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A rapid method of Complement Fixation test for Syphilis

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In spite of many good flocculation tests which have been developed during the past 30 years (Meinicke, Müller, Kahn, Kline, VDRL, etc.) in the field of sero-diagnosis of syphilis, the complement fixation reaction has retained its position.

In the U.S.A. however,the flocculation reaction is generally regarded as the reaction of choice, because it is less time consuming elsewhere the complement fixation reaction is still widely used for sero-diagnosis of syphilis.

The League of Nations has on two occasions recommended the simultaneous performance of one or preferably two flocculation reactions with each complement fixation test for syphilis (Copenhagen 1928, Montevideo 1930).

In some European countries, Germany for example; every flocculation reaction which is performed for the diagnosis of syphilis must be according to the low, accompanied by a complement fixation test.

✡This work is accomplished partly in Serology Department School of Medicine,University of Tehran, partly in « Serotherapeutisches Institute» Wien with collaboration of Frau Seibert and partly in «Forschungsanstalt für Psychiatrie» , Munich, Germany. I wish to express my appreciation to Dr. Kurt Meinicke, Dr. Gerhard Ehrmann, Dr. Gerd. Poetschke and Dr. Freidrich Kail, Miss G. Fakhrai, Dr. G. Nazari for assistance.

The complement fixation reactions are also in great use for the diagnosis of Rickettsiosis, Virosis, Toxoplasmosis and many other parasitic and infectious diseases.

The first complement fixation reaction carried out in 1906 by Wassermann, Neisser and Brück who had devised their inspirations from the Bordet-Gengou phenomenon.

Since then, modifications have been devised to improve both its sensitivity and specifity. These modifications specially concern the hemolytic systems, antigen preparation and the manner of incubating the antigen-serum-complement.

Hemolytic systems currently used comprise human, sheep or rabbit red cells together with their homologous antisera.

Native complement and anti-sheep hemolysin of human serum are also used by some workers (Hecht, Levaditi, Latapie Rubinstein, Gradwhole, Weinberg and Muttermilch).

Antigen preparation has been much improved and modified by the use of either acetone-ethêr insoluble lipids of human, animal heart (guinea pig, horse cow, etc.) or mixture of the alcoholic and acetone soluble lipids of the heart.

Since the discovery by Sachs in 1911 of the sensitizing effect of cholesterol, this agent has become more and more widly used for sensitizing the antigen.

A further improvement was made by Mary Pangborn (1941) who isolated a phospholipid, termed cardiolipid from heart tissue, when mixed with cholesterol and lecithin in suitable proportions, this phospholipid gives the so called «cardiolipin antigen» which is considered by some to give more specific results than lipid antigen.

Suspension of Nichols strain of treponema taken from infected rabbit tests, the growth of Reiter, s sample of spirochet or the isolated protein part of Reiter's treponema have also been used.

The actual technique of carrying out the complement fixation test for syphilis has also been the subject of considerable modifications and improvements, but the number of different methods is so great that it is difficult to enumerate them; however briefly.

Fortunately they can be classified into four groups. These are listed below.

Group 1. includes those which employ a temperature of 37° c. for one hour or more. The Nelson-Mayer T.P.I. test is included in this group.

Group 2. methods using a temperature of 6° c. for four hour or more.

Group 3. Both refrigerator and incubator are used.

Group 4. Agitation is used instead of incubation.

The stirring and rapid methods of complement fixation reaction have been used by Kadish, Navaro-Martin, Hombria, and recently by Portella. All these authors however, have employed vigourous shaking in the performance of their tests.

Now it seems certain that this vigourous manner of shaking the serum-antigen-complement may not only cause dissociation of antibody from antigen but also inactivation of complement wheras a more gentle manner of stirring first the serum antigen mixture and later on mixed with complement can with some advantage replace the short incubation at 37° c. or longer time at 6° e.

As a matter of fact there is no proof that a regular and uniform binding of antibody molecules occurs in either the refrigerator or incubator method but there is good reasons to believe that with gentle shaking of the ingredients rapid and uniform binding of antibody to the antigén will take place if the mixture is shaken of optimum time and optimum speed and temperature.

It is evident that the agglutination and precipitation reactions are enhanced by agitation and this is also true of complement fixation which is an adsorption phenomenon.

It has been recently shown that in Nelson test, the agitation has a good effect on the immobilisation of treponema by bringing about a more efficient and more uniform contact of immobilisin's molecules and the antigen cells.

Furthermore in all methods of complement fixation; the complement is added at or about the same time to the antigen-serum mixture.

These components react one with another not at random, but in an orderly manner, union between antigen and amboceptor must proceed reaction with the complement.

From a consideration of these facts, the author developed in 1953 a stirring method of complement fixation for the diagnosis of syphilis based upon a number of serological and immunological consideration. These are as follows:

1. A gentle manner of stirring that is rotation at the speed of 150-180 revolutions per minute is used in order that the complement is not damaged by too great anagitation.

2. The complement is not added to the other ingredients until the serum-antigen have been stirred for a period of 10 minutes; that is when antigen particles have been uniformly adsorbed and coated by reagin molecules.

3. Flat bottom tubes each containing a glass bead are used in order to bring about a quick and uniform sensitization of the antigen by reagin molecules.

4. To give more specific results, cardiolipin- lecithin- cholesterin antigen or the protein part of Reiter's Treponema are used.

5. A temperature of 35° c. for a period of 10 minutes is employed during which time there is no appreciable change in the complement and is the best for the complement fixation reaction.

6. The whole test is completed in 30 minutes with great economy of time, material and personnel.

7. Only 0, 1 cc. of serum is needed for a qualitative test. This makes it eminently suitable for routin use in public health laboratories and also for examination of samples from small children.

Procedure

Serum

The serum to be tested must be clear and free of hemoglobin. It is inactivated at 56° c. for one half hour.

Complement

Lyophilized and constituted or fresh or preserved guinea pig complement.

Antigen

Cardiolipin - lecithin - antigen is used. The antigen is titrated to determine how much it should be diluted with o,85'/. saline, freshly made and filtered. The purified protein antigen of Reiter's Treponema may also be used with some advantages in this test.

Hemolysin

Immune serum from a rabbit immunized against sheep red blood cells. This immune serum is glycerinated (50 per cent) and diluted 1:100 as follows:

0,85 per cent saline solution. ml. 94.
Phenol 5'/. in saline, ml. 4,0.
Glycerinized hemolysin, ml. 2.o

Saline

0,85'/. solution of pure sodium chloride plus o,1 gm. magnesium sulfate in one litre fresh distilled water.

Red celles

A 2- percent of washed and packed sheep red cells in saline solution.

Meethod of Complement Titration

Flat bottom tubes (fig 1) (9X1, 5 cm.) and Kolmer racks are used. Use tube racks which hold either 72 or 48 tubes in rows of 12 each.

Place 6 tubes, in every other space (starting with the first space) in the first row of tube rack, and in the second row, starting with the second space. a tube on every other space (fig 2)

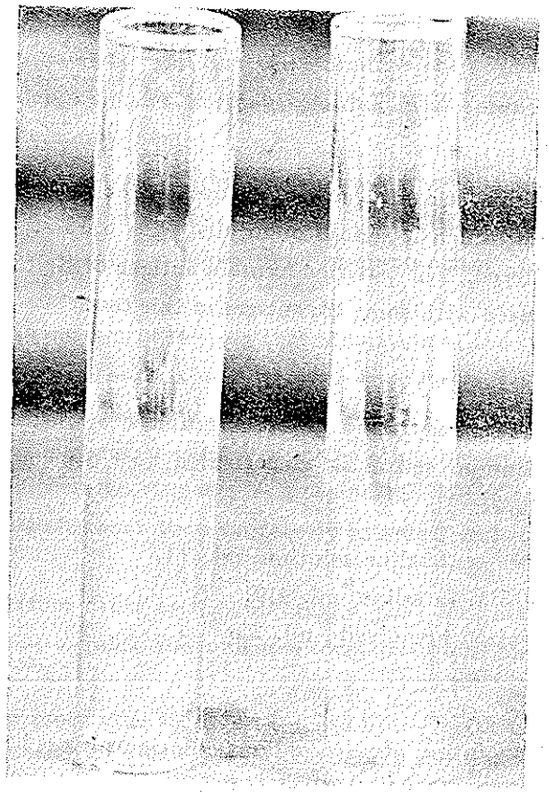

Fig. I. Flat bottom tubes with glass bead.

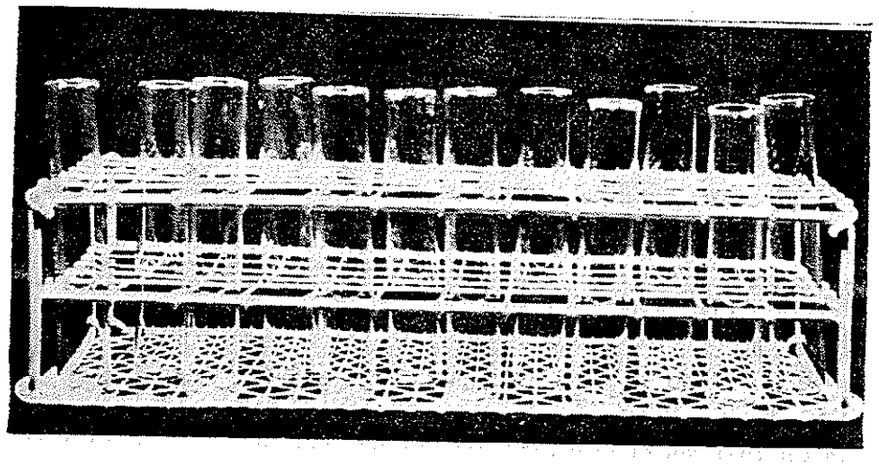

Fig. 2. Initial arrangement of tubes for double dilutions

Thus directly behind every blank space in the first row there will be a tube in the corresponding space in the second row. There are 12 tubes in all. Add o,1 cc. saline to each tube in the first row.

Add o,2 c.c. saline to the first tube in the second row and o,1cc. to every other one.

To the first tubes of the first and the second row, add 0,1 cc. of complement without touching the sides of the tubes. With a fresh two tenth cc. pipette, mix the contents of the first tube in the first row, then transfer o,1cc. from the first tube to the second tube in the first row. Mix well and transfer o,1 cc. from the second to the third tube' mix well and transfer o,1 cc. to the next tube. Repeat serial dilutions to sixth tube, mix the contents of sixth tube and discard o,1cc. cc. In the first row starting with the first tube; the complement will be diluted 1.2, 1.4, 1.8, 1.16, 1.32, 1.64.

Mix the contents of the first tube in the second row. Transfer o,1 cc. to the second tube and discard o,1 cc. Mix the contents of the second tube and transfer o,1 cc. to the third tube, mix well and transfer o,1 cc. to the fourth tube. Repeat serial dilutions to the sixth tube and discard o,1 cc.

In the second row, the complement dilutions will be 1.3, 1.6, 1.12, 1.24, 1.48, 1.96. Transfer the tubes of the second row to the first row, so that the complement dilutions will be in an orderly sequence as 1.2, 1.3, 1.4, 1.6, 1.8, 1.12, 1.24, 1.32, 1.48, 1.64 and 1.96.

Dilute (1.100) hemolysin 1.3000. Wash hemolysin measuring pipette thoroughly in diluent. Shake well. Use a fresh pipette to add o,1 cc. hemolysin to each tube of the complement titration. add one glass bead to each tube.

Antigen

Antigen for this test is an alcoholic solution containing 0,03'/. cardiolipin, 0,9'/. cholestrol and 0,2'/. lecithin which must be titrated according to usual method af antigen titration.

VDRL test antigen can also be used, the already antigen emulsion must be further diluted 1: 16.

Add 0,15 cc. antigen and 0,05 cc. saline to each tube of complement titration, place in 35 °c. incubator shaking machine (150-180 revolutions per minute) 10 minutes. In cold season it is advisable to keep the racks in water bath 37 °c. for 30 seconds before putting them in the incubator shaking machine.

Add o,1 cc 2'/. sheep cells suspension to each tube of the complement titration place in 35 °c. incubator shaking machine (fig 3) (150-180 revolutions per minute) 10 minutes.

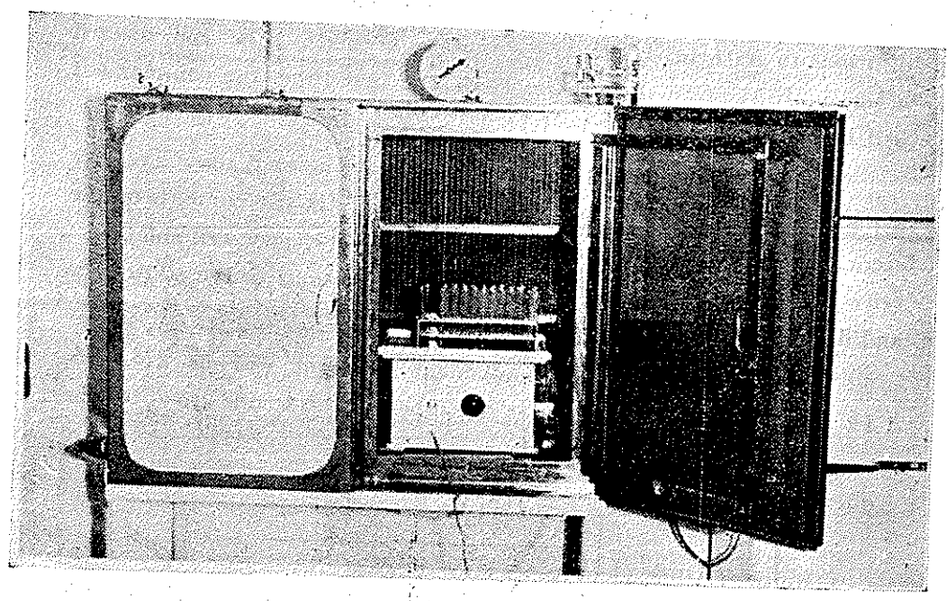

Fig. 3. Incubator shaking machin

Read, the unit of complement is the highest dilution giving sparkling hemolysis. The controls are as follows:

Hemolysin control

0,1 cc. 1: 100 hemolysin.
0,1 cc. 2'/. sheep cells.
0,3 cc. 0,85'/. saline.

Complement control

0, 1 cc. 1:3 complement.
0, 1 cc. 2'/. sheep cells.
0, 3 cc. saline.

Antigen control

0, 15 cc. antigen.
0, 1 cc. sheep cells.
0, 25 cc. saline.

Cells control

0, 1 cc. cells- 0, 4 cc. saline.

Hemolysin titration

Arrange 12 tubes in a rack in the same manner as was done in the complement titration.

Add 0, 1cc. saline to all the tubes of the first row. Add 0, 2 cc. 0, 85'/. saline to the first tube of the second row. Add o,1 cc. saline to the remainig tubes. Add o,1cc 1:100 hemolysin to the first tube of the first and the second row. Use fresh pipette and mix the contents of the first tube of the second row, transfer 0. 1 cc. to the second tube in the second row and discard 0, 1cc. then mix the content of the second tube, transfer 0, 1cc. to the third and continue these serial dilutions until the sixth tube, mix and discard 0, 1cc.

With fresh 0, 2 cc. pipette, mix the contents of the first tube in the first row. Transfer 0. 1cc. from the first tube to the second tube, and transfer 0, 1cc. to the third tube, continue these serial dilutions until sixth tube, mix and discard 0, 1cc.

The first row dilutions will be 1.200, 1.400, 1.800, 1. 1600,1. 3200. 1.6400.

The second row dilutions will be 1.300, 1.600, 1.1200, 1.2400 1.4800, 1.9600.

Combine the two rows of tubes to an orderly sequence of dilutions, in the first row add one glass bead to each tube.

Add o, 15 cc. antigen to every tube of hemolysin titration.

Add o, 1cc. complement diluted so that one unit is contained in o, 1cc. as determined by the complement titration which was completed just before the hemolysin titration was started.

Add 0.05 cc. saline to each tube. Shake 10 minutes. at 35°.

Add o, 1cc of 2% sheep cells to each tube.

Place rack in air inccubator at 35° c. and shake 10 minutes (150-180 revolutions per minute).

Read. The unit of hemolysin is the highest dilution which shows sparkling hemolysis.

Test proper

Pipette 0,05cc. of the inactivated serum into each of two tubes without touching the sides of the tubes (before pipetting the amounts of serum, it is best to mix the serum well by blowing up and down). The back tube will be the serum control. Add o, 15 cc. saline to the back tube. The antigen has already made up according to the titre.

Add o, 15 cc. antigen to front tube. Add one glass bead to every tube, shake well, place in 35° c. air incubator shaking machine(150.180 revolutious per miuute), 10 minutes. Make up complement to be used in the test according to the unit determined by the complement titration.

Add 0,1cc. complement to each tube. Shake in 35°c. incubator shaking machine for 10 minutes' Make up sensitized sheep cells as follows: using hemolytic unit (as determined by hemolysin titration) make up sufficient hemolysin for the test. One unit should be contained in o, 1cc. To one volume of hemolysin, add one volume 2% suspension of sheep cells. Mix well. Add 0,2cc. sensitized sheep cells to each tube. Shake in 35° c. air incubator shaking machine (150_180) revolutions per minute for 10 minutes.

Read if there are many tests to be read, the racks may be placed in 59° c. Water bath for 4 minutes directly after they are taken from the shaker to destroy the remaining complement which would change reading if tests are not read immediately.

For quantitative serum test arrange 7 tubes, add 0.15 cc. saline to the first, 0.15 cc. to the last and 0.1 cc. to the remaining tubes. Add 0.05 cc. inactivated serum to the first and 0.0 5 cc. to the last tube. Mix well the content of the first tube and transfer 0,1 to the second and so on until the sixth tube, discard 0.1 cc. The serum dilutions will be 1.4, 1.8, 1.6, 1.32, 1.64, 1.128.

Add 0.1 cc. antigen emulsion in 3/4 concentration (e.g. 1/12) to all tubes except the last one which will be serve as the control tube, add 0.1 cc. saline to the last tube and the test is then accomplished as the qualitative procedure. The reagin titre is the highest dilution of serum which shows no hemolysis at all, for ex. if it is the fifth tube the reagin unit will be 64.

For qualitative spinal fluid test, the method is the same with this difference that 0. 5 cc. of fresh and well centrifuged fluid is used.

For quantitative spinal fluid test, arrange 7 tubes, add 0.,1 cc. saline to the last and 0.5 cc. to the remaining 6 tubes. Add 0.5 cc. of fluid to the first and 0,5 cc. to the last tube.

Mix well the content of the first tube and transfer 0.5 cc. to the second and so on until the 6th tubes, discard 0.5 cc.

The spinal fluid dilutions will be 1/2, 1/4, 1/8, 1/16, 1/32, 1/64.

The test is then accomplished as the quantitative serum procedure using exactly the same quantities of antigen, complement, and hemolytic system. It is to be noticed here, that in both serum and spinal fluid quantitative tests the last tube, control tube, must be fully hemolysed, otherwise the result of quantitative test is worthless.

The method of complement fixation test for the diagnosis of Rickettsiosis, virosis, toxoplasmosis and echinococosis is exactly the same as for syphilis with this difference that a suitable and well titreted antigen is used in every respect.

Experiments:

a) Comparative results of the modified complement fixation test (MCFT) with Kahn, Müller, Meinicke and Müller-Wassermann. (Ka. Mu. Me. MuW.) on 901 sera.

Negative conformity 351 sera.

Positive conformity 483 sera.

Discordant results 67 sera.

30 sera of the discordant cases were positive in MCFT and negative in Ka Mu. Me. MuW.

23 of these sera showed a positive Nelson reaction. The diagnosis of the remaining were as follows:

1. Suspicion to syphilis (Nelson negative)
2. Syphilis III (Nelson negative : VDRL positive)
3. Syphilis (Nelson negative).
4. Cerebral insultus.
5. Intercostal neuralgia.
6-7. Tabes (Nelson negative).

35 sera were with MCFT negative and in Ka. Mu. MuW, positive. The two remaining were:

1) Latent syphilis (Nelson doubtfully positive).

2) Latent syphilis (Nelson positive).

3) Comparative results of MCFT with Ka, Mu, Me, MuW, and the Nelson test.

From 171 sera which were positive in Nelson test 102 were positive in Ka, Mu, Me, MuW, while 69 sera were negative. In MCFT 119 sera were positive and 52 were negative.

From 239 sera which were negative in Nelson test 44 showed positive results in Ka, Mu, Me, MuW, while 195 were negative.

In MCFT 17 sera were positive and 222 were negative.

C) Comparative results of quantitative MCFT and VDRL test on 1195 syphilitic sera.

MCFT = VDRLT	MCFT < VDRLT	MCFT > VDRLT
310 (26%)	183 (15, 3%)	702 (58. 7,%)

D) Comparative results of MCF and Kolmer tests on 171 sera of emigrant subjects.

	MCFT.	Kolmer test
Possitive (1)	65	42
Weakly positive	25	23
Negative	81	106

E) Comparative results of VDRL and MCF tests on 676 student's sera.

	VDRLT.	MCFT.
Weakly positive	3	3
Positive	0	0
Negative	673	673

By considering that these subjects, mostly medical students are generally considered as normal, one concluds that VDRL and MCF tests has given both 0.5'/. weakly flase positive results.

Summary

A rapid method for carrying out the complement fixation test for the diagnosis of syphilis involving stirring is described.

Flat bottomed tubes each containing a glass bead are employed and stirring is done at a rate of 150-180 revolutions per minute. The test is performed in three times periodes of 10 minutes at 35° c.

Resumé

Une methode rapide de fixation de complement pour le diagnostic de la syphilis a été decrit.

Dans cette methode, des tubes à fond plat ayant un perle de verre dedans sont utilisés.

La reaction s'effectue en 3 periodes de10 minutes et à la temperature de 35° c. touten donnant aux tubes des mouvements de rotation de 150-180 revolutions par minute.

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