeler found only 4 per cent of this group of patients to react to the RPCF test. Most observers believe that the RPCF test has its greatest usefulness in the differentiation of BFP reactors.

In view of the many false-positive reactions to the RPCF test in our personal experience, we believe that unqualified acceptance of the RPCF test results is not warranted, but that they should be checked further with the TPI test. One such study of discrepant RPCF and reagin results, which were then further checked by means of the TPI test, was conducted by Foster, Nicol, and Stone[19] in cooperation with Dr. P. J. L. Sequeira of the Central Serological Laboratory, Manchester, England. Of 150 samples of serum that showed discrepant reactions between a standard microflocculation test and the RPCF test, 20 per cent showed disagreement also between the results of the RPCF and the TPI tests. Most of these serums reacted negatively to the TPI and positively to the RPCF test. The authors suggest that these might have been cases of early stages of syphilis in which the TPI test result had not yet become positive; we suggest that some of their cases might just

as well have represented false-positive RPCF reactions. They also found that in a group of patients upon whom they performed repeated RPCF tests with successive serum samples, results were variable in 19 per cent. Almost half of their patients who gave variable RPCF results had positive results in TPI tests, suggesting to the authors that even a variable RPCF test could indicate a syphilitic infection. In our own series we found a 9.5 per cent variability rate, which was identical for the RPCF, Kolmer, and Kahn tests. Our belief is that one should not rely solely upon any single laboratory finding, particularly when successive serums from the same patient give varying results. The danger inherent in dependence upon the RPCF test alone, is evidenced in a comparative study[19] in which 132 serums were independently tested by two laboratories. Identical results were obtained in only 99 of the specimens. These findings further suggest that there is too much variability in the results of the RPCF test for this procedure to be used as the sole or ultimate criterion in the serodiagnosis of syphilis.

Conclusions

1. According to our experience the best testing method in the serodiagnosis of syphilis is the combination of the Kolmer test and the Reiter protein complement-fixation (RPCF) test.

2. The majority of patients with syphilis reacted positively to both serologic tests.

3. The RPCF test, alone, gave far more biologic false-positive (BFP) reactions than have been reported by other investigators using this test.

4. The diagnosis of syphilis should not be based upon a positive RPCF test alone.

5. To avoid error:

   All serologic test results should be interpreted in relation to a detailed clinical history.

   A serologic test that gives a positive result should be repeated.

   If the test result is at variance with the clinical impression, the result should be rechecked, first using the RPCF test, and subsequently, if indicated, the *Treponema pallidum* immobilization (TPI) test.

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