Studies on Bluetongue II. Complement-Fixing Activity of Ovine and Bovine Sera

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SUMMARY

Sera of sheep and calves infected with the California type 10 and Cyprus type 3 viruses of bluetongue were tested by the regular and modified direct complement-fixation tests. To obtain satisfactory complement fixation it was necessary to use the latter test. Cross reactivity was found, therefore, the California type 10 antigen could be used in testing sera of animals infected with the Cyprus virus.

RESUME

L'épreuve directe ordinaire et l'épreuve directe modifiée de la fixation du complément furent employées pour éprouver des sérums de moutons et de veaux infectés avec la souche de Californie, type 10 et la souche de Chypre, type 3 du virus de la fièvre catarrhale de mouton. Pour obtenir des résultats satisfaisants, il a fallu employer l'épreuve directe modifiée. Des réactions croisées furent observées et l'antigène préparé avec le virus de Californie type 10 put être employé pour éprouver les sérums d'animaux infectés avec la souche de virus de Chypre.

Bluetongue infection is present in many parts of the world including the United States. Pertinent literature has been reviewed by Cox (1) and by Browne *et al* (2). While the disease has not yet been recognized in Canada its presence in neighboring countries renders it desirable that a reliable *in vitro* diagnostic method be available to replace the original procedure of injecting suspected material into susceptible sheep.

In 1954, Van Den Ende *et al* (3) showed that suckling mice, 4 to 12 days of age, were susceptible to the Cyprus strain of bluetongue virus injected intracerebrally. These workers prepared antigens from mouse brains by the acetone and ether extraction method of Casals (4) and reported good fixation of complement with sera of hyperimmunized mice. In 1954, McKercher *et al* (5) using benzene-extracted suckling mouse brain antigen, obtained some complement fixation with convalescent sheep serum but concluded on the basis of the low titres recorded that antibody was either present in low concentration or had little affinity for guinea-pig complement. Furthermore, in support of this conclusion they showed that, in mice, bluetongue sheep antiserum possessed low virus-neutralizing potency. In 1956, Haig *et al* (6) studied virus neutralization in sheep kidney tissue cultures. They found serum of immune sheep capable of inhibiting the cytopathic effect of the homologous tissue-culture adapted virus, but that inhibition was slight and insignificant with a heterologous strain of bluetongue virus. Also in 1956 Shone *et al* (7) used serum-free tissue culture fluid for antigen in complement-fixation tests of sera from sheep affected with five strains of bluetongue virus. Serum titres of 1:16 to greater than 1:64 were reported.

Materials and Methods

INFECTIVE AGENTS

California type 10 bluetongue virus was received as freeze-dried mouse brain, tenth serial passage, from the Department of Agriculture, Onderstepoort, Union of South Africa. *Cyprus type 3 bluetongue virus,* also from Onderstepoort, was obtained as a freeze-dried ten per cent suspension of chick embryo tissues of the third egg passage.

ANTIGENS

A quantity of *California type 10* mouse brain antigen and normal mouse brain antigen was received from Onderstepoort to serve as controls.

As test material, 16 small lots of *California type 10* mouse brain antigen were produced from a total of 4,908 four-day-

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old suckling mice according to the acetone and ether extraction method of Casals (4). Two lots of the *Cyprus type 3* mouse brain antigen were also prepared by the acetone and ether extraction method. Normal mouse brain antigens were processed in parallel with the California and Cyprus antigens.

Attempts were also made to prepare antigens from brains of weaned mice and a lamb as well as from chorio-allantoic membranes, yolk sacs and fluids of embryonated chicken eggs inoculated with the virus.

Immune Sera

California type 10 sera. A quantity of California type 10 immune sheep serum and negative control sheep serum was obtained from Onderstepoort for use in titrations of our antigens. Antisera for this virus strain were also produced in four experimentally infected sheep and two calves.

Sheep 869 and 900 were pre-bled and inoculated intramuscularly and subcutaneously with 5 ml. of infective defibrinated sheep blood by each route. Six days post inoculation, when temperatures had risen to 106°F and 106.4°F respectively, blood was drawn and defibrinated for later challenge material. Seventeen days after peak of temperature blood samples were drawn and the animals inoculated as before using their own infective blood. Further serum samples were collected at intervals thereafter until 54 days after challenge.

Calf 227 was pre-bled and injected intravenously with 15 ml. of the defibrinated infective sheep blood used to infect the previously described sheep. Antibiotics were added to the infective material. At the same time a third sheep, 904, was inoculated intravenously with 5 ml. of the same infective blood. This sheep showed a temperature reaction of 105°F at nine days post inoculation, indicating virulence of the material. A 10 ml. volume of defibrinated blood drawn from calf 227 on the ninth day was inoculated intravenously into a second calf, 226. Inoculations were repeated on the two succeeding days. Seven days later when the temperature of the second calf was 103.4°F blood was collected and 5 ml. was inoculated intravenously into a fourth sheep, 77N, for evidence of transmission. Serum samples were taken at intervals from these animals for various periods. The second calf, 226, was kept for almost a year to determine the duration of complement-fixing antibody titre.

Cyprus type 3 sera. Antisera for this virus strain were produced in one experimental sheep and one calf.

Sheep 739 was pre-bled for serum and inoculated intramuscularly and subcutaneously with 5 ml. of infective defibrinated sheep blood by each route. Additional inoculations were made after approximately five and six months. On the seventh day after the first exposure a peak temperature of 106.8°F was reached and the animal developed slight swelling of the upper lip, hyperaemia of the oral mucosa, glossitis and conjunctivitis. Serum was collected at intervals over a period of approximately seven months.

Calf 113 was pre-bled for serum and inoculated intramuscularly and subcutaneously with 3 ml. of infective defibrinated sheep blood by each route. During the four months the animal was kept under observation the temperature remained within the normal range and no clinical signs of infection were observed. Blood was drawn at intervals throughout the observation period.

Complement-Fixation (CF) Tests

Direct CF test. At first, attempts were made to employ the direct CF method. The antigens were tested in the 1:2 to 1:64 dilutions against positive and negative sera in 1:10 dilution in line tests with three corrected 50 per cent haemolytic units of complement. Since a correction factor of 1.5 is used to allow for deterioration of complement during the 18 hours' incubation at 9°C, this complement dose represents 4.5 direct units. All reagents of the test were added in 0.1 ml. amounts. The period of primary incubation for fixation was 18 hours at 9°C., that of secondary incubation after the addition of 0.2 ml. of a 2.5 per cent suspension of maximally sensitized sheep red cells, was 30 minutes at 37°C. The titre of the antigen was recorded as the highest dilution with which 50 per cent haemolysis or less was obtained in the presence of a dilution of the positive serum supplying an excess of antibody.

Modified direct CF test. In later work both antigen and serum titrations were performed by the modified CF method. As previously described by Boulanger (9), the technique was the same as for the direct

method with the exception that the veronal buffer used in making the complement dilution contained 5 per cent normal unheated bovine serum fraction as supplementing factor. The fraction was prepared by dialysing fresh normal calf serum against phosphate buffer pH 6.2 for 1, 1.5 or 2 hours at 9°C. The precipitate was washed three times with phosphate buffer then restored to the original serum volume with veronal buffer and stored frozen. Tests of the supplementing activity of each preparation were made and the most active used in the tests. For use this was diluted 1:20 in veronal buffer containing 5 per cent heated guinea-pig serum and the mixture employed as the complement diluent. Antigen titrations were set up as described for the direct test. For serum tests serial dilutions from 1:5 to 1:160 of the heat inactivated serum (60°C for 30 minutes) were tested with two 50 per cent haemolytic units of antigen. Volumes of reagents, incubation times and temperatures were the same as for the antigen titrations. The serum titre was recorded as the highest dilution with which 50 per cent haemolysis or less was obtained in the presence of the antigen.

Table I — Results of modified direct CF tests of sera from sheep exposed to California type 10 bluetongue virus.

Time of Bleeding	Titre[1] with California type 10 antigen			
	869	900	904	77N
Pre inoculation..	—	—	—	—
1 wk. post inoc.	ND[2]	ND	ND	20[3]
3 " " "	10	10	—	80
4 " " "	ND	ND	5	ND
5 " " "	ND	ND	ND	160
6 " " "	80	10	ND	ND
7 " " "	40	20	ND	160[4]
8 " " "	20	20	>160	ND
9 " " "	40	20	ND	80[3]
11 " " "	40	20	ND	160[3]
12 " " "	ND	ND	>160	ND
14 " " "	ND	ND	>160	ND
16 " " "	ND	ND	>160	ND

1. Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
2. Not done. Animals were infected in different trials and bleeding intervals did not coincide. However, samples of all sera were stored and tested at one time.
3, 4. Sera reacted to 1:5 and 1:10 dilutions respectively, with normal mouse brain antigen but showed no anticomplementary activity. None of the other sera reacted nonspecifically.

Table II — Results of modified direct CF tests of sera from sheep 739 which was infected with Cyprus type 3 bluetongue virus

Time of Bleeding	Titre[1]			Serum Control
	California Antigen	Cyprus Antigen	Normal Antigen	
Pre inoculation	—	—	—	—
1 wk. post inoc.	—	—	5	—
2 " " "	20	20	5	—
3 " " "	160	>160	10[2]	—
4 " " "	>160	>160	20[2]	—
8 " " "	>160	>160	20[2]	—
12 " " "	>160	>160	10[2]	—
16 " " "	80	80	10[2]	—
21 " " "	80	40	10[2]	—
22 " " "	40	80	10[2]	5
26 " " "	40	40	20[2]	10

1. Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
2. These reactions were partial in all dilutions up to the titre indicated.

Results

Preliminary trials by the direct CF technique with the Onderstepoort reagents and those produced by us failed to show any trace of complement fixation. However, modification of the complement by addition of normal bovine serum fraction and heated guinea-pig serum resulted in satisfactory fixation of complement. This finding is in agreement with our experience using other viral antigens with bovine sera.

Reactivity of Antigens

The Onderstepoort reagents were employed in titrations as standards for comparison with those produced here. Generally our mouse brain antigens were comparable with the African material but the dilutions of various lots when titrated with sheep antiserum varied from 1:4 in earlier batches to 1:32 in later ones. A more reactive antigen was obtained when extractions were made with large volumes of solvent (1 part of brain to 20 parts of solvent) than with lower ratios. When testing calf serum it was necessary to use the antigen 2 to 4 fold more concentrated than when testing sheep serum.

Antigens prepared from brains of weaned mice, from the brain of a lamb and from embryonated chicken egg material failed to react. Attempts to passage the virus in

Table III — Results of modified CF tests of sera from calf 227 and second passage calf 226 infected with California type 10 bluetongue virus

Time of Bleeding	Titre[1] (California Type 10 Antigen)[2]	
	Calf 227	Calf 226
Pre inoculation	—	—
3 wk. post inoc.	10	ND
4 " " "	40	10
5 " " "	ND	80
6 " " "	80	80
8 " " "	40	80
10 " " "	40	80
12 " " "	20	80
14 " " "	20	ND
15 " " "	ND	80
16 " " "	20	ND
17 " " "	ND	40
19 " " "	"	20
22 " " "	"	20
28 " " "	"	20
31 " " "	"	20
36 " " "	"	10
40 " " "	"	10
45 " " "	"	10
50 " " "	"	10

1. Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
2. Non-specific reactions were not found with normal mouse brain antigen and the sera were not anticomplementary.

suckling and adult guinea pigs and in a kid with a view to antigen production, failed.

REACTIVITY OF SERA

Sheep Antisera: Those produced here tended to be anticomplementary and to react non-specifically with the normal antigen but sera received from Onderstepoort did not show these undesirable reactions. Inactivation of our sera at 60°C rather than at 56°C removed most of these difficulties. Dessication of the sera produced little improvement, whereas absorption with normal mouse brain powder increased the anticomplementary effect. "Warm tests" employing primary incubation at 37°C for 90 minutes failed to remove completely the non-specific activity and reduced the specific reactivity to an insignificant level.

The results of modified CF tests of sera from the two first sheep, 869 and 900, exposed to the *California type 10* virus at the same time, are shown in Table I. Tests were conducted using *California type 10* mouse brain antigen produced here, diluted 1:32. Sera collected from both animals 3 weeks after initial exposure reacted to a 1:10 dilution. In the case of 869 the titre increased sharply to 1:80 three weeks after the second inoculation of infective blood, that is, 6 weeks after primary inoculation and was still maintained at 1:40 when the animal was destroyed after the 11th week. Titres of sheep 900 rose to 1:20 following the second inoculation and stayed at that level until the end of the 11 week test period. None of the serum samples reacted with the normal mouse brain antigen.

Table I also shows serological titres obtained in tests of the third sheep, 904, which received a single inoculation of *California type 10* infective material. This animal developed high CF titres, over 1:160, by the 8th week post exposure and these were still high at the 16th week. Non-specific or anticomplementary reactions were not observed in these tests.

Reactions obtained in CF tests of sera from the fourth sheep, 77N, are listed in Table I. This animal, inoculated with blood from the second calf passage of *California type 10* virus, developed a serological titre of 1:160 five weeks post exposure. The final serum sample collected six weeks later still reacted to the 1:160 dilution. However, four sera reacted to a low degree with the normal mouse brain antigen.

Serum samples of all bleedings from

Table IV — Serological titres of calf 113 which had been inoculated with the Cyprus strain of bluetongue virus

Time of Bleeding	Titre[1, 2]	
	California Antigen	Cyprus Antigen
Pre inoculation	—	—
1 week post inoc.	—	—
2 " " "	—	—
3 " " "	10	10
4 " " "	10	10
5 " " "	20	20
6 " " "	20	20
7 " " "	20	20
8 " " "	20	40
10 " " "	10	10
12 " " "	10	20
14 " " "	5	10
16 " " "	10	10

1. Titre is expressed on the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
2. Non-specific reactions were not found with normal mouse brain antigen and the serum controls were not anticomplementary.

each of the above animals were stored frozen and were tested at the same time with the same antigen, complement and bovine serum fraction.

Sheep 739, which had been inoculated with *Cyprus type 3* infective material, was tested serologically using California and Cyprus antigens as shown in Table II. The California antigen appeared to be as satisfactory as the Cyprus antigen in these tests. Serum titres of 1:160 and above were found by the third week post inoculation with both antigens. Titres were maintained at this high level for a further 9 weeks and were still in the 1:40 range when the animal was sacrificed six months after inoculation. Serum from this animal tended to react non specifically to the 1:20 dilution with normal antigen.

Calf sera: Serological titres of calf 227, inoculated with *California type 10* infective sheep blood, are recorded in Table III. A low titre of 1:10 was present three weeks after inoculation. The titre increased to a peak of 1:80 by the sixth week and then declined to 1:20 by the 12th week. It remained at this level until the animal was destroyed 16 weeks post inoculation.

Table III also lists the serological titres of serial bleedings from calf 226 which had been inoculated with blood from calf 227 infected with *California type 10* virus. A peak titre of 1:80 was recorded from the fifth to the fifteenth weeks post inoculation after which there was a drop to 1:10 during the ensuing five months. The titre remained at this low level until the animal was destroyed approximately one year following inoculation.

Serological titres of calf 113, inoculated with *Cyprus type 3* infective sheep blood, are shown in Table IV. CF tests using *California type 10* antigen were as satisfactory as those with the *Cyprus type 3* antigen but titres were not high in either case.

Discussion

It appears that a supplementing factor present in normal unheated bovine serum is required to enable the bluetongue viral antigen-antibody reaction to fix guinea-pig complement at a detectable level. Calf sera, which did not appear to be as reactive as the sheep sera, required the use of the most actively supplementing, normal bovine serum fraction obtainable.

The supplementing activity of the normal bovine serum fraction was influenced by many factors. It varied from animal to animal and in an individual with age. Generally it was best to test several calves four or five months of age for suitability and to keep one or two satisfactory animals for fraction production. Feeding changes accompanying the season seemed to influence the activity of an animal's serum fraction. Fraction appeared to be most active in spring when animals were turned out on pasture. Technical variations in fractionation also influenced the activity of fraction and it was found best with each serum collection to test for optimum fractionation time. Fractions were usable for two or three weeks, and the serum used in their preparation for five or six weeks if stored frozen.

The modified CF test appeared to be a satisfactory technique for demonstration of bluetongue antibodies in ovine and bovine sera. However, its value was somewhat reduced by the technical aspects of maintaining stocks of usable bovine serum fraction. A further disadvantage was the very large number of suckling mice required for antigen production.

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REFERENCES

1. **COX, H. R.** Bluetongue. Bact. Rev. 18: 239-253, 1954.
2. **BROWNE, J. G., LUEDKE, A. J., JOCHIM, M. M.** and **FOSTER, B. S.** Current status of bluetongue in sheep. J. Amer. Vet. Med. Ass. 144: 759-764, 1964.
3. **Van Den ENDE, M., LINDER, ANNE,** and **KASCHULA, V. R.** Experiments with the Cyprus strain of bluetongue virus. Multiplication in the central nervous system of mice and complement fixation. J. Hyg. 52: 155-164, 1954.
4. **CASALS, D., OLITSKY, R.** and **ONSLOW, R. O.** A specific complement fixation for infection with poliomyelitis virus. J. Exp. Med. 94: 123-137, 1951.
5. **McKERCHER, D. G., McGOWAN, B.** and **SAITO, J. K.** Studies on bluetongue. I. Isolation, identification and typing of the bluetongue virus and a preliminary report on the serodiagnosis of the disease. Proc. Amer. Vet. Med. Ass. 167-177, 1954.
6. **HAIG, D. A., McKERCHER, D. G.** and **ALEXANDER, R. A.** The cytopathogenic action of bluetongue virus on tissue cultures and its application to the detection of antibodies in the serum of sheep. Onderstepoort J. Vet. Res. 27: 171-176, 1956.
7. **SHONE, D. R., HAIG, D. A.** and **McKERCHER, D. G.** The use of tissue culture propagated bluetongue virus for complement fixation studies on sheep sera. Onderstepoort J. Vet. Res. 27: 179-182, 1956.
8. **GRAY, D. P.** and **BANNISTER, G. L.** Studies on bluetongue. I. Infectivity of the virus in the sheep ked, **Melophagus** ovinus (L). Can. J. Comp. Med. 25: 230-232, 1961.
9. **BOULANGER, P.** Technique of a modified direct complement-fixation test for viral antibodies in heat inactivated cattle serum. Can. J. Comp. Med. 24: 262-269, 1960.