Bull. Org. mond. Santé } 1965, 33, 687-703
Bull. Wld Hlth Org. }

Fluorescent Treponemal Antibody Testing

K. KIRÁLY, M.D.,[1] A. JOBBÁGY [2] & T. MECHER, Sc.D.[2]

Contradictory reports have been published on the value of fluorescent protein tracing as a serological test for syphilis. The authors believe that this is due mainly to differences in technique and especially to variations in the quality of the conjugate used. They describe the preparation of a fluorescein isothiocyanate (FITC) immune serum conjugate, which they characterize by its antihuman-globulin titre, FITC/protein ratio and staining effect. The fluorescent treponemal antibody (FTA-50) test performed with this conjugate gave better agreement with the TPI test than any of four other serological tests with which it was compared. Its sensitivity is also good, being equal to that of the T. pallidum *complement-fixation (TPCF) test. Nevertheless, the FTA-50 test is not absolutely specific and, as a screening procedure before TPI testing, a combination of the TPCF test with the cardiolipin complement-fixation test appears to be simpler, more sensitive and cheaper. Further study is needed with a view to the elimination of non-specific staining and standardization of the conjugate and antigen before the FTA-50 test can replace the TPI test.*

Fluorescent protein tracing was first used in syphilis serology by Deacon, Falcone & Harris in 1957. Since then numerous papers have been published dealing with the specificity and sensitivity of the method in relation to other serological tests for syphilis and with changes in the response to the test in the course of syphilis. The results and technical modifications have been reviewed by Nielsen & Idsöe (1963). The present paper reports further studies of the method, with particular reference to the properties of fluorescein isothiocyanate protein conjugates.

METHODS

FLUORESCENT TREPONEMAL ANTIBODY (FTA-50) TEST

Antigen

Treponema pallidum, Budapest strain, extracted from the testes of rabbits with early orchitis and suspended in 0.85% saline, was used. The suspension was freed by triple fractionated centrifugation from tissue debris and proteins, the presence of which may cause disturbing background fluorescence. The final concentration aimed at was $60\text{-}70 \times 10^6$ treponemes per millilitre. Thiomersal 1: 5000 was added as preservative. The suspension can be stored at 4°C for three to four weeks.

Preparation of conjugate

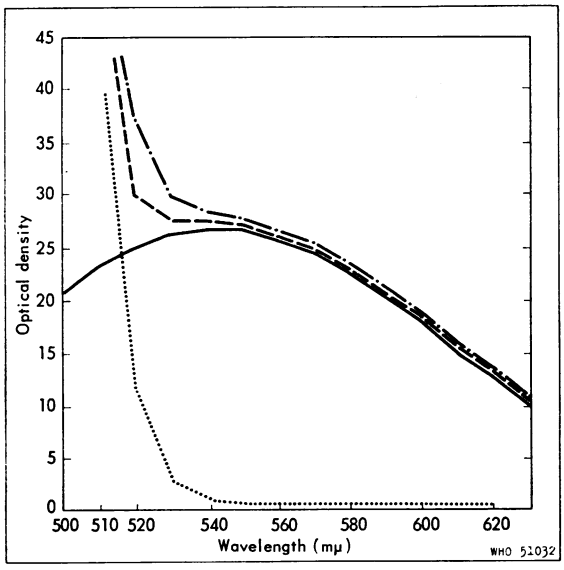
The procedures described below are those finally adopted in the light of experience (see page 690).

(*a*) *Gamma-globulin precipitation.* The immune serum was diluted 1: 1 with a mixture of 0.85% saline (eight parts) and M/15 phosphate buffer, pH 7.4 (two parts) (this mixture is referred to as buffered saline or BS). A 28% (W/V) solution of sodium sulfate was then added dropwise under continuous stirring, until a final concentration of 14% was obtained. After allowing to stand for 30 minutes at +4°C, the precipitate was centrifuged at 1500 *g* for 15 to 20 minutes. The sediment was dissolved in a volume of BS equal to that of the serum, and the precipitation repeated. Afterwards the redissolved protein was dialysed against 200 volumes

[1] Senior Research Associate, Hungarian State Institute for Dermatology and Venereology, Budapest, Hungary.

[2] Research Associate, Hungarian State Institute for Dermatology and Venereology, Budapest, Hungary.

FIG. 1
ABSORPTION SPECTRA OF PROTEIN AND FITC SOLUTIONS WITH BIURET REAGENT



biuret reagent and protein (1 mg/ml)
biuret reagent, protein (1 mg/ml) and FITC (20 μg/ml)
biuret reagent, protein (1 mg/ml) and FITC (50 μg/ml)
FITC (20 μg/ml)

of BS until traces of sulfate could be detected. Generally, a dialysis for 8-16 hours with 3-5 liquid changes was sufficient.

(*b*) *Conjugation.* The protein content of the solution was determined by the biuret method (Kingsley, 1939) and it was then diluted with BS to a final protein concentration of 10 mg/ml. Allowing 0.08 mg of fluorescein isothiocyanate (FITC) for 1 mg of protein, the necessary quantity was dissolved in 0.55 M carbonate buffer of pH 9 to give one-tenth of the volume of the protein solution. The two solutions were immediately mixed and gently stirred at 4°C for 20-24 hours. Afterwards the conjugate was dialysed against 200 volumes of BS until fluorescence was visible under ultraviolet light. Changing the saline twice a day, two-and-a-half to three days were sufficient. Then the conjugate was absorbed twice, each time by treatment for an hour at 4°C with rabbit liver powder (0.1 g/ml), as described by Coons, Leduc & Connolly (1955). The conjugate, preserved with thiomersal 1: 5000 and glycerol 1: 1, was stored at —10°C. Dilutions, according to the titre, were freshly prepared on every occasion. The diluted conjugate can be stored at 4°C only for a short time: its staining effect decreases significantly after a week and is lost completely after three weeks.

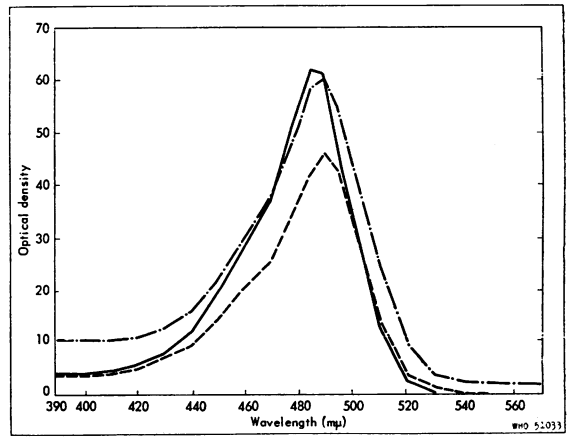
The FITC used in our experiments was in part supplied by Fluka (Buchs, Switzerland) and in part prepared by one of us (T.M.) as described by Riggs et al. (1958) and by Coons & Kaplan (1950). Both materials gave identical results.

Characterization of conjugates

(*a*) The protein content was determined by the biuret method (Kingsley, 1939) using a Hilger spectrophotometer at 560 mμ. The average extinction (E) of a protein solution containing 1.46 mg/ml is 0.346 (standard deviation ±0.0045 or 19 μg protein); in the presence of FITC it is slightly higher (Fig. 1). Under the experimental conditions used by the authors, the increase was within twice the standard deviation and therefore could be ignored.

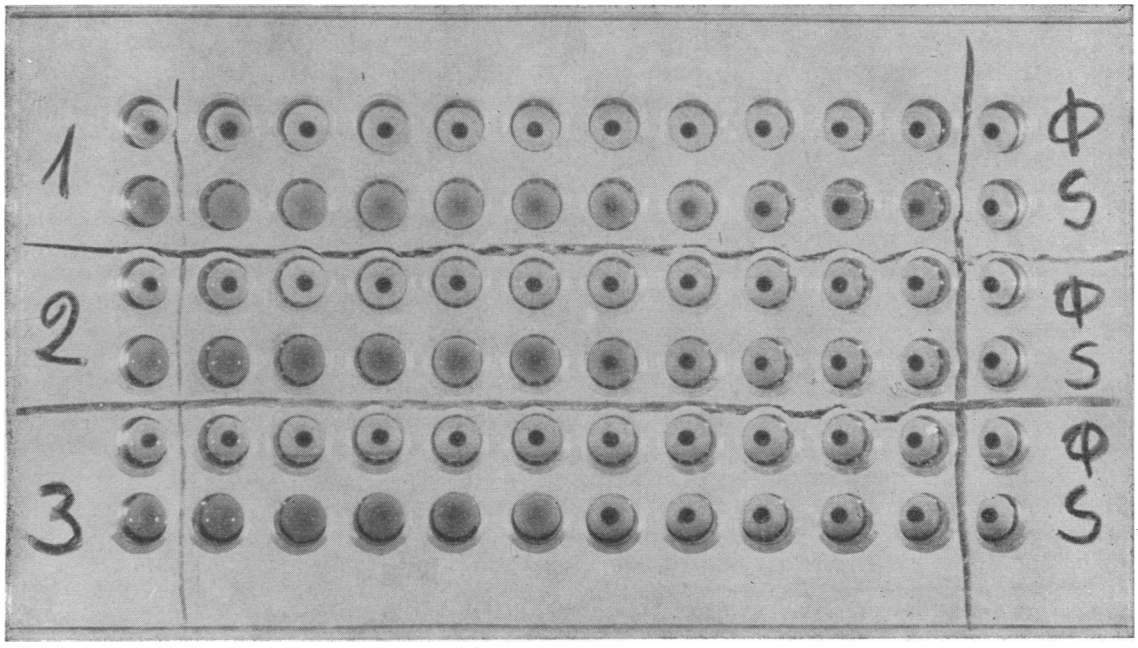
(*b*) The FITC content was measured using a Hilger spectrophotometer at 490 mμ by direct photometry. For FITC dissolved in buffered saline E_{max} was at 485 mμ, shifting to 490 mμ after conjugation with protein. The average extinction of a solution of FITC (Mecher's preparation) containing 2 μg/ml was 0.370 (standard deviation ±0.006 or 0.032 μg FITC). The extinction of an FITC-protein mixture was somewhat lower (Fig. 2), but the difference was negligible, being less than the standard deviation. It is presumed that this is the case with the conjugate too.

FIG. 2
ABSORPTION SPECTRA OF FITC AND CONJUGATE



FITC (5 μg/ml)
conjugate
FITC (5 μg/ml), protein (1.46 mg/ml)

FIG. 3
HAEMAGGLUTINATION TEST FOR DETERMINATION OF AHG TITRE



(*c*) The antihuman globulin (AHG) titre was determined by passive haemagglutination. D-positive O group red blood cells were washed once with 0.85% saline. The centrifuged sediment was sensitized with an equal volume of anti-D serum in a water-bath at 37°C for an hour. After washing three times, the packed cells were diluted to make a 2.5% suspension. After checking the agglutinability of the suspension with Coombs serum, haemagglutination was carried out on a Takácsy's slide. From the AHG serum diluted 1: 100 with saline, two rows of twofold dilutions were prepared. A sensitized suspension of red blood cells was added to the first row, non-sensitized suspension to the second. As controls, similar dilution rows were made from Coombs serum of known titre. The test was read after one hour's incubation in a thermostat at 37°C by the sedimentation pattern: the agglutinated red blood cells are evenly spread on the walls of the grooves, and the non-agglutinated cells collect at the lowest points (Fig. 3).

(*d*) The staining effect was measured by quantitative titration of the reactive control serum with three dilutions of the conjugate. The fluorescent antibody titre is the highest dilution of the conjugate that gives a result identical with that of the reference conjugate. The end-point of the titration is a weakly reactive (++) reading (see page 690).

Staining

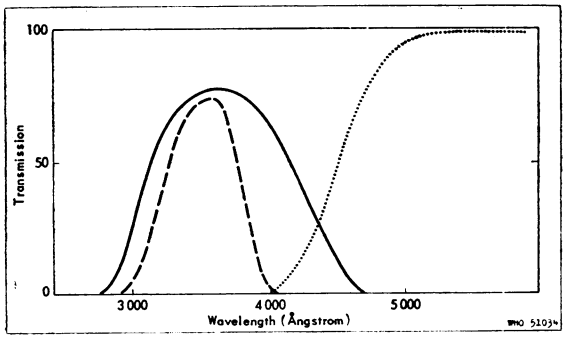
Three circles, about 10 mm in diameter, are scratched, on microscope slides by means of a diamond stylus. Both the slides and the circles are numbered. The slides can be used for several experiments.

The slides are cleaned by soaking for 16 hours in a solution of 5.0 g of $KMnO_4$ and 25.0g of NaOH in one litre of distilled water. After washing under tap water, they are bleached with 5% oxalic acid, washed with distilled water and then with acetone, and dried with a clean flannel cloth.

0.03 ml of the antigen is spread evenly within the marked area. After drying at room temperature, the smears are fixed by warming to 56°C for 10 minutes followed by immersion in acetone for 10 minutes. It is advisable to use only fresh smears; smears that have been stored stain less strongly.

The sera inactivated at 56°C for 30 minutes are diluted 1: 50 with BS and one drop of the dilution is added to the fixed treponemes. The slides are

FIG. 4
TRANSMISSION SPECTRA OF FILTERS USED



then placed in a humidified container and rotated at 100 rev/min at room temperature for 30 minutes. After blotting with filter-paper, the slides are rinsed with three changes of BS for 10 minutes. The adhering moisture is drained off and the smear covered with one drop of conjugate diluted according to its titre and incubated again for 60 minutes. After blotting and rinsing as before, the smears are mounted with coverslips, using BS with 10% glycerol as mounting medium. It is advisable to read the test immediately, although the fluorescence does not fade for 24 hours if the slides are kept at room temperature.

Optical equipment. The light source was an Osram HBO 200 high-pressure mercury vapour bulb, in a lamp-housing equipped with a condensing lens. The light was passed through a 10-mm thick trough, containing a 5% $CuSO_4$ solution to absorb infrared rays, and then through a Zeiss BG 2 exciter filter. A monocular microscope was used with Zeiss cardioid dark-ground condenser, high dry-field objective and 10× eyepiece fitted with a GG 5 barrier filter. The magnification was 400. The transmission spectra of the filters used in this work are shown in Fig. 4. To eliminate the disturbing fluorescence of immersion oil, distilled water is used between the slide and the condenser. The strength of illumination is controlled on each occasion with a standard preparation.

The treponemes are thick and yellow for reactive, and thin and blue for non-reactive sera. Using a Zeiss UG 2 filter transmitting only the invisible ultraviolet light, the intensity of fluorescence is graded as follows:

++++	very thick, bright yellow	with UG2 strongly fluorescent treponemes
+++	moderately thick, bright yellow	
++	yellow, with UG2 clearly fluorescent treponemes	
+	pale yellow	
0	blue	

++++ and +++ are designated reactive, ++ weakly reactive, + and 0 non-reactive.

The following controls are set up:

(*a*) reactive serum in a twofold dilution row (the sensitivity of the test is adequate if the deviation is not more than ± 1 dilution from the titre);

(*b*) non-reactive serum;

(*c*) *T. pallidum* smear.

OTHER METHODS

The TPI test was performed with the Budapest pathogenic strain of *T. pallidum*, using 40% complement by volume. If a previous TPI test had shown a weak reaction or if other serological tests for syphilis had given discordant results, a double quantity of serum was taken.

All sera examined by the FTA-50 test were also examined by the complement-fixation reaction, using cardiolipin (CKVI Moscow), pathogenic *T. pallidum* protein and Reiter protein antigens, and by the VDRL test, using Difco antigen. The complement-fixation reaction was performed according to the modification described by Király (1959). The Portnoy-Magnuson antigen, used for the examination of a few sera, and the *T. pallidum* and Reiter protein antigens were prepared in the laboratory.

EXPERIENCE WITH THE FTA-50 TEST

The conjugate was characterized by the FITC/protein ratio, the AHG titre and the fluorescent-antibody titre, all related to the original volume of the serum. Data on the conjugates prepared and used by the authors are given in Table 1.

For adequate sensitivity of the FTA test, a minimal FITC/protein ratio is necessary. If it is less than 3, the staining effect is weak and is uninfluenced by a high immune titre (see Table 1, notes on conjugates Nos. A-8-59 and A-8-60). Good conjugate had a

TABLE 1

CHARACTERISTICS OF CONJUGATES FOR INDIRECT FLUORESCENT STAINING, PREPARED BY VARIOUS METHODS

Conjugate No.	Immune serum	Protein content (mg/ml)	FITC content (mg/ml)	FITC/ protein ratio [a]	Anti-human globulin titre		Titre of conjugate in the FTA-50 test	Notes
					before conjugation	after conjugation		
A-8-59	Horse anti-human serum No. 249 [b]	15.3	0.113	7.4	596 000	525	105	These figures refer to a second conjugation; after the first conjugation, the FITC/protein ratio was 2.4; the anti-human globulin titre 596 000; and the fluorescence in FTA-50 test very weak
A-5-10		11.1 [c]	0.04 [c]	3.6	596 000	16 000 [c]	200 [c]	
A-5-15		31.6	0.316	10	596 000	3 200	160	
A-5-18	Serum No. 249 absorbed with human albumin	16.25	0.479	29.1	51 200	436	87	Strongly fluorescent precipitate in the field of the microscope; thin, hardly visible treponemes in positive control
A-8-60	Rabbit anti-human serum No. 46 [b]	25.5	0.211	8.2	64 000	348	17.5	These figures refer to a second conjugation; after the first conjugation, the FITC/protein ratio was 1.62; the anti-human globulin titre 64 000; and the fluorescence in the FTA-50 test very weak
A-5-11		7.19	0.0288	4.0	64 000	3 200	160	
A-5-17	Mixed pool of rabbit anti-human serum	25.7	0.327	12.7	3 200	600	120	
A-5-19	Rabbit anti-human globulin No. 420	27.5	0.284	10.3	102 400	3 450	108	
A-5-20	Rabbit anti-human globulin No. 429	21.2	0.236	11.1	102 400	2 800	90	
A-5-21	Rabbit anti-human globulin No. 914	32.9	0.482	14.6	102 400	4 200	130	
A-5-16	Goat anti-rabbit globulin [d]	60	0.426	7.1	—	—	240	

[a] Calculated as $\frac{\text{FITC content}}{\text{protein content} \times 10^{-3}}$.

[b] Received by courtesy of Dr R. Backhaus, Human Serum and Vaccine Institute, Budapest.

[c] Not calculated to original volume.

[d] Received by courtesy of Dr P. Geck, Hungarian State Institute of Public Health and Hygiene, Budapest.

ratio of about 10 and an AHG titre of about 3000. Staining was, however, excellent with conjugates of lower FITC/protein ratio (3.6 to 4.0) if the AHG titre remained high. Above a certain limit, the FITC causes protein denaturation, as was the case with the conjugate No. A-5-18, which had an FITC/protein ratio of 29.1.

The simplest method of increasing the ratio is to add more FITC to the protein. The optimal ratio (14.6) was achieved by adding 0.08 mg of FITC for each mg of protein.

The FITC/protein ratio depends greatly on the quantity of protein to be labelled. The protein concentration must be chemically determined and cannot be calculated from the original serum volume. The original globulin level of immune serum is variable, the conditions of precipitation are ill-defined and the protein loss and dilution during dialysis are also variable. It is not possible to allow for any of these factors by calculation. After determination of the protein content, the solution was diluted to 10 mg/ml, although the concentration did not influence the FITC/protein ratio significantly.

Between 10% and 15% of the FITC is bound, forming *N-N'* di-substituted thiourea derivatives with the free amino groups of the proteins. The rest remains unbound and participates in secondary reactions. The degree of conjugation can be increased by prolonging the reaction time and by gentle stirring. The FITC deteriorates quickly. Delay in adding it to the protein solution diminishes the efficiency of conjugation.

In our experiments, the unreacted fluorescent material caused non-specific staining, in contrast to the experience of Niel & Fribourg-Blanc (1962). Unfortunately, part of it was so strongly adsorbed by the proteins that it could not be removed, either by very long dialysis or by absorption by rabbit liver powder, as recommended by Chadwick & Nairn (1960). The dialysis as used by the authors caused a significant loss of proteins, amounting to 15-30%. The more efficient Sephadex treatment (Nairn, 1962) for the removal of unbound FITC was not used.

All conjugates were purified by absorption by rabbit liver powder, which was originally designed for the elimination of non-specific staining caused by electrostatic binding between the proteins of the conjugate and the microscopical preparation. For this purpose, this method is presumably satisfactory: the disturbing background fluorescence mentioned by Fry & Wilkinson [1] was hardly noticed. Otherwise, no practical purpose could be ascertained.

Commercial antihuman sera (rabbit or horse) and rabbit antihuman gammaglobulin prepared by the authors were used. Although the use of antigammaglobulin sera seems logical, the antibodies formed against other serum proteins did not disturb the microscopic picture. The original AHG titre was 64 000 to 600 000. In parallel testing there was practically no difference between horse and rabbit immune sera.

Glycerol, added as preservative, stabilizes the conjugate and eliminates the undesirable continuous precipitation of fluorescent material during storage.

Three cultivable treponemal immune sera and one syphilitic serum were labelled for direct staining. Their characteristics are shown in Table 2. The antibody titre of the syphilitic serum was otherwise fairly high (FTA test, 800; TPI test, 300; cardiolipin and *T. pallidum* complement-fixation reactions, 20) but the treponemes stained weakly. They appeared thinner than the treponemes stained by the indirect method.

The various cultivable treponemal immune sera cross-reacted with *T. pallidum*. This agrees with the finding of Deacon & Hunter (1962) and also with the earlier observation (D'Alessandro & Dardanoni, 1953) that cultivable and pathogenic treponemes have a common antigenic determinant, the treponemal group antigen. Several attempts have been made to replace *T. pallidum* as antigen in the FTA test by cultivable treponemes (Covert et al., 1961; Fife et al., 1961; Kent et al., 1962). An objection to this modification is that it involves only one antigenic determinant on the treponemal surface.

The suspension of *T. pallidum* can be used immediately after extraction. The preparation of a stable antigen has not yet been accomplished. Using freeze-dried *T. pallidum* the sensitivity of the FTA test decreases by about 50%. After three to four weeks of storage, the antigen deteriorates, the first sign being the decrease of reactivity titre in the quantitative test and a higher frequency of non-specific positive results. This spontaneous change in the treponemal surface on storage seems to be analogous to that observed in agglutination reactions with *T. pallidum* (Hardy & Nell, 1957).

The surface antigens may be occupied by rabbit antibodies, which inhibit the reactivity with human sera. The FTA antibodies appear as early as 6 days after infection: of 10 rabbit sera, one was weakly

[1] Unpublished mimeographed document WHO/VDT/RES/35.

TABLE 2

CHARACTERISTICS OF CONJUGATES FOR DIRECT FLUORESCENT STAINING, PREPARED BY VARIOUS METHODS

Conjugate No.	Immune serum	Protein content (mg/ml)	FITC content (mg/ml)	FITC/protein ratio [a]	Agglutination test			FTA-50 test	
					Antigen	Agglutinin titre		Antigen	Antibody titre
						Before conjugation	After conjugation		
A-5-14	Human syphilitic serum No. A-7-187	11.9	0.0998	8.4	*T. pallidum*	640	0	Pathogenic treponemes	34
A-8-61	Rabbit antiserum to Reiter treponemes	4.0	0.0447	11.1	Reiter treponemes	1 280	385	Reiter treponemes Pathogenic treponemes	6 200 24
A-8-62	Rabbit antiserum to Budapest-4 treponemes	19.0	0.205	10.8	Budapest-4 treponemes	2 560	190	Budapest-4 treponemes Pathogenic treponemes	6 200 192
A-8-63	Rabbit antiserum to Kazan-5 treponemes	13.3	0.152	11.4	Kazan-5 treponemes	2 560	185	Kazan-5 treponemes Pathogenic treponemes	12 000 93

[a] Calculated as $\frac{\text{FITC content}}{\text{protein content} \times 10^{-3}}$.

reactive and four strongly reactive with a homologous serum dilution of 1: 50; with lower dilutions all were positive. For that reason, the antigen was prepared only from rabbits with early orchitis.

The *T. pallidum* complement fixation test was more sensitive than the FTA-50 test by about 13%. Attempts were made to increase the sensitivity of the FTA test by treatment with trypsin or with lysozyme to make the deeper antigens accessible. Neither gave satisfactory results.

Longer drying is disadvantageous in any phase: e.g., if the test is performed with smears prepared 11 days previously or with antigen-antibody complexes dried for 18 hours, its sensitivity is reduced to one-tenth.

Opinions on the optimal degree of serum dilution differ widely. Some authors observed hardly any non-specific staining even with serum dilutions of 1: 5 (Censuales & Garofalo, 1959), 1: 10 (Ovčinnikov et al., 1961), 1: 20 (Delacrétaz & Frenk, 1961), 1: 30 (Borel et al., 1961), 1: 50 (Mannucci & Spagnoli, 1961; Vaccari et al., 1961), or 1: 100 (Vaisman & Hamelin, 1961). Deacon et al. (1960) had to use a dilution of 1: 200 to eliminate non-specific staining; Nielsen & Idsöe (1963) were unsuccessful even with this dilution. In spite of the unfavourable previous experiences, a 1: 50 serum dilution was used in the current work for two reasons: (*a*) the FTA test was used as a screening test; therefore maximal sensitivity was aimed at; (*b*) sensitivity and specificity are mutually antagonistic; it seems illogical to strive for better specificity by the use of a higher serum dilution while increasing the sensitivity by other methods (e.g., rotation, use of Tween, etc.).

Otherwise, no particular claims are made in respect of the serum: both dried blood [1] and contaminated, anti-complementary sera can be tested. The test proceeds as well at room temperature as at 37°C. Provided the glassware is clean, the use of Tween when diluting solutions and rotation of the slides may be omitted.

Reproducibility was tested in 134 cases. The reason for repetition was disagreement between the TPI test and one of the other tests performed, or disagreement between the TPI test and clinical supposition. The correlation between the two consecutive FTA tests is shown in Table 3, and their correlation with the serum reactivity pattern in Table 4. The two tests gave identical results in

[1] Vaisman, A., Hamelin, A. & Guthe, T.—unpublished mimeographed document WHO/VDT/RES/36 (1963).

TABLE 3
REPRODUCIBILITY OF THE FTA-50 TEST

		First testing					Totals
		0	1+	2+	3+	4+	
Second testing	0	70	4	6		1	81
	1+	1		1			2
	2+	10	3	3	5	3	24
	3+			4	2	3	9
	4+		1	2		15	18
Totals		81	8	16	7	22	134

90 cases (67%); the difference between them was within one degree in 111 cases (83%). When the readings were classified as either reactive or non-reactive, agreement was noted in 112 cases (84%). In 20 cases, one test was read as non-reactive and one as weakly reactive, while in 2 more cases, one was non-reactive and one definitely reactive.

The reproducibility was worst in the group where all tests were read as reactive with the exception of the FTA test (Table 4). Considering that it was the most difficult cases that were repeated, the reproducibility of the test under routine laboratory conditions was fairly good, although not as high as was found in the WHO collective study.[1]

TABLE 4
REPRODUCIBILITY OF THE FTA-50 TEST IN GROUPS OF SERA WITH DIFFERENT PATTERNS OF REACTIVITY

Result of other tests	Number of cases	Result of two consecutive FTA-50 tests				
		Identical readings	Readings differing by less than one degree	Both readings reactive or both non-reactive	One reading non-reactive, one weakly reactive	One reading non-reactive, one definitely reactive
All +	48	25 (52%)	36 (75%)	38 (79%)	10	—
TPI and treponemal tests + Lipoidal antigen tests 0	9	7	7	7	1	1
TPI and lipoidal antigen tests + Treponemal tests 0	1		1	1	—	—
Lipoidal antigen tests + TPI and treponemal tests 0	6	5	6	5	1	—
Treponemal tests + TPI and lipoidal antigen tests 0	46	34 (74%)	39 (85%)	39 (85%)	7	—
All 0	24	19 (79%)	22 (92%)	22 (92%)	1	1
Totals	134	90 (67%)	111 (83%)	112 (84%)	20	2

SEROLOGICAL RESULTS

Four hundred and forty-seven sera are included in this study. All were problem sera, sent for TPI testing from all over Hungary. They comprise: (*a*) sera showing unexpected serological reactivity; (*b*) negative sera from patients who had received adequate treatment; (*c*) negative sera from patients without any specific history, but clinically suspected of active late syphilis. Two-thirds of the sera belonged to the first two categories, one-third to the third category. The sera were screened by the VDRL test, and by the complement-fixation reaction using as antigens *T. pallidum* protein (TPCF), Reiter protein (RPCF) and cardiolipin (CCF); if the sera were found to be non-reactive in the screening tests, no TPI test was performed, except in the case of sera from patients suspected of

[1] Unpublished mimeographed documents WHO/VDT/RES/30, WHO/VDT/RES/31 and WHO/VDT/RES/32.

TABLE 5
COMPARATIVE RESULTS OF THE FTA-50 TEST AND OTHER TREPONEMAL TESTS

FTA-50	Number of specimens	TPI test				TPCF test				RPCF test				Portnoy-Magnuson complement fixation test			
		+	±	−	Not examined	+	±	−	Not examined	+	±	−	Not examined	+	±	−	Not examined
++++	113 (25%)	104 (28%)	2 (0.5%)	7 (1.9%)	0	97 (22%)	7 (1.6%)	9 (2.0%)	0	77 (17%)	10 (2.3%)	24 (5.4%)	2	63 (22%)	5 (1.7%)	7 (2.4%)	38
+++	49 (11%)	39 (11%)	1 (0.3%)	8 (2.2%)	1	31 (7.0%)	4 (0.9%)	13 (2.9%)	1	24 (5.4%)	6 (1.4%)	18 (4.1%)	1	23 (7.9%)	3 (1.0%)	5 (1.7%)	18
++	62 (14%)	38 (10%)	6 (1.6%)	14 (3.8%)	4	31 (7.0%)	10 (2.2%)	20 (4.5%)	1	19 (4.3%)	14 (3.2%)	28 (6.4%)	1	21 (7.2%)	5 (1.7%)	8 (2.7%)	28
0 or +	223 (50%)	15 (4.1%)	12 (3.3%)	123 (33%)	73	36 (8.1%)	22 (4.9%)	165 (37%)	0	8 (1.8%)	12 (2.7%)	200 (45%)	3	42 (14%)	24 (8.2%)	85 (29%)	72
Totals	447 (100%)	196 (53%)	21 (5.7%)	152 (41%)	78	195 (44%)	43 (9.7%)	207 (46%)	2	128 (29%)	42 (9.5%)	270 (61%)	7	149 (51%)	37 (13%)	105 (36%)	156

syphilis on clinical grounds. Classification of the material according to the stage of syphilis would be unreasonable, as the diagnosis is based on the TPI test.

COMPARISON BETWEEN THE FTA-50 TEST AND OTHER SEROLOGICAL TESTS

Using the above-mentioned criteria, the TPI test was not carried out on 78 specimens. These had all been found reactive in other laboratories when examined by the classical tests (MKR II, Kahn R, Citochol R, etc.). As a result of this screening, biologically false positive sera were practically absent from the material. Of those giving false positive reactions, only five (6.4%) were found reactive in the FTA-50 test. Detailed clinical data on these patients were not available.

The correlation of the results of the FTA-50 test with those of other treponemal tests is shown in Table 5; the FTA-50 test and tests using cardiolipin antigens are compared in Table 6. The reactivity rates of the various tests made with nearly equal numbers of sera were as follows (Fig. 5): TPCF, 53.5%; FTA-50, 50.1%; CCF, 49.0%; VDRL, 42.5%; RPCF, 39.6%. The percentage agreement with the FTA-50 test (Fig. 6) was: TPI, 85.1; VDRL, 80.3; CCF, 80.2; RPCF, 79.5; TPCF, 78.5.

On 369 specimens, all the tests were carried out. Their correlation with the TPI test is shown in Table 7; the agreement rate was as follows: FTA-50, 85.0%; VDRL, 82.5%; CCF, 80.9%; TPCF, 80.5%; RPCF, 73.2%. Detailed clinical and serological data on cases where there was disagreement between the results of the FTA-50 and TPI tests are shown in Tables 8 and 9. The disagreement was not consistent, since in a number of cases the results of the TPI test obtained with subsequent serum samples showed more reduction than increase in immobilization.

In 27 cases, the sera were found non-reactive by the FTA-50 test and reactive by the TPI test (Table 8). In one of these (the second in the table) a second specimen was weakly reactive in the FTA-50 test. In 12 patients there was clinical evidence or a history of syphilis. For 15 patients the clinical information was incomplete; these need further study, even serologically. The results of the TPI test were: + and − in 3 cases, ± and − in 6 cases, ± and + in 2 cases, + (but never attaining complete immobilization) in 12 cases and ± in 4 cases. The other tests gave rather discordant results. Four of the sera were found to be reactive in all the tests,

TABLE 6

COMPARATIVE RESULTS OF THE FTA-50 TEST AND SEROLOGICAL TESTS PERFORMED WITH LIPOIDAL ANTIGENS

FTA-50 test	Number of specimens	Cardiolipin complement-fixation test				VDRL test			Combined reactivity		
		+	±	−	Not examined	+	±	−	+	±[a]	−
++++	113 (25 %)	98 (22 %)	3 (0.7 %)	12 (2.7 %)	0	76 (17 %)	18 (4.0 %)	19 (4.2 %)	92 (21 %)	11 (2.5 %)	10 (2.2 %)
+++	49 (11 %)	31 (7.0 %)	3 (0.7 %)	15 (3.4 %)	0	23 (5.1 %)	11 (2.5 %)	15 (3.4 %)	29 (6.5 %)	10 (2.2 %)	10 (2.2 %)
++	62 (14 %)	34 (7.6 %)	6 (1.3 %)	22 (4.9 %)	0	20 (4.5 %)	15 (3.4 %)	27 (6.0 %)	32 (7.2 %)	11 (2.5 %)	19 (4.2 %)
0 or +	223 (50 %)	31 (7.0 %)	12 (2.7 %)	178 (40 %)	2	10 2.2 %	17 (3.8 %)	196 (44 %)	15 (3.4 %)	40 (8.9 %)	168 (38 %)
Totals	447 (100 %)	194 (44 %)	24 (5.4 %)	227 (51 %)	2	129 (29 %)	61 (14 %)	257 (57 %)	168 (38 %)	72 (16 %)	207 (46 %)

[a] Discordant results, or both weakly positive.

3 in the TPCF and RPCF tests, 10 in the TPCF test and in lipoidal antigen tests, 8 in the TPCF test alone, and 2 in lipoidal antigen tests only. This lack of agreement and the poor reproducibility of the TPI test may be explained by the cessation of specific antibody production.

Twenty-nine sera were found to be reactive by the FTA-50 test and non-reactive by the TPI test (Table 9). Syphilis could be assumed in 19 cases: in 9, specific treatment had been given previously; in 4, clinical symptoms of syphilis were present; in 3, there was a family history of syphilis; and in the other 3, the serological findings indicated syphilis (positive Wassermann reaction in the cerebrospinal fluid, and a second serum specimen reactive to the TPI test). The remaining 10 patients are to be considered as biologically false positives. Two of them were pregnant, one suffered from spondylitis, and 5 had previously been classed as biologically false positives; the case histories of two were incomplete. A positive result was obtained with the FTA-50 test only in 7 of the presumed syphilitic patients and in 3 of those considered to be false positives.

TABLE 7

COMPARATIVE RESULTS OF THE TPI TEST AND OTHER SEROLOGICAL TESTS

	FTA-50 test		TPCF test		RPCF test		VDRL test		CCF test	
	Number of cases	%	Number of cases	%	Number of cases	%	Number of cases	%	Number of cases	%
TPI and other tests agree (both + or both −)	313	85	297	80	270	73	304	82	298	81
TPI test −, other tests +	29	7.9	45	12	28	7.6	21	5.7	34	9.2
TPI test +, other tests −	27	7.3	27	7.3	71	19	44	12	37	10
Totals	369	100	369	100	369	100	369	100	369	100

TABLE 8. DETAILS OF 27 PATIENTS FOUND REACTIVE BY THE TPI TEST AND NON-REACTIVE BY THE FTA-50 TEST

Clinical diagnosis	FTA-50	SLA[a]	TPI[b]	TPCF	RPCF	Remarks
Latent syphilis; chronic alcoholism	—	±	100	+	+	Wife syphilitic.
Latent syphilis	—	—	42 13 8	+	+	Daughter congenital syphilitic. TPI test became reactive after provocation with *T. pallidum* vaccine. FTA-50 test performed with another serum specimen was weakly reactive.
Tabes dorsalis	—	±	74	±	—	
Tabes dorsalis	—	±	35 52 9 87	±	—	
Meningovascular syphilis	—	—	50 0 0 0	+	+	
Congenital syphilis; atrophy of optic nerve; chorioretinitis	—	±	43 8	±	—	
Congenital syphilis; atrophy of optic nerve	—	—	29	+	—	
Congenital syphilis (?)	—	—	96	+	—	
Congenital syphilis	—	—	58	+	+	
Treated syphilis	—	±	61	+	—	Specific treatment in 1944.
Treated syphilis	—	+	58	+	±	8 courses of penicillin since 1955.
Treated syphilis	—	—	87	+	+	Six courses of arsenobenzene + bismuth between 1946 and 1949
	—	±	62	+	+	
	—	+	22	+	—	
	—	+	82	+	—	
	—	+	37	+	—	
	—	—	39 0	+	—	
	—	±	37 12 4 13	+	—	
	—	±	92 6 13	±	—	
	—	—	33 75	+	—	
	—	—	42 17 4	+	—	
	—	—	87	+	—	
	—	—	52	+	—	
	—	—	100 52 9	+	—	
	—	—	35 25	±	—	
	—	+	61 100 96	—	—	
	—	±	58 48	—	—	

[a] Serological tests with lipoidal antigens.

[b] % immobilization.

TABLE 9.

DETAILS OF 29 PATIENTS FOUND REACTIVE BY THE FTA-50 TEST AND NON-REACTIVE BY THE TPI TEST

Clinical diagnosis	SLA [a]	FTA-50	TPI	TPCF	RPCF	Remarks
Latent syphilis	—	±	0 0 0 42	+	+	Daughter congenital syphilitic. TPI test reactive after provocation with *T. pallidum* vaccine
Latent syphilis	—	+		—	—	Daughter congenital syphilitic.
Cardiovascular syphilis	—	+	13 22	+	—	
Syphilitic aortitis	—	+	0 0	+	—	Specific treatment for many years.
Neurosyphilis (?)	±	+	4	—	—	
Tabes dorsalis with minor symptoms	—	±	0 8	—	—	
Congenital syphilis	±	±	4 4	+	—	SLA [a] positive during pregnancy.
Treated syphilis	—	+	8	+	—	Six courses of arsenobenzene + bismuth in 1930.
Treated syphilis	—	+	0	—	—	Three courses of arsenobenzene + bismuth between 1943 and 1945.
Treated syphilis	—	+	17	—	—	Six courses of arsenobenzene + bismuth between 1948 and 1950.
Treated syphilis	—	+	8	—	+	Specific treatment in 1946.
Treated syphilis	—	+	13	—	+	Five courses of arsenobenzene + bismuth for primary syphilis between 1942 and 1945.
Treated syphilis	—	±	4	+	+	Two courses of arsenobenzene + bismuth in 1945.
Treated syphilis	—	±	8	+	+	Specific treatment in 1947.
Treated syphilis	±	±	0	—	+	Specific treatment in 1951.
?	—	+	4	—	—	Wassermann reaction in cerebrospinal fluid positive.
?	—	±	4	—	—	Syphilitic history in family.
Pregnancy	—	+	13 79	+	—	
?	—	±	17 42	—	—	
Pregnancy	+	+	20 0	—	—	
Pregnancy	±	±	0 0	+	+	
Cerebral arteriosclerosis; cervical and lumbar spondylitis; osteochondrosis	±	+	0 4	+	+	
	—	±	0 8	+	+	
	—	±	5	—	±	
	—	+	4 13	—	±	
	+	±	12 4 4	—	—	
	—	±	5	—	—	
	—	+	13 0	—	—	
	—	±	0	—	—	

[a] Serological tests with lipoidal antigens.

FIG. 5

COMPARISON OF REACTIVITY RATES OBTAINED WITH VARIOUS TESTS

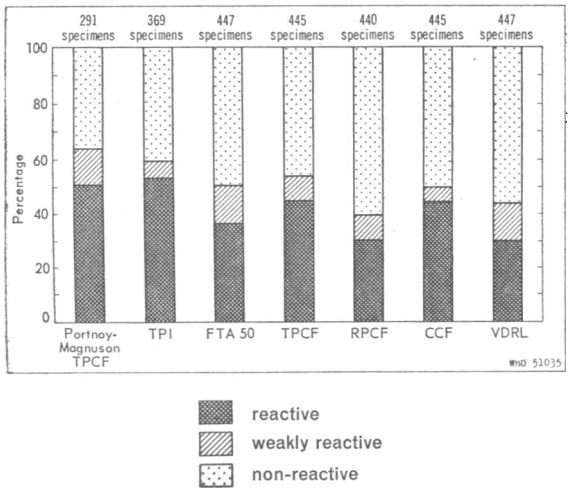
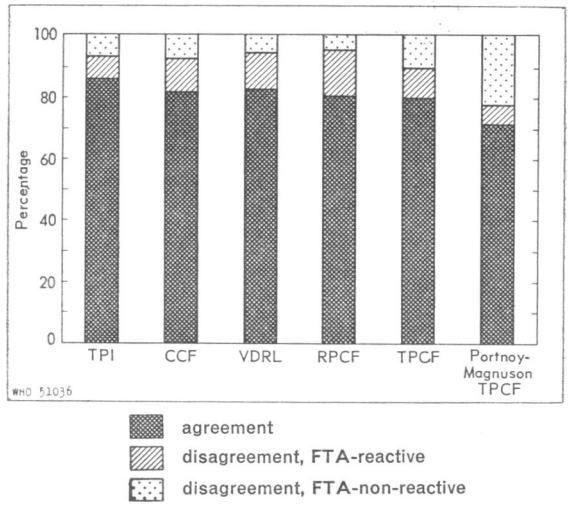


FIG. 6

PERCENTAGE AGREEMENT BETWEEN FTA-50 TESTS AND OTHER TESTS



DISCUSSION

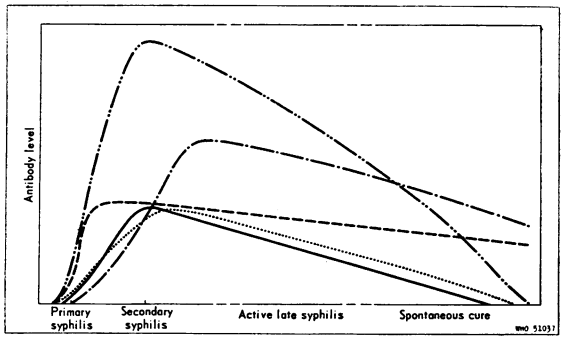
It seems that the FTA test detects antibodies that are not indentifiable, or only partly identifiable, by other methods.

The antigens involved are not identical with the lipoidal antigens of *T. pallidum*. The sera of rabbits immunized with VDRL antigen are non-reactive in the FTA test (Deacon & Freeman, 1960; Pillot & Borel, 1961; McLeod & Garson, 1962). The FTA titre of sera absorbed with VDRL antigen does not alter (Deacon & Freeman, 1960; Mannucci & Spagnoli, 1961; Pillot & Borel, 1961; Wilkinson, 1961). The dissimilarity of the FTA and VDRL antigens is also confirmed by the practical results, since biologically false positive sera are non-reactive in the FTA test (Delacrétaz & Frenk, 1961; Wilkinson, 1961).

It is unlikely that the FTA antigens are identical with those involved in TPI testing. This question cannot be solved by serum absorption, since all the antibodies reacting with the surface antigens of the treponemes are removed along with the immobilizing antibodies. Decisive evidence in favour of the separate identity of the antigens is given by the difference in speed of reaction between the two tests: immobilization is a slow process in contrast to the rapidity of the FTA test. There is also a difference in the rate of production of the two antibodies following syphilitic infection. The FTA and TPI titres do not show parallel changes: the FTA titre correlates better with that of reagin (Wilkinson, 1961). Incongruities noticed by the authors in latent syphilis cannot be explained by the difference in sensitivity between the two tests, but rather by the formation of at least two independent antibodies.

So far, two antigens participating in the FTA test have been successfully identified. One of them is the treponemal group antigen also demonstrated in the RPCF test. This explains why *T. reiter* can be used as an antigen in the FTA test (Poetschke & Killische, 1959; Covert et al., 1961). In addition, *T. zuelzerae* and *T. microdentium* (Deacon & Hunter, 1962) and, according to our investigations, the cultivable treponeme strains Kazan 5 and Budapest 4 also contain the common treponemal antigenic determinant, as evidenced by immunofluorescent staining. The other antigen(s) identified are characteristic of *T. pallidum*. The FTA titre of reactive sera absorbed with Reiter treponeme either remains unchanged (Pillot & Borel, 1961; Wilkinson, 1961), or decreases only slightly (Deacon & Hunter, 1962). The specific antigen is present in the thermolabile component of Portnoy-Magnuson antigen, as is shown in immunofluorescent staining by its blocking

FIG. 7
CHANGES IN REACTIVITY TO VARIOUS TESTS DURING THE COURSE OF SYPHILIS



—— serological tests with lipoidal antigens
RPCF
TPCF
TPI
FTA

effect, and with the immune serum prepared with the antigen (McLeod & Garson, 1962).

A few attempts have been made to isolate the fluorescent antibody by serum protein fractionation using continuous-flow paper electrophoresis. Bonelli et al. (1961) found the antibody only in the gamma fractions. Portnoy et al. (1963) detected it by the FTA-5 test in the area covering the beta and gamma zone.

The changing response to the FTA test during the course of syphilis and its relation to other serological tests have been the subject of several papers. The behaviour of the various tests is expressed schematically in Fig. 7. As may be seen, the FTA and the TPCF tests are the first to become reactive after infection. This is particularly evident when low serum dilutions are used in the FTA test. The FTA titre rises rapidly, reaching its peak in the secondary stage before the response to the TPI test reaches a maximum (Montgomery et al., 1960; Borel et al., 1961; Vaisman & Hamelin, 1961). It is about five to six times higher than the titre of immobilizing antibody (Wilkinson, 1961). The FTA antibody level later decreases and—in contrast to the TPI test—the test finally becomes spontaneously non-reactive, even in the majority of untreated syphilitic patients.[1] The sequence in which the tests become negative is as follows: serological tests with lipoidal antigens, RPCF, FTA; the TPI and TPCF tests remain reactive practically throughout life. Treatment commenced in late syphilis does not influence significantly the immunological events. If treatment is commenced in early syphilis, it modifies the serological picture in two respects: (*a*) the antibodies appearing subsequently (immobilizing antibody and antibody binding the treponemal group-specific antigen) may remain non-reactive; (*b*) the other antibodies disappear within a shorter period than in untreated patients.

The sensitivity of the FTA test is greatly influenced by differences in technique: i.e., the serum dilution used, the quality of the TP antigen and conjugate, and the optical equipment. The contradictions in the literature are partly due to such differences. The only attempt to standardize the method was the collective study organized by WHO.[2]

The FTA titre is very high, presumably because fluorescence microscopy is highly sensitive. Its median range, expressed in terms of serum dilution, is from 1:800 to 1:7000 (Deacon et al., 1960; Borel et al., 1961; Wilkinson, 1961; Kent et al., 1962), which many times surpasses the TPI titre, and even with less sensitive techniques it is five to eight times the VDRL titre (Montgomery et al., 1960; Vaisman & Hamelin, 1961). The higher antibody titre does not necessarily mean, however, that the absolute sensitivity of the FTA test is greater than that of other serological tests in all clinical stages of syphilis.

For purposes of comparison, the results obtained by other authors who applied the FTA test to sera from patients with latent syphilis and to " problem " sera were arranged in an analogous manner to our own results: (*a*) according to the percentage of reactive specimens (Table 10); and (*b*) according to the agreement between the results of the serological tests with lipoidal antigens and those of the TPI test (Table 11). With a few exceptions, where lower serum dilutions (1:5 and 1:20) were used (Delacrétaz & Frenk, 1963), the sensitivity of the FTA test was lower than that of the TPI test. The difference is about 12% to 18% in favour of the TPI test (Wilkinson, 1961; Kent et al., 1962). The smaller sensitivity difference of 7.1% found in our studies is explained by the fact that we worked with a serum dilution of 1:50. Thus, previous experience also confirms that the FTA test does not replace the TPI test. It is apparent both from Table 10 and from our own data that the FTA test is more sensitive than the RPCF test; the only exception is

[1] Eng, J., Nielsen, H. A. & Wereide, K.—unpublished mimeographed document WHO/VDT/RES/29 (1963).

[2] Unpublished mimeographed documents WHO/VDT/RES/30, WHO/VDT/RES/31 and WHO/VDT/RES/32.

TABLE 10

RESULTS OF SEROLOGICAL TESTS ON "PROBLEM" SERA AND SERA FROM PATIENTS WITH LATE LATENT SYPHILIS: SUMMARY OF PUBLISHED FINDINGS

Author	Description of sera	FTA method	Number of specimens	Percentage found reactive			
				FTA	TPI	SLA [a]	RPCF
Bellone & Bonelli (1961)	Submitted with comments for TPI testing	FTA-200	106	25	25	25	
	Submitted without comments for TPI testing	FTA-200	43	56	85	46	
Csermely et al. (1962)	From patients with late latent syphilis	?	110	86		43	79
Delacrétaz & Frenk (1963)	Unexpected reactivity with SLA [a]	FTA-20	340	73	74	100	
Fife et al. (1961)	Unclassified; submitted for TPI testing	FTA-5 FTA-5 FTA-100	745	64	62		
Kent et al. (1962)	Unclassified sera and sera from late latent syphilis	FTA-200	119	82	100		87
Tucker et al. (1962)	From patients with late latent syphilis	FTA-5 FTA-200	144	94		100	71
Wilkinson (1961)	"Problem" sera	FTA-200	144	30	40	82	22

[a] Serological tests with lipoidal antigens.

TABLE 11

FREQUENCY OF AGREEMENT AND DISAGREEMENT IN REACTIVITY OF SERA AS DETERMINED BY THE TPI TEST AND BY OTHER SEROLOGICAL TESTS: SUMMARY OF PUBLISHED FINDINGS

Author	Description of sera	FTA method	Number of specimens	FTA test			SLA [a]			RPCF test		
				agreement (%)	disagreement (%)		agreement (%)	disagreement (%)		agreement (%)	disagreement (%)	
					TPI+ FTA−	TPI− FTA+		TPI+ STS−	TPI− STS+		TPI+ RP−	TPI− RP+
Delacrétaz & Frenk (1963)	Unexpected reactivity with SLA [a]	FTA-20	340	97	2	1	74	—	26	—	—	—
Fife et al. (1961)	Unclassified; submitted for TPI testing	FTA-5 FTA-5 FTA-100	745	90	4	6	—	—	—	—	—	—
Kent et al. (1962)	Unclassified sera and sera from late, latent syphilis	FTA-200	119	82	18	—	—	—	—	87	13	—
Wilkinson (1961)	"Problem" sera	FTA-200	144	87	12	1	42	6	52	71	24	5

[a] Serological tests with lipoidal antigens.

the series of Kent et al. (1962), who used *T. reiter* as antigen.

The last 10 cases in Table 9 without any history or clinical evidence of syphilis indicate that the FTA-50 test is not absolutely specific. The probability of obtaining non-specific positive results with " problem " sera is 2.7%. The non-specific staining of *T. pallidum* is not eliminated by the tissue powder treatment of conjugate; it is an intrinsic property of the serum, or possibly of the antigen. There are three possible explanations:

(1) The serum of healthy persons, as shown for the first time by Beck (1939), contains treponemal antibodies; according to our experiments, cultivable treponemes can be agglutinated down to a serum dilution of 1 : 200. When oral or genital treponemes invade the tissues (as in balanitis, or Plaut's and Vincent's angina), the complement fixation test performed with treponemal antigens has been observed to give a transitory positive reaction. Deacon & Hunter (1962) were able to absorb the antibody responsible for the non-specific staining by treating the serum with a suspension of *T. pallidum* or *T. reiter*.

(2) It is possible that a component of the rabbit testicle adheres to the surface of *T. pallidum* and reacts with rabbit antibodies present in the serum to be tested. The correctness of this assumption is made probable by the fact that *T. pallidum* is immobilized by anti-rabbit-testicle goat-immune serum (Julian et al., 1963). It was also noticed that treponemes cultured in media containing human albumins could not be freed from these proteins, and after staining with homologous conjugate a bright fluorescence was observed. The sera of some healthy persons contains complement-fixing antibodies that react with rabbit-tissue antigens (Muschel et al., 1961). If this assumption is correct, it should be possible to eliminate non-specific staining by absorbing the sera with dried powdered testicles of healthy rabbits (Thivolet & Cherby-Graspiron, 1961).

(3) The lipid component of intact *T. pallidum* is serologically inactive; for that reason biologically false positive sera are non-reactive in the FTA test. However, if the *T. pallidum* suspension used is treated as described by Hardy to enhance its agglutinability (Deacon & Freeman, 1960) or if it has deteriorated as the result of prolonged storage, biologically false positive sera may give rise to non-specific staining.

RÉSUMÉ

La valeur sérologique de la réaction de mise en évidence des anticorps tréponémiques par immuno-fluorescence (IF) a été jugée de façon très diverse. Les auteurs pensent que les différences de technique en sont responsables et, particulièrement, que les conjugués utilisés sont une source de variabilité. Ils décrivent la préparation du conjugué immunsérum-isothiocyanate de fluorescéine qu'ils caractérisent par son titre en globuline anti-homme (l'optimum paraît être de 3000 environ), le rapport isothiocyanate de fluorescéine/protéine (10 mg/g) et son action colorante. Au cours de ce travail, la valeur sérologique de l'IF a été étudiée sur 447 sérums posant des problèmes diagnostiques (réactivité sérologique inattendue, séroréactions négatives après traitement, et sérums de malades sans antécédents spécifiques mais cliniquement suspects de syphilis tardive active). Le test d'immobilisation du tréponème (TIT) a été pris comme référence. L'IF effectuée avec ce conjugué a donné une concordance meilleure que celle de chacune des quatre autres réactions sérologiques effectuées en comparaison (VDRL, réactions de fixation du complément avec *Treponema pallidum* et le tréponème de Reiter, sérologie cardiolipidique), et s'est montrée en accord avec le TIT dans 85% des cas. L'IF a également témoigné d'une sensibilité satisfaisante, égale à celle de la réaction de fixation du complément avec *T. pallidum*. Mais elle n'est pas d'une spécificité absolue: les colorations, dans 10 cas, n'étaient vraisemblablement pas spécifiques. Un premier examen des sérums avant le test d'immobilisation paraît réalisable plus simplement et à moindres frais en combinant la réaction de fixation du complément avec *T. pallidum* et la sérologie cardiolopidique.

Bien que procédé largement spécifique et sensible, l'IF, sous sa forme actuelle, ne peut remplacer le test d'immobilisation du tréponème. Elle nécessite d'autres études pour éliminer les colorations non spécifiques et standardiser le conjugué et l'antigène.

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