Studies of Johne's Disease in Canada.

X. A More Sensitive Complement-Fixation Test

by Christine E. Rice, H. Konst and J. Carrière

The modified implemented direct complement-fixation test developed by Brumfield and Pomeroy (1, 2) for the detection of psittacosis antibody in turkey sera, has been adapted to the titration of antibodies in bovine antiviral sera (3, 4). Vesicular stomatitis cattle sera after heating for 30 minutes at 56°C usually fail to fix complement with homologous viral antigen in the standard form of direct complement-fixation test, but may give marked fixation with this antigen when the guinea-pig complement is supplemented with diluted fresh bovine serum (4). In the case of serum from cattle immunized with infectious rhinotracheitis virus, more marked fixation was obtained when the test was supplemented with a globulin fraction of fresh bovine serum rather than with the whole serum itself. Furthermore, these serum fractions had less tendency than the whole serum to fix complement nonspecifically with tissue antigens.

It seemed possible that this supplemented direct test might be more sensitive than the standard direct test in the detection of small amounts of antibacterial antibody in cattle serum. In preliminary studies with sera of brucellosis cattle, no significant increase in complement-fixing titres with *Brucella abortus* antigen was observed when fresh unheated normal bovine serum was added to the complement. On the other hand, sera of cattle with Johne's disease gave definitely stronger reactions with Johne's bacillus antigens in the supplemented than in the standard direct test. Complement-fixing activity develops slowly in cattle known to be infected with Johne's bacilli and may never reach titres beyond 1:20 in the standard test. Hence, to increase the sensitivity of the complement-fixation test will increase its diagnostic value provided this is accomplished without loss of specificity. Further examination of the supplemented test appeared worthwhile therefore from the practical standpoint.

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Experimental Methods

Technique of Test

The supplemented test was carried out in exactly the same manner as the standard direct test (5), except that the guinea-pig complement was made up in a 1:20 dilution of fresh bovine serum or a globulin fraction thereof. Two Johne's bacillus antigens, bacillary suspension (JS) and carbohydrate fraction (JC), were employed in these tests in the same dilutions as used routinely (5, 6). Serum titres were expressed in terms of the highest dilution showing 50 per cent haemolysis or less.

Preparation of Norman Bovine Serum Fraction

Cattle, preferably under one year of age, were bled and the sera tested immediately for anticomplementary properties and nonspecific complement-fixing activity with the JS and JC antigens. Sera that appeared satisfactory were pooled, dispensed in 5 ml. amounts and frozen immediately. Such frozen pools retain their supplementing activity for at least a month, but tend to become anticomplementary on prolonged storage.

Alternatively, 5 ml. amounts of fresh serum were dialyzed in the cold for three hours in cellophane sacs against phosphate buffer, pH 5.8, ionic strength 0.02. More prolonged dialysis led to the precipitation of anticomplementary as well as supplementing substances. The precipitate was removed by centrifugation in the cold, washed, made up to 5 ml. in veronal buffer, pH 7.0, and used immediately or frozen. Extensive tests showed the reconstituted globulin precipitates to be more active as complement supplements than whole serum. These were used in all of the tabulated experiments.

On the day of the test, the whole serum or reconstituted precipitate was diluted 1:15, 1:20 or 1:30 in veronal buffer containing calcium and magnesium, the re-

TABLE I

Per cent haemolysis recorded in standard (stand.) and supplemented (suppl.) direct complement-fixation tests of five bovine sera * with two Johne's bacillus antigens: bacillary suspension and carbohydrate fraction

Serum Number	Test	Suspension Antigen						Carbohydrate Antigen					
		Serum Dilution											
		1:2	1:5	1:10	1:20	1:50	1:100	1:2	1:5	1:10	1:20	1:5	
1	Stand.	90	70	50	60	80	80	35	50	55	65	85	95
	Suppl.	5	0	0	5	40	80	0	0	0	0	0	5
2	Stand.	—	90	75	70	75	85	—	90	80	85	90	—
	Suppl.	80	50	30	35	80	90	30	0	5	5	5	20
3	Stand.	50	30	45	50	80	90	25	20	25	60	75	90
	Suppl.	20	30	50	50	65	75	5	0	0	5	5	30
4	Stand.	50	20	10	20	30	60	5	0	0	5	15	80
	Suppl.	0	0	0	10	15	40	0	0	0	0	0	20
5	Stand.	95	95	—	—	—	—	—	—	—	—	—	—
	Suppl.	95	95	—	—	—	—	—	—	—	—	—	—

*Sera Nos. 1, 2, 3 and 4 were from known infected cattle, No. 5 from a normal cow.

quisite dilution having been determined by preliminary titrations of its anticomplementary and supplementing properties.

Preparation of Guinea-Pig Complement

The guinea-pig complement was diluted 1:50 in the diluted bovine serum or fraction and titrated in the usual manner to determine the amount required for 50 per cent haemolysis. For the test it was made up in the same diluent to contain three 50 per cent haemolytic units in 0.1 ml.

Results

Preliminary comparative tests of sera from animals known to be infected with Johne's bacilli were made in parallel by the standard and supplemented tests with JS and JC antigens. With both antigens, a greater degree of fixation occurred in the supplemented test. Moreover, the prozones observed in lower serum dilutions were almost eliminated with the JC antigen and reduced with the JS antigen. This is illustrated in Table I for sera numbers 1, 2, 3 and 4. The increased sensitivity of the test with known positive sera was not accompanied by a decrease in specificity with known negative sera; that is, the addition of the supplement did not result in an increase in non-specific fixation with sera from animals known to be free from infection with Johne's bacilli, such as No. 5, Table I.

Experimentally Infected Cattle

Serial monthly bleedings from five experimentally-infected cattle were tested in parallel by both methods. On July 3, 4, 7 and 9, 1958, when they were less than six months of age, four of these calves had received oral doses of suspensions of mucosal scrapings from an animal that had died of Johne's disease. All four animals have since died with confirmed Johne's disease. The other animal, No.

TABLE II

Comparison of titres obtained with serial bleedings from four experimentally infected cattle in standard and modified direct complement-fixation tests with Johne's bacillus carbohydrate antigen

Date of Bleeding	Serum Titres							
	No. 27615		No. 27616		No. 27617		No. 27620	
	Stand.	Suppl.	Stand.	Suppl.	Stand.	Suppl.	Stand.	Suppl.
1958								
24/6	—	—	—	—	—	—	—	—
11/7	—	—	—	—	—	—	—	—
1/8	—	—	—	—	—	—	—	—
5/9	—	—	—	—	—	—	—	—
7/10	—	tr.	—	5	—	—	—	—
5/11	—	2	—	10	—	20	—	20
4/12	—	20	—	20	—	20	—	50
1959								
6/1	tr.	100	tr.	—	—	50	—	20
3/2	20*	100	10	50	—	50	—	100
6/3	20*	100	tr.	100	20	50	10	100
7/4	20*	100	20	100	20	100	20	100
6/5	50	100	10	100	100	>100	20	100
9/6	50	100	20	100	100	>100	50	100
9/7	50	>100	20*	100	50	>100	50	>100
11/8	20*	>100	20*	100	20	100	20	>100
10/9	20	>100	50*	>100	50	100	20	>100
9/10	20*	>100	50*	>100	100	>100	20	>100
10/11	10	>100	20*	>100	50	>100	10	>100
10/12	20	>100	20*	>100	100	>100	20	>100
1960								
13/1	100	>100	20*	100	50	>100	100	100
10/2	100	>100	**	**	>100	>100	100	100
11/3	20	100			>100	>100	20	100
4/4	**	**			50	>100	10	100
6/5					100	100	10	100
1/6					100	100	10	100
6/7					>100	>100	5	20
4/8					>100	>100	**	**
1/9					100	>100		

**Dead * Irregular test. tr. (trace) indicates that a weak reaction, 90 to 70 per cent haemolysis, was recorded with the 1:2 and 1:5 dilutions.

24703, on February 5, 1960, was given *per os* one dose of 180 ml. of a suspension of mucosal scraping from a case of Johne's disease. The results of intradermal tests and faeces examination on these five experimentally-infected animals, will be described by Konst and Carrière in a subsequent paper (7).

Each serial serum specimen had been tested when fresh by the standard complement fixation method, frozen immediately afterward, and stored at —20°C. Neither form of complement-fixation test detected antibody in the serial bleedings of any of the animals collected during the first three months after oral dosage. By the fourth month, No. 27616 showed a complement-fixing titre of 5 with JB and JC antigens in the supplemented test; by the fifth month, sera from Nos. 27615, 27617 and 27620 were also exhibiting a reaction in this test (Table II). It was not till 3 or 4 months later, that is 7 to 8 months after dosing, that fixation of complement was observed in the standard test. From then on reactions occurred in both tests, but titres in the supplemented test were usually higher. Serum titres were still elevated in the specimens collected a few weeks prior to death. The fifth animal, No.

TABLE III

Comparison of titres obtained for serial bleedings from naturally-infected cattle in standard and modified direct complement-fixation tests with Johne's bacillus carbohydrate antigen

Tag Number	Bleeding Date	Titre		Tag Number	Bleeding Date	Titre		Tag Number	Bleeding Date	Titre	
		Stand.	Suppl.			Stand.	Suppl.			Stand.	Suppl.
	1958				**1958**				**1958**		
40046	5/3	10	100	40059	7/11	—	—	40054	7/11	—	—
	2/4	20	100		4/12	—	—		4/12	—	—
					1959				**1959**		
	9/5	50	100		6/1	—	—		6/1	—	—
	12/6	100	100		2/2	tr.	10		2/2	—	—
	7/7	100	100		6/3	—	5		6/3	—	—
	6/8	50	100		7/4	—	2		7/4	—	—
	9/7	**	**		5/5	—	—		5/5	—	—
	1958										
40062	7/11	20	50		9/6	10	20		9/6	—	—
	4/12	10	20		9/7	tr.	10		9/7	—	—
	1959										
	6/1	20	20		11/8	—	2		11/8	—	—
	2/2	20	20		10/9	tr.	20		10/9	—	—
	6/3	20*	20		9/10	tr.	20		9/10	—	—
	7/4	20*	50		10/11	tr.	5		10/11	—	—
	5/5	20*	50		10/12	—	—		10/12	—	—
					1960				**1960**		
	9/6	20*	50		13/1	—	10		13/1	—	—
	9/7	20	50		10/2	tr.	20		10/2	—	—
	11/8	10*	50		11/3	tr.	50		11/3	—	—
	10/9	50*	50		4/4	—	20		4/4	—	—
	9/10	100	100		6/5	5	10		6/5	—	—
	10/11	20	100		1/6	10	5		1/6	—	—
	10/12	20	100		1/6	10	100		6/7	tr.	tr.
	1960										
					4/8	20	100		4/8	tr.	5
	18/1	**	**		1/9	20	100		1/9	tr.	5

**Dead.
*Irregular test

24703, began to react in both tests about three months after treatment and has continued to do so with titres somewhat higher in the supplemented test.

Naturally Infected Cattle

In this group are included five cows purchased in 1958 from the owner of a herd in which a cow died of proven Johne's disease. Each animal reacted initially in the intradermal johnin test or the complement-fixation test or both and acid-fast organisms indistinguishable from Johne's bacilli were demonstrated in its faeces. All animals have been housed in isolation at the Animal Diseases Research Institute since their purchase. Monthly bleedings have been made and the serum tested and stored in the frozen state. Hence they were still available for parallel testing by the supplemented and standard complement-fixation methods.

Nos. 40046 and 40062 showed moderately high complement-fixing titres when tested initially by the standard test and these high titres were maintained until the animals died of proven Johne's disease. When these stored serial bleedings were retested by the standard test, little or no loss in titre was evident; the titres in parallel supplemented tests were higher (Table III). No. 40059 showed little or no reaction in the standard test and fluctuating low titres in the supplemented test during the first year and a half of testing. Then a marked rise in titre in both tests occurred about three months after the last calving. This animal now shows clinical symptoms of Johne's disease. No. 40054 was negative in both tests from November 7, 1958, to June 1, 1960, then began to exhibit weak complement-fixing activity. The fifth cow, No. 40060, had a titre of 1:20 in the supplemented test with JS antigen from January 6, 1959 to January 13, 1960 inclusive, then became negative. It was consistently negative with the JS antigen in the supplemented test and with the JC antigen in both tests.

In addition to serum samples from these five cows, all blood specimens submitted during this period for the serological diagnosis of Johne's disease, were tested in parallel by both complement-fixation methods. The results for one group of 380 specimens are shown in the accompanying summary. These specimens were from 12 herds in which the presence of Johne's disease was confirmed by gross and microscopic examination of specimens of intestine taken at slaughter from animals reacting in either the intradermal or serological test or in both. It would seem warranted to assume therefore that the serological reactions obtained are indicative of the presence of infection in the respective animals.

Among the 380 cattle, 24.7 per cent reacted in one or both of the complement-fixation tests, the titres being consistently higher and the degree of fixation stronger in the supplemented test. Examination of the above values will show that on the basis of the latter test, 72 animals would be considered serologically "questionable" (titres 1:5 or 1:10) and 22 serologically "positive" (titres 1:20 or higher). By contrast, on the basis of the standard test, 64 would be classified as "questionable" and 8 as "positive". The reactions of these 8 animals were strong enough in both tests to be interpreted as a definite indication of the presence of complement-fixing antibody for Johne's bacillus antigens.

At the present time, all cattle sera submitted for diagnosis of Johne's disease are being tested on the first day by the supplemented direct method and all reactors retested the following day by the standard technique. This dual testing will be continued until sufficient comparative data have been accumulated to indicate that the gain in sensitivity has not been accompanied by any decrease in specificity.

Titres			
Standard Test	Supplemented Test	Number of Cattle	Per cent of Total
Negative	Negative	286	75.3
Negative	5 or 10	22	5.8
2 or 5	5 cr 10	50	13.2
5 or 10	20 or over	14	3.7
20 or over	50 or over	8	2.1

TABLE IV

The effect of the time of addition of bovine serum supplement (1:20) on the fixation of complement in tests of serum of an infected cow with Johne's bacillus carbohydrate antigen

Incubation Temperature	Time of Addition of Supplement	Antiserum Dilution						
		1:5	1:10	1:20	1:50	1:100	1:200	1:500
37°C. 90 min.	No supplement	65	—	—	—	—	—	
	Added before	0	0	20	90	—	—	
	Added with	0	0	20	90	—	—	
	Added 30 min. after	5	15	45	95	—	—	
	Added 60 min. after	15	40	95	—	—	—	
	Added 90 min. after	40	90	—	—	—	—	
4-8°C. 18 hrs.	No supplement	0	0	0	40	90	—	—
	Added before	0	0	0	0	0	50	90
	Added with	0	0	0	0	0	45	95
	Added 15 min. after	0	0	0	0	5	55	95
	Added 30 min. after	0	0	0	0	5	55	95
	Added 60 min. after	0	0	0	0	10	60	95
	Added 2 hrs. after	0	0	0	0	10	65	—
	Added 18 hrs. after	0	0	5	35	95	—	—

Time of Addition of Bovine Serum Supplement

In view of the encouraging results in the preliminary trials, more detailed studies are being made to determine whether further improvements may be made. Brumfield and Pomeroy (1, 2) emphasized the importance of adding the chicken serum supplement before complement in their "implemented" direct complement-fixation test for ornithosis viral antibodies in turkey serum; less fixation of complement was obtained when it was added subsequently. In our bovine antibody systems, no differences in results were observed when the bovine serum supplement was added before, with, or immediately after complement. In the 37°C test, lower complement-fixing titres were recorded when the intervening interval was extended to 30 or 60 minutes (Table IV). In the refrigerator test with a fixation period of 18 hours at 4 to 8°C, addition of the normal bovine serum as long as 8 hours after complement appeared as effective as its simultaneous addition, since the remaining 10 hour period was long enough for maximum complement fixation by the various antiserum-antigen mixtures. In either test, when the normal bovine serum was added at the end of the fixation period, just prior to the sensitized sheep-red-cell suspension, it had no evident supplementing effect.

These findings were confirmed in more precise tests in which the amount of complement required for 50 per cent haemolysis was determined. Twelve to 24 serial dilutions of complement were used in titrating the complement-fixing activity of each serum-antigen mixture with and without supplement and the 50 per cent value obtained by plotting percentage haemolysis against ml. complement. The results of a refrigerator test of 0.1 ml. 1:40 bovine antiserum with 0.1 ml. of JC antigen are given below:

Amount of Complement Required for 50 per cent Haemolysis.	
No bovine serum supplement	0.0030 ml.
Supplement added before complement	0.0061 ml.
Supplement added with complement	0.0064 ml.
Supplement added 30 min. after	0.0063 ml.
Supplement added 60 min. after	0.0065 ml.
Supplement added 2 hours after	0.0062 ml.
Supplement added 18 hours after	0.0039 ml.

Effect of Supplement on Antigen Titre

The same dilutions of JS and JC antigens were used in the standard and modified complement-fixation tests after initial titrations had indicated these amounts to be adequate. Further tests were made, however, to verify this conclusion. When tested by a serial dilution method, both antigens appeared of higher titre in the presence of bovine serum supplement than in its absence. Table V gives the results of representative water bath and refrigerator tests of JC antigen with an excess of bovine

TABLE V

The effect of the addition of bovine serum supplement (1:20) on the complement-fixing activity of Johne's bacillus carbohydrate antigen with bovine antiserum (0.05 ml. 1:5) in 37°C and refrigerator tests

Incubation Temperature	Supplement Added	Antigen Dilution					
		1:50	1:100	1:200	1:400	1:800	1:1600
37°C, 90 min.	None	40	45	65	80	—	—
	Supplement (1:20)	0	0	0	0	15	50
4-8°C, 18 hrs.	None	15	0	0	30	90	95
	Supplement (1:20)	0	0	0	0	10	35

The antigen, antiserum and complement controls with and without supplement showed 100 per cent haemolysis.

antiserum. The stock solution of JC antigen contains 2 mg. carbohydrate per ml., so that the amounts in these particular tests ranged from 0.004 to 0.000125 mg. The dilution used routinely contains 0.0004 mg. in 0.1 ml.

Fractionation of Normal Bovine Serum

As stated above, all of the normal bovine sera were carefully pre-tested and any that proved to be anti-complementary or fixed complement non-specifically with JB or JC antigens were discarded. Only a small proportion of fresh normal bovine sera meet these requirements. Accordingly, attempts were made to fractionate normal bovine sera to see if it would be possible to remove these undesirable activities without too great a reduction in the supplementary property. The dialysis procedure employed in simple fractionation of guinea-pig complement was followed.

Fresh normal bovine serum, in 5 ml. amounts, was dialyzed in the cold against phosphate buffer pH 5.4, ionic strength 0.02. The serum was removed from the dialysis bags after periods of 1 to 18 hours, centrifuged in the cold, the supernate removed and the precipitate resuspended in 5 ml. veronal buffer pH 7.0. The pH of the supernate was determined and adjusted to 7.0 by the addition of dilute sodium hydroxide. Each fraction was tested in serial dilutions for supplementing effects with constant amounts of bovine antiserum and JC antigen. The amounts of antiserum selected for the warm and cold tests gave only traces of complement-

TABLE VI

Supplementing activity of bovine serum fractions in 37°C complement-fixation tests Johne's bacillus carbohydrate antigen (1:250) and bovine antiserum (0.05 ml. 1:160)

Period for Dialysis	Test System	Precipitate					Supernate					No Fraction
		1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:80	1:160	
1 hour	JC +	35	85	90	—	—	0	0	0	0	50	95
3 hours	bovine	0	0	0	5	95	0	0	0	45	90	
4 hours	antiserum	0	0	0	0	60	0	0	0	60	98	
18 hours		0	0	0	0	0	0	45	70	85	90	
24 hours		0	0	0	0	0	60	70	90	95	—	
1 hour	Control*	—	—	—	—	—	—	—	—	—	—	—
3 hours		90	—	—	—	—	—	—	—	—	—	
4 hours		30	95	—	—	—	—	—	—	—	—	
18 hours		0	70	—	—	—	—	—	—	—	—	
24 hours		20	25	60	95	—	—	—	—	—	—	

*Fraction + Complement

fixation with the JC antigen in the absence of supplement.

The material precipitated during the first hour of dialysis was found to have some degree of supplementing activity, particularly in refrigerator tests, but the greater part of this activity remained in the supernate. The supernates became progressively less active as dialysis proceeded, while the precipitates became more active. Unfortunately, however, the latter also showed an increase in anticomplementary properties (Table VI). Precipitates formed during 18 to 24 hours dialysis were too anticomplementary for use.

Summary

Preliminary results with a more sensitive direct complement-fixation test for the detection of antibody in Johne's disease in cattle are presented. This test is similar in all respects to the standard direct test except that a dilution of unheated bovine serum or a globulin fraction of the same is added as a supplement to the guinea-pig complement. Higher titres were obtained with this supplemented test for sera from naturally infected cattle and reactivity was detected two to three months earlier in experimentally infected animals.

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Investigation into the Incidence and Causes of Infertility in Dairy Cattle: Brucella abortus and Vibrio fetus Infections

This paper represents an extensive study into factors affecting fertility in dairy herds served by two artificial insemination centres in southwest England — Dartington Hall and Somerset. The results of a field study of brucellosis and vibriosis are presented. Individual milk samples were taken from 3,935 cows and tested for Brucella abortus infection. The incidence of ring positive reactions was 5.4% of 1,166 at Dartington Hall, and 4.7% of 2,769 samples from Somerset. There were 0.2% whey positive reactors at Dartington Hall, and 1.5% in Somerset. Of 2,670 vaginal mucus samples collected in Somerset 0.9% were positive with the vaginal mucus agglutination test for Brucella abortus.

The ring test was influenced by various factors other than infection. The most important were the stage ingestation at the time of sampling and the use of strain 19 vaccine. Strain 19 vaccination, particularly in adults, was also associated with a higher incidence of positive reactors. Samples taken in the first week after calving had a higher incidence of positive reaction presumably due to the presence of colostrum. The whey agglutination test was affected to a lesser extent than the ring test by gestation stage and strain 19. The vaginal mucus agglutination test for Brucella abortus appeared to be less affected by extrinsic factors. In Somerset the herds were divided into infected and non-infected on the basis of herd history and the clinical tests used. Fertility measurements were made for the gestation prior to and current with sampling. Infected herds had a poorer conception rate than non-infected ones. Within the infected herds the positive individuals had poorer fertility than the non-reactors but these differences were not significant.

Vibrio fetus infection.

The incidence of positive reacters to Vibrio fetus with the vaginal mucus agglutination test was 5.7% of 1,605 samples and 5.3% of 677 samples in two rounds of sampling in the Dartington area; in Somerset it was 2.7% of 2,682 samples. In Dartington practically all positive reacters had been served naturally. In the whole material at both centres only 0.9% of the positive reacters had been serviced by artificial insemination exclusively. It is suggested that these are sporadic reactions as there was no significant fertility difference between positive and negative individuals and no other suggestion of vibriosis was present.

H. Boyd and H. C. B. Reed, The Veterinary Record 72:836, 1960