Studies on Bluetongue
III. Comparison of Two Complement-Fixation Methods

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SUMMARY

The complement-fixation test used at Onderstepoort was compared with the method used at A.D.R.I. on infected calf and sheep sera. In the first method, the tests are incubated at 37°C for 90 minutes and the test sera are inactivated at 53°C; whereas in the A.D.R.I. method, the test sera are inactivated at 60°C for 30 minutes, incubation is at 9°C for 18 hours, and guinea-pig complement is supplemented with 5 per cent fresh, non-inactivated, normal calf serum. Serial serum samples from one of six experimentally infected calves were negative in the Onderstepoort test, three calves gave only trace reactions and two showed maximum titres of 1:10 whereas all six had maximum serum titres of 1:10 to 1:80 in the A.D.R.I. test. A good correlation was obtained, however, between the results of the two methods with the sera of experimentally inoculated sheep although titres 3 to 8 times higher were obtained with the A.D.R.I.'s test. Post inoculation bleedings from each sheep reacted in both tests.

RESUME

L'épreuve de la fixation du complément en usage à Onderstepoort a été comparée avec celle employée à A.D.R.I. sur des sérums de veaux et de moutons infectés avec le virus de la fièvre catarrhale du mouton. Dans la première méthode les épreuves sont incubées à 37°C pour 90 minutes et les sérums à éprouver sont inactivés à 53°C; toutefois dans la méthode employée à A.D.R.I. les sérums à éprouver sont inactivés à 60°C pour 30 minutes et les épreuves sont incubées à 9°C durant 18 heures. En plus, le complément de cobaye est fortifié avec 5 pour cent de sérum non inactivé de veau normal.

La série d'échantillons de sérum provenant de l'un des six veaux expérimentalement infectés furent négatifs dans l'épreuve d'Onderstepoort, ceux provenant de trois de ces veaux ont montré que des traces de réactions et chez les deux autres veaux un taux maximum d'anticorps de 1:10 fut observé. Toutefois chez ces six veaux le taux maximum d'anticorps a varié entre 1:10 et 1:80 dans l'épreuve de l'A.D.R.I.

Une bonne corrélation a semblé exister entre les résultats des deux méthodes appliquées aux sérums de moutons expérimentalement inoculés même si des taux d'anticorps de 3 à 8 fois plus élevés furent obtenus avec la méthode de l'A.D.R.I. Chez chaque mouton, des échantillons prélevés après inoculation ont réagi dans chaque épreuve.

Antibodies in the serum of cattle and sheep recovering from viral infection are, in general, not detectable by ordinary complement-fixation (CF) tests. We have shown, however, that such so-called non-complement fixing antibodies can be demonstrated by a modified direct CF test (1-3). In this method, guinea-pig complement is supplemented by the addition of 5 per cent fresh normal non-inactivated calf serum. Howell at Onderstepoort has standardized a CF technique for the testing of sheep sera for bluetongue antibodies in which the test serum is inactivated at 53°C rather than 56°C to avoid or reduce what was thought to be the destruction of antibodies at the higher temperature (4).

In most viral infections complement-fixing antibodies are of short duration; they disappear soon after the acute stage of the disease. In bluetongue in cattle the infection is often subclinical (5). However, bovines may serve as carriers of the virus for long periods. Amongst other means, infection in these animals can be shown by inoculation of their blood into susceptible sheep, which are later subjected to serological tests and procedures for isolation of the infective agent (6).

The present paper will describe briefly the CF methods used at Onderstepoort and at our laboratory, and will compare the results obtained by the two methods on sera of experimentally infected cattle and sheep.

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TABLE I. Comparison of the results of complement-fixation tests on experimentally infected cattle sera by Onderstepoort and A.D.R.I. methods

Calf number	Days of exposure	Complement-fixation titres: Onderstepoort test, Positive antigen	Onderstepoort test, Normal antigen	A.D.R.I. test, Positive antigen	A.D.R.I. test, Normal antigen
506520	0	–	–	–	–
”	63	± 1:5	–	1:40	–
”	84	± 1:5	–	1:20	–
”	133	± 1:5	–	1:20	–
”	168	–	–	–	–
29546	0	–	–	–	–
”	56	–	–	1:5	–
”	84	–	–	1:10	–
”	105	–	–	1:10	–
”	154	–	–	1:5	–
506602	0	–	–	–	–
”	28	± 1:5	–	1:10	–
”	35	1:5	± 1:5	1:40	–
”	42	1:5	–	1:40	–
”	49	1:10	± 1:5	1:80	–
”	56	1:10	± 1:5	1:80	–
”	63	1:10	± 1:5	1:40	–
”	70	1:10	± 1:5	1:40	–
”	77	1:5	–	1:40	–
”	84	1:5	± 1:5	1:20	–
”	98	± 1:5	± 1:5	1:10	–
3	0	–	–	–	–
”	21	–	–	–	–
”	35	± 1:5	–	1:40	–
”	56	± 1:5	–	1:20	–
506113	0	–	–	–	–
”	21	± 1:5	–	1:10	–
”	42	± 1:5	–	1:20	–
506226	0	N.D.	N.D.	–	–
”	35	1:10	–	1:80	–
”	105	1:10	–	1:80	–

–: complete haemolysis which indicates a negative reaction.
±: insignificant trace reaction in the dilution concerned.
N.D.: not done because the serum was not anymore available.

Materials and Methods

Sera used in the present experiment, as listed in Tables I and II, were from six calves and five sheep experimentally infected in connection with other projects. A full description of the experiment will be found in other publications (3, 7). These animals were proven to have been infected by inoculation of blood to other animals or to tissue culture followed by demonstration of the virus by immuno-fluorescence (7). A brief summary of the procedure used in the infection of animals will be given below.

Cattle — Calf 506520 was infected with the Cyprus (type 3) virus by the intravenous and subcutaneous inoculation of a pool of 15.0 ml. of infective calf blood and the same amount of infective sheep blood.

— Calf 29546 received intravenously 100.0 ml. of infective blood obtained by pooling samples collected from calf 506520 on day 1 through day 10 after inoculation.

— Calf 506602 received intravenously and subcutaneously 5.0 ml. of blood by each route, collected from calf 29546 on the 7th day after inoculation.

— Calf 3 received 10.0 ml. intravenously and the same amount subcutaneously of infective blood collected on the 5th, 6th and 8th post-inoculation days from calf 506602.

— Calf 506113 received 3.0 ml. intramuscularly and the same amount subcutaneously of blood from a sheep infected with Cyprus (type 3) virus.

TABLE II. Comparison of the results of complement-fixation tests on experimentally infected sheep sera by Onderstepoort and A.D.R.I. methods

Sheep number	Days of exposure	Complement-fixation titres			
		Onderstepoort test		A.D.R.I. test	
		Positive antigen	Normal antigen	Positive antigen	Normal antigen
161	0	–	–	–	–
"	22	–	–	–	–
"	45	1:25	± 1:5	1:80	1:10
"	57	1:50	± 1:5	1:80	1:5
"	64	1:25	± 1:5	1:160	1:5
"	78	1:25	± 1:5	1:160	1:5
"	85	1:25	± 1:5	1:80	1:5
"	113	1:10	–	1:40	–
"	127	1:10	± 1:5	1:10	–
"	141	1:10	± 1:5	1:10	–
5	–	± 1:5	± 1:5	–	–
"	32	± 1:5	± 1:5	1:10	± 1:5
"	74	1:5	± 1:5	1:40	1:5
"	88	1:10	± 1:5	1:80	1:5
"	95	1:10	± 1:5	1:40	–
34904	0	N.D.	N.D.	–	–
"	56	1:50	–	> 1:160	–
"	84	1:50	–	> 1:160	–
"	112	1:50	–	> 1:160	–
68R	199	1:50	–	> 1:160	–
34869	74	1:25	–	1:80	–

–: complete haemolysis which indicates a negative reaction.
+: insignificant trace reaction in the dilution concerned.
N.D.: not done because the serum was not anymore available.

— Calf 506226 was inoculated intravenously on three successive days with 3.0 ml. of blood from a calf infected with the California (type 10) virus.

Sheep — Sheep 161 was inoculated intravenously and subcutaneously with 5.0 ml. of infective blood collected from a sheep on the 7th day after inoculation with the Texas Agricultural Experimental Station strain of 1962.

— Sheep 5 received intravenously and subcutaneously 10.0 ml. of Cyprus (type 3) infective blood collected from calf 29546 on the 7th day post-inoculation.

— Sheep 34904 was inoculated intravenously with 5.0 ml. of infective blood from a sheep inoculated with California (type 10) virus.

— Sheep 68R was experimentally infected by exposure to keds which had been feeding on sheep infected with Cyprus (type 3) virus. In addition it received 3 months later 5.0 ml. of infective blood intravenously.

— Sheep 34869 was inoculated intramuscularly and subcutaneously with 5.0 ml. of California (type 10) infective defibrinated sheep blood by each route. Fourteen days later the inoculations were repeated using this time its own defibrinated blood collected at height of febrile response.

Complement-Fixation Methods

Onderstepoort's test — The Onderstepoort laboratory's CF method is a serum dilution test with constant antigen and two 100 percent haemolytic units of complement (4). The test serum is heat-inactivated at 53°C for 30 minutes and the unitage of guinea-pig complement is determined in the presence of the standard dose of antigen. The bluetongue viral antigen consists of an acetone-ether extract of infected mouse brain. An antigen control is also prepared with normal mouse brain.

To 0.1 ml. amounts of serial dilutions of serum and 0.1 ml. amounts of diluted antigen, is added 0.2 ml. of guinea-pig complement containing two complete (100%) haemolytic units. The period for fixation is 90 minutes at 37°C., after which

0.2 ml. of a mixture of equal parts of a 2 percent suspension of sheep red blood cells and diluted amboceptor is added. Tests are incubated for a further period of 30 minutes at 37°C., or until the complement-antigen controls are appropriately haemolysed. The amount of haemolysis is read and recorded. Serum titres are based on the highest serum dilution showing a 2+ reading i.e., 40-60 percent haemolysis.

Parallel tests were also performed by the Onderstepoort method on test sera inactivated at 60°C for 30 minutes using guinea-pig complement supplemented by the addition of 5 percent non-inactivated fresh normal calf serum.

A.D.R.I. test — In our laboratory, the general procedure described in the standard Methods of the New York State Department of Health (8), which is based on a 50 percent haemolytic unit, is followed closely for the routine titration of complement and amboceptor. As in the South African method, all dilutions of serum and other reagents are made in veronal-buffered saline containing magnesium and calcium ions. The test serum is inactivated at 60°C for 30 minutes. The bluetongue viral antigen is an acetone-ether extract of infected mouse brain. As in the previous method, a normal control antigen is used in testing each serum. A line type of serum titration is employed in which 0.1 ml. of serial dilutions of serum ranging from 1:5 to 1:160 and 0.1 ml. of properly diluted antigen is mixed with 0.1 ml. of a complement dilution containing 4.5 fifty percent haemolytic units. Tests are held overnight at 7-9°C to allow for fixation. After the addition of 0.2 ml. of a mixture of equal parts of a 5 percent suspension of sheep red blood cells and diluted amboceptor, the tests are incubated for 30 min. at 37°C. The percent haemolysis is read with the aid of a colour standard prepared from the same days' reagents. Titres of the sera are expressed in terms of the highest dilution with which 50 percent haemolysis or less occurred with antigen.

In addition to the ordinary direct CF test, a modified method was used in which complement to be incorporated into the tests is diluted in veronal buffered saline containing 5 percent of a selected, unheated, pretested, normal calf serum (1-3) as a supplementing factor. In winter months, it has often been difficult to find a calf with a serum of satisfactory suplementary activity, but this difficulty was not encountered during the testing reported here.

Results

Onderstepoort's Test

Standard — Pre-inoculation sera from the 6 experimentally infected calves listed in Table I, did not react in the standard Onderstepoort CF test in the presence of bluetongue viral antigen. Fifteen other sera amongst the 31 serial bleedings from these calves gave only trace reactions in the 1:5 dilution. Of the remaining 10 sera, 4 gave a complete reaction in 1:5 and 6 in the 1:10 dilution. Seven of the 11 sera collected from one of these calves (506602) gave trace reactions in the 1:5 dilution with normal mouse brain control antigen indicating a low degree of non-specific reactivity.

A greater degree of reaction was obtained with the sera from the 5 sheep listed in Table II. The sera collected from the first 3 sheep on the 45th, 88th and 56th day post-inoculation had titres of 1:25, 1:10 and 1:50 respectively. These titres remained at diagnostic levels for the remainder of the observation period. Only the 199th and the 74th post-inoculation sera from the fourth (68R) and fifth sheep (34869), respectively, were available for testing, and gave titres of 1:50 and 1:25.

Of the 21 sheep sera tested, 12 gave trace reactions in the 1:5 dilution in the presence of normal mouse brain antigen.

Modified — Modification of the Onderstepoort CF test by the addition of 5 percent fresh normal calf serum to guinea-pig complement did not enable detection of bluetongue antibodies. In this case, calf and sheep sera were previously inactivated at 60°C for 30 minutes instead of at 53°C, as performed in the standard test.

A.D.R.I. Test

Non-modified direct — No reactions were observed by this procedure in any of the 31 calf and 21 sheep sera listed in Tables I and II.

Modified direct — None of the pre-inoculation sera from the 6 calves listed in Table I reacted in the modified direct CF test. In addition, serum collected 21 days post-inoculation from the fourth calf (3) was also negative but the titre had risen to 1:40 by the next bleeding collected on the 35th day. The first post-inoculation bleed-

ings collected from the other calves between the 21st to 63rd day respectively had titres ranging from 1:5 to 1:80. These titres remained at diagnostic levels for the remainder of the experiment.

The 5 sheep listed in Table II developed comparatively higher serum titres than the calves. Pre-inoculation sera from the first three sheep and the 22nd post-inoculation serum from the first sheep were negative. The following bleedings, collected respectively on the 45th, 32nd and 56th days, had serum titres of 1:80, 1:10 and 1:160. These titres in certain cases increased and eventually decreased, but all remained at diagnostic levels for the duration of the experiment. The only available bleedings from the last 2 sheep, the 199th and 74th day bleedings, had serum titres of 1:160 and 1:80 respectively. Five sera collected from the first sheep and three from the second, gave low-grade reactions with normal mouse brain antigen.

An inspection of the Tables indicates that the A.D.R.I. method gave titres which were in general, 3- to 8- fold higher than those obtained by the Onderstepoort method. This is not an unusual observation. It is known that tests incubated for 18 hours at 9°C generally give higher titres than those incubated for 1 to 1½ hours at 37°C. In both methods a positive reaction could be suspicious of current infection, but a negative reaction in calf serum would not exclude past infection. This consideration is supported by the fact that serological titres, when present, remain at a diagnostic level for a few months only. This is the reason for recommending that blood of suspected calves be inoculated four times at 2-week intervals into susceptible sheep and testing sheep sera for antibody three weeks after final inoculation (6). It is important that an interval of a few weeks elapse after exposure in order to permit the development of a significant titre.

Discussion

Before selecting a procedure for the diagnosis of bluetongue, two points should be considered. Bluetongue in cattle is often sub-clinical (5) and when complement-fixing antibodies are present in the serum of an exposed animal, they remain at a detectable level for only a short period following infection (3). This should be taken into consideration if one is relying on the complement-fixation test only, for diagnosis of the infection in calves. As shown by Du Toit (6), a more accurate diagnosis is achieved by inoculation of suspected calf blood into isolated, known susceptible sheep, followed by the complement-fixation tests of their sera for bluetongue antibodies. Four inoculations are given at 2 week intervals and the animals bled after the necessary post-inoculating waiting period of 3 weeks. Our results suggest that either the Onderstepoort test on serum inactivated at 53°C for 30 minutes, or the A.D.R.I. modified direct complement-fixation test would be useful in establishing a presumptive serological diagnosis with serum samples from these inoculated sheep. The presumptive serological test could then be confirmed by inoculation of blood, collected from the test sheep at height of pyrexia, into appropriate tissue culture followed by staining with immunofluorescent conjugates (9-10). However, since the serological methods, except serum-neutralisation testing with living virus (9), are group reactive, final identification of the serotype involved can be done only by means of neutralisation test in tissue culture.

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