

# *Treponema pallidum* immune adherence test for serodiagnosis of syphilis

## 1: An improved method of the TPIA test

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**SUMMARY** Clinical investigations using the *Treponema pallidum* immune adherence (TPIA) test as reported by Nelson (1953) were performed only by Olansky *et al* in 1954. In this paper an improved method of the TPIA test is described. The test was simple to perform and was highly specific for antitreponemal antibody. Furthermore, as it was possible to use heated *T. pallidum* as antigen the antigen suspension could be preserved for long periods. The TPIA test for syphilis was evaluated by examining the sera from 40 healthy subjects and from 166 patients with syphilis.

### Introduction

Of the serological tests for the diagnosis of syphilis the first using *Treponema pallidum* as antigen, the *T. pallidum* immobilisation (TPI) test, was originally reported by Nelson and Mayer (1949). The *T. pallidum* agglutination (TPA) test was described by McLeod and Magnuson (1953). The *T. pallidum* complement-fixation (TPCF) test (Portnoy and Magnuson, 1955) and the Reiter protein complement-fixation (RPCF) test (Cannefax and Garson, 1957) were then developed. Further progress included the development of the fluorescent treponemal antibody-200 (FTA-200) test (Deacon *et al*, 1960) and the improved fluorescent antibody absorbed (FTA-ABS) test (Hunter *et al*, 1964). The *T. pallidum* haemagglutination (TPHA) test developed by Tomizawa *et al* (1969) is specific and sensitive and has been evaluated throughout the world.

The *T. pallidum* immune adherence (TPIA) test was described by Nelson (1953) as the immune adherence phenomenon. This was defined as adherence of specific antigen-antibody and complement-complex to primate erythrocytes or to non-primate platelets; *T. pallidum*, syphilitic sera, and complement are incubated and group O human erythrocytes are added. After being centrifuged, the number of organisms remaining in the super-

natant are counted. A decreased number of organisms is found with positive sera but not with negative sera, because of their adherence to erythrocytes.

This phenomenon has an excellent capacity for antibody detection, and it is widely applied in virology (Ito and Tagaya, 1966; Mayumi *et al*, 1971). However, since Olansky *et al* (1954) reported the results of the TPIA test compared with those of the TPI and the Venereal Disease Research Laboratory (VDRL) tests there has been no wide-ranging evaluation of the test with respect to syphilis. In this paper an improved method of the TPIA test is reported and its value in the diagnosis of syphilis is discussed.

### Material and methods

#### TEST SERA

Sera from 40 healthy persons which gave negative results to the glass plate (see below) and TPHA tests were used as normal controls. Sera from syphilitic patients were obtained from the Tokyo Metropolitan Okubo and Taito Hospitals. All sera were inactivated at 56°C for 30 minutes before use.

#### ANTIGEN PREPARATIONS

The antigen was isolated from syphilitic rabbits which were inoculated in the testicles with *T. pallidum* (Nichols strain). The rabbits, weighing 3 kg, were inoculated intratesticularly with 0.5 ml of a suspension of *T. pallidum* containing 10<sup>8</sup> organisms/ml. After 13-16 days the rabbits were

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killed by exsanguination, and discs with a thickness of 0.2–0.3 mm were prepared from the testes. The tissue slices were suspended in 20–30 ml of sterile saline per testicle. The suspensions were agitated gently and the *T. pallidum* organisms in the tissue were extracted. The extract was centrifuged at 1500–1700 r/min for 30 minutes to remove blood cells and tissue particles. The supernatant was again centrifuged at 12 000 r/min for 60 minutes. The precipitated organisms were washed twice with sterile saline and suspended in a suitable amount of the saline so that a uniform suspension was obtained. The suspension was then heated at 65° C for one hour and stored at 4° C.

#### BLOOD CELLS

Group O human red cells, from fresh blood from healthy persons and from blood stored for transfusion, were used.

#### COMPLEMENT

Pooled, fresh serum from normal guinea pigs was used.

#### TPIA TEST

A modification of the method described by Nelson (1953) was carried out; 0.3 ml of the treponemal antigen suspension, 0.05 ml of the test serum, and 0.05 ml of the complement were reacted at 37° C for 30 minutes (the first phase). To this mixture was added 0.1 ml of red cell suspension. After the mixture was incubated for 30 minutes at 37° C (the second phase) 5 µl of the supernatant obtained by centrifugation at 800–1000 r/min for three minutes was placed on a slide and the number of organisms was counted in 10–15 fields using a darkfield condenser and ×400 magnification. Gelatin veronal buffer (0.07 mol/l) containing 0.15 mol/l NaCl (pH 7.3) instead of test serum was used as the control. The results were expressed as the immune adherence (IA) index calculated by the following formula: IA index (%) =

$$\frac{\text{No. of organisms in the supernatant for each sample}}{\text{No. of organisms in the supernatant for the control}} \times 100$$

#### SEROLOGICAL TESTS

The cell suspension of the TPHA reagent (Fujizoki KK, Tokyo) was used at a 6.5× dilution of the manufacturer's recommendation. For the glass plate test (Mizuoka *et al*, 1973) a modification of the VDRL test was used. Antigen for this test is an alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and 0.3% purified lecithin (Sumitomo Kagaku KK, Tokyo). Antigen-emulsion

(1/60 ml) was added to 0.05 ml inactivated serum on a 1.4 cm diameter circle on a slide and rotated for five minutes at 120 r/min. The degree of precipitation was read by means of a microscope at a magnification of ×100.

#### Results

##### ESTABLISHMENT OF CONDITIONS FOR TPJA TEST

###### *Treatment of T. pallidum*

In the method reported by Nelson (1953) non-heated organisms were used, but in our experiments the organisms were heated at 65° C for one hour and similar results were obtained by both methods. Furthermore, the heated *T. pallidum* could be preserved for longer.

###### *Concentration of T. pallidum in suspension*

At high concentrations of *T. pallidum* counting the organisms at the final step of the test is difficult, and at low concentrations an error of one to two cells may greatly affect the result. Our findings indicated that the optimum antigen concentration was 70–100 cells per visual field for final counting in 10–15 visual fields, since there is a decrease of *T. pallidum* due to the non-specific adherence.

###### *Concentration of red cells*

In the report by Nelson (1953) fresh, human, whole blood was used but this is difficult to preserve, and when whole blood is used non-specific adherence may occur even in the control tube. The effect of using washed cells on the concentration was investigated.

Figure 1 shows the results when various concentrations of red cells were used with sera giving positive results to both the TPHA and glass plate tests. The degree of adherence increased in proportion to the concentration of the cells. The IA indices of sera giving strong or medium positive results attained a minimum value at the concentration of 40% or more. However, as the concentration of 40% was insufficient for some sera giving positive as well as for those giving doubtful-positive results, we concluded that the concentrations of 50% or more were suitable for any serum to be tested.

###### *Concentration of complement*

Nelson (1953) did not need complement because fresh whole blood was used. In our experiments complement had to be added because washed blood cells were used. The optimal concentration of complement, therefore, was investigated with fresh pooled, guinea-pig serum.

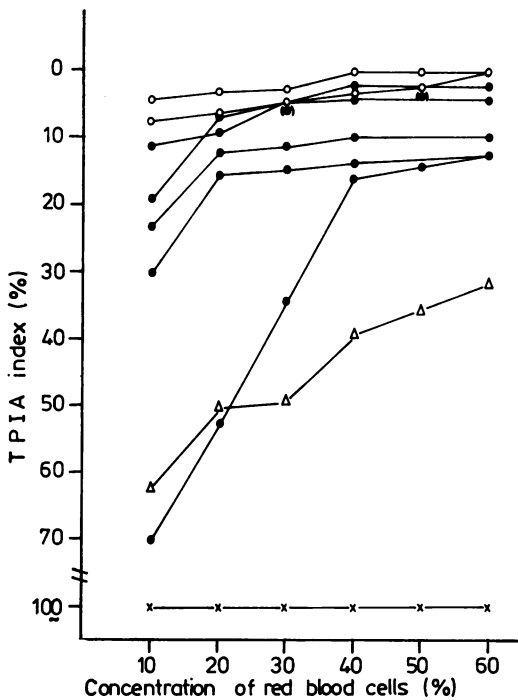


Fig. 1 Relationship between IA index and concentration of human erythrocyte type-O  
 x—x negative sera; o—o and ●—● positive sera; △—△ doubtful-positive sera

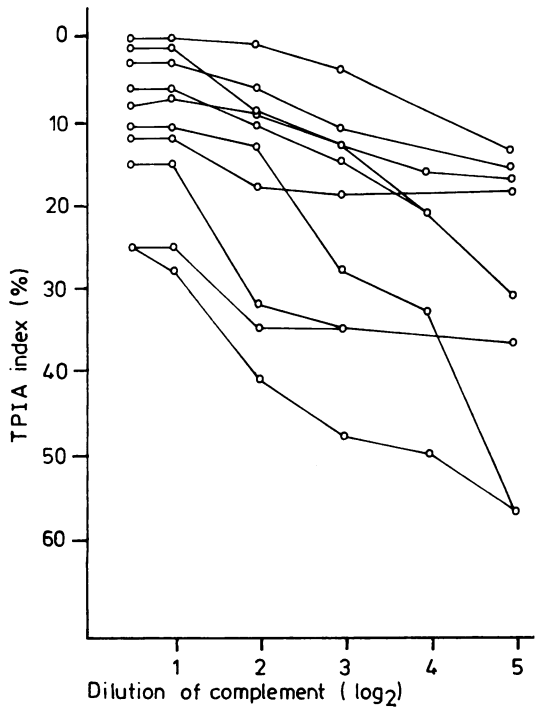


Fig. 2 Relationship between IA index and concentration of complement (Pooled guinea pig serum was used for complement, and its titre measured by 50% haemolysis was 130 CH<sub>50</sub>)

Figure 2 shows the results when various dilutions of guinea-pig serum were used. The complement titre of the serum measured by 50% haemolysis was found to be 130 CH<sub>50</sub> (Kabat and Mayer, 1961). There were no differences in the results for any of the test sera in twofold dilutions of the complement. In cases of dilutions of fourfold or more the degree of adherence decreased in proportion to the concentration of the complement. It was also clear that the amount of complement required increased as the antibody concentration decreased.

*Length of incubation time*

As described above the incubation time in the original method (Nelson, 1953) was in two steps, but we used a single step in order to shorten the test time. The blood cells were added simultaneously to the mixture of the test serum, complement, and antigen. No differences could be observed between the two methods, and we, therefore, concluded that the procedure using the single step was satisfactory.

Figure 3 shows the relation between the incubation time and the degree of adherence in the single-

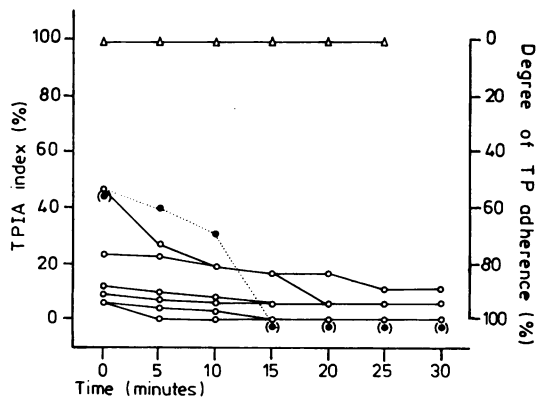


Fig. 3 Relationship between incubation time and degree of immune adherence in the single-step method  
 △—△ negative sera; o—o positive sera; ●—● the degree of adherence of *T. pallidum* in control tube

step method. It is clear that the adherence occurs quicker when the antibody titre is high. In most sera studied the reaction was completed within 20 minutes. However, an incubation time of 30 minutes was used in these experiments.

#### ESTABLISHMENT OF CRITERIA OF REACTIVITY

The TPIA test with the modifications described was performed on sera from 151 cases with positive results to the glass plate test, from 166 cases with positive results to the TPHA test, and from 40 cases with normal sera giving negative results to both tests. The results are shown in the Table. All but one of the healthy controls showed IA indices of 50% or more. However, of the cases with positive sera, 9.3% of sera with positive results to the glass plate test and 4.8% of those to the TPHA test showed values of 50% or more. IA indices of 25% or less were obtained for 83% of the sera giving positive results to the glass plate test and for 88% of the sera giving positive results to the TPHA test. For the evaluation of syphilis by the IA index, we concluded, therefore, that sera with an IA index of 50% or more were negative and those with one of 25% or less were positive. Sera with values between 25% and 50% were interpreted as doubtful-positive.

#### Discussion

Of the serological tests which use *T. pallidum* as antigen the TPI test presents many technical

difficulties and is very expensive. For these reasons the test has not been adopted in some countries. We studied the TPIA test, which is thought to be comparable to the TPI test and for which no systematic research on its clinical applications for syphilis has been reported.

Our results showed that the TPIA test was simple to perform; that, as it was possible to use heated *T. pallidum* as antigen, the antigen suspension could be preserved for long periods; and that the test showed a high specificity. Consequently, we concluded that the TPIA test should be studied as an appropriate technique for serological diagnosis of syphilis.

Large quantities of complement are required for the TPIA phenomenon. In a complement with a titre of 130 CH<sub>50</sub> the adherence activity was stable up to a twofold dilution. When a titre of 230 CH<sub>50</sub> is used no difference was found in the results up to a fourfold dilution. As pooled, fresh guinea-pig serum generally has a titre of about 250 CH<sub>50</sub> it is better to use undiluted serum as complement. In addition, the lack of adherence when commercially available lyophilised complement is used is probably due to the very low titre of the complement. Consequently, when such products are used it is necessary to measure the titre beforehand and to use only samples with a high titre.

It is known that the immune adherence activity of human blood cells varies with different individuals on account of the variation in the amount of C<sub>3</sub> receptor on the cells. The activity of red cells

Table Distribution of IA index of human syphilitic sera

IA index	Results of serological tests							
	TPHA (positive)		Glass plate (positive)		TPHA (positive) Glass plate (negative)		TPHA (negative) Glass plate (negative)	
	No.	%	No.	%	No.	%	No.	%
0	98	59	87	57.6	59	59	0	0
5	20	12	12	7.9	17	17	0	0
10	16	9.6	12	7.9	7	7	0	0
15	5	3	5	3.3	2	2	0	0
20	5	3	7	4.6	4	4	0	0
25	2	1.2	1	0.7	4	4	0	0
30	6	3.6	7	4.6	2	2	0	0
35	2	1.2	2	1.3	0	0	0	0
40	2	1.2	0	0	0	0	0	0
45	1	0.6	1	0.7	1	1	1	2.5
50	1	0.6	3	2	1	1	1	2.5
55	1	0.6	1	0.7	0	0	0	0
60	0	0	0	0	0	0	1	2.5
65	0	0	0	0	0	0	1	2.5
70	0	0	0	0	0	0	3	7.5
75	0	0	2	1.3	0	0	2	5
80	1	0.6	0	0	0	0	3	7.5
85	0	0	0	0	0	0	3	7.5
90	0	0	0	0	0	0	5	12.5
95	2	1.2	3	2	0	0	6	15
100	4	2.4	8	5.3	2	2	14	35
Total	166	100	151	100	100	100	40	100

from 15 healthy subjects was examined. In three cases the cells gave less than half the maximum adherence found with the other samples. When cells from a single donor are used their reactivity should be checked before the test is performed. The use of pooled cells from several persons is recommended as a routine procedure.

The IA phenomenon appears in a short time. As shown in Figure 3 the reaction was completed in five minutes when there were large amounts of antibody, but 20 minutes or more was necessary for small amounts of antibody. To achieve maximum sensitivity incubation should be for 30 minutes at 37° C.

Nelson (1953) set an IA index of 50% as the standard for the diagnosis of syphilis. In these experiments almost all the normal subjects showed IA indices of 50% or more, while more than 90% of the patients' sera showed a value of less than 50%. Although the standard of Nelson (1953), therefore, might be applied directly 93% of the sera from 100 patients with positive results to both the glass plate and the TPHA tests showed IA indices of 25% or less; thus a new standard with an adherence point of 25% was taken to indicate reactivity. Although further research is necessary on the point of disagreement (between 25% and 50%) with the standards established by Nelson (1953) we interpreted the disagreement range as doubtful-positive.

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