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The Use of Direct and Indirect Complement-Fixation Tests for the Demonstration of Antibodies for Vesicular Viruses in Cattle

CHRISTINE E. RICE, Ph.D., and
PAUL BOULANGER, M.Sc., D.M.V.

Hull, Quebec

Early diagnosis of foot-and-mouth disease and vesicular stomatitis (VS) in cattle can be readily made in the majority of cases by means of complement-fixation (c.f.) tests using extracts of infected tongue epithelium and hyperimmune guinea pig antiserum for these respective viruses.[1-8] At later stages when this material is no longer available, c.f. tests have relatively little value for two reasons. In the first place, the material from foot lesions in foot-and-mouth disease has not proved satisfactory as a source of antigen for c.f. tests for the presence of virus. The second and more important difficulty is that only a very small proportion of cattle convalescent from either of these viral diseases develop antibodies that fix guinea pig complement with homologous viral antigen.[9,10] When such serums are tested without inactivation at 56 C.,[11] or after treatment with 1.5 per cent sodium chloride,[12] an increase in the percentage of serums exhibiting fixation has been reported.

The first part of this paper will deal briefly with our use of the direct c.f. test in

From the Division of Animal Pathology, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

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identifying the agent responsible for the recent Canadian outbreak of foot-and-mouth disease, and with our attempts to apply the salt method of Serra and Guarini[12] in the titration of serums of cattle experimentally infected with VS virus. The second part of the report will describe the results of preliminary investigations of the indirect c.f. test in the detection of antibody in serums of cattle convalescent from foot-and-mouth disease of the A type, and from vesicular stomatitis.

METHODS

Direct Complement-Fixation Tests.—Direct c.f. tests for the presence of virus in infected tongue epithelium were made by the method described by Brooksby,[2] and by a modification of the New York State quantitative methods,[13] using hyperimmune foot-and-mouth disease viruses, types A, O, and C, and New Jersey and Indiana types VS guinea pig serums received from Dr. I. A. Galloway of the Foot-and-Mouth Disease Research Station at Pirbright, Surrey, England.

The salt treatment of the convalescent bovine serums was performed as suggested by Serra and Guarini.[12] The active serums were diluted serially with 1.5 sodium chloride solution and allowed to stand for sixty minutes at room temperature. The diluted serums were pipetted in 0.1-ml. amounts in duplicate into 11 by 75 mm.-tubes, and 0.1 ml. of a dilution of guinea pig complement containing 3 50 per cent hemolytic units, and 0.1 ml. of diluted vesicular stomatitis virus antigen added. One set was incubated for thirty mniutes at 37 C., the other held for eighteen hours in the refrigerator. At the end of the fixation period the hemolytic indicator was added—0.2 ml. of a mixture of equal parts of a 5 per cent suspension of sheep red blood cells and a dilution of amboceptor containing sufficient antibody to maximally sensitize the cells. At the end of thirty minutes at 37 C., the tests were centrifuged and read in comparison with prepared color standards.[13] Serum, antigen, and complement controls were included in each titration. Antigens were previously standardized to determine their anticomplementary and specific activities. Antibody titers were expressed in terms of the highest serum dilution showing 50 per cent hemolysis, or less, in the presence of antigen.

Normal and convalescent bovine serums, after inactivation at 56 C. for thirty minutes, were titrated as described above, except for the omission of the salt treatment.

Indirect Complement-Fixation Test.—Indirect c.f. tests[14] may be made in two general ways. In the first method, serial dilutions of serum are mixed with a constant dilution of antigen and, after a suitable period for combination, tested undiluted for residual complement-fixing activity with an excess of homologous guinea pig antiserum in the presence of a constant dose of complement. In the second method, mixtures are prepared with serial dilutions of serum and a constant dilution of antigen and, after the period allowed for neutralization, tested in serial amounts for residual antigen with an excess of homologous antiserum and a constant dose of complement. Both tests can be rendered more precise by the use of graded doses of complement for studies of strain differences. The first method in its simpler form was used in the present study.

Heat-inactivated bovine serums were serially diluted in 0.85 per cent salt solution or barbitol buffer containing calcium and magnesium,[15] and pipetted in 0.1-ml. amounts in triplicate. An equal volume of salt solution was added to the first set, the same amount, 0.1 ml., of the standard dilution of antigen to the second and third sets. The mixtures were held for one hour at 37 C. and overnight in the refrigerator. To each tube, 0.1 ml. of the 3-unit dilution of guinea pig complement was added. In addition, the first set received 0.1 ml. of salt solution, the second set 0.1 ml. of a 1 : 20 dilution of inactivated normal guinea pig serum, the third set 0.1 ml. of a 1 : 20 dilution of hyperimmune guinea pig serum corresponding to the residual antigen to be titrated. A period of thirty minutes at 37 C. was allowed for fixation, followed by the customary thirty minutes at 37 C. after the addition of sensitized sheep red blood cells.

When tests were being made with more than one antigen, the test was proportionately increased, two additional sets being required for each of the extra antigens.

RESULTS

Foot-and-Mouth Disease.—The direct c.f. test was used in the identification and typing of the strain of virus responsible for the outbreak of foot-and-mouth disease in Canada in 1952. As stated above, hyperimmune guinea pig serums received from Pirbright, England, were employed. Specimens of tongue epithelium from infected cattle exhibited strong fixation with foot-and-mouth disease type A antiserum but no significant reaction with foot-and-mouth disease types O or C antiserums, or with VS New Jersey or Indiana types antiserums. The titer of these epithelial extracts in type A, foot-and-mouth disease virus, was usually high. Specimens of epithelium from cattle showing tongue erosions of a nonvesicular nature, and presumably of bacterial origin, showed no increase in fixation in the presence of any of these antiserums beyond that noted in the antigen controls, which usually appeared somewhat anticomplementary.

We were also presented with the problem of determining whether antibodies were

present in serums from 34 cattle on farm W where it was suspected the Canadian outbreak had originated. Vesicular disease of a mild nature had been noted in some of these animals shortly after the arrival of an immigrant laborer. In view of its mild nature and because of the fact that VS has occurred in horses in this general area, the condition was diagnosed as this disease. Later, as the disease continued to spread and increase in virulence, specimens were submitted to our laboratory for examination and shown by c.f. tests and animal inoculation to contain foot-and-mouth disease virus of the A type. Epidemiological investigation traced the outbreak to this farm and revealed the information that the immigrant laborer had come directly from a farm in Germany where foot-and-mouth disease had been previously diagnosed.

These W herd serums were tested by direct complement-fixation tests with the Canadian type A strain of foot-and-mouth disease virus and with VS antigens of the New Jersey and Indiana strains. No fixation was obtained which, as stated above, would be in agreement with the general experience of other investigators that convalescent foot-and-mouth disease bovine serums only infrequently fix guinea pig complement with foot-and-mouth disease viral antigen. In view of the satisfactory results we had previously obtained with an indirect c.f. test in demonstrating antibody in noncomplement-fixing avian and equine antiserums,[16, 17] it seemed worthwhile to assay its possibilities in the detection of combining activity with foot-and-mouth disease or VS antigen in these W herd serums. Included as controls were serums from 26 normal cattle at the Central Experimental Farm, Ottawa, that had never had any contact with either virus.

These indirect c.f. tests were made with three viral antigens and an extract of normal bovine tongue epithelium; eight sets of serum dilutions were therefore required, one each for the serum and antigen controls, and two for each of the viral antigens with normal and homologous hyperimmune guinea pig serum, respectively. None of the serums were appreciably anticomplementary, none showed significant fixation in the presence of normal guinea pig serum and any of the three viral antigens. In the sets containing foot-and-mouth disease type A antigen and homologous guinea pig antiserum, a few of the normal bovine serums in 0.1 ml. of 1 : 2 and 1 : 5 dilutions showed some reducing effect on fixation. All but four of the 34 W herd serums displayed definitely more inhibition of fixation than was shown by any of the normal serums. In 0.1 ml. of a 1 : 10 dilution, 25 of the 34 showed complete inhibition, seven showed complete or almost complete inhibition with 0.1 ml. of a 1 : 100 dilution. In the tests with the two vesicular stomatitis antigens and their respective guinea pig antiserums, the W herd serums showed no more than the very slight inhibitive effect displayed by the normal serums in 1 : 2 and 1 : 5 dilutions. Representative results for five normal and five W herd serums with the foot-and-mouth disease antigen are given in table 1.

Absorption of all serums with a heavy suspension of sheep red blood cells to remove

TABLE I—Indirect Tests of Serum Dilution Form with Foot-and-Mouth Disease Virus Type A Antigen* and Five Serums from a Herd of Cattle in which an Outbreak of Vesicular Disease Had Occurred About Three Months Previous to Date of Bleeding, and from 5 Normal Cows

Serum	Set 1**				Set 2**				Set 3**						
	Dilution of Serum														
	1:2	1:5	1:10	0	1:2	1:5	1:10	0	1:2	1:5	1:10	1:20	1:50	1:100	0
W-1	–	–	–		–	–	–		–	–	–	–	95	90	
W-2	–	–	–		–	–	–		–	–	–	95	90	60	
W-3	–	–	–		–	–	–		–	–	90	80	25	10	
W-4	–	–	–		–	–	–		–	90	45	10	5	0	
W-5	–	–	–		–	–	–		–	–	90	40	20	10	
N-1	–	–	–		–	–	–		80	70	60	0	0	0	
N-2	–	–	–		–	–	–		65	40	20	0	0	0	
N-3	–	–	–		–	–	–		60	30	20	0	0	0	
N-4	–	–	–		–	–	–		65	30	20	0	0	0	
N-5	–	–	–		–	–	–		90	55	25	0	0	0	
None				–				–							0

*Prepared from tongue epithelium of a calf experimentally infected with the Canadian strain of foot-and-mouth disease, type A virus. – — 100% hemolysis.

**Set 1—serum, complement, and antigen mixture plus saline.

Set 2—above mixture plus normal guinea pig serum 1:20.

Set 3—above mixture plus type A, foot-and-mouth disease guinea pig antiserum 1:20.

natural sheep antibody, removed most of the inhibitive effect of the normal bovine serums with foot-and-mouth disease antigen and of all serums with the VS antigen but had no appreciable influence on the inhibitory properties of the W herd serums with foot-and-mouth disease antigens.

The results obtained in these indirect tests suggested, therefore, that the majority of serums from this suspected herd of cattle were combining with the foot-and-mouth disease type A antigen but not with either of the VS antigens. Without further experimental studies of the specificity of the test, no definite statement in regard to the significance of the results was felt to be justified. Moreover, some years ago, Palacios and Rodriguez[4] had reported briefly on the detection by means of a c.f. test of the inhibitive form of neutralizing activity in foot-and-mouth disease convalescent bovine serums but did not consider the method to be of practical value. In the light of our results with the W herd serums, we felt that further study of the method with a view to exploring its possibilities as an epidemiological tool was well worthwhile.

When the opportunity was afforded to one of us to visit the Pirbright laboratories for several months in the autumn of 1952, these studies were therefore continued and further evidence obtained indicating the usefulness of the indirect c.f. method in detecting foot-and-mouth disease antibody in convalescent bovine serums. These later data will be reported in detail elsewhere.[18]

Vesicular Stomatitis. — Serial bleedings from 3 cows experimentally infected with VS virus of the New Jersey strain, and of 1 infected with the Indiana strain have been tested for activity with these two viral antigens by both the direct and indirect c.f. methods. None of the pre- or postinfection bleedings from these animals after inactivation for thirty minutes at 56 C., showed any fixation in the direct tests. An attempt was made to apply the salt-inactivation method of Serra and Guarini[12] but without any conspicuous success.

The use of active serums in c.f. tests of bovine antiserums was originally proposed because of certain evidence suggesting that their complement-fixing properties were reduced by heating at 56 C. Because of the anticomplementary properties of the majority of bovine serums and, more particularly, their tendency to exhibit "nonspecific" fixation even with homologous noninfected tissue extracts, the results of tests with such unheated serums have never been very satisfactory. Treatment of these active serums with hypertonic salt solution[12] has been thought to alter the serum proteins in some way and to inactivate the complement. The latter change is apparently reversible, however. Although no hemolytic activity was observed when bovine complement was titrated for hemolytic activity for sensitized rabbit red blood cells using 1.5 per cent salt solution as a diluent throughout, when the salt-treated bovine serum was tested for hemolytic properties using 0.85 per cent salt solution in all subsequent dilutions, the hemolytic titer, 0.0082 ml., was found to be comparable with that of a corresponding sample of the same serum that had not been salt-treated.

If 0.1 ml. of this salt-treated serum was added to 0.1 ml. of a 1 : 50 dilution of guinea pig complement, conglutination but no hemolysis occurred; with a 1:10 dilution hemolysis was observed. Thus the slight degree of fixation, which was recorded when the salt-treated VS bovine serum was titrated by a direct c.f. method with VS antigen (table 2), was probably related to the inhibitive effects of the hypertonic salt concentration rather than to combination with specific antigen-antibody complexes, particularly as the reaction of the postinfection

TABLE 2—Complement-Fixing Activity with Vesicular Stomatitis—New Jersey Viral Antigen of Salt-Treated Serums from a Cow Experimentally Infected with this Virus

Serum—Cow No. 3	Fixation period	Serum control			With antigen					
		Dilution of Serum								
		1:2	1:5	1:10	1:2	1:5	1:10	1:20	1:50	1:100
Preinfection	37 C. 30 min.	80	75	95	75	85	90	95	—	—
Postinfection 2 days	37 C. 30 min.	80	90	95	75	90	95	—	—	—
Postinfection 4 days	37 C. 30 min.	85	90	95	85	95	95	98	—	—
Postinfection 8 days	37 C. 30 min.	80	90	95	90	95	95	98	—	—
Postinfection 14 days	37 C. 30 min.	80	90	95	65	70	75	80	95	—
Postinfection 23 days	37 C. 30 min.	80	85	98	45	65	75	80	95	—

– — 100% hemolysis.

bleedings was comparable to or only slightly stronger than that of the preinfection bleeding from the same animal. The nonspecific inactivation of complement was augmented in tests held for eighteen hours in the refrigerator.

Definitely better results were obtained in indirect c.f. tests of these vesicular stomatitis serums. No inhibitory activity was shown by the preinfection or early postinfection bleedings from any of the 3 cows infected with the New Jersey strain of this virus in a test with homologous antigen and using a VS New Jersey strain guinea pig antiserum in the detection of residual antigen. Definite inhibition was observed in bleedings collected at fourteen to eighteen days after infection. A bleeding taken from the third animal twenty-eight days after inoculation showed a slight drop in inhibitory titer. Weak inhibitory activity was demonstrated, however, in 1 animal bled about four months after a second virus inoculation (table 3). The bleedings from a calf infected with VS Indiana strain virus showed no inhibition in these indirect tests with VS New Jersey strain antigens. It appeared of low titer in indirect tests with homologous antigen, possibly because the degree of infection established was slight, only a very limited vesiculation developing.

SUMMARY

Complement-fixation tests of the direct type were applied in identifying the agent responsible for the 1952 outbreak of foot-and-mouth disease in Canada and establishing it as type A. The virus was differentiated from foot-and-mouth disease viruses of types O and C, and from the New Jersey and Indiana types of vesicular stomatitis virus through the use of hyperimmune guinea pig antiserums received from Dr. I. A. Galloway of the Animal Virus Diseases Research Institute, Pirbright, Surrey, England.

Serums from cattle on a farm where an outbreak of mild vesicular disease had occurred three months earlier failed to fix complement in direct tests with foot-and-mouth disease virus type A or VS antigens. Evidence of combining activity with the former, that is with an antigen prepared with the Canadian type A strain, was however obtained in complement-fixation tests of the indirect form. No inhibition was observed in indirect complement-fixation tests with VS antigens of the New Jersey or Indiana types.

Indirect complement-fixation tests were also used in demonstrating the development of antibody in the serums of cattle experimentally infected with the New Jersey type of vesicular stomatitis virus.

In testing serums of cattle convalescent from a vesicular disease of unknown nature, it is suggested that complement-fixation tests of both direct and indirect forms be carried

TABLE 3—Indirect Complement-Fixation Tests with Vesicular Stomatitis—New Jersey Viral Antigen of Serial Bleedings, from Cattle Infected with the Same Strain of Virus, using Homologous Guinea Pig Antiserums as the Indicator of Residual Antigen

No. of animal	Date of bleeding	Set 2		Set 3							
		Dilution of serum									
		1:2	1:5	1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1000
1	4/16/52 (N)	60	—	20	5	0	0	0	0		
	4/26/52	—	—	70	—	—	95	80	50	10	0
	5/1/52	—	—	—	—	—	98	70	60	20	0
	5/16/52	—	—	—	—	—	95	75	55	25	10
	5/22/52	—	—	—	—	—	98	45	30	10	0
2	8/22/52 (N)	85	—	10	0	0	0	0	0		
	9/6/52	—	—	—	—	—	95	85	20	15	10
	9/22/52	—	—	—	—	—	—	98	90	80	30
	9/29/52	—	—	—	—	—	95	90	80	45	30
	1/18/53	—	—	—	98	55	40	0	0	0	0
3	2/5/53 (N)	—	—	10	5	0	0	0	0		
	2/7/53	—	—	10	0	0	0	0	0	0	0
	2/9/53	—	—	15	0	0	0	0	0	0	0
	2/13/53	—	—	—	—	—	98	80	35	25	10
	2/19/53	—	—	—	—	—	90	65	40	0	0
	3/5/53	—	—	—	—	—	90	35	25	0	0

— — 100% hemolysis.
Set 2—Mixture of serum and VS—New Jersey antigen with normal guinea pig serum.
Set 3—Mixture of serum and VS—New Jersey antigen with VS—New Jersey hyperimune guinea pig serum.

out in parallel with foot-and-mouth disease antigens of types A, O, and C, and with VS antigens of the Indiana and New Jersey types, using hyperimmune guinea pig antiserums of the respective types in the titration of residual antigen in the indirect test.

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