Reiter haemagglutination test: a screening test for syphilis

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SUMMARY Using an ultrasonicate of the Reiter treponeme as antigen the Reiter haemagglutination test (RHA) was evaluated as a serological test for syphilis. Comparison of the results of the cardiolipin Wassermann reaction, Reiter protein complement-fixation test, the fluorescent treponemal antibody-absorbed (FTA-ABS) test, the *Treponema pallidum* haemagglutination test (TPHA) (at dilutions of 1/16 and 1/80), and the Venereal Disease Research Laboratory test with those of the RHA showed that the RHA was sensitive (85·8%) and agreed well (85·8%) with the FTA-ABS test result. Simplicity, sensitivity, availability of the antigen, and the very low cost of this test support its use as a first-line screening test for syphilis.

Introduction

The changes that have recently taken place in the serology of syphilis have fortunately tended to make the subject less complicated and more rational than it was in the past. The routine diagnosis of syphilis is still hampered, however, by the inability of investigators to culture *Treponema pallidum* in vitro. Furthermore, the cutaneous manifestations of primary and secondary syphilis are inconstant. In some patients, the first warning of the disease is the appearance of tertiary lesions. Since those of late syphilis may be irreversible, it is important to recognise the disease early.¹ To achieve this we must rely mainly on serological methods to detect antibodies in the patient's serum or cerebrospinal fluid.

Infection with *T pallidum* results in the production of several distinct types of antibodies, those directed against treponemal antigens, which may be specific and group-specific, and those directed against lipoidal antigens, which are non-specific.²

New methods of identifying *T pallidum*, as well as serological tests and modifications of standard tests, continue to be developed. Consequently a large number of tests using treponemal or lipoidal antigens have been developed, but the usefulness of many of these procedures needs confirmation.³

Of the newer methods the *T pallidum* haemagglutination (TPHA) test has been evaluated

widely^{2 4-14} and represents an important development in the field.

The simplicity and reproducibility of the test have been accepted by almost all workers in the field. In principle, the test is very attractive, but the difficulties encountered in growing pathogenic treponemes in rabbit testicles, the time and labour needed, and the consequent high cost of the antigen make the commercially available TPHA kit unsuitable for many routine serological laboratories. We investigated the possibility of coating the red cells for the haemagglutination test with material derived from the Reiter treponeme, which is readily cultivated in the laboratory.

Materials and methods

SERA

Of 208 selected sera, 113 were from patients who attended the special treatment clinic, Manchester Royal Infirmary, and were diagnosed clinically and serologically as having syphilis, and 95 were from presumed healthy persons. Before testing each sample was inactivated by heat at 56°C for 30 minutes.

ANTIGEN

Reiter treponemes (seeding cultures received from Wellcome Company) were grown on Christiansen's¹⁵ modification of Brewer's thioglycollate medium with the omission of agar and resazurin: trypticase 15 g,

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sodium chloride (analar) 2.5 g, D-glucose 5.0 g, yeast extract (Difco) 5.0 g, sodium thioglycollate 0.5 g, L-cysteine HCl 0.75 g, and distilled water to make one litre. After the pH of the medium had been adjusted to 7.2, the medium was autoclaved at 121°C for 15 minutes at 15 lb, cooled at 37°C, supplemented with 10% v/v sterilised inactivated calf serum, inoculated with a 5-10% v/v inoculum, and incubated at 37°C for 5-6 days.

After centrifugation the treponemes were washed four times in 0.1% w/v azide saline and resuspended in distilled water to give a density on Browns opacity standards of 3. The container was immersed in ice and the bacterial suspension sonicated in a Rapidis 300 ultrasonic machine for 10-15 minutes. The preparation was then centrifuged at $500 \times g$ for five minutes or left overnight at 4°C, after which the supernatant was collected and sodium azide added to a final concentration of 0.1% w/v. This cell lysate was divided into 5-10 ml aliquots and kept at -20°C until required. The working dilution of the antigen was determined by sensitising small quantities of formalised tanned red cells with serial dilutions of antigen and testing them with known positive and negative sera in a chess-board titration. Each well of the U microtitre plate contained a mixture of 0.075 ml of serum dilution and 0.075 ml of 0.94% test or control cells in 1.5% w/v normal rabbit serum. The working dilution of the antigen was the one which allowed maximum differentiation between positive and negative sera and which gave the highest titre with the positive serum; the dilution of antigen used was 1/10.

SENSITISATION OF FOWL RED BLOOD CELLS WITH ANTIGEN

Fowl erythrocytes (RBC) were formalised by the method described by Sequeira and Eldridge.⁶ The cells were then suspended in saline to a concentration of 3.75% and mixed with an equal volume of 1/8000 tannic acid solution; the mixture was incubated at 37°C for 15 minutes, the cells were then washed twice with saline and resuspended in phosphate-buffered saline (PBS), pH 6.4, to their original concentration (3.75%). To one volume of cells diluted in four volumes of PBS one volume of a 1/10 dilution of the antigen in PBS was added. The mixture was incubated for 30 minutes at 37°C with occasional shaking. These were termed the test cells. Simultaneously another volume of RBCs was treated exactly as the test cells except that the antigen was replaced by PBS; these were termed the control cells. Test and control cell suspensions were centrifuged at $1000 \times g$ for 4-5 minutes, washed once with saline, and resuspended in two volumes of 1.5% v/v rabbit serum in saline (at a final concentration of 1.875%).

RHA TEST PROCEDURE

For each inactivated serum specimen three wells of a U microtitration plate were filled with serum diluted in PBS (pH 7.2) to final dilutions of 1/4, 1/16 and 1/16. Using a standard 0.025-ml dropper, one drop of test cells was added to well number 2 and one drop of control cells to well number 3. The plate was covered, shaken for 30 seconds, and incubated at 37°C for 45-60 minutes. Positive and negative control sera were included in each group of samples tested and were treated exactly as the sera under test.

Reading of results

Results were read and recorded as follows: strongly positive, smooth carpet of agglutinated cells on the well surface; positive, smooth carpet of agglutinated cells surrounded by irregular margin of cells; and negative, button or definite ring of cells. Where it was difficult to decide whether a sample was positive or negative, the serum was retested at a 1/8 dilution. Serum samples with which control cells did not give a button were absorbed by the addition of 0.1 ml serum to 0.3 ml control cell suspension in a small plastic tube. After incubation at 37°C for 30 minutes the mixture was centrifuged and the supernate used as a 1/4 dilution of the original serum sample. Further test dilutions were made and tested again. If the control cells again did not settle to a button, the test was reported as "no valid result."

OTHER TESTS

Different conventional serological tests for syphilis were carried out on the same serum samples. Cardiolipin Wasserman reaction (CWR) and Venereal Disease Research Laboratory slide test (VDRL) to detect antilipoidal antibodies, Reiter protein complement-fixation (RPCFT) test to detect group antitreponemal immunoglobulin, *T pallidum* haemagglutination test (TPHA) (at 1/80 and 1/16 serum dilutions) and the fluorescent treponemal antibody-absorbed (FTA-ABS) test to detect the specific antitreponemal antibodies were performed. Most of the reagents were obtained from Wellcome Company and the techniques followed were according to the recommendations of the manufacturers; the FTA-ABS test was performed according to the procedure described by Wilkinson.¹⁶

Results

Several serological tests were performed on the same serum specimens. For VDRL tests sera were used undiluted; for the CWR, the RPCFT, and the FTA-ABS test sera were diluted to 1/5; for the TPHA sera were diluted to 1/80 and 1/16 and for the RHA test to 1/16.

The results are summarised in table I. The FTA-ABS test was performed twice independently. The RHA test result was positive in sera from 23 patients who did not have syphilis; in sera from this group of non-infected patients the VDRL and TPHA (at a 1/16 dilution) test results were positive in 16 and 15 patients respectively. The results of the testing of sera from patients with syphilis are shown in the figure.

The percentage sensitivities of each test in the different stages of syphilis and in treated and untreated patients are shown in table II. Both tables show clearly that in all stages of syphilis, both treated and untreated, the results of the TPHA (1/16) and the RHA tests were nearest to those of the FTA-ABS test.

The results of screening with different combinations of two tests are shown in table III. The total number of sera in which either one or both tests gave positive results was compared with the FTA-ABS test results on the same serum samples. From this, truly positive and false-positive results were calculated while false-negative results were determined by subtraction of the number of true positives from 113, which was the number of confirmed positives. The RPCFT and RHA test

FIGURE Results of different serological tests on 113 sera from patients with syphilis (CWR = cardiolipin Wassermann reaction; RPCFT = Reiter haemagglutination assay; FTA-ABS = fluorescent treponemal antibody-absorbed (test); TPHA = *T pallidum* haemagglutination assay).

TABLE I Results of different serological tests for syphilis on 208 human (113 syphilitic and 95 non-syphilitic) sera

Test	Syphilitic sera			Non-syphilitic sera	
	No positive	No negative	% Agreement with FTA-ABS (sensitivity)	No positive	% False-positive
CWR	56	57	49.6	3	3.2
RPCFT	81	32	71.7	6	6.3
RHA	97	16	85.8	23	24.2
FTA-ABS	113	0	100.0	0	0
TPHA (1/16)	97	16	85.8	15	15.8
VDRL	65	48	57.5	16	16.8
TPHA (1/80)	24	89	21.2	2	2.1

CWR = cardiolipin Wassermann reaction; RPCFT = Reiter protein complement-fixation (test); RHA = Reiter haemagglutination (test); FTA-ABS = fluorescent treponemal antibody-absorbed (test); TPHA = *T pallidum* haemagglutination assay; VDRL = Venereal Disease Research Laboratory (test)

TABLE II Comparison of sensitivity of CWR, FTA-ABS, RPCFT, TPHA (1/80), TPHA (1/16), RHA, and VDRL tests* in 113 sera from treated and untreated patients at different stages of syphilis

Stage of syphilis	Treatment	No of cases	% Sensitivity						
			CWR	FTA-ABS	RPCFT	VDRL	TPHA (1/80)	TPHA (1/16)	RHA
Primary	Treated	12	41.7	100	58.3	25.0	16.7	83.3	83.3
	Untreated	4	75.0	100	75.0	75.0	00.0	50.0	75.0
Secondary	Treated	22	50.0	100	81.8	63.6	22.7	90.9	81.8
	Untreated	3	100.0	100	100.0	100.0	66.6	100.0	100.0
Latent	Treated	39	51.3	100	69.2	69.2	28.2	84.6	82.0
	Untreated	8	25.0	100	50.0	25.0	00.0	75.0	75.0
Congenital	Treated	14	42.9	100	71.4	35.7	14.3	92.9	100.0
	Untreated	11	45.5	100	81.8	72.7	18.2	90.9	100.0
Stage not specified	Treated	95	46.3	100	69.5	53.7	21.0	86.3	84.2
	Untreated	18	61.1	100	83.3	77.8	22.2	83.3	94.4

* For meaning of abbreviations see table I

TABLE III Results of screening with different combinations of two tests in 208 selected human (113 syphilitic and 95 non-syphilitic) sera

Type of results	CWR/ RPCFT	RPCFT/ TPHA (1/80)	RPCFT/ TPHA (1/16)	RHA/ RPCFT	VDRL/ RPCFT	CWR/ VDRL	CWR/ TPHA (1/80)	CWR/ TPHA (1/16)	CWR/ RHA	VDRL/ TPHA (1/80)	VDRL/ TPHA (1/16)	VDRL/ RHA
No positive*	92	89	120	131	106	92	67	111	127	87	131	136
Truly positive	89	81	103	104	85	72	63	85	103	68	102	101
False-positive	3	8	17	27	21	10	4	26	24	19	29	35
False-negative	24	32	10	9	28	41	50	28	10	45	11	12

*Either one or both test results were positive
For meaning of abbreviations see table I

combined detected 104 truly positive sera, but there were 27 false-positive and nine false-negative results. The RHA and CWR combined detected 103 truly positive, 24 false-positive, and 10 false-negative results, while the RPCFT and TPHA (1/16) combined detected 103 positive but 17 false-positive and 10 false-negative results.

Discussion

In this series the sensitivities of the CWR, RPCFT, FTA-ABS, and VDRL tests were similar to those reported by other workers, but the most unexpected results were found with the TPHA (1/80) test, an overall sensitivity of 21.2%. Compared with the FTA-ABS test, the TPHA (1/80) has been shown to be highly sensitive by Garner and co-workers¹⁷ (97%), Johnston⁵ (97.8%), Young *et al*⁷ (96.1%), Le Clair⁴ (92.3%), and Luger *et al*¹⁸ (nearly 100%). These workers used the kit from the Fujizoki Company of Japan. Blum *et al*¹⁹ used a kit supplied by Canalco Inc, Rockville, Md, USA, based on the method of Tomizawa and Kasamatsu,²⁰ and found agreement of 90.2% between the FTA-ABS and TPHA tests; they were disappointed by the false-positive and false-negative rates and questioned the reliability of the TPHA. Later work by Garner *et al*²¹ on "problem sera" led them to be less enthusiastic than before. MacFarlane and Elias-Jones⁹ compared Wellcome and Fujizoki kits at 1/80 serum dilutions and found that the former lacked sensitivity. Using a 1/40 serum dilution the Wellcome kit gave improved sensitivity, but both kits gave a high false-positive rate. Using the Fujizoki reagents Alessi and Scioccati¹⁰ found that the percentage sensitivity decreased with increasing serum dilution from 91.1% at 1/20 to 74.4% at 1/80. The use of a macromethod improved sensitivity to 91.6% at 1/80.¹⁰ All other workers have used the micro-technique.

Thus, materials for the TPHA test are not always of comparable quality, and this probably explains our low sensitivity rate with the TPHA test at a serum dilution of 1/80. Since the Reiter treponeme

and *T pallidum* possess common antigens the TPHA test will detect both group-specific and *T pallidum*-specific antibodies unless the group antibodies are absorbed out completely. Except in early infection group antibody activity may equal or even exceed that of the specific antibodies.²² A high serum dilution may result in failure to detect specific antibodies. At a low serum dilution both types of antibody may be present. Using sera diluted 1/80 in the TPHA some positive sera may not be identified; at a lower serum dilution mainly group antibody is detectable.

If the haemagglutination test is detecting group antibody there should be no need to use the expensive *T pallidum* antigen, which can be replaced with Reiter treponeme antigen. The RHA test showed an 85.8% agreement with the FTA-ABS test (and 100% agreement with the Wellcome TPHA). The false-positive rate for the RHA test (24.2%) was high but the false-negative rate (14.2%) compared very favourably with that of the CWR, RPCFT, and VDRL test. The combination of two tests giving the highest rate of truly positive results and least false-negative results was the RHA test and the RPCFT. If the TPHA is used as a screening test only, or if good quality reagents are not available, then it might be more economic to use the RHA test instead. In any case the RHA is a screening test that misses fewer cases than the other screening tests at present in use.

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