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Radioimmunoassay for antibodies against *Brucella abortus*: a new serological test for bovine brucellosis

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SUMMARY

A radioimmunoassay (RIA) has been developed to measure antibodies against *Brucella abortus* in bovine serum and can be used in the diagnosis of bovine brucellosis. The RIA measures the amount of specific antibody of the IgG_1 and IgG_2 subclasses but is insensitive to IgM, a characteristic which may make it more suitable than the complement fixation test (CFT) or the serum agglutination test for distinguishing infected animals from those which have been vaccinated with *Br. abortus* strain 19. The RIA is not subject to prozoning or ambiguous reactions, both of which interfere with the interpretation of the CFT.

INTRODUCTION

Commonly used serological tests for bovine brucellosis have a number of limitations. The serum agglutination test (SAT) gives negative results on sera from some animals from which *Br. abortus* can be isolated (Alton, Maw, Rogerson & McPherson, 1975), yet false positive reactions can be caused by non-specific agglutinins in serum (Hess, 1953). The Rose Bengal plate test (RBPT) gives many false positive reactions (Alton *et al.* 1975) but it is useful as a screening test because it is rapid and simple. The complement fixation test (CFT) is probably the most accurate test in widespread use but prozoning and ambiguous reactions sometimes make interpretation of the results difficult. Prozoning is at least partly attributable to an abnormally high ratio of specific IgG_2 to specific IgG_1 and a sufficiently high proportion of IgG_2 can, in fact, block complement fixation altogether (Plackett & Alton, 1975; McNaught *et al.* 1977).

Radioimmunoassay (RIA) has been widely applied in estimating the concentrations of antigens but it is equally applicable to the measurement of specific antibody concentrations. This method is potentially more sensitive and specific than existing serological tests for bovine brucellosis.

MATERIALS AND METHODS

Antigen

Antigen was obtained from Commonwealth Serum Laboratories, Melbourne, as the Standardized *Brucella abortus* Agglutination Concentrate (Alton & Jones, 1967).

Antigen dilutions quoted below refer to this concentrate. Antigen was washed before use in phosphate buffered saline (PBS) pH 7·2 and diluted in the same buffer.

Albumin diluent

Albumin diluent was made by adding bovine albumin to PBS at a concentration of 5 mg./ml.

Sera

Sera for the isolation of immunoglobulins with antibody activity against *Br. abortus* were obtained from cows judged to be infected on the basis of high titres to the CFT or SAT or from adult cows which had been vaccinated 11–13 days previously with *Br. abortus* strain 19. Sera for testing were taken from those submitted to the Attwood Veterinary Research Laboratory for testing in connexion with a brucellosis eradication programme.

Immunoglobulins

IgG_1, IgG_2 and IgM were purified from sera, and their purity and identity established, as described by Allan, Chappel, Williamson & McNaught (1976).

Immunoglobulins were labelled with iodine-125 by the lactoperoxidase method (Marchalonis, 1969). Specific activities obtained were in the range 0·4–1·4 μci./μg.

The percentage of each immunoglobulin preparation directed specifically against *Br. abortus* was estimated by adsorbing radioiodinated antibody onto excess antigen (Allan *et al.* 1976). This was called the *Brucella*-specific percentage. Protein concentration was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using a commercial preparation of bovine immunoglobulin as a standard. Using the protein concentration and the *Brucella*-specific percentage, the concentration of *Brucella*-specific antibody in each preparation was calculated. Immunoglobulin concentrations quoted below refer, unless otherwise stated, to *Brucella*-specific antibody rather than total immunoglobulin.

Serological tests

The SAT was performed as described by Alton & Jones (1967).

The RBPT was performed as described by Allan *et al.* (1976).

The CFT was performed by a micro-method (Alton *et al.* 1975) using warm fixation (37° C, 40 min.) and a dilution of added antigen of 1/100. The reaction at each dilution was read on a scale from 1 to 4 (complete fixation), trace reactions being also recorded. Occasional results occurred which could not be confidently interpreted. These were termed ambiguous, and included those cases in which a low reaction occurred at each of several dilutions.

Radioimmunoassay

A typical RIA was set up as follows. To polystyrene tubes were added, in the following order, 50 μl. each of:

(1) albumin diluent,

(2) serum (diluted 1/4 in PBS) or IgG_1 standard (diluted in PBS) or PBS,

(3) [^{125}I]IgG_1 (diluted in albumin diluent),

(4) washed antigen (diluted in PBS) or PBS.

Standard curves were replicated four to six times, using typically 200 ng. of specific [^{125}I]IgG_1 and antigen added at a dilution of 1/300. Standards covered the approximate range 100–10,000 ng. of specific IgG_1. A zero point with no unlabelled antibody was always included. A zero point control, without antigen, was usually included but was not essential. Sera were normally assayed in duplicate without controls.

Tubes were capped and incubated at 37° C. overnight. To each tube was then added 1 ml. of albumin diluent between 0 and 4° C., and tubes were immediately centrifuged at about 10,000 *g* for 10 min. at 4° C. Tubes were then placed in an ice bath and the supernatants were removed as quickly as possible. Precipitated radioactivity was counted in a well-type scintillation counter.

Serum levels of IgG were read from the standard curve. The value obtained for a serum was accepted if the duplicate counts agreed within 10% of their mean, or where both duplicates lay outside the working range of the standard curve.

Competition by immunoglobulins isolated from different sera

Experiments were performed to compare the abilities of IgG_1, IgG_2 and IgM from the sera of different animals to displace radioiodinated IgG_1 or IgG_2 from antigen.

Precision of the assay

Experiments were performed to assess the precision of the assay. Firstly, a single serum was included three times in each of ten different assays. Secondly, in another series, 113 sera found to contain the equivalent of between 500 and 2000 ng. of the standard used were repeated once in subsequent assays. Thirdly, 26 sera which had values less than 200 ng. and 26 other sera which had values more than 10,000 ng. were retested in subsequent assays.

Comparison with other serological tests

Five hundred sera from female cattle, all of which were positive to the RBPT, were tested by RIA, CFT and SAT.

RESULTS

A typical standard curve for IgG_1 is shown in Fig. 1. One hundred nanograms of added unlabelled IgG_1 sometimes caused a slight rise in precipitated radioactivity. Sera negative to the RBPT usually gave more precipitated radioactivity than the zero point. It was nearly always possible to read from the standard curve unknown serum values of 200 ng. and above.

About 1·0–1·5% of added radioactivity was left in zero point control tubes. This could be reduced to about 0·5% by washing once or twice with 1 ml. of albumin diluent but washing was not found to be worthwhile in routine assays.

Albumin diluent was necessary to prevent complete adsorption of [^{125}I]IgG_1

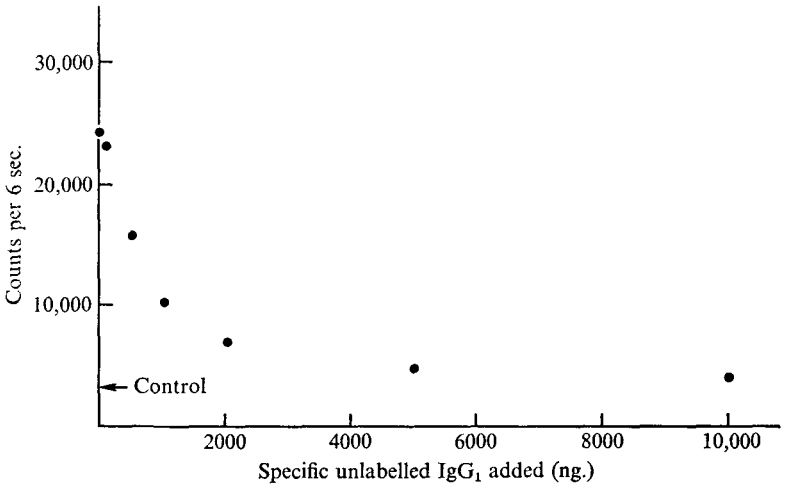

Fig. 1. Typical standard curve for the radioimmunoassay. The incubation mixtures contained 200 ng. of specific [^{125}I]IgG$_1$ and *Br. abortus* antigen at a final dilution of 1/1200, in a total volume of 200 μl.

Table 1. *Displacement (%) of [^{125}I]IgG$_1$ and [^{125}I]IgG$_2$ from* Br. abortus *antigen by batches of unlabelled IgG$_1$ and IgG$_2$ derived from different sera*

(A constant amount of specific [^{125}I]IgG$_1$ or [^{125}I]IgG$_2$ was incubated separately with 10 μg. (specific) of each batch of IgG$_1$ and IgG$_2$. Antigen was added at a final dilution of 1/4000 in a total volume of 200 μl.)

Serum from which IgG$_1$ and IgG$_2$ were isolated*	Expt. 1: [^{125}I]IgG$_1$ from serum H (205 ng.)		Expt. 2: [^{125}I]IgG$_1$ from serum D (216 ng.)		Expt. 3: [^{125}I]IgG$_2$ from serum G (64 ng.)		Expt. 4: [^{125}I]IgG$_2$ from serum H (27 ng.)	
	IgG$_1$	IgG$_2$	IgG$_1$	IgG$_2$	IgG$_1$	IgG$_2$	IgG$_1$	IgG$_2$
a	—	15	—	25	—	22	—	18
b	8	25	28	39	1	16	24	28
C	26	—	57	—	35	—	49	—
D	38	34	66	63	50	33	54	35
E	44	—	70	—	54	—	54	—
F	54	50	82	83	64	69	65	66
G	66	55	85	79	74	73	55	61

* Sera a and b were from recently vaccinated animals. Sera C–H were from animals presumed to be infected because of high serological titres.

to the polystyrene tubes. However, the concentration of albumin was not critical above about 0·5 mg./ml. Controls without antigen for points on the standard curve or for sera did not differ significantly from the zero point control and were therefore considered unnecessary.

Incubation for 2–4 hr. at 37° C. was sufficient to bring the reaction to equilibrium. It was important to store tubes on ice after centrifuging and to remove the supernatants promptly. If these were not done, less precipitated radioactivity was recovered, probably because of dissociation of antibody from the antigen pellet.

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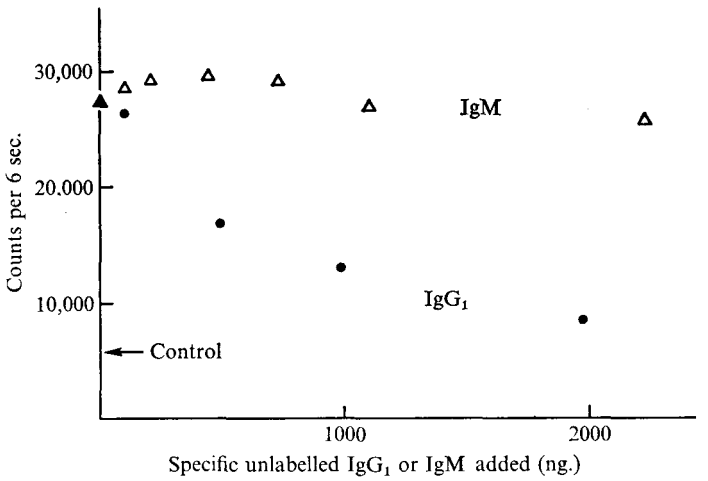

Fig. 2. Displacement of [^{125}I]IgG$_1$ from *Br. abortus* antigen by unlabelled IgG$_1$ and by unlabelled IgM. The incubation mixtures contained 200 ng. of specific [^{125}I]IgG$_1$ and *Br. abortus* antigen at a final dilution of 1/1600, in a total volume of 200 μl.

Competition by different batches of IgG$_1$ and IgG$_2$

Different batches of IgG$_1$ and IgG$_2$, when incubated with radioiodinated IgG$_1$ or IgG$_2$ of a given batch, competed with differing effectiveness for antigen (Table 1). IgG$_1$ and IgG$_2$ from the sera of infected cows competed more effectively than IgG$_1$ and IgG$_2$ from the sera of recently vaccinated cows. The relative ability of a series of IgG$_1$ and IgG$_2$ preparations to displace labelled antibody remained the same within experimental error, irrespective of the IgG$_1$ or IgG$_2$ used for radioiodination. In general, the competing abilities of the IgG$_1$ and IgG$_2$ derived from a given serum were similar.

Throughout this study, sera were assayed using a single batch of IgG$_1$ for radioiodination and as a standard. This was from serum H from a cow believed to be infected.

Competition by IgM

IgM was less effective than IgG$_1$ or IgG$_2$ in competition with [^{125}I]IgG$_1$ or [^{125}I]IgG$_2$ for antigen. IgM could only be obtained from sera of recently vaccinated cows but even IgG$_1$ and IgG$_2$ derived from such sera usually competed more effectively than IgM. Fig. 2 compares displacement curves obtained with IgG$_1$ from serum H and with a typical IgM. Within a low range, the relative competing ability of batches of IgM varied with the IgG$_1$ or IgG$_2$ used for iodination.

Precision of the assay

A single serum, tested three times in each of ten assays, gave a mean value of 1200 ng. and individual values ranged from 900 to 1600 ng. Analysis of variance allowed the calculation of a standard deviation within assays of 91 ng. (coefficient of variation 8 %) and a standard deviation between assays of 198 ng. (coefficient of variation 17 %).

Table 2. *Comparison between the results of radioimmunoassay and the complement fixation test for* 500 *sera positive to the Rose Bengal plate test*

(Complement fixation titres include any reaction (1–4) at the dilutions given.)

	CFT titre *				
RIA (ng. IgG)	Negative	4	8	>8	Ambiguous
< 200	302	12	7	12	25
200–400	10	2	2	10	1
500–900	5	2	1	11	0
1000–10,000	4	2	2	30	3
> 10,000	3	0	0	53	1

* The minimum diagnostic value is considered to be 4.

Table 3. *Comparison between the results of radioimmunoassay and the serum agglutination test for* 500 *sera positive to the Rose Bengal plate test*

	SAT (international units) *			
RIA (ng. IgG)	< 50	50–99	100–200	> 200
< 200	297	43	16	2
200–400	16	1	5	3
500–900	10	3	3	3
1000–10,000	10	4	8	19
> 10,000	2	1	8	46

* The minimum diagnostic result is considered to be 100 i.u.

Of 113 sera which had values between 500 and 2000 ng., two had values more than 10,000 ng. in a second assay and none had less than 200 ng. Only one serum out of 49 in which the first value was 1000 ng. or more had a value of less than 500 ng. in the second assay.

Of 26 sera which had values less than 200 ng. in one assay, 25 again showed less than 200 ng. in a second assay and one showed 200 ng. Of 26 other sera which had values more than 10,000 ng., 24 gave the same result in a second assay, one showed 5400 ng. and one 9200 ng.

Comparison with other serological tests

A general relationship existed between RIA results and the results of the CFT and SAT (Tables 2, 3).

DISCUSSION

A practical radioimmunoassay has been developed for measuring the concentrations of antibodies against *Br. abortus*. It is sufficiently sensitive and reproducible for use in the diagnosis of bovine brucellosis.

It has been shown (Table 1) that different batches of IgG_1 and IgG_2 differ in their effectiveness in the RIA. This may be due to differences in the average association constants of different antibody populations, particularly as IgG from infected cows was more effective than IgG from recently vaccinated animals. The RIA thus

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possibly measures some combination of antibody concentration and average association constant. This may give it an advantage over existing serological tests as some chronically infected animals can be expected to have IgG antibody in low concentrations, but with high average association constant because of prolonged antigenic stimulation.

Residual titres after vaccination with *Br. abortus* strain 19 are a major cause of confusion in brucellosis diagnosis. Residual antibodies to vaccination are believed to be mainly IgM while in infected animals IgG predominates (Elberg, 1973). It follows that the RIA, which is insensitive to IgM, is unlikely to be influenced by residual vaccination titres. In contrast, the RBPT and SAT are each ten times as sensitive to IgM as to IgG on a weight basis (Allan *et al.* 1976). The CFT is at least as sensitive to purified IgM as to IgG_1. However, because IgM in serum is partly destroyed when serum is heated to inactivate complement and because the extent of this destruction may differ according to the inactivating procedures adopted in different laboratories (Allan *et al.* 1976) the efficiency of the CFT in detecting IgM is variable.

The fact that IgM populations compete more poorly than IgG in the RIA may be partly due to the lower average association constants of the former. It may be, however, that IgM is also directed against a different range of antigenic determinants on the bacterial surface. This may relate to the possibility that IgM is responsible for cross-reactions with distantly related organisms, as was found by Corbel & Wray (1975) in the case of *Salmonella urbana*.

RIA has the advantage over the CFT that it measures both subclasses of IgG. IgG_2 does not fix complement and can, if it makes up a sufficient proportion of the total *Brucella*-specific IgG, cause prozoning or completely suppress the CFT reaction (Plackett & Alton, 1975; McNaught *et al.* 1977).

The minimum diagnostic value for any serological test is to some extent arbitrary and must be determined by field evaluation. In the case of the RIA it will differ with the preparation of antibody used as a standard. Allan *et al.* (1976) have estimated that about 10 μg. of *Brucella*-specific IgG_1 per ml. is needed to give a diagnostic CFT reaction (1 at 1/4). This is equivalent to 125 ng. of IgG_1 per 12·5 μl. of serum, or roughly 200 ng. of total IgG as measured in the RIA. This argument, however, ignores the effect of average association constant. The data in Tables 2 and 3 suggest that the equivalent of perhaps 500 ng. of IgG_1 from serum H could be considered diagnostic.

Although RIA has several theoretical advantages, its usefulness in routine diagnosis has yet to be determined. Comparison of the results of different tests on a series of sera are of value but are open to different interpretations due to the lack of an absolute criterion of infection. Testing sera from animals proved by culture to be infected with *Br. abortus* is also of restricted value as culture is only successful in a proportion of infected cases. This approach can prove false negative diagnoses but cannot prove that false negatives do not occur, nor does it give any information about false positives. The ultimate criterion for the usefulness of a serological test is its performance in the field in a test-and-slaughter eradication programme.

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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. (1967). Laboratory techniques in brucellosis. *World Health Organisation Monograph Series*, no. 55, Geneva.

ALTON, G. G., MAW, J., ROGERSON, B. A. & MCPHERSON, G. G. (1975). The serological diagnosis of bovine brucellosis: an evaluation of the complement fixation, serum agglutination and Rose Bengal tests. *Australian Veterinary Journal* **51**, 57.

CORBEL, M. J. & WRAY, C. (1975). The effect of natural infection with *Salmonella urbana* on the serological status of cattle in relation to tests for brucellosis. *British Veterinary Journal* **131**, 324.

ELBERG, S. S. (1973). Immunity to *Brucella* infection. *Medicine, Baltimore* **52**, 339.

HESS, W. R. (1953). Studies on a non-specific *Brucella*-agglutinating substance in bovine serum. 1. The differentiation of the specific and non-specific agglutinins by heat treatment. *American Journal of Veterinary Research* **14**, 192.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265.

MCNAUGHT, D. J., CHAPPEL, R. J., ALLAN, G. S., BOURKE, J. A. & ROGERSON, B. A. (1977). The effects of IgG_2 and of antigen concentration on prozoning in the complement fixation test for bovine brucellosis. *Research in Veterinary Science* (in the Press).

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

PLACKETT, P. & ALTON, G. G. (1975). A mechanism for prozone formation in the complement fixation test for bovine brucellosis. *Australian Veterinary Journal* **51**, 374.