Treponema pallidum haemagglutination test for syphilis

Evaluation of a modified micro-method

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Passive haemagglutination of erythrocytes sensitized with antigen is an extremely sensitive method for the detection of antibody. Its application to the serological diagnosis of syphilis was first reported by Rathlev (1965, 1967), using a haemagglutination technique for the detection of specific antitreponemal antibody in which formalized tanned sheep cells sensitized with ultrasonicated material from pathogenic *Treponema pallidum* (Nichols strain) were used as antigen. This was modified by Tomizawa and Kasamatsu (1966) and Tomizawa, Kasamatsu, and Yamaya (1969), who introduced a different absorbing reagent to remove cross-reacting antibodies. The *T. pallidum* Haemagglutination Assay (TPHA) was originally described as a macro-method, reagents for which are available commercially. An automated quantitative micro-haemagglutination assay, the AMHA-Tp test, was developed and evaluated by Cox, Logan, and Norins (1969) and Logan and Cox (1970). Tringali (1970), Cox, Logan, and Stout (1971), Uete, Fukazawa, Ogi, Takeuchi (1971), Le Clair (1971), and Garner, Backhouse, Daskalopoulos, and Walsh (1972) have also reported on the clinical usefulness of the TPHA test. Its overall specificity and sensitivity are thought to be comparable to those of the FTA-ABS and TPI tests, although the latter has been used in only a minority of the series reported; it is thought to be less sensitive than the FTA-ABS test in primary syphilis.

In this study an evaluation of a modified qualitative micro-TPHA technique was undertaken to assess its usefulness as a qualitative screening test for the diagnosis of treponemal infection and its specificity and sensitivity compared with the TPI and FTA-ABS tests.

The technique was a manual method based on the automated micro-haemagglutination test for detection of specific treponemal antibodies described by Cox and others (1969). It was found that economy of serum and reagents, so reducing the cost per test, was possible without loss of sensitivity or specificity.

The unit volume (10 μ l. serum) and 30 μ l. reconstituted cell suspension, diluted 1 in 5 in absorbing diluent, was used. This was the critical volume of sensitized and control cells which elicited a full response visible to the naked eye.

Material and methods

SERA

The following groups were examined:

- (1) 1,100 sera selected from those sent to the laboratory for verification tests including both TPI and FTA-ABS tests.
- (2) 1,409 sera falling into various diagnostic categories:
 - (i) Syphilis (309)
 - (ii) Yaws (107). These sera were obtained from pygmies from an endemic yaws area in the Congo.
 - (iii) Presumed normal sera from blood donors (528)
 - (iv) Sera with a high heterophil antibody titre from patients with infectious mononucleosis (65)
 - (v) Biological false positive (BFP) reactors (400)

TESTS

Cardiolipin WR, Reiter protein complement-fixation, and VDRL slide tests were performed by the methods described by Wilkinson (1969).

The FTA-ABS technique was based on that described by the Venereal Disease Research Laboratory (1968); the sorbent and antigen were prepared in the laboratory. Sera giving doubtful (+) results or results at variance with those of the other tests were re-tested after absorption with an ultrasonicate of Reiter treponemes in place of sorbent against both *T. pallidum* and Reiter treponemes as antigens (Wilkinson and Wiseman, 1971).

Micro-TPHA technique

ANTIGENS

Sensitized (test) and unsensitized (control) cell suspensions were reconstituted according to the makers' directions and stored at 4°C. for not more than 7 days. For use, portions of these stock suspensions were diluted

1 in 5 in the absorbing diluent provided, so as to give 30 μ l. diluted suspension for each serum to be tested.

10 μ l. inactivated serum was added by an Eppendorf pipette to 190 μ l. absorbing diluent to give a 1 in 20 dilution which was left at room temperature for 30 min. 10 μ l. amounts of this dilution were transferred to two wells of U-type microtitre plates and 30 μ l. volumes of the diluted test and control cell suspensions were added to each with a Hamilton dispensing syringe, giving a final dilution of serum of 1/80. A non-reactive control serum and quantitative tests on a reactive control serum were included in each batch of tests. The plates were tapped gently to suspend the cells evenly, covered to prevent evaporation, and left on a flat surface away from direct sunlight and sources of vibration. A preliminary reading was made after 3 to 4 hours and a final reading after standing overnight. The sedimentation patterns were examined with a Coldlite illuminator giving a magnification of $\times 2$ against a white background. Results were assessed as positive if there was an evenly distributed carpet of agglutinated cells covering the whole of the bottom of the well, as negative if there was a compact button of cells with a clear-cut regular edge, and as doubtful if there was a central ring of cells with a markedly irregular edge. Doubtful results were uncommon and were disregarded in assessing the reactivity of the test. For results to be valid, the individual serum control and the non-reactive serum control should show compact buttons of unagglutinated cells and the positive control serum should be reactive to the expected titre.

QUANTITATIVE TEST

Doubling dilutions of serum in absorbing diluent were prepared from 1 in 20 to 1 in 1,280 in unit volumes of 10 μ l.; 30 μ l. sensitized cells were added to each, giving final dilutions of serum of 1 in 80 to 1 in 5,120, and a similar volume of unsensitized cells to 10 μ l. of the lowest (1 in 20) dilution. The subsequent procedure was as described above. The titre was taken as the highest dilution giving definite agglutination.

Results

In tests on 1,100 problem sera, the reactivity of the TPHA test compared favourably with that of the TPI and FTA-ABS tests (Tables I and II).

TABLE I *Comparison of TPHA and TPI results on 1,100 problem sera*

TPI results	TPHA		Total
	Positive	Negative	
Reactive	332	415	425
Positive	93	10	
Doubtful			
Negative	25	650	675
Total	440	660	1,100

Agreement 96.8 per cent.
Disagreement 3.2 per cent.

TABLE II *Comparison of TPHA and FTA-ABS tests on 1,100 problem sera*

FTA-ABS results	TPHA		Total
	Positive	Negative	
Positive	420	17	437
Negative	7	656	663
Total	427	673	1,100

Agreement 97.8 per cent.
Disagreement 2.2 per cent.

The TPHA test results agreed with those of the TPI test in 96.8 per cent. and with those of the FTA-ABS test in 97.8 per cent.

There were 35 discrepancies (3.2 per cent.) between the results of the TPHA and TPI tests. In 25 of these the TPHA test was positive but the TPI negative; examination of the clinical data supplied showed that eighteen were from patients who had a previous history of treated or untreated syphilis or yaws; the remaining seven had no history or signs of treponemal disease. Ten sera gave a negative TPHA result although the TPI test was reactive; investigation of the records showed doubtful evidence of treponemal disease in four. Thus, among the 35 sera giving discrepant results, there were thirteen from patients with no clinical signs or history of treponemal infection. In six of these the TPHA and FTA-ABS tests were positive but the TPI negative, and in four TPI positive sera the other two tests were negative.

A summary of the findings in the second group of 1,409 sera is shown in Table III (overleaf). The greatest discrepancy between the TPHA and FTA-ABS tests was seen in the 27 patients with darkfield positive primary syphilis; in these, the TPHA test was positive in nineteen and the FTA-ABS test in 23. Both tests were positive in all of 27 sera from patients with darkfield positive secondary syphilis. The tests also showed good agreement on sera from patients with latent and late syphilis. In tests on 107 FTA-ABS positive sera from pygmies from an endemic yaws area in the Congo, presumably untreated infections, the TPHA test was positive in all. A comparison of the VDRL and TPHA titres of this group of yaws sera is shown in the Figure (overleaf).

The group of non-syphilitic sera included 528 from presumed healthy blood donors with negative VDRL slide and FTA-ABS tests, and 400 from patients with positive lipoidal antigen tests but negative TPI and FTA-ABS tests who had no history of treponemal disease; the latter were classed as biological false positive (BFP) reactors.

TABLE III *Reactivity of the TPHA test in 1,409 sera of various clinical categories*

Clinical category	No. of sera	TPHA		
		Positive	Negative	Per cent. reactivity
Primary syphilis (untreated)	27	19	8	70
Secondary syphilis (untreated)	27	27	0	100
Secondary syphilis (treated) ^a	85	85	0	100
Early latent syphilis (treated) ^a	90	81	9	90
Late and congenital syphilis (treated)	80	76	4	95
Other treponemal disease (untreated yaws)	107	107	0	100
Total	416	395	21	94.8
Blood donors	528	3	525	0.57
BFP reactors	400	9	391	2.2
Infectious mononucleosis	65	0 ^b	60	0
Total	993	12	976	1.2

^aMost had been treated recently

^bFive sera with high heterophil antibody titres (>1,000) gave inconclusive results as they agglutinated both control and test cells.

FIGURE *Comparison of TPHA and VDRL titres in 107 sera from Congolese pygmies presumed to have untreated yaws*

Three of the sera from donors gave positive TPHA tests; one was from a man with chronic bronchitis and a previous history of a positive Wassermann reaction, one from a homosexual with a history of treated gonorrhoea, and no information was available regarding the third.

Nine sera in the BFP group gave reactive TPHA tests; four were from coloured immigrants and no clinical history was obtainable for the other five.

65 sera were examined from patients with confirmed infectious mononucleosis; all were checked for the presence of heterophil antibody (HA) and had non-reactive FTA-ABS tests. Five sera with a HA titre of more than 1 in 1,000 gave inconclusive results as they agglutinated both sensitized and

control cell suspensions. The other sixty specimens gave negative TPHA tests.

Reproducibility of the micro-TPHA technique was checked by repeating tests on 200 sera (100 syphilitic, 100 non-syphilitic). Concordant results were obtained in 97 per cent. No prozone reactions were found on quantitative testing of 150 sera, so that a qualitative screening test at a dilution of 1 in 80 is unlikely to miss any reactive specimens.

The micro-TPHA technique was found to give positive results in tests on a small number of sera from mice and rabbits experimentally infected with *T. pallidum*.

Discussion

In the past decade numerous technological advances in syphilis serology have been made using more sophisticated equipment and systems. In assessing the value of yet another test there is need to exercise discrimination. 'The question is', to quote Humpty Dumpty, 'which is to be master—that's all!' Specific treponemal tests are not designed for routine use, but as verification procedures to be carried out in problem cases. They are of value in distinguishing between specific treponemal reactions and BFP reactions, when the standard tests are positive in the absence of any clinical evidence of infection, and as an aid to diagnosis of treponemal disease in patients whose sera show negative or inconclusive results with lipoidal antigen tests. To-day the accepted confirmatory tests for specific treponemal antibodies are the TPI and FTA-ABS tests. The FTA-ABS test is technically simpler and more economical than the TPI test; it is probably the most sensitive specific test available for the detection of syphilis,

particularly early syphilis, and has the added advantage that it can be used to detect the different classes of immunoglobulins. But, as the results of this survey have shown, the very high sensitivity, specificity, economy, and ease of performance of the TPHA technique merit consideration of this test as a useful alternative in the panel of confirmatory tests available for serological diagnosis. Expensive equipment and skilled staff are necessary for the execution of the TPI and FTA-ABS tests and for the interpretation of results. Criticisms have been made of the immunological specificity of the sorbent used in the FTA-ABS test by several workers (Cannefax, Hanson, and Skaggs, 1968; Rathlev, 1968; Wilkinson and Ferguson, 1968), and of its failure to remove all group reactive antibody from some sera (Király, Jobbágy, and Kováts, 1967; Wilkinson and Wiseman, 1971). False positive FTA-ABS tests have also been reported (Fife, 1964; Buchanan and Haserick, 1970; Kraus, Haserick, and Lantz, 1970).

The results obtained in this survey confirm the findings of the earlier workers already cited that the TPHA test is slightly less sensitive than the FTA-ABS test in early primary syphilis; this is also true of the TPI test. Table III shows that the TPHA test has a very high reactivity at other stages of the disease. It is probably slightly more sensitive than the TPI test in late and congenital syphilis.

From tests on 150 sera, the practical information gained from quantitation as an indication of the progress of the disease or of response to treatment seems to be limited. In general it was found that the TPHA titre in untreated primary syphilis was of the order of 1 in 80 to 1 in 1,280. Thereafter the titres in secondary and early latent syphilis, untreated or treated, are greater than those of late syphilis. In practice, because of the overlap in the range of titres in untreated and treated groups, quantitation of this test is of limited use in follow-up. However, quantitative tests are easy to perform and give reproducible results, unlike quantitative tests with the more complex TPI and FTA-ABS tests. The estimated cost of reagents for a single qualitative test using the micro-TPHA technique described is about 2 pence per test. Unfortunately, the antigens, once reconstituted, have to be used within a relatively short time.

Summary

Qualitative TPHA tests were performed by a micro-method on:

- (a) 1,100 sera presenting serological problems; in all these cases TPI and FTA-ABS tests were done in parallel.

- (b) 1,409 sera comprising specimens from patients with treponemal infection, a presumed normal control group, and sera showing confirmed BFP reactions.

The sensitivity and specificity of the TPHA test compared favourably with those of the TPI and FTA-ABS tests. The test gave reproducible results, was technically easy to perform, and may be said to provide a simple alternative specific treponemal test for laboratories not equipped to carry out the more elaborate TPI or FTA-ABS tests.

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Le test d'hémagglutination au *Treponema pallidum* pour la syphilis. Evaluation d'une micro-méthode modifiée

SOMMAIRE

Des tests TPHA qualificatifs furent effectués par une micro méthode sur:

(a) 1.100 sérums posant des problèmes sérologiques; dans tous ces cas, les tests TPI et FTA-ABS furent faits en parallèle.

(b) 1.409 sérums comprenant des spécimens provenant

de malades atteints d'une infection tréponémique, d'un groupe de témoins supposés normaux et des sérums montrant des réactions biologiquement faussement positives confirmées.

La sensibilité et la spécificité du test TPHA se compare favorablement avec celles des tests TPI et FTA-ABS.

L'épreuve donne des résultats reproductibles, elle fut techniquement facile à effectuer et elle peut être considérée comme offrant un test tréponémique spécifique de remplacement simple pour les laboratoires qui ne sont pas équipés pour effectuer les tests plus compliqués TPI ou FTA-ABS.