16681

Conglutinating Complement Absorption Test Compared with Hemolytic Complement-Fixation Reactions Using Q Fever Immune Bovine Serum.

DON M. WOLFE AND LOTTIE KORNFELD. (Introduced by Herald R. Cox.)
(With technical assistance of Lois Gallik.)
From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

The difficulty of evaluating serodiagnostic tests performed on animal serums of various species is well known. In complement-fixation tests sensitivity is largely sacrificed for specificity, and modifications, such as alterations of the units of complement, varying the time and temperature of the incubation period, have not proved entirely satisfactory.

Hole and Coombs[1] reinvestigated the phenomenon of conglutination, first described by Bordet and Streng,[2] and developed a conglutinating complement absorption test[3] which they applied to a study of glanders infection in horses.[4] This test is based on the observation that when heat-inactivated bovine serum and fresh (unheated) horse, cat or pig serum are added to a suspension of sheep red cells, the cells clump together, or conglutinate. Bovine serum plays a dual role by furnishing a natural antibody to the sheep cells for their sensitization, and also a substance termed conglutinin. The reaction of the antibody and sheep cells has the property of absorbing complement. Obviously, the complement in this case must be of a nonhemolytic nature, otherwise the sheep cells would be lysed rather than conglutinated. Fresh horse, cat or pig serum provides such a complement. Thus, conglutinin acts on the sensitized cells which have also absorbed complement and causes them to conglutinate. It is necessary that all 4 of the above components be present before conglutination occurs. In the test proper a known antigen and a suspected immune serum are mixed in the presence of a conglutinating complement. Later the conglutinating system (inactivated bovine serum and sheep red cells) is added to detect the presence of unabsorbed complement. In this test, conglutination may be considered analogous to hemolysis in the hemolytic complement-fixation test.

In the report which follows, the test was applied to Q fever infections in cattle and the results were compared with those obtained by two widely used technics of the hemolytic complement-fixation reaction.

Technics of the Tests. Conglutinating Complement Absorption Test (C.C.A.T.) Diluent. Physiological salt solution adjusted to pH 7.3 with 0.005 *M* phosphate buffer was used throughout the study.

Sheep red cell suspension. Defibrinated sheep's blood was stored in modified Alsever's solution.[5] Before use the cell suspension was standardized spectrophotometrically and diluted to 0.25%.

Conglutinin. As mentioned previously, bovine serum served as the source both of conglutinin and antibody for sheep red cells. Routinely bovine serum is titrated for conglutinin only. Since the sheep cell antibody is always much higher in titer, a sufficient amount of antibody to sensitize the cells is present in the quantity of serum required to cause conglutination.

Conglutinin is not destroyed at ordinary inactivation temperatures. It is easily preserved by sealing small volumes in glass ampoules and, without adding chemical preservatives, storing in the CO_2 icebox. For titration, the serum is thawed, inactivated for 30

[1] Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, **45**, 480.

[2] Bordet, J., and Streng, O., *Zbl. Bakt.*, I. Orig., 1909, **49**, 260.

[3] Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, **45**, 490.

[4] Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, **45**, 497.

[5] Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, **53**, 37.

minutes at 56°C and diluted 1:30 with the diluent. Graduated amounts are placed in tubes, brought to total volumes of 1.2 ml with diluent, and complement (0.4 ml of a 1:40 dilution), and cells (0.4 ml) are then added. Following an incubation period of 30 minutes at 37°C, the tubes are centrifuged for a few minutes at a low speed. After shaking the tubes, the conglutinated cells appear as small clumps in a clear supernate. The absence of conglutination is evident from the homogeneous suspension of cells. The smallest quantity of conglutinin giving complete conglutination is taken as one unit. Four units, contained in 0.4 ml, are employed in the test.

Conglutinating complement. Fresh horse serum, preserved by storing small amounts in sealed glass ampoules in the CO_2 icebox, was used. Its activity was satisfactory even after a period of several weeks. For titration, increasing amounts of complement (in increments of 0.05 ml) of an appropriate dilution are added to 0.4 ml antigen, with sufficient diluent to bring the total volumes to 1.2 ml. After incubation for 30 minutes at room temperature, 0.8 ml of a mixture of equal parts 0.25% sheep cells and bovine serum (adjusted to contain 4 conglutinating units) is added to each tube. Following a 30 minute incubation period at 37°C the tubes are centrifuged, shaken and read. The smallest amount of complement giving complete conglutination is taken as one unit. Two units, contained in 0.4 ml, are employed in the tests.

Antigens. The Q fever antigen, consisting of washed, formalinized suspension of the American Nine Mile strain of *Coxiella burnetii,* was prepared according to the method described by Plotz[6] but with certain modifications, the details of which have been presented elsewhere.[7] The antigens of *Rickettsia typhi* (murine typhus) and *Rickettsia akari* (rickettsialpox) were similarly prepared and used to control specificity. These two were used because at the time they were the only washed rickettsial body antigens available. The 3 antigens were originally titrated with their respective immune guinea pig serums. The Q fever antigen was also titrated with a positive bovine serum which gave titers comparable to those obtained with the guinea pig serum.

The "optimal" antigen dose must be used with the C.C.A.T. because of the tendency for prozone reactions. This dose was determined by cross titration of various dilutions of the antigen in the presence of various dilutions of the immune serum. The smallest amount of antigen showing maximal conglutination titers, without demonstrating prozones, is the optimal dose.

Serums. Immune serums from 34 Q fever infected cattle were obtained through the courtesy of Dr. R. J. Huebner of the National Institute of Health, Bethesda, Md.

Technic of the C.C.A.T. Twofold dilutions of the inactivated serum to be tested are prepared. Two units of complement and the optimal antigen dose are added and the tubes incubated at room temperature for 30 minutes. After the addition of the conglutinating system the tubes are incubated at 37°C for an additional 30 minutes. The tubes are then centrifuged and read. Appropriate controls for the test serum, antigen and complement are always included.

In the original technic of Hole and Coombs[3] 0.4 ml of each test component was used, thus making a total volume of 2.0 ml per tube. In the present study one-half these volumes were used and no interference with the sensitivity, specificity or the reading of the results was observed. Also, preliminary trials indicated that the results were equally satisfactory when stored cells and buffered diluent were used, instead of the fresh cells and unbuffered physiological salt solution suggested in the original method.

Hemolytic Complement-Fixation Methods. Two methods were used, the so-called warm-bath method, suggested by Huebner,[8] and the icebox method.

Warm-bath method. Test components: serum, antigen, complement, amboceptor and fresh sheep red cells in 0.2 ml quantities each.

[6] Plotz, H., *Science,* 1943, **97**, 20.

[7] Wolfe, D. M., Kornfeld, L., and Cox, H. R., to be published.

[8] Huebner, R. J., personal communication.

TABLE I.
Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Cattle Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

No. of serums tested	C.C.A.T. titers*				Complement-fixation titers*			
					Warm-bath method		Ice-box method	
	Q fever	Murine typhus	Rickett-sialpox	Control†	Q fever	Control	Q fever	Control
1	1:16	—	—	—	—	—	1:16	—
1	1:64	—	—	1:8	—	—	1:8	—
2	1:64	—	—	—	—	—	1:8 1:16	—
1	1:128	—	—	1:32	—	—	1:16	—
1	1:128	1:16		1:64	—	—	1:32	1:8
1	1:128	—	—	—	1:16	—	1:64	—
2	1:128	—		—	1:8	—	1:32	—
1	1:256	—		1:8	1:16	—	1:32	—
3	1:256	—		—	1:16	—	1:32 1:64(2)	—
1	1:512	1:8		1:128	—	—	1:64	—
2	1:512	—		—	1:4 1:8	—	1:64	—
1	1:512	1:16		1:32	1:8	—	1:128	—
1	1:512	—		—	1:8	—	1:128	—
4	1:512	—	—	—	1:16	—	1:64(3) 1:128	—
5	1:512	—		—	1:32	—	1:64(2) 1:128(3)	—
1	1:1024	—		—	1:16	—	1:256	—
2	1:1024	—		—	1:32	—	1:128 1:256	—
3	1:1024	—		—	1:64	—	1:128 1:256(2)	—
1	1:2048	—		1:16	1:32	—	1:256	—

* The highest dilution of the serum in which complete, or near-complete (3+ or 4+) fixation was observed is shown.

† The dose of complement used is not always the same for each antigen. Serum controls were always tested with the highest dilution of complement used. It is reasonable to assume that if a serum is not anticomplementary in the presence of a small amount of complement it would not be anticomplementary with larger quantities of complement.

Unbuffered physiological salt solution, and 2% suspension of red cells are used. Varying amounts of amboceptor are titrated in the presence of a 1:30 dilution of complement for one hour at 37°C. Complement is titrated in the presence of 2 units of antigen after fixation for the same period of time and temperature; 2 units of amboceptor and cells are added and a period of one hour at 37°C is allowed for hemolysis. Two "full units" of complement contained in 0.2 ml are used in the test.

The test consists of mixing twofold serial dilutions of the suspected immune serum with 2 units of antigen and 2 "full units" of complement. Fixation is allowed to take place for one hour at 37°C. The sensitized cells are then added and the tubes reincubated for one hour at 37°C. All tubes are stored overnight at 4 to 6°C and readings are made the following morning.

Icebox method. This method is similar to that described above except that 2 "exact units" of complement are used and a period of 24 hours at 4 to 6°C is allowed for fixation. The tubes are read after the addition of the sensitized cells, followed by incubation of one hour at 37°C.

In both complement-fixation tests the antigens were titrated with their respective immune guinea pig serums.

On each serum specimen all 3 titrations, the C.C.A.T. and the 2 hemolytic complement-fixation tests, were performed on the same day with the identical dilutions. The C.C.A.T., using Q fever, murine typhus and rickettsial-

TABLE II.

Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Human and Guinea Pig Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

Serum	Lowest serum dilution tested	C.C.A.T. titers				Complement-fixation titers: Warm-bath method		Complement-fixation titers: Ice-box method	
		Q fever	Murine typhus	Rickett-sialpox	Control	Q fever	Control	Q fever	Control
Human 1		1:1024	1:128	1:128	1:128	1:8	1:8	1:64	1:32
" 2		1:256*	—	—	1:8	1:64	—	1:128	—
" 3		1:512	—	—	—	1:64	—	1:128	—
" 4		1:512*	—	—	1:16	1:32	—	1:64	—
" 5	1:8	1:1024*	—	—	—	1:256	—	1:512	—
" 6		1:256	—	—	—	1:32	—	1:128	—
Guinea pig No. 3 Nine-Mile		1:2048	—	—	1:8	1:512	—	1:1024	—
Guinea pig No. 39 Nine-Mile		1:2048	—	—	—	1:512	—	1:1024	—
Guinea pig No. 5 Nine-Mile		1:2048	—	—	—	1:512	—	1:1024	—
Guinea pig Henzerling		1:2048	—	—	1:32	1:512	—	1:1024	—

* Prozone.

TABLE III.

Results of C.C.A.T. and Complement-fixation Titrations of Serums from Cattle Having No Known Contact with Q Fever, with Murine Typhus, Q Fever and Rickettsialpox Antigens.

Serum	No. serums tested	C.C.A.T. titers				Complement-fixation titers: Warm-bath method		Complement-fixation titers: Ice-box method	
		Q fever	Murine typhus	Rickett-sialpox	Control	Q fever	Control	Q fever	Control
Brucella	2	—	—		—	—	—	—	—
negative	6	—	—		—	—	—	1:4	—
	2	—	—		—	—	—	1:8	—
Brucella	3	—	—	—	—	—	—	1:8	—
positive	3	—	—	—	—	—	—	1:16	—
	1	—	—	—	—	—	—	1:32	—

pox antigens, and the 2 types of hemolytic complement-fixation tests, using Q fever antigen only, were performed on human, guinea pig and cattle serums. With the C.C.A.T. pools of normal human serums, human syphilitic serums and normal guinea pig serums (all used in dilutions of 1:4) were negative with the 3 antigens. Murine typhus and rickettsialpox guinea pig serums, tested with their respective antigens, gave titers comparable to those obtained with the icebox method for complement fixation test.

Discussion of Results. Considering the results of the Q fever conglutinating complement absorption test from the point of specificity, it is seen from the data shown in Tables I and II that, unless also anticomplementary, no Q fever immune serum (cattle or otherwise) reacted with the murine typhus or rickettsialpox antigens. Of the 17 serums obtained from cattle having a negative history of Q fever (Table III), none were positive when examined by the C.C.A.T.

It will be noted from the data summarized in Table I that with the positive Q fever cattle serums the order of increasing sensitivity is: the warm-bath method, the icebox method, and the C.C.A.T. Serums submitted

to the C.C.A.T. show titers from 4 to 8 times higher than those obtained with the icebox complement-fixation method, and these in turn are from 4 to 8 times higher than those given by the warm-bath method. A similar picture is noted in Table II with human and guinea pig Q fever immune serums. Here, however, the differences in titers are of an order of from 2 to 4 times greater rather than 4 to 8. This apparently greater increase in sensitivity when testing cattle serum is, as yet, unexplained.

The question arises whether the use of cattle serum, as source of the immune body, causes irregularities in the results of the C.C.A.T. One would expect that conglutinin and normal antibodies for sheep cells introduced in excess of the standard amounts would tend to lower the antibody titer rather than cause the observed increase. Normal sheep amboceptor probably was present in the serums studied but no attempt was made to adjust the amount of amboceptor employed in the test. It is doubtful whether any conglutinin persisted until the date of testing since storage conditions are unfavorable for that particular component, and bearing in mind its relatively low titer (1:40 or 1:48), dilution of the serums beyond that point undoubtedly removed any effects which may be attributed to the introduction of additional conglutinin into the reaction mixture.

It is noteworthy that the titers of the Q fever, rickettsialpox and murine typhus antigens, as determined by the icebox complement-fixation method, were 1:32, 1:32 and 1:16 respectively, while the "optimal" C.C.A.T. titers on the same antigens were 1:120, 1:4 and 1:4 respectively. The Q fever antigen titer of 1:120 was determined with both bovine and guinea pig immune serums.

From the icebox complement-fixation results obtained with the Q fever negative-Brucella negative cattle serums (Table III) it would seem possible to adopt a lower limit of test significance, *i.e.*, a positive result exhibited in serum dilutions only as high as 1:8 would not be considered significant. But the inadvisability of imposing such an arbitrary limitation is demonstrated by the higher titers offered by the Q fever negative-Brucella positive serums (Table III).

The data presented indicate that the Q fever conglutinating complement absorption test may prove superior, both in sensitivity and in specificity, to either of the hemolytic complement-fixation tests employed to date. It is realized that numerous specimens of serum from cattle suffering from infections other than Q fever and Brucellosis should be examined before placing too much confidence in the results of the C.C.A.T. Such a study is now being undertaken and serums of goats, sheep, swine, dogs and horses will be included.

Conclusions. Preliminary studies in testing Q fever immune bovine serums indicate that the conglutinating complement absorption test is both more sensitive and more specific than are either the warm-bath or icebox methods for carrying out hemolytic complement-fixation tests.