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A MODIFIED COMPLEMENT FIXATION TEST FOR BOVINE CONTAGIOUS PLEUROPNEUMONIA FOR LARGE-SCALE LABORATORY USE

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SUMMARY

Rapid testing of large numbers of cattle serum samples for complement fixing antibodies for bovine contagious pleuropneumonia was achieved by initially testing at a 1 in 10 dilution only. This screen test was carried out in a known order and only those sera showing fixation were identified, recorded and retested at dilutions of from 1 in 10 to 1 in 160.

Testing was facilitated by prior dispensing of preserved sheep red cells to give a standard concentration when made up to a predetermined volume, by the use of stored complement of known titre, by the premixing of complement and antigen, and by the addition of the reagents using an automatic pipetting machine.

Introduction

Since 1962, as part of a national campaign for the eradication of bovine contagious pleuropneumonia, large numbers of bovine blood samples have been tested at the Animal Research Institute for antibodies indicative of the disease. Certain modifications to the complement fixation (CF) test of Campbell and Turner (1936, 1953) have facilitated the testing of these large numbers of sera. The test incorporating these modifications is described in this article.

Materials

Blood samples.—These were collected in 1-oz pomade jars and despatched to the laboratory in cartons containing 16 samples. Most samples were from cattle at slaughter and were identified only by property of origin. The others were collected by tail-bleeding of cattle on properties. These were individually

labelled, and the identified stock were generally held until the results were known. Samples from distant centres were usually forwarded by air. If it was anticipated that samples would be in transit for more than 2 days, the sera were poured off by the field officers into clean bottles before despatch.

Diluent.—Dilutions were prepared in buffered saline (Cohen *et al.* 1953) or, since early 1964, prepared from "Oxoid" complement fixation test diluent tablets.

Complement.—Pooled fresh serum from a large number of guinea pigs was frozen rapidly in 10-ml ampoules in an alcohol-solid CO_2 bath and stored at a temperature below minus 40°C. Prior to use, the serum was thawed and an equal volume of complement preservative, prepared by dissolving 12 g of sodium acetate in 100 ml of 4% boric acid solution (Seelemann 1940), was added.

Antigen.—The titrated antigen was supplied by the Animal Health Research Laboratory of the Commonwealth Scientific and Industrial Research Organization at Parkville, Victoria. Its preparation has been described by Campbell and Turner (1953).

Red cells.—Sheep blood was collected aseptically from the jugular vein into an equal volume of modified Alsever's solution (Anon. 1957). It was dispensed into sterile bottles in quantities which would yield 50 or 75 ml of 6% washed red cells. The Alsever's solution consisted of 20·5 g dextrose (D glucose), 12 g sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), 0·55 g citric acid and 4·2 g sodium chloride dissolved in 1 l of distilled water. This was dispensed in appropriate amounts and autoclaved at 10 lb/sq in for 10 min.

Haemolysin.—Haemolysin, obtained from the Commonwealth Serum Laboratories, preserved with glycerol, was used after titration.

Methods

The haemolysin and complement titration methods were developed at the C.S.I.R.O. Animal Health Research Laboratory and the details made available to us.

The titrations and the CF tests were done in 3-in. x ½-in. Pyrex glass test-tubes in wire racks containing four rows of 12 holes. One volume of the reagents was taken as 0·25 ml.

Standardization of red cells.—A single standardization was done on each collection of preserved red cells. A known volume (usually 40 ml) was washed three times without loss of cells. Diluent was added to the packed cells until the suspension had a haemoglobin value of 2 g per 100 ml, which was taken as equivalent to a 6% suspension of red cells. From the volume of this suspension the volumes of preserved red cells which would yield 50 and 75 ml of 6% washed cells were calculated.

Haemoglobin values were estimated as oxyhaemoglobin (Dacie 1956, p. 29). Using a Sahli pipette, 20 cmm of the red cell suspension was added to 5 ml of approximately 0·05% ammonia in distilled water and the resulting solution allowed to stand for 10 min for complete colour development. The haemoglobin values were estimated in a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 545 mμ, the instrument having been calibrated by means of haemoglobin solutions of known concentration.

Haemolysin titration.—Haemolysin was initially diluted 1 in 250 and a series of 15 dilutions in which tenfold dilutions were achieved in each eight dilution steps were prepared as follows: to a row of 15 1-oz, wide-neck Universal containers (Cruickshank 1960, p. 432) 24·9 ml of diluent was added to the first and 1·5 ml to the subsequent bottles. By means of a 0·25 ml tuberculin syringe, 0·1 ml of haemolysin was added to the first. Using a graduated 10 ml pipette, 4·5 ml of the mixture was transferred to the next bottle, mixed, and 4·5 ml carried on to the next bottle, using the same pipette. This procedure was continued to the end of the row.

Bottle	1	2	3	4	5	6	7	8
Haemolysin (ml) ..	0·1 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →
Diluent (ml)	24·9	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Dilution of haemolysin*	$^1/_{250}$	$^1/_{330}$	$^1/_{440}$	$^1/_{590}$	$^1/_{790}$	$^1/_{1,100}$	$^1/_{1,140}$	$^1/_{1,900}$

Bottle	9	10	11	12	13	14	15
Haemolysin (ml) ..	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5
Diluent (ml)	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Dilution of haemolysin*	$^1/_{2,500}$	$^1/_{3,300}$	$^1/_{4,400}$	$^1/_{5,900}$	$^1/_{7,900}$	$^1/_{11,000}$	$^1/_{14,000}$

* To 2 significant figures

Starting with the highest dilution, 0·5 ml quantities of each haemolysin dilution were mixed with an equal volume of 6% washed red cells in test-tubes and allowed to react at room temperature for 30 min.

The haemolysin titration was carried out by transferring 0·25-ml quantities of each suspension of sensitized red cells to further corresponding test tubes each containing:—

diluent .. .. 0·5 ml

2·5 units, or excess complement .. .. 0·25 ml

These were incubated in a thermostatically controlled 37°C water-bath with shaking at 10-min intervals and the titration read after 30 min. The endpoint was taken as the tube showing complete haemolysis.

Six minimal haemolytic doses (MHD) were used to sensitize an equal volume of 6% washed red cells in the CF tests.

Complement titration.—Complement was diluted in two overlapping series with initial dilutions of 1 in 10 and 1 in 15 as shown below. The number of dilutions prepared in each series varied according to the estimated strength of the complement, but was never more than nine.

Series (1)

Bottle	1	2	3	4	5	6	7	8	9
Preserved complement (ml)	0·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5
Diluent (ml)	4·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Dilution of preserved complement*	$^1/_{10}$	$^1/_{13}$	$^1/_{18}$	$^1/_{24}$	$^1/_{32}$	$^1/_{42}$	$^1/_{56}$	$^1/_{75}$	$^1/_{100}$

Series (2)

Preserved complement (ml)	0·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5 →	4·5
Diluent (ml)	7·0	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Dilution of preserved complement*	$^1/_{15}$	$^1/_{20}$	$^1/_{27}$	$^1/_{36}$	$^1/_{48}$	$^1/_{64}$	$^1/_{85}$	$^1/_{100}$	$^1/_{150}$

* To 2 significant figures

Two parallel titrations were made: (i) in the presence of antigen, and (ii) without antigen. For each series, two rows of test-tubes were set up corresponding to the complement dilutions prepared. Each contained:—

	Row (i) (with antigen)	Row (ii) (no antigen)
Diluent (ml)	0·25	0·5
Corresponding dilution of complement (ml)	0·25	0·25
Antigen (ml)	0·25	..

These were incubated in the 37° water-bath for 30 min and a volume of sensitized red cells added. After re-incubation at 37°C, with shaking at 10-min intervals, for 30 min, the titration was read. The unit of complement was taken as the quantity producing 90–95% haemolysis. If this fell between two tubes, a dilution value midway between was used. One volume containing 2·5 units was used in the tests.

Previous titrations had shown that after removal from the deep freeze the preserved complement maintained its activity for longer than a week at 5°C. Consequently, the quantity of serum thawed and mixed with complement preservative at a particular time was usually that amount estimated to be required for a week's testing. This was normally titrated only once and stored at 5°C. Subsequent titrations were done only if it was kept for longer than a week at 5°C or if a fresh batch of red cells was being used.

The CF test.—Sera were screened at a single 1 in 10 dilution and reacting sera retested to titre. Procedures were usually carried out in the order in which they are described.

(i) *Preparation of sensitized red cells.*—Red cells which had been stored for at least 3 days in Alsever's solution, and which showed no trace of haemolysis during washing, were used. The amount of preserved red cells required for the testing on the day was washed three times in 0·85% sodium chloride solution in 15 ml centrifuge tubes, each centrifuge tube representing 25 ml of the final 6% washed red cell suspension. The 6% suspension was prepared by making the washed red cells up to the predetermined volume in diluent. An equal volume

of haemolysin containing 6 MHD in diluent was added and mixed quickly, and the suspension allowed to stand at room temperature for at least 15 min. The sensitized red cells were used on the day of preparation only.

(ii) *Dilution of sera.*—If the individual samples had not been labelled, the cartons of blood samples from each property were numbered and marked on the side with an arrow pointing to the right to indicate the order in which the samples were to be tested—always working from left to right, starting with the nearest row.

For screening the sera, 1 in 10 dilutions were prepared as follows: using an automatic pipetting machine, 0·45 ml volumes of diluent were dispensed into test-tubes. Where whole-blood samples were submitted for testing, the serum was taken directly off the clot. In most instances 0·05 ml of clear serum was taken with a calibrated Pasteur pipette, but occasionally, where poor separation of the serum from the clot had occurred, red cells were included with the sample. Allowance was made for the volume of these red cells by taking excess serum, up to a maximum of twice the normal 0·05 ml aliquot. The red cells normally settled out during standing and inactivation and only occasionally did a diluted serum sample have to be centrifuged. It was sometimes convenient to dilute the sera and store them overnight at 5°C the day before testing. The sera were inactivated in a 56°C water-bath for 30 min on the day of testing.

One volume of the two volumes of 1 in 10 inactivated serum was discarded from all tubes, leaving one volume for testing. This step enabled the removal of any contaminating red cells; this was accomplished by either sucking them up from the bottom of the test-tube or transferring a volume of the clear supernatant to a new test-tube.

(iii) *Screening.*—Equal quantities of antigen and complement (2·5 units) were mixed immediately before use and two volumes (0·5 ml) of the mixture added to the volume of clear, inactivated serum using an automatic pipetting machine. The tests were incubated in a water-bath at 37°C for 30 min. A volume of sensitized red cells was added, and after a further 30 min at 37°C with at least one shaking, the tests were read. In most instances the sera were read by eye against normal light. Sometimes reading was facilitated by the use of a fluorescent desk lamp covered with a sheet of clear perspex.

Sera giving a 1+ or greater reaction (more than 25% non-haemolysed cells) were set aside for retesting to determine their titre and to detect any anti-complementary samples. Only the samples which had a 1+ or greater reaction were identified and recorded.

(iv) *Retesting to titre.*—A 1 in 10 dilution of the serum was prepared by diluting 0·1 ml of clear serum, measured accurately with a 0·2 ml graduated serological pipette, with 0·9 ml of CF diluent. After inactivation, 0·25 ml doubling dilutions from 1 in 10 to 1 in 160, were carefully prepared and tested along with a serum control at 1 in 10. Sera found to be not anticomplementary

and showing 50% or more non-haemolysed cells at the 1 in 10 dilution were interpreted as suspect. Those showing 50% or more non-haemolysed cells at a 1 in 20 dilution were regarded as positive.

Control sera.—At least three positive control sera were included with each lot of screen tests and retests and were read to titre. The control sera, as in all such tests, should be checked against a reference. In the testing for CF antibodies of bovine contagious pleuropneumonia the C.S.I.R.O. Animal Health Research Laboratory has acted as the reference centre and positive control sera supplied diluted 1 in 5 in phenol saline have been used. It has been found satisfactory to dilute a small quantity of each of these sera with diluent to give approximately 5-ml lots which show an endpoint at a 1 in 2 dilution. Quantities of 0·6 ml of each diluted control serum were inactivated and the dilutions of 1 in 1, 1 in 2 and 1 in 4 tested so that in each case the endpoint was in the middle tube.

Discussion

The favourable position regarding bovine contagious pleuropneumonia in Queensland has been referred to by Ladds (1965), who reported an incidence of 0·39% reacting sera in 76,000 tested between June 1961 and July 1964. At least 76·6% of these reactions could be attributed to recent vaccination. Few anticomplementary sera have been received, and consequently it has been necessary to retest to titre only a relatively small number of samples. Under these conditions, the use of the modified CF test of Campbell and Turner has enabled a trained staff of three to test, at least for short periods, up to 1,000 sera per day.

Hindmarsh, Webster, and Stewart (1943), in examining sera during an "extensive and virulent" outbreak of bovine contagious pleuropneumonia in Victoria, discovered on rare occasions sera from animals displaying symptoms of the disease which gave negative reactions at a 1 in 10 dilution and yet reacted with higher serum dilutions. As a consequence they tested the two serum dilutions of 1 in 10 and 1 in 20. This 2-tube test was used initially during outbreaks of the disease in New South Wales but was later replaced by a test employing a single 1 in 10 serum dilution (Golding 1958). The 1-tube test was adopted in this laboratory after considerable experience with the 2-tube test had indicated that if a prozone did occur at the 1 in 10 dilution it must be extremely rare.

In most cases the test was carried out in the test-tube in which the serum was diluted. A criticism of the technique may be levelled where poor separation of the serum from the clot had occurred and it can only be assumed that in every case allowance was made for the contaminating red cells taken up with the serum aliquot. Risk of error here was largely overcome by the retesting of all sera which give at least a +14 reaction. Any false positive reactions resulting from over-compensation for the volume of the red cells during screen-testing would have been detected when retesting was carried out using an accurately

measured volume of the clear serum. The low percentage (less than 0·15%) of anticomplementary samples is indicative of the good condition of most of the samples arriving at the laboratory as well as the good keeping quality of cattle serum. The positive control sera diluted to give an endpoint at a dilution of 1 in 2 and kept at 5°C have remained apparently stable for periods of at least 2 weeks. Certainly no significant loss of antibody was observed in positive control sera included with batches of diluted sera stored overnight at 5°C.

The pooling and freezing of complement from a large number of guinea pigs is more convenient than more frequent bleeding to meet the immediate requirements. Little variation in complement activity was observed in serum stored at below −40°C. for at least 9 weeks (the longest period tried), and this has simplified the titration of each week's supply of thawed, preserved complement. A close day-by-day check on the complement activity could be kept by means of the titres of the positive control sera included with the tests. Slight deterioration of the complement served to increase the sensitivity of the test but did not significantly decrease the value of the screen-testing on a particular day.

The mixing of complement and antigen before their addition to the serum saved time in that one manipulation of the tests was avoided, but presupposes that appreciable inactivation of the complement did not occur during the interval between mixing and dispensing. Control sera and sera being retested to titre were always set up before screen-tests, so with the sera taken to titre the time interval was quite short, less than 2 min, though it may have been up to 10 min for the screen tests. Experience has shown that at room temperatures appreciable inactivation of complement does not occur for at least 10 min after mixing with CPP antigen, and this has been supported by complement titrations which have consistently shown that the antigen exhibits relatively little anticomplementary activity. Furthermore, as sera which reacted during screenings were retested to titre as a matter of course, at least in this test the mixing of complement and antigen just prior to dispensing appears justifiable.

The setting up of the tests was facilitated and it was evident that reduced technical variation resulted from the use of commercial CF diluent tablets, prediluted control sera, an automatic pipetting machine which allowed the rapid delivery of small, accurately measured volumes, and the prior standardization and dispensing of preserved red cells.

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