A modified radioimmunoassay for antibodies against *Brucella abortus*

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SUMMARY

The radioimmunoassay for brucellosis previously reported from this laboratory was a sensitive and useful method for detecting antibody against *Brucella abortus* in bovine serum. Changes in the procedure have improved sensitivity, have apparently increased interassay precision, and have made the assay easier to perform.

INTRODUCTION

Our original radioimmunoassay (RIA) for antibodies against *B. abortus* (Chappel *et al.* 1976) showed promise as a serological test for bovine brucellosis. It measured antibody of the IgG_1 and IgG_2 subclasses but not IgM. Because the assay method involved competition for antigen between populations of antibodies, IgG antibody of higher avidity was measured with greater sensitivity. The original RIA gave fewer false negative reactions under field conditions than either the complement fixation test (CFT) or the serum agglutination test (Chappel *et al.* 1978*a, b*).

The RIA procedure has been modified to make it more suitable for routine use. In the modified method a serum standard is used instead of purified immunoglobulin, there are fewer steps and results are calculated by computer.

The RIA result is a measure of a combination of antibody concentration and avidity. The results of our original method were expressed as the number of nanograms of IgG_1 standard that was equivalent to the antibody in 12·5 μl of test serum. The results of the modified assay are expressed in arbitrarily defined units to avoid giving the misleading impression that the RIA measures absolute amounts of antibody.

MATERIALS AND METHODS

Antigen was the Standardized *B. abortus* Agglutination Concentrate (Alton, Jones & Pietz, 1975) obtained from the Commonwealth Serum Laboratories, Melbourne. It was washed by centrifugation and diluted in sodium phosphate buffered saline (0·116 M phosphate, 0·139 M chloride), pH 7·2 (PBS). Albumin diluent was PBS containing 5 mg/ml bovine serum albumin (Sigma Chem. Co.).

IgG_1 and IgG_2 were purified for bovine sera positive to the CFT (Anon., 1977) as described by Allan *et al.* (1976). Pools of bovine sera negative to the Rose Bengal

plate test (Allan *et al.* 1976) or to the modified RIA were made. Control sera were made by mixing serologically positive and negative serum.

Immunoglobulins with antibody activity against *B. abortus* were labelled with iodine-125 (The Radiochemical Centre, Amersham, Bucks) using lactoperoxidase (Marchalonis, 1969). The specific activity of labelled immunoglobulin was in the approximate range 0·5 to 1·0 μCi/μg.

Radioimmunoassay standards

The immunoglobulin standard for the original method was an IgG_1 preparation (Chappel *et al.* 1976). Working standards covered the approximate range 100 to 10000 ng of brucella-specific IgG_1.

The serum standard for the modified RIA was a pool of sera from cattle with high CFT titres. It was stored in small volumes without preservative below -18 °C. The activity of this serum was arbitrarily defined as 1200 units (u.), where the unit is a measure of both the concentration and the avidity of antibody. A 1/30 dilution of the standard serum gave a reaction of 1000 ng equivalents in the original RIA, so that one unit approximately equalled 25 ng equivalents. Standard serum was diluted periodically in pooled negative serum to give working standards of 5, 10, 20 and 40 u. Negative pooled serum represented 0 u. Working standards were stored frozen in volumes sufficient for a single assay.

Radioimmunoassay procedure

The following additions were made to 5 ml polypropylene (Type DWT-1P, Medical Plastic Supplies, Melbourne) or polystyrene tubes:

Original RIA

(1) 50 μl albumin diluent, (2) 50 μl test serum (diluted 1/4 in PBS) or immunoglobulin standard (in PBS) or PBS, (3) 50 μl labelled immunoglobulin in albumin diluent, (4) 50 μl washed antigen (1/300 in PBS) or PBS.

Modified RIA

(1) 500 μl labelled immunoglobulin in albumin diluent, (2) 25 μl serum (test, standard or control), (3) 50 μl washed antigen (1/300 in PBS) or PBS.

Tubes were capped and were incubated at 37 °C for 3 to 24 h. They were then placed on ice and to each was added 1·0 ml of cold albumin diluent. They were immediately centrifuged for 10 min. at 10000 r.p.m. at 4 °C using a JA-14 head of a Beckman J-21B centrifuge. Inserts for the head were made to hold 11 tubes each. Tubes were next placed on ice, the supernatants were removed by aspiration, and precipitated radioactivity was counted in a Packard gamma scintillation counter. Immediate removal of supernatants was necessary to minimize dissociation of labelled antibody from the antigen pellet.

The use of polypropylene tubes was adopted as the polystyrene tubes occasionally cracked during centrifugation. The same amount of specific anti-brucella antibody per tube, estimated to be 0·2 μg, was used in both procedures, and the same preparation of IgG_1 was used for radioiodination. Standard sera were replicated four to six times in each assay and test sera were in duplicate.
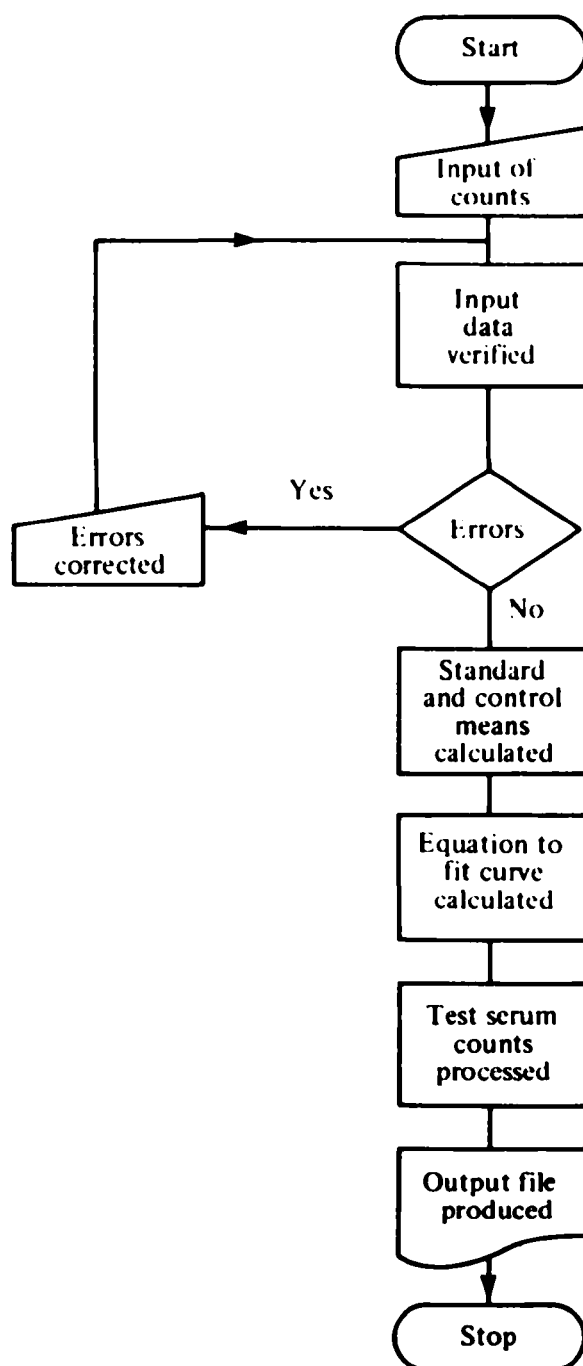
Fig. 1. Flow chart showing main steps in the computer processing of
radioimmunoassay data.

Computer calculation of radioimmunoassay results

The results of the modified RIA were usually calculated by computer. The
computer program was designed for a standard assay in which 105 unknown sera
were tested and working standards and control sera were each replicated six times.

Rather than transforming the data to give a linear plot, the actual standard
curve was approximated using a polynomial equation. Data were processed on a
Data General Nova 3 minicomputer. The main programming steps are outlined
in Fig. 1:

(*a*) A disk file was created manually using a text editor, by typing in the gamma
counts for each assay tube. A subprogram ordered the file into a form suitable for
subsequent calculations, checked it for errors, and printed it out. The counts in the
file were then checked by the operator, and any errors were corrected.

(b) The main program calculated the mean, standard deviation and coefficient of variation (CV) of the six counts for each standard and control serum. If the CV exceeded 10% the count with the greatest deviation from the mean was rejected until the CV was less than 10%.

(c) The coefficients of the polynomial equation that best fitted the standard curve were calculated from the mean counts of the standards, using the gaussian-elimination method of least squares. When a fourth degree polynomial was determined from the five standard points alone, the gaussian-elimination method sometimes produced an irregular S-shaped curve between 0 and 5 u., a region in which the true curve is virtually linear. The problem was overcome by including in the calulation, counts corresponding to 1, 2 and 3 u. of antibody, assuming linearity in this part of the curve. A good approximation of the actual standard curve was usually obtained from the resulting seventh degree polynomial equation.

(d) The units of antibody corresponding to the mean counts for each test serum were calculated from the equation. Control serum values were determined from three pairs of counts as well as from the set of six. Where duplicate counts differed by more than 10% of their mean they were rejected.

(e) The output file showed mean counts and the corresponding units of antibody for each test, standard and control serum, along with all individual counts that had been rejected. It also plotted the points of the standard curve.

Quality control

Individual test sera whose duplicate counts were rejected as described above were retested. Poor precision between duplicate counts was occasionally caused by the accidental removal with the supernatant of part of an antigen pellet.

Whole assays were rejected only occasionally. The decision to accept or reject an assay was made by the operator, who used a subprogram to validate computer results for counts falling between the standard points. The subprogram calculated the polynomial equation and gave the number of units for any count fed in. Values corresponding to a set of appropriately spaced counts were plotted on a manually drawn curve, and the computer calculation was accepted if all calculated values were within 0·5 u. of that curve.

The operator took into account the control serum values, the CV of the standard serum counts, and the number of standard serum counts rejected. The usual reason for rejecting an assay was that one standard gave an unusually high or low mean value relative to the other standards, causing distortion of the calculated standard curve.

Variations in the modified radioimmunoassay method

A number of variations were made to the modified method, to see whether standard curves and the values given by control sera remained satisfactory.

The conditions varied were the concentration of labelled antibody, the batch of immunoglobulin used for labelling (IgG_1 and IgG_2 were represented), the dilution of antigen, the batch of antigen, the volume of serum, the incubation time and the incubation volume. The latter was varied by changing the volume of diluent in which the same amount of labelled antibody was added. In other experiments the 1·0 ml of albumin diluent normally added after incubation was omitted or was added after the labelled antibody.
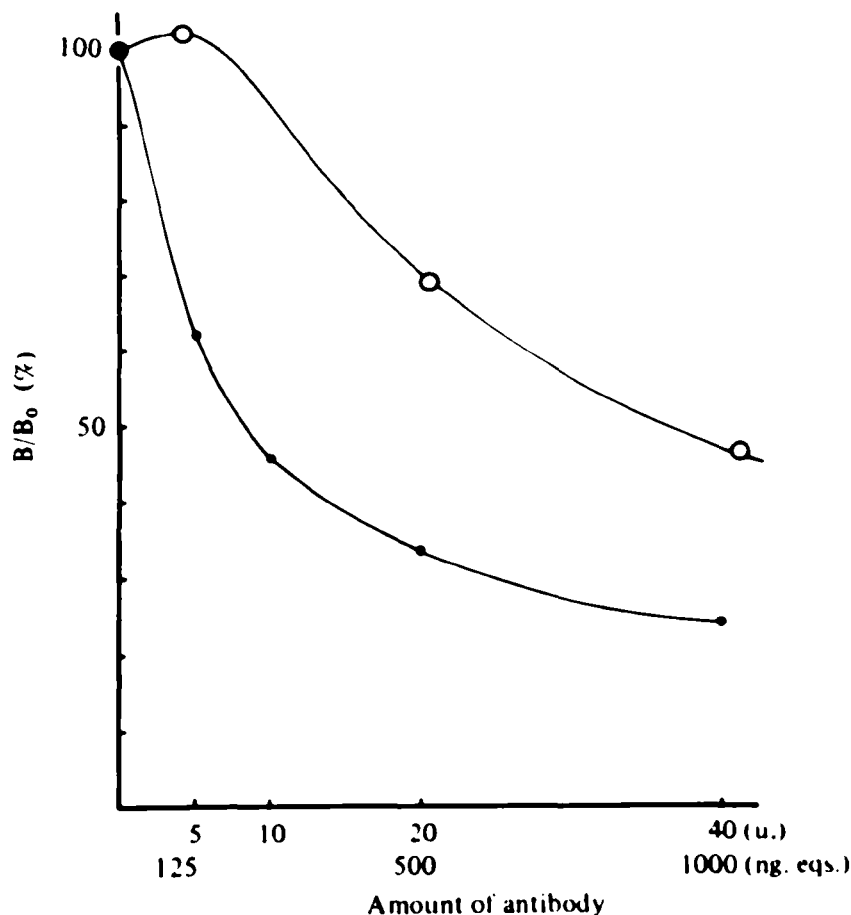
Fig. 2. Comparison of the original (○) and modified (●) radioimmunoassay standard curves. B/Bo = counts bound/zero point counts.

RESULTS

Sensitivity

Figure 2 shows typical standard curves obtained with our original RIA using purified immunoglobulin standards, and with the modified RIA using serum standards. They are plotted on the same scale, assuming one unit to equal 25 ng equivalents.

The modified RIA was considerably more sensitive than the original method, partly because the irregularity that was sometimes present at the start of the standard curve did not now occur. Owing to this irregularity, the minimum value distinguishable from zero in the original method was about 200 ng equivalents. In the modified RIA, 1 u. could be distinguished from zero, representing an eight-fold increase in sensitivity.

The range of the modified assay, up to 40 u. or about 1000 ng equivalents, is one tenth that of the original assay.

Precision

Table 1 shows the precision of control serum values in the modified RIA, compared with original assay results obtained by Chappel *et al.* (1976). There were three values for every control serum from each assay, determined from each of the three pairs of duplicate counts generated. Variance of radioimmunoassay results is greatest at the extremities of the standard curve (Woo & Cannon, 1976) and control serum A, with the least antibody activity, gave the greatest variation.

Table 1. *Precision of the modified radioimmunoassay*

Control serum	Modified RIA							Original RIA
	A	B	C	D	E	F	G	
Number of assays	12	34	52	38	52	37	48	10
Number of estimations	36	102	156	114	156	111	144	30
Mean*	3	6	7	15	15	21	25	1200
Range*	2–4	4–8	5–12	11–19	11–22	17–28	16–33	900–1600
CV (%)								
Intraassay	17	11	13	7	10	7	11	8
Interassay	13	4	9	5	6	7	6	17

* The original RIA results are expressed in ng equivalents and the modified RIA results in units.

Table 2. *Effect of varying assay conditions on control serum values
in the modified RIA*

Aspect of assay varied			Control serum (u.)		
			C	E	G
Labelled antibody added					
(μg brucella-specific)					
0·04			8	16	22
0·1			6	13	22
0·2*			7	17	24
0·4			6	18	24
Labelled antibody preparation					
	brucella-specific percentage of immunoglobulin	Amount added (μg brucella-specific)			
IgG₁ 1*	21	0·2	5	16	21
2	5	0·2	8	19	ND
3	17	0·2	8	18	26
4	10	0·2	7	15	26
5	28	0·3	8	16	24
6	22	0·2	7	15	24
7	12	0·2	7	17	28
IgG₂ 1	6	0·2	7	15	25
2	6	0·2	9	16	27
Antigen dilution					
1/50†			—	—	ND
1/100†			7	12	ND
1/200			8	17	ND
1/300*			7	17	ND
1/500			8	17	ND
1/1000			8	16	ND
Batch of antigen					
1			7	17	23
2			9	17	21
Serum volume (μl)					
5†			—	—	—
10			6	16	23
15			7	13	25
20			7	17	25
25*			8	17	22
25*			8	16	25
50			8	16	26
100			8	17	28
Incubation time (h)					
2			8	16	25
4			6	14	29
6			7	17	27
24			7	15	26
Incubation volume (μl)					
175			7	15	28
575*			5	15	22
1075			7	17	24
2075			8	14	18
Addition of albumin diluent					
Before incubation			7	14	21
After incubation*			8	16	26
None			7	19	21

* Routine assay conditions. † Irregular standard curve produced. ND = not done.

Although the precision of the original RIA was only determined at one point on the standard curve, the results suggest that interassay precision was much improved in the modified RIA. Intraassay precision appeared to be comparable for both methods.

Effects of variations in modified assay conditions

The results of experiments in which the conditions of the modified RIA were varied are given in Table 2. In most cases the control serum values were little influenced by variations in procedure within the limits tested, and standard curves were satisfactory. The two lowest antigen dilutions and the lowest serum volume gave rise to irregular standard curves and unsatisfactory control serum values.

DISCUSSION

Modification of the RIA increased sensitivity approximately eight-fold, and eliminated an irregularity sometimes found at the start of the standard curve. It also appeared to increase interassay precision. These improvements are probably attributable to the adoption of a serum standard.

The practical sensitivity of a serological test depends not only on its inherent sensitivity, the minimum result that can be confidently distinguished from zero, but also on the minimum diagnostic value selected. We have adopted a value of 5 u. for the modified RIA, and this has proved satisfactory in studies of vaccinated or experimentally infected cattle (Chappel *et al.* 1981; Hayes & Chappel, 1981). A minimum diagnostic value of 200 ng equivalents, or about 8 u., was proposed for the original RIA (Chappel *et al.* 1978a, b).

The modified assay is more suitable for routine use than the original and its range has proved adequate in practice.

The modified RIA is effective in detecting early infection in vaccinated animals (Hayes & Chappel, 1981) but gives no more persistent reactions after strain 19 vaccination than the CFT (Chappel *et al.* 1981). It has been used in our laboratory for more than 3 years, and its use has helped to eliminate infection in some herds in which eradication by test-and-slaughter has proved difficult.

REFERENCES

ALLAN, G. S., CHAPPEL, R. J., WILLIAMSON, P. & McNAUGHT, D. J. (1976). A quantitative comparison of the sensitivity of serological tests for bovine brucellosis to different antibody classes. *Journal of Hygiene* **76**, 287.

ALTON, G. G., JONES, L. M. & PIETZ, D. E. (1975). Laboratory techniques in brucellosis, 2nd ed. *World Health Organization Monograph Series* no. 55. Geneva.

ANON. (1977). Standardised complement fixation test for bovine brucellosis. *Australian Veterinary Journal* **53**, 394.

CHAPPEL, R. J., HAYES, J., ROGERSON, B. A. & SHENFIELD, L. J. (1981). The serological response of cattle to vaccines against brucellosis, as measured by the brucellosis radioimmunoassay and other tests. *Journal of Hygiene* **88**, 11.

CHAPPEL, R. J., McNAUGHT, D. J., BOURKE, J. A. & ALLAN, G. S. (1978a). Comparison of the results of some serological tests for bovine brucellosis. *Journal of Hygiene* **80**, 365.

CHAPPEL, R. J., McNAUGHT, D. J., BOURKE, J. A. & ALLAN, G. S. (1978*b*). The diagnostic efficiency of some serological tests for bovine brucellosis. *Journal of Hygiene* **80**, 373.

CHAPPEL, R. J., WILLIAMSON, P., McNAUGHT, D. J., DALLING, M. J. & ALLAN, G. S. (1976). Radioimmunoassay for antibodies against *Brucella abortus*: a new serological test for bovine brucellosis. *Journal of Hygiene* **77**, 369.

HAYES, J. & CHAPPEL, R. J. (1981). A comparison of the results of the brucellosis radio-immunoassay and other serological tests in experimentally infected cattle. *Journal of Hygiene* **88**, 21.

MARCHALONIS, J. J. (1969). An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochemical Journal* **113**, 299.

WOO, J. & CANNON, D. C. (1976). A quality control program for the radioimmunoassay laboratory. *American Journal of Clinical Pathology* **66**, 854.