Technique of a Modified Direct Complement-Fixation Test for Viral Antibodies in Heat Inactivated Cattle Serum

by Paul Boulanger*

Many investigators (1-4) have reported failure to demonstrate viral antibodies in heat-inactivated serum of cattle by conventional direct complement-fixation methods. A modified direct complement-fixation test based on the observations of Brumfield and Pomeroy (5) with ornithosis turkey antiserum has been found to be a simple and efficient means to obviate this difficulty with heated viral cattle anti-serum (6). A detailed description of the modified complement-fixation technique used in this laboratory will be presented together with the methods of preparation and standardization of the reagents. Aside from the addition of the unheated bovine serum supplement, these methods follow closely those described in Standard Methods of the New York State Department of Health (7).

Preparation and Standardization of Reagents

All reagents, test serum, antigen, complement, amboceptor and sheep red blood cells suspension, are used in 0.1 ml. amounts, making a total volume of 0.5 ml. in the test.

Sheep Red Blood Cells

Preliminary tests of their red blood cells are made before selecting sheep as donors. The cells should not haemolyse during washing or upon standing for 4 or 5 days in a packed form in buffered salt solution. They should not be agglutinated by the standard dilution of amboceptor nor, when sensitized, be unusually resistant or susceptible to haemolysis in the presence of complement.

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Blood is drawn aseptically from the jugular vein into sterile Alsever's solution (8) and stored in the refrigerator at 6-9°C. It should be allowed to stabilize for approximately a week before use and will keep for 5 or 6 weeks if sterile.

Washing of Red Blood Cells: The red blood cells in Alsever's solution are washed three times in veronal buffered physiological saline (9). The washed packed cells are kept in 15 ml. calibrated centrifuge tubes in the refrigerator for not more than five days. If haemolysis is observed in the supernatant fluid, the cells are discarded.

Preparation of 5 per cent Suspension: The volume of the packed red blood cells is recorded, the supernatant fluid siphoned off and sufficient buffered saline added to make an approximately 5 per cent suspension. The cell concentration is further adjusted by means of a Coleman Junior spectrophotometer. One ml. of the 5 per cent suspension is lysed with 19 ml. of distilled water. The intensity of the colour is determined in the spectrophotometer. The concentration used in the test gives 45 per cent T when the wave length is adjusted at 550 Mu and the transmittance scale at 100 per cent T against distilled water. The necessary adjustments in cell concentration are made by removing or adding diluent.

Sensitization of Red Blood Cell Suspension: To the required quantity of cell suspension is added an equal volume of the optimal amboceptor dilution. The suspension is mixed and allowed to stand for 15 minutes at room temperature before use.

Rabbit Antisheep Amboceptor

This antiserum is prepared in healthy adult rabbits by repeated intravenous in-

jections of a 10 per cent buffered saline suspension of freshly-washed sheep red blood cells in increasing doses of 1 to 5 ml. at 4-day intervals. Fourteen days after the last injection, the rabbits are bled from the ear vein. The serum is heated at 56°C. for half an hour and titrated for haemolysin activity, in serial two-fold dilutions with a 1:50 dilution of guinea-pig complement. Rabbits with a haemolytic serum titre of 1:10,000 or higher are exsanguinated. The serum is preserved by the addition of 1 ml. of 5 per cent phenolysed glycerine to each 9 ml. of serum. A more exact titration is then made to determine the amount required to maximally sensitize the standard volume of 5 per cent sheep red blood cells employed in the test (7).

Titration of Amboceptor: A range of amboceptor dilutions from 1:500 to 1:10,000 is used to sensitize an equal volume of sheep red blood cell suspension. A complement-titration is run as in Table I with each mixture of sensitized cells. The amount of complement for 50 per cent haemolysis is estimated for each titration as shown in Figure I. These amounts are plotted as in Figure II against the respective dilution of amboceptor used. A smooth curve is fitted as closely as possible to the points. The optimum dilution of amboceptor is defined as that containing an amount

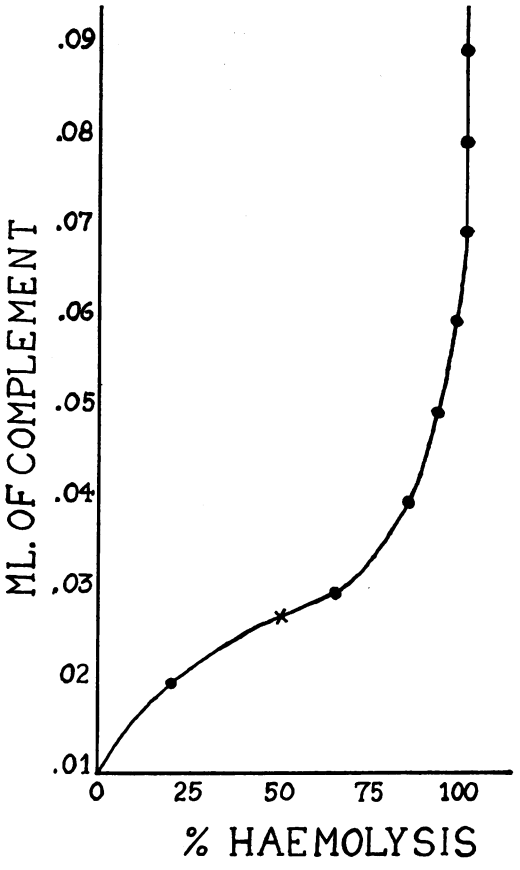

Figure 1.—Estimation of the amount of complement for 50 per cent haemolysis.

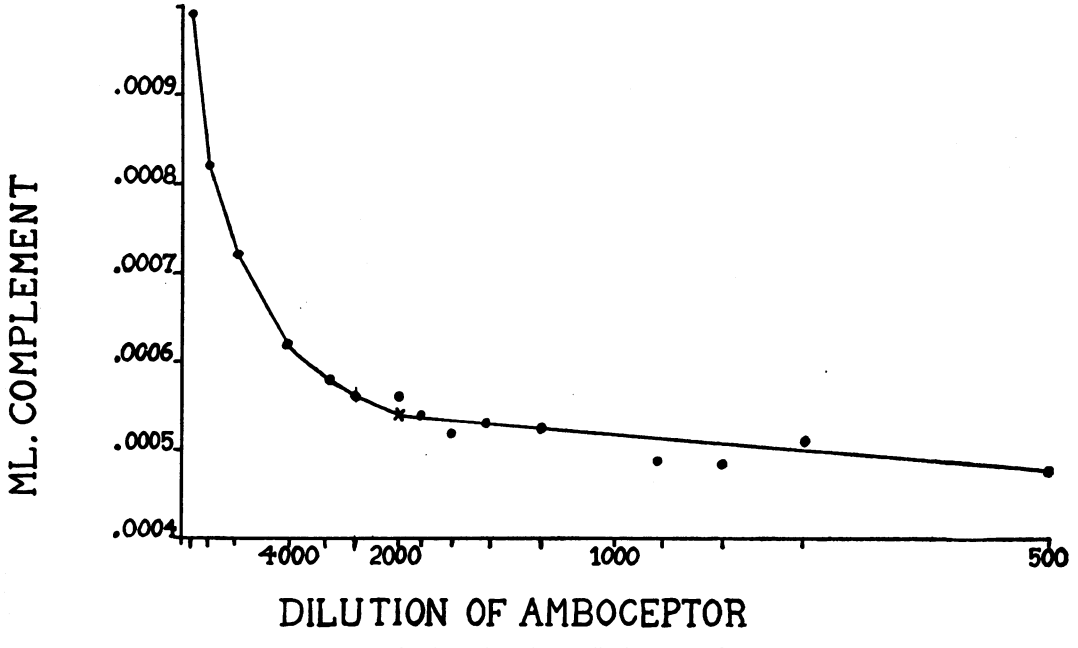

Figure II.—Estimation of optimum dilution of amboceptor.

TABLE I

Complement Titration

Tube No.	Suppl. Compl. (1:50)[x]	Veronal Buffered Saline	Sens. Sheep R.B.C.	Period for Haemol.	% Haemol.[xx]
	ml.	ml.	ml.		
1	0.1	0.2	0.2	30 min.	100
2	0.09	0.21	"	at 37°C.	100
3	0.08	0.22	"	in	100
4	0.07	0.23	"	water-	100
5	0.06	0.24	"	bath	95
6	0.05	0.25	"		95
7	0.04	0.26	"		85
8	0.03	0.27	"		65
9	0.02	0.28	"		20
10	0.01	0.29	"		0

[x]: This test is performed in duplicate.
[xx]: These are representative of the % haemolysis obtained in a typical test.

of this rabbit antiserum beyond which further increase fails to diminish the quantity of complement required for 50 per cent haemolysis. This is 1:2000 in Figure II.

PREPARATION OF COLOUR STANDARD

The percentage of haemolysis in each tube of the test is estimated by comparison with a colour standard containing known proportions of haemolysed and non-haemolysed cells. To prepare the haemoglobin solution, 10 ml. of the sheep red blood cell suspension made for the day's test is centrifuged in a graduated 15 ml. centrifuge tube. The supernatant fluid is removed and the sedimented cells lysed by adding distilled water to the 9.5 ml. mark. The haemoglobin solution is made isotonic by adding 0.5 ml. of 17 per cent sodium chloride solution. The colour standard is then prepared in duplicate as outlined in Table II.

TABLE II

Preparation of colour standard

% Haemolysis	Haemoglobin Solution	Sheep R.B.C.	Veronal Buff. Saline[x]	Reading
	ml.	ml.	ml.	
90	0.09	0.01	0.04	Read
80	0.08	0.02	"	in
75	0.075	0.025	"	suspension
70	0.07	0.03	"	
65	0.065	0.035	"	
60	0.060	0.04	"	Read
55	0.055	0.045	"	
50	0.05	0.05	"	after
45	0.045	0.055	"	
40	0.04	0.06	"	centrifuging
35	0.035	0.065	"	
30	0.03	0.07	"	
25	0.025	0.075	"	
20	0.02	0.08	"	
15	0.015	0.085	"	
10	0.01	0.09	"	

[x]: If either of the reagents is cloudy of slightly coloured, 0.1 ml is added to one row of the colour standards in place of the same volume of saline.

TABLE III

Test on the non-specific reactivity of the non-inactivated cattle serum supplying the necessary heat-labile factor.

Cattle Serum		Anti-gen[x]	Compl. (4.5) Units	Period for Fixation	Sens. Sheep R.B.C.	Period for Haemol.	% Haemol.[xx]
Dilutions	Amounts						
	ml.	ml.	ml.		ml.		
1:5	0.1	0.1	0.1	18 hrs,	0.2	30 min.	45
1:10	"	"	"	at	"	at 37°C.	90
1:20	"	"	"	4-9°C.	"	in	100
1:40	"	"	"		"	water- -	100
1:80	"	"	"		"	bath	100
1:160	"	"	"		"		100

[x]: The antigen is used in the concentration optimum for the test.
[xx]: These are representative of the % haemolysis obtained in a typical test.

Guinea-Pig Complement

Pooled serum from approximately 10 healthy adult guinea pigs, males or non-pregnant females, is the source of complement. The serum from each animal is kept in an ice-water bath while short preliminary tests for haemolytic activity, natural anti-sheep amboceptor and non-specific fixability with the antigen, are made individually. Satisfactory sera are pooled and stored in 2 to 5 ml. quantities in an electric refrigerator at —25°C. Sera should not be stored in a dry ice chest unless adequately sealed.

Titration of Pooled Complement: A sample of pooled guinea-pig serum is thawed, well mixed and a 1:50 dilution made by adding 0.2 ml. of serum to 9.8 ml. of buffered saline supplemented with 5 per cent pretested unheated normal bovine serum. The diluted complement which is kept in an ice-water bath, is pipetted in duplicate in serial amounts, as outlined in Table I. At the end of the incubation period estimations of per cent heamolysis are made in comparison with the colour standard. Tubes with more than 70 per cent haemolysis are read in suspension, the others after centrifugation. The percent haemolysis is plotted for each amount of diluted complement as in Figure I. The unit value, that is the amount which gives 50 per cent haemolysis, is read from the graph. In routine testing, 4.5 haemolytic units of complement are usually added. This amount is sufficient to allow for deterioration during primary incubation and still provide approximately 3 units in the complement controls at the end of the incubation period.

The complement titration is not made in the presence of antigen as has been recommended by other workers as a means of compensating for possible anticomplementary activity. Instead, the anticomplementary activity of the antigen is determined and the product diluted beyond this point or purified by fractionation.

Normal Bovine Serum Factor

A factor in fresh normal bovine serum has been found necessary to promote fixation of guinea-pig complement by certain heat-inactivated cattle serum antibodies with homologous antigen. Sera of cattle under one year of age are preferred as a source of the normal factor because they usually show less non-specificity with tissue antigens than those of adult cattle. The test on non-specific reactivity is illustrated in Table III. Since a 5 per cent concentration of the normal serum is used in the test, no reaction should be observed with this dilution and the optimum amount of antigen. The normal bovine serum is frozen and stored at —25°C immediately after its collection. In this state, it keeps its activity for about three weeks.

When setting up a test, the supplementing bovine serum is added in 5 per cent concentration to the veronal buffered saline which is used to make the required dilution of complement. With each test, a control on the non-specific reactivity of the normal factor with the test antigen is set up in duplicate.

TABLE IV

Test of anticomplementary activity of the antigen.

Antigen		Veronal Buff. Saline	Suppl. Compl. (4.5 units)	Period for Fixation	Sens. Sheep R.B.C.	Period for Haemol.	% Haemol[x]
Dilution	Amount						
	ml.	ml.	ml.		ml.		
Undil.	0.1	0.1	0.1		0.2	30 min.	45
1:2	"	"	"	18 hrs.	"	at 37°C.	100
1:4	"	"	"	at	"	in	100[xx]
1:8	"	"	"	4—9°C.	"	water-	100
1:16	"	"	"		"	bath	100
1:32	"	"	"		"		100
1:64	"	"	"		"		100

[x]: These are representative of the % haemolysis obtained in a typical test.
[xx]: This undiluted antigen being somewhat anticomplementary, should not be used in dilution below 1:4 in the test.

ANTIGEN

The antigens are suspensions or extracts of tissues infected with particular viruses. These infected tissues should be harvested when the viral antigen content is most likely to be at its peak. Most tissues suspensions or saline extracts show some degree of anticomplementary or non specific complement-fixing activity with normal cattle serum. These activities must be removed as completely as possible by methods of purification which do not reduce the specific titre appreciably.

The preparation of vesicular stomatitis viral antigens from chorio-allantoic membranes of chicken embryos will be given as an example. Each membrane is macerated in 3 ml. of infected egg fluid, subjected to sonic vibration for 10 minutes, desiccated and extracted by an acetone-ether method (10). Normal antigens are also prepared in a similar way from non-inoculated egg embryos to serve as control for tissue reactivity in the test serum.

Test of Anticomplementary Activity of Antigen: Serial two-fold dilutions of antigen are tested with 4.5 units of complement as in Table IV. In the test the antigen should not be used in greater concentration than one-fourth its anticomplementary dose.

Test of Haemolytic Activity of Antigen: Some antigens are haemolytic for sheep red blood cells. To check the presence of this undesirable characteristic, a test is set up as in Table IV, except that the red blood cells are not sensitised, the amboceptor being replaced by 0.1 ml. of veronal buffered saline.

Test of Specific Activity of Antigen: This test is set up with serial two-fold dilutions of antigen and an excess of homologous antiserum as shown in Table V. The titre of the antigen is taken as the highest dilution that shows 50 per cent haemolysis or less. Two or three times this amount is used in serum titrations.

Test of Non-Specific Activity of Antigen: The antigen is tested with a series of supposedly-normal cattle sera. This test is set up as in Table V except that normal serum replaces the specific antiserum. A normal control antigen is titrated in similar way.

STANDARD ANTISERUM

Standard antisera are prepared in cattle through experimental infection with the particular virus. Infected bovine tissue should be used as the source of virus tissue. If tissue from another species of animal must be used, it should not be the same as the one from which the antigen is prepared, since the animal is likely to produce antibodies both to the viral agent and to the tissue constituents. The serum is stored at —25°C. Before testing it is inactivated at 56°C for 30 minutes.

Titration of Antibodies: Serial two-fold dilutions of the antiserum are titrated with an optimum amount of antigen as illustrated in Table VI. The anticomplementary activity of the antiserum is determined by replacing the antigen dose with 0.1 ml.

TABLE V

Test of specific activity of antigen

Antigen[x]		Suppl. Compl. (4.5 units)	Anti-serum[xx]	Period for Fixation	Sens. Sheep R.B.C.	Period for Haemol.	% Haemol.[xxx]
Dilution	Amount						
	ml.	ml.	ml.		ml.		
1:2	0.1	0.1	0.1		0.2	30 min.	0
1:4	"	"	"	18 hrs	"	at 37°C	0
1:8	"	"	"	at	"	in	0
1:16	"	"	"	4-9°C.	"	water-	0
1:32	"	"	"		"	bath	0
1:64	"	"	"		"		5
1:128	"	"	"		"		45
1:256	"	"	"		"		95

[x]: With anticomplementary antigens the initial dilution used is the lowest non-anticomplementary and non-haemolytic dose.
[xx]: Antiserum is used in some excess as determined by preliminary tests.
[xxx]: These are representative of the % haemolysis obtained in a typical test.

buffered saline. The titre of the serum is taken as the highest dilution that shows 50 per cent haemolysis or less in the presence of the antigen. Three to four times this amount is used in titrating the antigen. A control of the presence in the antiserum of reactivity to normal tissue, is set up by duplicating the test with antigen prepared from normal tissue. A serum should not be used in a concentration showing reactivity with normal tissue.

In the outline just given for titration of reactivity of antigen or antiserum, one reagent is tested in constant amount with increasing dilutions of the other. Then the test is repeated reversing the position of the reagents. The "block" form of titration in which increasing dilutions of one reagent are tested against increasing dilutions of the other has the advantage that it gives simultaneously the optimum dilution of each reagent in relation to the other.

Technique of Diagnostic Test

Detection of Antibodies in Serum

The antibodies are detected through their complement-fixing activity with the standard viral antigen. The serum-dilution technique is the simplest and most widely used for routine testing. Serial dilutions of serum are titrated as in Table V in the presence of 4.5 units of supplemented complement and the standard dilution of antigen. The titre is expressed as the highest dilution of serum giving 50 per cent haemolysis or less. A control of the activity of each dilution of serum in the absence of antigen and in the presence of normal antigen should be included for each serum.

In addition, with every test, controls of the complement activity and of the anticomplementary and specific properties of the antigen should be set up in duplicate. The complement controls consist of 0.1 ml. of 1.5, 3 and 4.5 units of complement plus 0.2 ml of buffered saline solution. The control mixture containing 1.5 units of complement should show approximately 50 per cent haemolysis.

By this method, complement-fixing antibodies for vesicular stomatitis virus have been shown to persist in the serum of convalescent cattle at a 1:20 to 1:80 level for approximately three months. They were not demonstrated at the acute stage of the infection but made their appearance at 7 to 9 days following infection. Before that time the diagnostic tests must be oriented toward the detection of virus in extracts of infected tissue.

Detection of Viral Antigen

The serum of convalescent cattle, standardized as described in the foregoing, can be utilized in demonstrating corresponding virus in animal tissue (6). The test is performed as outlined in Table VI except that the antigen is diluted instead of the serum.

More precise evaluation of serum or antigen titre or assay of cross reactivity is preferably made by a more precise quantitative technique (11). This type of test is also usable with supplemented comple-

TABLE VI

Test of specific and anticomplementary activities of serum together with complement and antigen controls

Serum Dilution	Amount	Suppl. Compl. (4.5 units)	Antigen	Veronal Buff. Saline	Period for Fixation	Sens. Sheep R.B.C.	Period for Haemol.	% Haemol.*
	ml.	ml.	ml.	ml.		ml.		
Test Serum								
1:5	0.1	0.1	0.1	0		0.2		0
1:10	"	"	"	"		"		0
1:20	"	"	"	"		"		0
1:40	"	"	"	"	18	"	30	0
1:80	"	"	"	"		"		10
1:160	"	"	"	"	hrs	"	min.	60
1:320	"	"	"	"		"		80
1:640	"	"	"	"	at	"	at	100
1:5	"	"		0.1		"		100
1:10	"	"		"	4-9°C.	"	37°C.	100
1:20	"	"		"		"		100
Pos. Serum							water-	
1:20	0.1	"	"	0		"	bath	0
Neg. Serum								
1:20	0.1	"	"	0		"		100
Antigen Control		"	"	0.1		"		100
Compl. Control		"	0	0.2		"		100
R.B.C. Control		0	0	0.3		"		0

*: These are representative of the % haemolysis obtained in a typical test.

ment (6). In this method, serial dilutions of antiserum are titrated in the presence and absence of antigen with varying amounts of complement. The reverse type of test in which serial dilutions of antigen are tested against a constant amount of antiserum is also possible. All reactions are expressed in terms of the number of units of complement required for 50 per cent haemolysis under the conditions of the test.

Discussion

The modified direct complement fixation technique described was used successfully in demonstrating antibodies in heat-inactivated serum of cattle infected with vesicular stomatitis virus (6). In addition, the test was shown to be sufficiently specific to determine the type of vesicular stomatitis virus causing infection in the animal. It indicated the type of virus in epithelial tongue tissue collected at the acute stage of infection and revealed type-specific antibodies in the serum of convalescent animals.

The main difficulty to overcome in applying the modified direct complement-fixation test, is to maintain a supply of satisfactory, fresh, normal bovine serum. It is essential to utilize a normal bovine serum devoid of non-specific activity with the test antigen.

This method, if applied under other test conditions, might require certain modifications in technical details. The present technique has been described in full detail to illustrate one set of conditions under which the test can be performed successfully.

Summary

A modified direct complement-fixation test which has been found effective for the demonstration of antibodies in heat-inactivated serum of cattle infected with vesicular stomatitis virus, is described in detail. The modification of the test consists in adding a 5 per cent concentration

of unheated normal bovine serum to the diluted complement before its addition to the test. This normal bovine serum must be devoid of non-specific complement-fixing activity with the test antigen. Serum from normal cattle under one year of age is more satisfactory than that of older animals. This serum factor is heat-labile; it may be preserved frozen at —20°C. for about three weeks. It is hoped this test will prove useful in the study and diagnosis of other viral infections in cattle.

Résumé

Une modification de l'épreuve directe de la fixation du complément est décrite en détails. Cette épreuve a été effective pour démontrer les anticorps dans les sérums inactivés par la chaleur provenant de bovins infectés de stomatite vésiculeuse. La modification consiste à ajouter au complément avant de l'incorporer à l'épreuve, un sérum bovin normal non inactivé à 5 pour cent. Le sérum normal de bovin doît être dépourvu de réactions non spécifiques avec l'antigène employé dans l'épreuve. Les bovins de moins d'un an ont été préférés aux sujets plus vieux pour l'obtention de ce sérum. Le facteur présent dans le sérum est thermolabile et peut-être préservé par congélation à —20°C pour une période d'environ trois semaines. Il est espéré que cette épreuve sera utile pour l'étude et le diagnostique de divers infections à virus chez les bovins.

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Studies in Immunity to Experimental Staphylococcal Mastitis in the Goat and Cow

The high incidence of bovine staphylococcal mastitis and the difficulty in controlling this disease has emphasized the need for an evaluation of the degree of protection which might be conferred by prophylactic vaccination. This study was undertaken to examine antibody production in response to various staphylococcal vaccines and to compare the degree of protection which these vaccines would confer against mastitis produced experimentally by the homologous strain of staphylococcus.

On economic grounds, goats were used for the greater part of the experimental work. The results indicated that serum staphylococcal antibody levels were higher and persisted longer in two goats vaccinated with staphylococcus aureus strain 201 formolized cell-toxoid with aluminum hydroxide gel added as an adjuvant than in pairs of goats vaccinated with simple formolized cell toxoid, cells alone, toxoid alone, or with a formolized whole-broth culture.

The adjuvant cell-toxoid conferred a higher level of protection in five goats against challenge by the intermammary innoculation of 10^{-9} living staphylococci of the homologous strain, the goats showing only a mild transient reaction compared with gangrenous mastitis in the controls. This vaccine conferred a similar degree of protection on four cows challenged in a similar manner. A test of separate preparations of cells alone, toxoid alone, adjuvant cells, adjuvant toxoid, cell-toxoid, and adjuvant cell-toxoid for immunizing power against experimental mastitis showed that only those vaccines containing toxoid produced immunity.

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