A new rapid method for automatic complement-fixation tests

R. VARGUES, C. STUDIEVIC, AND Ph. MAUPAS

From the Faculty of Medicine, Tours, France

SYNOPSIS Several automatic methods have been described for complement-fixation tests. However, each of these methods presents some inconvenience either due to cross contamination or to limited sensitivity. The new method consists basically in separating clearly the fixation stage from the haemolytic stage by using two different sampler plates placed on the same sampler. By this procedure, it becomes possible to obtain a very high sensitivity. The serologist may set the temperature and the duration of the reaction according to the properties of the tests under study. In addition, the sampling rate can be raised to 120 analyses per hour.

Automation of the Wassermann test has already been described. In 1965, a method was proposed simultaneously in Great Britain by Pugh and Gaze and in France by Vargues, Studievic, and Ripault (1965). These initial methods were later improved (Irvine, 1966; Pugh and Gaze, 1966; Smith, Read, and Prytula, 1967) but cross contamination of normal sera by strongly positive sera was still troublesome. In 1968, Vargues proposed a second method which presented many advantages such as the absence of contamination, good reproducibility, and a sufficiently rapid rate of analysis (70 samples per hour). Consequently, the second method, using fixation on the sampler plate, was adopted by several laboratories not only to detect syphilis but also for the diagnosis of brucellosis (Valette and Joubert, 1967) or for antigen titrations (Roumiantzeff, 1969; Vargues, 1965).

Some inconveniences persist in the second method: the fixation phase is very brief (17 minutes at 37°C), and it is difficult to increase the sensitivity because of the small size of the sampler plate where the complement-fixation test is carried out.

Other methods were experimented with (Taylor, Karshaw, and Heimer, 1968) but they did not work properly.

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A new technique has been introduced presenting many advantages. Tests may be run at 120 samples per hour, and the technique increases flexibility in choosing the temperature and duration of the complement-fixation stage so that the true Kolmer technique may be automated (fixation for 16 hours at 6°C).

Equipment

The AutoAnalyzer (Technicon) is used with certain modifications.

The sampler II is modified by the addition of a second plate which is placed above the normal plate by means of a support 13 cm high. The sampler II is provided with a specially made cam. The sampling rate is 120/hour. The sample-to-wash ratio is 1:1, that is, 15 seconds for sampling and 15 seconds for washing. It is possible to use a (90 1:1) cam or a (150 1:1) cam without changing anything in the manifold. There are two inputs on the sampler. First is a drop-by-drop flow bringing antigen and complement into the cups of the upper plate. The second input corresponds to the normal probe which pumps off the contents of cups placed on the lower plate.

The proportioning pump is provided with

Fig. 1 Flow diagram of the automated method of complement fixation showing the transfer system.

Tube 1 (2.9 ml/min) aspirates buffer solution and sends it towards the washing receptacle of the sampler II.

Tube 2 (2.5 ml/min) re-aspirates liquids from the flow cell.

Tube 3 (1.2 ml/min) pumps titrated complement.

Tube 4 (1.2 ml/min) pumps antigen dilution.

Tubes 3 and 4 are joined together and the antigen-complement mixture is directed towards the cups of the upper plate.

Tube 5 (2.9 ml/min) aspirates the contents of the cups placed on the lower plate.

Tube 6 (1.2 ml/min) aspirates red cells suspension.

Tube 7 (1.2 ml/min) aspirates haemolysin dilution.

Tube 8 (1.8 ml/min) delivers air for bubbling.

Tubes 5, 6, and 7 are joined by h-fittings.

several pumping tubes as indicated on the flow diagram (Fig. 1).

A delay coil immersed in a heating bath at 37°C is made of two 40-feet glass coils. It is used for the haemolytic stage, which must last for 10 minutes.

The colorimeter has a 15-mm flow cell and filter of 630 nanometers. The debubbler has to be large enough to prevent the passage of air through the flow cell.

The recorder is equipped with a special motor allowing the chart to unroll at a speed of 45 inches per hour instead of at 18 inches per hour. A motor, rotating at 10 rpm, is substituted for the normal motor rotating at 4 rpm.

Figure 1 shows the equipment.

Technique

Series of analyses for syphilis using two antigens,

namely, cardiolipid and Reiter treponemic protein, are performed as follows.

The sera under test are inactivated at 56°C for 30 minutes. Then, into each AutoAnalyzer standard sample cup 0.1 ml of serum is introduced. Two cups are necessary for each serum, one for the cardiolipid test and the other for the Reiter test.

REAGENTS

These are veronal buffer at pH 7.4 (Mayer and Levine solution), cardiolipid antigen diluted 1/500 to a final dilution of 1/1,000, and treponemic protein antigen diluted in 180. These two antigens must have been titrated either by conventional methods or preferably by the automatic continuous method of Vargues (1965). For each 100 sera to be analysed, 80 ml of each antigen dilution is prepared.

Sheep red blood cell suspension is adjusted to give a turbidity of 50% light transmission corresponding approximately to 0.5/100 ml of packed red cells; rabbit anti-sheep haemolytic serum is diluted 1/1,000; and a complement dilution is prepared according to the results of complement titration.

ADJUSTMENT OF RED CELL SUSPENSIONS

The apparatus is switched on. The delay bobbin is shunted out. Tubes 5, 6, and 7 aspirate buffer solution. The optical zero is adjusted (100% light transmission), then tube 6 aspirates red cell suspensions at 0.5/100 ml. Turbidity is adjusted at 50% light transmission (0.30 optical density by adding buffer or red cells in the suspension).

COMPLEMENT TITRATION

The delay coil is put back in the circuit. Tube 6 aspirates erythrocytes and tube 7 haemolysin. Complement dilutions are prepared manually according to the following scheme:

	Complement Concentration (per 100 ml)									
	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2
Guinea pig serum diluted at										
2/100 ml	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Buffer solution	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Antigen	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

The mixtures are put into cups which are placed on the sampler plate. The automatic complement titration begins by switching on the sampler. Ten minutes later results are recorded as indicated in Figure 2. The correct complement concentration must be of 1 full unit, that is, a concentration slightly greater than the minimal haemolytic dose. Generally, that corresponds to a guinea pig serum diluted at about 1.4/100 ml.

AUTOMATED ADDITION OF ANTIGEN AND COMPLEMENT

Tube 3 aspirates titrated complement. Tube 4 aspirates cardiolipid antigen. The first plate (plate A) receiving the 40 prime sera (nos. 1-40) is placed in the upper position of the sampler, which is switched on. The plate will rotate a complete turn in 20 minutes. The 40 cups will be filled with antigen and complement. Each cup receives 0.6 ml of antigen and 0.6 ml of complement. If a (90 1:1) cam were employed, each cup would receive 1.8 ml of antigen-complement mixture. If a (150 1:1) cam were employed, each cup would receive 0.96 ml of mixture. Consequently the final serum dilution will not be the same with the different cams. The true dilution must be taken into account to express the results by applying the formula given in the discussion.

PLATE TRANSFER

When plate A is filled up, it is placed in the waiting position at room temperature. The

second plate (plate B), with sera from nos. 41-80, is substituted for plate A in the upper position. The filling operation starts again. When plate B has made a complete rotation, it passes into the waiting position, and plate C replaces it in the upper position on the sampler, and the process is repeated.

FINAL COMPLEMENT DETECTION

When it is time to put plate C in the upper position, the fixation phase of plate A is finished, and it is placed in the lower position, and plate B is put above plate A. Simultaneously, the contents of the cups of the lower plate are pumped off for complement detection, while the cups of the upper plate are filled with antigen and complement. Liquids aspirated from the cups of plate A are automatically mixed with erythrocytes and haemolysin. The mixtures pass through the delay coil at 37°C for about 10 minutes. Finally, samples pass through the colorimeter and then to waste.

REGULAR TRANSFERS

When the analyses of plate A are finished, plate B replaces it in the lower position and plate C is put in the waiting position. Plate D is placed in the upper position. In this way, transfers will take place every 20 minutes realizing regular permutations of plates. When all the cups are filled for analysis with cardiolipid antigen, the Reiter antigen is aspirated instead of cardiolipid, and the operation continues until all the plates are filled with Reiter antigen and complement.

RESEARCH OF ANTICOMPLEMENTARY SERA

When cardiolipid tests are performed, it is possible to detect on the chart the sera which completely inhibit haemolysis. Such sera must be analysed to demonstrate a final anticomplementary effect. A plate is prepared with these sera, each cup receiving 0.1 ml of serum. At the time of anticomplementary control, tube 4 aspirates buffer

Fig. 2 Recording of a complement titration followed by a series of complement-fixation tests with cardiolipid antigen.

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instead of antigen. The plate will be filled and treated as the previous plates.

When the anticomplementary plate has been filled, the upper part of the sampler is no longer necessary. The last plate, corresponding to the Reiter antigen, is placed in the lower position, and the anticomplementary plate put in the waiting position. Then, as the last Reiter antigen plate is empty, the anticomplementary control is placed on the sampler (lower position). After that the test is complete.

To sum up, the sampler plates are transferred regularly from place to place. First a plate is filled with 0.1 ml serum cups. Secondly, this plate is placed in the upper position of the sampler for addition of reagents (antigen and complement). Thirdly, the plate is put in a waiting position to increase the duration of the fixation phase. Fourthly, the plate is placed in the lower position of the sampler for the haemolytic stage. Finally, the plate is taken away and cups are thrown away.

As usual, dilutions of positive sera are incorporated in the series of sera under test.

The reaction time for complement fixation is about 40 minutes. The temperature is room temperature but can be raised to 37°C by placing sampler and plates in a hot room.

INTERPRETATION OF RECORDS

The degree of haemolysis is measured by colorimeter. The base line, which corresponds to the washing period between two cups, is at 50% light transmission. Negative sera determine a complete haemolysis, consequently the optical density falls to 90%, which gives a deep valley; positive sera determine no haemolysis, and there is no valley. With weakly positive sera, the valley is not very deep. There is never contamination after a positive serum because the fixation phase is carried out in separate cups. Figure 2 is a representative record.

Discussion

The rapid transfer method presents several advantages compared with the previous methods.

VERY HIGH SENSITIVITY

The antibody activity of a serum can be expressed by calculation of the reaction rate constant at 37°C, according to the formula of Vargues (1966):

$$k_{37^{\circ}} = 2 \left(\frac{37 - T}{10} \right) \cdot 2.3 \cdot \frac{D}{t} \cdot \log \frac{C_o}{C_t}$$

where T = temperature (degree C)

D = serum dilution into the final volume (for example, if serum is positive at 1/100, D = 100)

t = duration of the fixation phase (in minutes)

C_o = initial quantity of complement (in C'H100 units)

C_t = concentration of complement in C'H100 units at the end point of the test.

This formula allows the calculation of the value for D obtained with different techniques versus the same serum (characterized by k_{37°}).

$$D = \frac{k_{37^{\circ}} \cdot t}{2 \left(\frac{37 - T}{10} \right) \cdot 2.3 \cdot \log \frac{C_o}{C_t}}$$

The sensitivity of a technique is proportional to the value of D. The greater is D, the greater is the sensitivity for the same value for k_{37°}. By studying the same serum, D is increasing in four circumstances: when t is increasing; when T is close to 37°; when C_o is close to 1 unit C'H 100; and when C_t is close to C_o. Consequently, to obtain a very high sensitivity the fixation time must be lengthened. The reaction has to be carried out at 37°C (minimal value of 37 - T), only 1 unit of complement should be used, and the reading made at 50% haemolysis.

With the transfer method, sensitivity can be chosen as desired. The values of t, T, C_o and C_t will be modified to obtain maximum sensitivity.

RAPIDITY OF THE SAMPLE RATE

The technique has been described for a sample rate of 120 analyses per hour. The same flow diagram can be used for 90 analyses per hour or even for 150 analyses per hour. The following data give some information concerning the rapidity of analysis:

	Sample Rate		
	90/Hour	120/Hour	150/Hour
Number of teeth of the cam	9	12	15
Duration of washing and sampling	20 sec	15 sec	12 sec
Quantity of antigen-complement mixture in cups	1.8ml	1.2ml	0.96ml
Duration of one rotation of plate	26 min 40sec	20 min	16 min
Interval between 2 sera on the graphs	12.5 mm	9.0 mm	7.2 mm

Employing the (120:1) cam, one Auto-Analyzer alone with a single pen can analyse 400 sera in eight hours with two antigens. A two-pen recorder allows of study of about 800 sera a day.

Whatever the sample rate, the duration of the haemolytic phase remains identical, because the flow volume is invariable.

FACILITY OF GRAPH READING

Among the advantages of serological automation the recording of the results is very interesting. On the graphs it is easy to see immediately the

positive sera because they determine no haemolysis, consequently no valley. The counting of sera is done by series of 40 sera. In fact, at each transfer, the sampler is stopped for one or two minutes. During this time the sample probe aspirates the washing buffer without complement. On the graph this period will be indicated by a durable return to the base line at 50% transmission. Each series of 40 sera is very well distinguished.

RELIABILITY OF THE RAPID TECHNIQUE

The automatic apparatus produces perfect reproducibility because each parameter is exactly defined. The times of the fixation and haemolytic stages are constant; temperature is fixed. For these reasons, it becomes possible to use only 1 unit of complement while many manual techniques employ 2 units for safety.

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