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EVALUATION OF THE T. PALLIDUM HAEMAGGLUTINATION (TPHA) TEST FOR SYPHILIS ON "PROBLEM SERA"

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Abstract. The sensitivity and specificity of the T. pallidum haemagglutination (TPHA) reaction was compared with other serological tests for syphilis in qualitative (35 samples) and quantitative (42 samples) tests on syphilitic "problem" sera and 32 biologically false positive (BFP) sera. Half of the latter were also reactive with Reiter protein antigen. The sensitivity of TPHA in qualitative and quantitative assay was higher than that of the other treponemal tests (RPCF, FTA and FTA-ABS). In primary syphilis however, it was less sensitive. This fact could be explained by a decreased reactivity of the antigen with the IgM-type immunoglobulins which form the bulk of antibodies at the onset of syphilitic infection. In one-fourth of BFP sera the sorbent furnished with the kit failed to absorb the treponemal group antibodies. Based on a previous study, the frequency of false-positive results with the TPHA test among BFP reactors was calculated as 1–2.3 %, a rate agreeing well with other observations. Depending on the geographical location of the study area, however, essentially higher non-specifically positive rates were reported. The TPHA with reagents available commercially is highly sensitive with syphilitic "problem" and, in Europe, specific with BFP sera. It is easy to perform and it requires neither highly skilled personnel, nor expensive equipment. Therefore, the TPHA can replace the TPI or FTA-ABS test in the verification of "problem" sera.

The serological diagnosis of syphilis is generally confirmed by detection, in sera positive with reagin tests, of antibodies to treponemal antigens. The reference treponemal test is the T. pallidum immobilization test or, when this is not feasible, the absorbed fluorescent treponemal antibody (FTA-ABS) test. Neither is simple to perform. The TPI-test is expensive and laborious; it is therefore restricted to large reference laboratories with well trained personnel. The FTA-ABS test, which is simpler, can be performed only with a small number of specimens at one time and by employing elaborate and expensive equipment.

The T. pallidum haemagglutination (TPHA) test developed by Rathlev (31) and Tomizawa & Kasamatsu (34) is a simple procedure for examining large numbers of specimens without costly equipment. The reagents are available commercially. The test is essentially the haemagglutination reaction as described by Boyden in 1951 (3) using tanned sheep red blood cells coated with a sonicate of pathogenic T. pallidum harvested from rabbit testes. To eliminate false-positive reactions and cross-reacting antibodies, sera to be examined are treated with a sorbent containing sheep red blood cell membrane components, normal rabbit testes, and T. reiteri cell components (35). Using this antigen, an automated quantitative microhaemagglutination assay—the AMHA-TP test—was developed, and evaluated immunologically by Cox et al. (4). The test, though simple to perform, is not designed for routine use but as a verification procedure.

The results of the majority of the authors show that the TPHA test, whether carried out either by a manual or by an automated method, has in general a favourable specificity and sensitivity in comparison with the TPI-test and/or the FTA-ABS test (2, 5, 7, 24, 25, 26, 32, 35, 36, 39) or with clinical diagnoses (13, 27). The TPHA test is easier to perform than either the TPI or FTA-ABS tests and is therefore an attractive simple confirmatory test for syphilis.

Abbreviations:

BFP	= biologically false-positive
CCF	= cardiolipin complement fixation reaction
CF	= complement fixation reaction
FTA	= fluorescent treponemal antibody
FTA-ABS	= FTA procedure using sera absorbed with sonicate of T. reiteri
Ig	= immunoglobulin
PS	= polysaccharide
RPCF	= Reiter protein complement fixation
STS	= serologic test(s) for syphilis
T	= treponema
TPHA	= T. pallidum haemagglutination
TPI	= T. pallidum immobilization
UPR	= Unheated plasma reagin-test

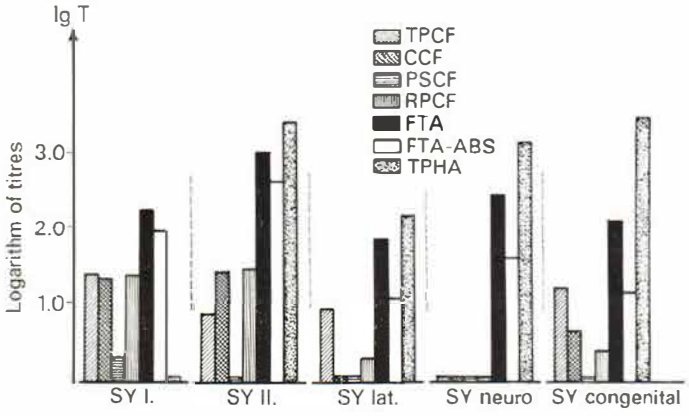

Fig. 1. Geometrical means of logarithmic titres of sera from patients with different clinical forms of syphilis in different STS.

The TPHA test remains positive after specific treatment even after lipoidal tests become negative (37). It is therefore suitable for the retrospective verification of syphilis. The TPHA test is thought, however, to be less sensitive than the FTA-ABS test in primary syphilis (5, 25, 35).

First experience regarding the specificity of the test in biologically false-positive (BFP) reactors with lipoidal tests was very favourable. Later, some doubts arose regarding the absolute specificity of the TPHA-test. First Garner et al. (8) observed that only TPHA was reactive in 17.2% of patients from a yaws endemic area. They explained the findings either as burnt-out yaws or as insufficient absorption of treponemal group antibodies. In a later work (9) they again obtained only in TPHA an impressive ratio of reactive results in BFP (11.3%) and a less impressive one in seronegative leprosy (2.8%) patients. The false-positive results of TPHA and BFP sera seem to be related somehow to the geographical area. In a mainly European population Johnston (13) found only a 2.2% positivity among 400 BFP reactors. Having in view these equivocal results regarding the specificity of this test, the objectives of the present study were:

1. to compare the qualitative results of TPHA and TPI tests on syphilitic sera which several times gave equivocal results with lipoidal tests;

2. to assess the sensitivity of the TPHA test by comparing titres obtained with those of other treponemal tests;

3. to assess its specificity with sera showing a BFP result in lipoidal tests as well as in the Reiter protein complement fixation (RPCF) test;

4. to analyse the antigens and immunoglobulin classes of antibodies involved in the TPHA.

MATERIALS AND METHODS

1. *TPHA reaction*

The antigen used was that manufactured by Fujizoki Pharmaceutical Co. Ltd. It consists of freeze-dried tanned formolized sheep erythrocytes that have been sensitized with a lysate of T. pallidum, Nichols strain. Unsensitized tanned sheep erythrocytes were used as control antigen. In the present study two kinds of kit, which differed in the method of absorption, were used:

(*a*) Lyophilized sorbent consisting of healthy rabbit testicular tissue adsorbed to tanned sheep erythrocytes, homogenate of T. reiteri, rabbit serum, and arabic gum, was dissolved. To 0.05 ml serum, 0.95 ml reconstituted sorbent was added; the mixture was incubated at 2–6°C for 30 minutes and sedimented by centrifugation. The TPHA was performed with the clear supernatant.

(*b*) The diluent contained the sorbent (sonicated sheep and cow red blood cell membrane components, normal rabbit testis and cell components of T. reiteri) in homogenized form. Sera were diluted as directed by the manufacturer for the micro method.

Reconstitution of the sensitized red blood cell suspension was as directed by the manufacturer. The test was performed with Takatsy's microtitrator equipment with cups of V-shaped bottom to allow a proper settling pattern of the erythrocytes to occur. For the qualitative test, the dilutions of sera were 1:80 and 1:160.

The reaction was read after an incubation at room temperature of 22°C (±3°) for 4 hours, followed by an incubation at 4°C for 16 hours. Positive and negative serum controls furnished by the manufacturer were used. In the quantitative test, the titre was the reciprocal of the highest dilution giving a 2-plus sedimentation pattern.

2. *Other serological tests*

TPI test was performed with the Budapest pathogenic strain of T. pallidum with an incubation time of 42 hours (15) as described earlier (20). FTA and FTA-ABS reactions were performed as described (19, 21) with chemically and immunologically well characterized conjugates (method of characterization, see 12). Sonicates of T. reiteri for the absorption of sera and conjugates were produced in our laboratory. The antigens and methods of unheated plasma reagin (UPR)

Table I. *Distribution of results of STS with problem sera according to clinical diagnoses*

Diagnosis	Number of cases	Result	Unheated plasma reagin (UPR) test	Complement fixation		T. pallidum immobilization (TPI) test		*T. pallidum* haem-agglutination (TPHA) test
				Cardiolipin (CCF)	Reiter protein (RPCF)		Not performed	
Syphilis primary (treated and untreated)	11	Reactive	—	3	6	—	6	2
		Weakly Reactive	1	2	5	—		1
		Negative	10	6	—	5		8
Syphilis secondary (treated)	5	Reactive	—	—	2	2	1	2
		Weakly Reactive	—	1	2	—		1
		Negative	5	4	1	2		2
Syphilis latent (treated and untreated)	16	Reactive	4	2	2	11	2	11
		Weakly Reactive	3	2	2	2		4
		Negative	9	12	12	1		1
Syphilis congenital	2	Negative	—	—	—	2		1
		Weakly Reactive	—	—	—	—		—
		Negative	2	2	2	—		1
Syphilis of the central nervous system	3	Reactive	1	—	1	3		2
		Weakly Reactive	—	—	—	—		1
		Negative	2	3	2	—		—
Chronic biologically false positive reactors	32	Reactive	14	20	8	—		3
		Weakly Reactive	4	4	9	—		5
		Negative	14	8	15	32		24
"Problem sera" tested currently	21	Reactive	—	—	—	—	20[a]	—
		Weakly Reactive	2	3	1	—		1
		Negative	19	18	20	1		20

[a] TPI-tests performed are not shown in the tables.

(38) and Reiter protein complement fixation (RPCF) tests were used as described (22). The complement fixation (CF) test used was as described by the author (14).

3. *Examination of immunoglobulin (Ig) class of antibody involved in the reaction*

(*a*) *Precipitation of IgG or IgM from serum.* To a serum pool, strongly reactive in TPHA, and under constant stirring an equal volume of saturated solution of $(NH_4)_2SO_4$ was added. The precipitate was sedimented by centrifugation at 8 000 *g* for 10 minutes. The sediment was dissolved in an equal volume of saline containing phosphate buffer (pH 7.4) and dialysed against the same solution for 36 hours. To a 10-fold dilution of globulin solution an equal volume of commercial anti-IgG or anti-IgM immune serum was added. The mixture was incubated for 30 minutes in a 37°C waterbath. The completeness of precipitation was checked in immunoelectrophoresis by the disappearance of the relevant precipitation lines.

(*b*) *Fractionation* on Sephadex G-200 column of a serum from a patient with treated secondary syphilis and positive in STS was performed as described by Jobbagy (11).

4. *Absorption of sera with polysaccharide (PS) antigen from pathogenic T. pallidum*

100 mg wet weight of T. pallidum was suspended in 1 ml of saline homogenized in Potter and sonicated in a Lehfeld sonicator at 24.3 W/cm² at 0°C for 6–12 minutes. The disruption was controlled under the darkfield microscope. The suspension was autoclaved at 114°C for 30 minutes and an equal volume of 1.0 M NaOH was added. The mixture was kept at 4°C overnight and, after neutralization with 5 N KH_2PO_4, it was centrifuged at 6 000 *g* for 30 minutes. The clear supernatant was dialysed for 48 hours at 4°C against saline containing 1:5 000 merthiolate, changing the dialysing solution twice a day. The activity of antigen was checked in CF with syphilitic serum. 0.1 ml of sera was absorbed by adding an equal volume of dialysate and incubating the mixture under constant stirring for 1 hour in a water bath at 37°C.

5. *Specimens*

Sera used in this study were "problem sera" from our lyophilized serum bank giving equivocal results with different

Table II. *Comparison of TPI and TPHA results on 61 "problem" sera*

TPI results	TPHA results		
	Positive	Negative	Total
TPI positive (among them 6 weakly reactive)	19	1	20
Negative	10	31	41
Total	29	32	61

types of serologic tests for syphilis (STS). Clinical antecedents and epidemiological data were well known. Half of them were of BFP reactors giving positive results with lipoidal and/or Reiter protein antigens. To assess the overall performance of the TPHA test, 21 sera were tested from the current problem material sent to our laboratory for verification.

RESULTS

1. *Sensitivity of TPHA*

1.1. *Comparison of serum titres*

Quantitative tests were performed with 48 sera (primary syphilis 9, secondary 16, latent 15, syphilis of the nervous system and congenital syphilis 4 cases each). The geometrical means of titres are shown in Fig. 1. Except for primary syphilis, the titres in other forms of syphilis were essentially higher than those of other tests, the means exceeding even that of FTA, showing a high sensitivity of the method and good quality of antigen batches examined.

Table III. *Titre of sera in TPHA before and after absorption with polysaccharide treponemal antigen*

Number of serum	Diagnosis	Titre in TPHA	
		Before absorption	After absorption
102	Syphilis II treated	5 120	5 120
103	Syphilis II treated	5 120	5 120
106	Syphilis II treated	1 280	1 280
107	Syphilis II treated	320	320
5-8	Syphilis latent treated	5 120	3 200
S-22	Syphilis latent treated	80	80
S-28	Syphilis latent treated	80	80
S-10	Syphilis of the nervous system treated	1 280	1 280
S-74	Syphilis of the nervous system treated	80	80
8-87	Biologically false-positive	80	80

1.2. *Comparison in qualitative testing*

Qualitative tests were performed with 37 syphilitic sera (Table I). TPHA was reactive with 25, RPCF with 20, cardiolipin CF with 10 and UPR with only 9 sera. Except for primary syphilis, the TPHA was more frequently reactive than the other tests compared.

1.3. *Comparison with TPI-test*

The results of the TPHA and TPI tests are compared in Table II. Of the 21 sera positive in the TPI-test, the TPHA was non-reactive with the serum of a patient with congenital syphilis. This serum was also non-reactive with the other STS. Taking the sensitivity of the TPI-test as 100%, the sensitivity of the TPHA test in the verification of problem sera was 95%.

2. *Specificity of TPHA*

32 BF sera, 17 of which were positive in RPCF procedure, were examined (Table I). The TPHA was reactive in 8 (3 + + + and 5 + +). With these sera the sorbent (diluent) did not block the antibodies reacting with the treponemal group antigen. The rate of false-positive TPHA results occurring in "problem" sera was estimated—as based on 21 sera sent currently to our Laboratory where syphilis had been excluded by earlier TPI-testing and also by epidemiological investigation. Of these, TPHA was positive in only one patient; TPI was non-reactive. The exact diagnosis of the patient was not established. The probability of false-positive reactivity with our problem sera may have been 5%.

3. *Immunochemical examinations*

3.1. *Titres of sera after absorption with treponemal PS antigen*

These remained the same as before absorption (Table III). There was only one dilution step difference in a single case. It could be concluded that the bulk of antigen coating the surface of red blood cells was of proteic nature.

3.2. *Ig class of antibodies reacting in TPHA*

As shown with the pooled serum absorbed with anti-IgG and anti-IgM globulin (Table IV), as well as

in the fractions of gel-filtered serum (Fig. 2), the bulk of reactivity in TPHA is with the IgG class of globulins. With the gel-filtered serum the TPHA titre in the second IgM-containing fraction was only 1:80, while it was very high with the other fractions containing IgG. The reactivity in fractions 3 and 4 may be due to a reactivity with IgA-type globulins.

3.3. *Kinetics of TPHA-test in primary syphilis*

It is well known that the primary immune response in syphilis is in the IgM class of globulins. IgG-type antibodies appear only later. A follow-up to TPHA in treated primary syphilis may be an additional proof for the IgG nature of antibodies involved in the TPHA test. Mean titres of three sera are shown in Fig. 3 before treatment, and 3 and 6 months after penicillin treatment. The TPHA was non-reactive in untreated primary syphilis. It became reactive, however, (mean titre 300) 3 months after treatment. In the serum samples taken 6 months after treatment, the TPHA test was already non-reactive.

DISCUSSION

There are two crucial criteria for the performance of a new confirmatory serological test: its sensitivity as measured in comparison with the TPI-test and its specificity with BFP sera.

1. *Sensitivity* is expressed in the present study as percentage of TPI positives. Taking this criterion, the performance of the test was 95.2%, with confidence limits ranging between 76.2 and 99.9% (at 95% probably level). From the studies published (7, 8, 13, 23, 35) the sensitivity calculated by us according to the criterion mentioned above was between 88.9 and 99%. The lowest sensitivity (88.9%) was found by Tomizawa et al. (35). On the basis of the sensitivity found, and on the comparison of geometrical means of titres, the TPHA seems to be adequately sensitive to replace the other treponemal confirmatory (TPI, FTA, FTA-ABS) tests.

2. *Specificity* is measured by the non-reactive results found with BFP sera. The performance of TPHA in the majority of studies was excellent, giving a range of false positivity rates between 1.7 and 2.4% only (5, 9, 13, 26). The present study cannot be compared with those previously cited studies, however, as specimens also reactive with lipoidal and Reiter protein antigens were examined. As was shown earlier in a fairly large series (17) that the probability of coincident positivity of both tests in

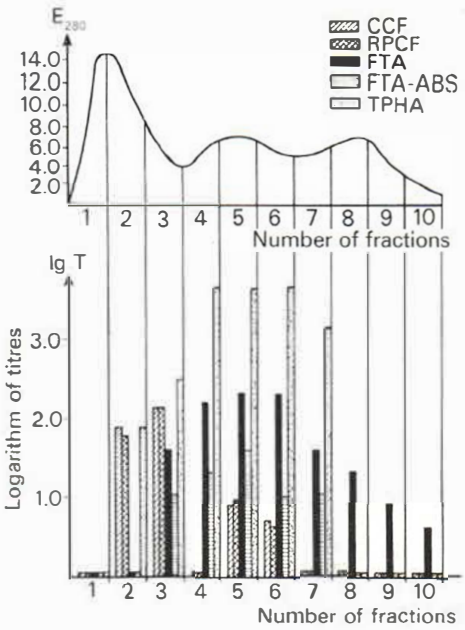

Fig. 2. Upper curve: absorbancy of serum fractions collected from Sephadex G200 column measured at 2 800 Å. As identified by immunoelectrophoresis, fractions 1 and 2 (first peak) contain mainly IgM; fractions 3 and 4, IgA; fractions 4, 5, 6 (second peak) and partly 7, IgG; the remainder, albumin. Lower diagram: reactivity of fractions in different STS expressed as logarithm of titres.

BFP sera is 6.2%, with confidence intervals between 3.7 and 9%. In our present study it was found that the absorption of sera reduced the non-specific result of TPHA to one-fourth, reducing the confidence intervals to 1–2.3%, thus agreeing well with values found in other studies. The ratio of non-specific TPHA results was essentially higher, however, if BFP specimens from ecological conditions essentially different from those in Europe were examined, as was found by Tomizawa et al. (35): 8.3% in Japan; Garner et al. (9): 11.3% in Papua, New Guinea; Ghinsberg et al. (10): 38% in Israel. Regarding the

Table IV. *TPHA titres after blocking of sera with antihuman globulins*

Material tested	TPHA titre
Serum pool	1 280
Globulin solution	5 120
Globulin solution blocked by anti-IgM serum	1 280
Globulin solution blocked by anti-IgG serum	80

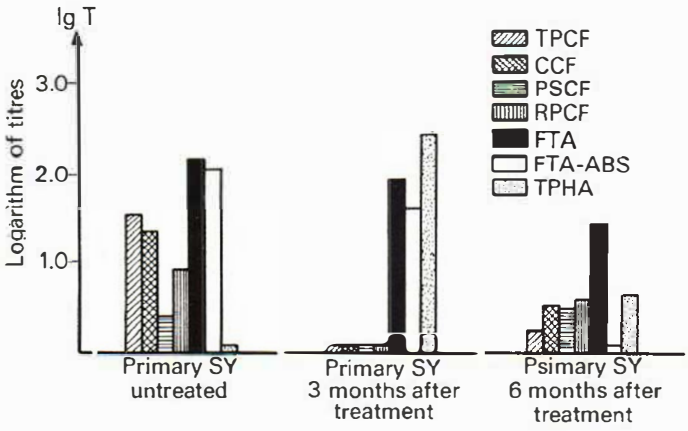

Fig. 3. Geometrical means of logarithmic titres of sera from three patients with primary syphilis examined before and after penicillin treatment with different STS.

FTA-ABS test it was shown (18) that absorption of sera with Reiter treponemes cannot remove all treponemal antibodies. In the present study this possibility was confirmed regarding the TPHA test.

The overall non-specific result rate of TPHA (reactives among healthy populations non-reactive in STS including possibly TPI or FTA-ABS) ranged between 0.3 and 3.2% (2, 5, 7, 13, 26, 35). As the TPHA has not been developed with the aim of serving as a routine STS, this non-specific result is of no practical importance. The rate of non-specific results was very high, however: 11.3% in rural communities in Senegal (33) and 14% in a multipurpose serological survey in Kenya (28). The rate of non-specific positive TPHA results with "problem" sera sent for confirmation was 4.8% in our series (confidence limits 0.12–23.8). The data compiled from different studies containing a larger material are within these confidence limits: 7.5% (6), 11.3% (23), the highest rate (20%) being reported from Israel (10).

As non-specific reactivity in the RPCF test is due partly to the polysaccharidic component, an attempt was made to discover whether polysaccharidic antigens of T. pallidum are involved in the TPHA-test. Under the experimental conditions used, their role could not be proven.

As the TPHA is not proposed as a diagnostic procedure in symptomatic syphilis, its lower sensitivity in primary syphilis is of theoretical importance only. The study regarding the distribution of antibodies according to Ig class gives a clue to the understanding of this shortcoming. The bulk of antibodies produced in early syphilis are found in the IgM class of immunoglobulins. Thereafter a gradual shift takes place to the IgG fraction. This type of immune response was shown for antilipoidal (1), FTA (16, 29), and other types of antibodies, with the exception of TPI (11). The inadequate sensitivity of TPHA in primary syphilis may be explained by a decreased reactivity of human IgM molecules as the preponderant antibody in primary syphilis in the TPHA test. Okamoto & Tanabe (30) made mention of their similar observation: in patients with early syphilis, 19S (IgM) fractions of sera were less sensitive in the TPHA assay. Both observations are at odds with the rule of high sensitivity of haemagglutination in detecting IgM-type antibodies in humans, and with experiments performed on rabbits infected with T. pallidum. Rabbit sera fractionated by ultracentrifugation and gel filtration (30) or sucrose gradient centrifugation (31) displayed TPHA antibodies in the early stages of infection exclusively in the IgM class of immunoglobulins.

CONCLUSION

From previous evidence available and the data obtained from the present study, and under ecological conditions similar to those prevailing in Europe, the TPHA can be considered as a simple and reliable confirmatory reaction for the diagnosis of syphilis to replace the more sophisticated TPI and FTA-ABS tests. When it is used without discrimination to test all sera, however, its specificity is less satisfactory.

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