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The diagnostic efficiency of some serological tests for bovine brucellosis

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SUMMARY

Results obtained from 1887 sera using three serological tests for bovine brucellosis were compared with a serological classification of sera described as the 'probable infection status'. Sera showing apparent false positive and apparent false negative reactions were identified, and were subjected to supplementary testing as appropriate.

The serum agglutination test (SAT) gave 35 % apparent false negative reactions and 5 % apparent false positives. The complement fixation test (CFT) gave 12 % apparent false negative reactions using warm fixation (CFTW) and at least 5 % using cold fixation (CFTC). The routine diagnostic system used in Victoria, in which the CFTW is supplemented by the CFTC and the SAT, gave 9 % apparent false negative reactions and 2 % apparent false positive reactions. The radioimmunoassay gave 1 % or 6 % apparent false negative reactions, depending on the minimum diagnostic value used.

Atypical reactions in the CFT sometimes caused difficulties in diagnosis.

INTRODUCTION

In the previous paper (Chappel, McNaught, Bourke & Allan, 1978) we have compared the results of three serological tests for brucellosis, performed on a group of bovine sera. Studies of this type suffer from the limitation that, when serological tests are compared against one another, there is no absolute criterion of infection.

A common experimental approach is to compare serological results with the results of attempts to isolate *Brucella abortus* from the animals concerned. Unfortunately, attempts to culture the organism sometimes fail (Green, 1969) and successful isolation is more probable in more heavily infected animals. Such a study cannot, therefore, yield an estimate of the frequency of false negative reactions, although it can show that such reactions occur. Furthermore, it can yield no information about false positive reactions.

The present paper uses an alternative approach, which has limitations of its own, but which should be at least complementary to comparisons with the results of culture.

The approach is based on the assumptions that the serum of infected animals contains IgG antibody and that the presence of IgM antibody does not necessarily indicate infection (Elberg, 1973). It involves three steps.

(1) Two serological tests, each of which is primarily sensitive to IgG antibody, are used to classify sera by a criterion described as 'probable infection status'. The tests are the complement fixation test (CFT) using warm fixation (CFTW), and the radioimmunoassay (RIA). The following specific assumptions were made:

(*a*) A serum which shows no detectable reaction in either the CFTW or the RIA is unlikely to be from an infected animal.

(*b*) A serum which gives a reaction above a specified level in either the CFTW or the RIA is likely to be from an infected animal. The specified level was assessed from field experience in the case of the CFTW: in the case of the RIA, it was determined by comparing the results of the CFTW and the RIA (Chappel *et al.* 1978).

(2) Sera are identified which, using a particular test or diagnostic strategy, gave apparent false positive or apparent false negative reactions by comparison with the 'probable infection status'.

(3) These sera are, where possible, subjected to supplementary tests to discover whether the test is, as indicated, in error, or whether the criteria of 'probable infection status' are at fault.

Because of the assumptions made, it is not possible to identify sera giving apparent false positive reactions in either the CFTW or the RIA. It is, however, possible to identify sera giving apparent false negative reactions in either test. It is known that the CFTW can give false negative reactions when the serum ratio of IgG_2 to IgG_1 antibody is high, although it is not known how frequently this occurs (Plackett & Alton, 1975; McNaught, Chappel, Allan, Bourke & Rogerson, 1977).

The aims of this study were to compare and evaluate the diagnostic efficiency of the CFTW, the RIA, the serum agglutination test (SAT) and the routine diagnostic strategy used in Victoria.

MATERIALS AND METHODS

Sera

The sera were the 1887 studied in the previous paper (Chappel *et al.* 1978). All were positive to the Rose Bengal plate test (RBPT).

Serological tests

The techniques for performing the RBPT, the RIA and the SAT were outlined in the previous paper (Chappel *et al.* 1978).

In the SAT, a reaction of 100 i.u./ml or more was taken as positive.

For the RIA, two alternative minimum diagnostic reactions were considered: 200 ng as suggested by Chappel *et al.* (1978), and 500 ng.

Mercaptoethanol test

The mercaptoethanol test (MET), a modification of the SAT, was performed as described by Alton, Jones & Pietz (1975*a*). The diluent used was 0·1 M 2-mercaptoethanol in 0·85 % sodium chloride, and a control for each test was included in which the diluent was 0·85 % sodium chloride alone. Antigen was diluted with 0·85 % sodium chloride for both the test and the control.

A reduction in titre of at least one complete tube compared with the control was arbitrarily taken to indicate that the serum contained a significant proportion of IgM antibody.

Indirect haemolysis test

The indirect haemolysis test (IHLT) was performed as described by Plackett, Cottew & Best (1976). The result was expressed as the highest dilution at which 50 % lysis occurred. A 50 % reaction at a dilution of 1/8 was taken to be positive.

Complement fixation test

The CFTW, and the complement fixation test using cold fixation (CFTC), were performed as described for the CFTW by Chappel *et al.* (1978), who also explain the method of expressing the results and the basis on which certain reactions were defined as 'atypical'.

The minimum diagnostic reaction in the CFTW was taken to be 50 % haemolysis at a serum dilution of 1/4 (a reaction of 2), while the minimum diagnostic reaction in the CFTC was taken to be 50 % haemolysis at a dilution of 1/8 (4, 2).

Interpretation of atypical reactions in the complement fixation test

Weak atypical reactions in the CFT are not always repeatable and are sometimes read as negative. Because no procedure is laid down for deciding whether an atypical reaction is positive, an arbitrary rule was devised for the purposes of this study.

The CFTW was classed as positive if a reaction of 2 or above was observed at any single dilution between 1/4 and 1/128. For example, a reaction of 1, 1, *T*, *T*, *T* was classified as negative but a reaction of *T*, 3, *T* was classified as positive.

Similarly the CFTC was classified as positive if a reaction of 2 or above was observed at any single dilution between 1/8 and 1/128.

Routine diagnostic system

The 'routine diagnostic system' used in Victoria is designed to reduce the impact of IgG_2-induced false negatives in the CFTW. Sera negative to the CFTW but giving 'strong' agglutination in the RBPT are retested using the CFTC and the SAT, provided that the RBPT reaction is confirmed as 'strong' in a second test. If the serum gives a positive reaction in either of the supplementary tests, the animal is classified as a reactor.

Table 1. *Criteria for the classification of sera by probable infection status*

CFTW reaction	RIA reaction (ng)	'Probable infection status'
Any	500 and above	'Positive'
4, 2 and above*	Any	
2 to 4, 1*	200–400	
Negative*	200–400	'Doubtful'
2 to 4, 1*	< 200	
Atypical	< 500	
Negative*	< 200	'Negative'

* And not atypical.

Probable infection status

As described in the Introduction, each serum was assigned a 'probable infection status' on the basis of the results of the CFTW and the RIA. The criteria used are shown in Table 1. The 'probable infection status' allowed the identification of sera giving apparent false negative or apparent false positive results in any individual test or in the routine diagnostic system.

Supplementary serological testing

Sera giving apparent false positive or apparent false negative reactions were, where appropriate, subjected to supplementary testing using any of the following: the CFTW (repeated), the CFTC, the RIA (repeated), the MET and the IHLT.

RESULTS

Atypical reactions in the complement fixation test

Of the 1887 sera, 257 gave atypical reactions in the CFTW. These were excluded from comparisons in the previous paper (Chappel *et al.* 1978), as they would have complicated the comparisons between the results of the CFTW and those of the SAT and the RIA, but they must be considered here.

Of these 257 sera, 224 were classed as positive to the CFTW by the criterion given in the Materials and Methods. That most of these reactions are true positives is suggested by the fact that 204 of the 224 gave RIA reactions of 500 ng or more. Most of these had CFTW reactions which appeared quite unambiguous, such as 0, 0, 3, 3, 4, 4 and 0, *T*, 1, 3, 4, 4.

On the other hand, some atypical reactions were ambiguous. Despite the previously observed relationship between the results of the CFTW and the RIA, it was quite impossible to predict from these CFTW reactions whether the RIA would be negative or positive. Reactions considered to be ambiguous included all those atypical reactions classified as negative (e.g. *T*, *T*, *T*) and some weaker reactions classified as positive (e.g. 2, 1, *T*). This ambiguity is illustrated in Table 2.

Table 2. *Examples of ambiguous reactions in the complement fixation test (warm fixation)*

CFTW reaction	
RIA reaction < 200 ng	RIA reaction ⩾ 500 ng (shown in parentheses)
T, *T*, *T**	*T*, *T*, *T* (> 10000)*
1, 1, 1, *T**	*T*, 1, *T*, *T*, *T* (> 10000)*
2, 1, *T*	2, *T*, *T* (3100)
0, 0, 4	0, 4 (800)
0, 0, 2, 3, 4	2, 2, 3, 1 (2200)
0, 4, 4, 4	0, 0, 1, 4, 4, 4 (> 10000)

* These reactions were classed as negative by the criterion given in the Materials and Methods.

Table 3. *The serological basis of the routine diagnostic result and comparison with the results of the radioimmunoassay*

Serological bases of routine diagnostic result					Routine diagnostic result		RIA reaction (ng)		
RBPT	CFTW	RBPT repeat	CFTC	SAT	Negative	Positive	< 200	200–400	⩾ 500
'Weak'	Negative				1163	—	991	111	61
Positive	Positive				—	642	44	34	564
'Strong'	Negative	'Not strong'			15	—	14	1	0
'Strong'	Negative	'Strong'	Negative	Negative	18	—	17	0	1
'Strong'	Negative	'Strong'	Positive	Negative	—	7	3	2	2
'Strong'	Negative	'Strong'	Negative	Positive	—	23	18	2	3
'Strong'	Negative	'Strong'	Positive	Positive	—	19	3	1	15
Totals					1196	691	1090	151	646

Routine diagnostic system

The routine diagnostic result was positive for 691 of the 1887 sera in the study. Of these, 642 sera were classed as positive on the basis of positive results in the CFTW, and 49 on the basis of supplementary testing. Table 3 compares the routine diagnostic result with the results of the RIA which, unlike the RBPT, the CFTW and the SAT, plays no role in determining the diagnostic result.

Of the 49 sera classed as positive on the basis of supplementary testing, only 25 gave RIA reactions of 200 ng or more. Of the 49, 23 were classed as positive on the basis of the SAT alone, and only five of these gave RIA reactions of 200 ng or more.

Probable infection status

The 'probable infection status', defined by the criteria given in Table 1 and determined for each serum, was used as a basis for the evaluation of the results of the CFTW, the RIA and the SAT, and of the routine diagnostic result (Tables

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Table 4. *Assessment of serological tests by comparison with probable infection status*

(The criteria of 'probable infection status' are given in Table 1.)

(*a*) Probable infection status: negative (1026 sera)

Procedure	Negative	Positive	Percentage apparent false positive
CFTW	1026	0	0*
RIA	1026	0	0*
Routine diagnostic system	1006	20	2
SAT	970	56	5

(*b*) Probable infection status: doubtful (177 sera)

Procedure	Negative	Positive
RIA, read at 500 ng	177	0
RIA, read at 200 ng	57	120
SAT	157	20
CFTW	137	40
Routine diagnostic system	128	49

(*c*) Probable infection status: positive (684 sera)

Procedure	Negative	Positive	Percentage apparent false positive
RIA, read at 200 ng	7	677	1
RIA, read at 500 ng	38	646	6
Routine diagnostic system	62	622	9
CFTW	82	602	12
SAT	239	442	35

* These results follow from the criteria of 'probable infection status'.

4*a–c*). This allowed the identification of apparent false positives and apparent false negatives.

In almost every case, sera were classified by 'probable infection status' on the basis of the first CFTW and RIA tests performed. However, three sera were given a status of 'doubtful' rather than 'positive' on the basis of a negative second CFTW test.

Apparent false positives

The SAT gave 56 (5%) apparent false positives (Table 4*a*). Of these, 44 gave reactions of less than 200 i.u./ml and all gave less than 400 i.u./ml. Every serum but one was tested by the MET to determine whether IgM was making a major contribution to the SAT titre. Forty-five sera (82%) showed a significant reduction in titre due to 2-mercaptoethanol, and among these were 11 of the 12 sera giving reactions of more than 200 i.u./ml.

Twenty sera (2%) gave routine diagnostic results which were apparent false positives. It follows from the criteria of 'probable infection status' that all were negative to the CFTW but gave 'strong' reactions in the RBPT. Eighteen of the

20 were classed as positive because of positive reactions in the SAT, although three of these were also positive to the CFTC. Two were classed as positive on the basis of the CFTC alone. The highest reactions in the CFTC were 4, 4, 1 and 4, 4, *T*, *T*. The MET showed, in 14 out of 19 sera (74%), a significant reduction in titre due to 2-mercaptoethanol.

Apparent false negatives

(*a*) *Serum agglutination test.* The SAT gave 239 (35%) apparent false negatives (Table 4*c*). Most of these sera gave positive reactions in both the CFTW and the RIA, often strongly positive.

(*b*) *Complement fixation test.* The CFTW gave 82 (12%) apparent false negatives. It follows from the criteria of 'probable infection status' that all these gave RIA reactions of 500 ng or more. To try to discover which of the CFTW and the RIA was incorrect, these sera were subjected to supplementary testing.

In the case of six sera there was insufficient volume to complete all the supplementary testing desired, and their positive status was not confirmed. The other 76 sera were tested by the CFTC and the SAT and were retested by the CFTW. Sometimes a test was performed more than once. Fifty-four sera were positive to one or more of these three tests. Of these, 51 were positive to the CFTC, 26 to the SAT and 24 to the repeated CFTW. Only one of these was positive to the SAT alone.

The remaining 22 sera were tested by the IHLT and 17 were positive. The five IHLT-negative sera had given RIA reactions of 500 ng and 600 ng. These were retested by the RIA: three still gave reactions of 500 ng or more and the other two gave reactions of 300 ng and 400 ng.

In total, therefore, 71 out of 76 sera (93%) were confirmed as false negatives in the CFTW by supplementary testing.

Forty-six out of 81 sera were positive to the CFTC the first time this test was used. This indicates the proportion of apparent false negatives which would have been avoided had the CFTC been used instead of the CFTW.

Of the 82 sera which gave apparent false negatives in the CFTW, 62 gave negative diagnostic results by the routine system. Of these, 51 out of 56 (91%) were positive to one or more of the supplementary tests as described above.

Table 5 shows serological results obtained with eight representative sera among the 71 apparent false negatives for which supplementary serological evidence of positive status was obtained.

(*c*) *Radioimmunossay.* The RIA gave 38 (6%) apparent false negatives assuming a minimum diagnostic value of 500 ng. Assuming a minimum diagnostic value of 200 ng, however, this number was reduced to seven (1%). Of these seven sera, six gave RIA values, in repeat assays, of 200–900 ng. A seventh serum twice gave RIA reactions of less than 200 ng. It gave CFTW reactions of 4, 2 and 4, 4 and a CFTC reaction of 4, 4, 1, 4.

Table 5. *Serological results obtained with eight representative sera giving negative reactions in the complement fixation test (warm fixation) but giving reactions of 500 ng or more in the radioimmunoassay*

Serum no.	RBPT reaction	CFTW reaction	CFTW reaction (repeat)	RIA reaction (ng)	SAT reaction i.u./ml	CFTC reaction	IHLT* reaction	Routine diagnostic result
174	'Weak'	0	4	1900 1500	34	4, 4, 4, *T*	n.d.	Negative
347	'Strong' 'Strong'	0	0, 0, *T*, *T*	> 10000	372 424	0, 0, *T*, *T* *T*, 4, 4, 4, 4, 2	n.d.	Positive
495	'Weak'	0	0	500 400	27	0 0	1/8	Negative
816	'Strong' 'Strong'	0	4, 1	500	160 268	4, 4, *T*	n.d.	Positive
1370	'Weak'	0	4, *T*	800	53	4, 4, 2	n.d.	Negative
1431	'Strong' 'Strong'	0, 0, 0, *T*, *T*, 1	0, 0, 0, 0, 0, 4	> 10000	2560	4, 4, 4, 4, 4, 4	n.d.	Positive
1620	'Weak'	0	0	4100 3300	20	3, *T*	1/32	Negative
1624	'Weak'	0	*T*	1800	17	4, 4, 4, 4, 3	n.d.	Negative

* n.d., Not done.

Sera with a probable infection status of doubtful

A total of 177 sera were given a 'probable infection status' of 'doubtful' (Table 4*b*). The largest group of these was 137 sera negative to the CFTW, of which 115 showed no low level atypical reactions in the CFTW. Of these, 110 were submitted to the CFTC and 17 (15%) were positive.

DISCUSSION

In this study, a new approach to the evaluation of serological tests has been used, an approach which is complementary to the usual approach of comparing serological results with the results of attempts to culture the infective organism.

The chief disadvantage of the 'probable infection status' approach was that there was no absolute criterion of infection. The advantages were that, unlike comparisons with the results of culture, it allowed estimates to be made, with some degree of confidence, of the frequency of false negative reactions, and it allowed the frequency of false positives to be estimated except for the RIA and the CFTW.

The frequencies of apparent false positive and apparent false negative reactions (Tables 4*a–c*) apply only to the 1887 cattle whose sera were included in this study. They should, however, be a guide to the frequency of incorrect diagnosis in any area in which vaccination of calves with strain 19 is compulsory.

Serum agglutination test

The SAT gave 5% apparent false positive reactions (Table 4*a*). The fact that, in these sera, the SAT results differed from those of both the CFTW and the RIA, suggests that IgM antibody was responsible. The results of the MET support this idea in most cases.

The SAT gave 35% apparent false negative reactions. This result could be predicted from the low sensitivity of the SAT to IgG antibody (Chappel *et al.* 1978). Alton, Maw, Rogerson & McPherson (1975*b*) found that 11% of animals from which *B. abortus* could be isolated gave SAT reactions of less than 100 i.u./ml. The present study has shown that an even greater problem may exist. The different results of the two studies may reflect the limitations of the approach of comparing serological results with those of culture.

The minimum diagnostic result in the SAT was taken to be 100 i.u./ml. Had a value of 200 i.u./ml been used as has been suggested for vaccinated animals (Alton *et al.* 1975*a*), most of the false positives would have been eliminated but the number of false negatives would have been even higher than it was. If, on the other hand, 30 i.u./ml had been taken as positive, as is accepted in the European Economic Community (Alton *et al.* 1975*a*) there would have been far more false positive reactions but the test would still have been less sensitive to IgG than the CFTW.

Complement fixation test

The CFTW gave 82 (12%) apparent false negative reactions (Table 4*c*). An elevated ratio of IgG_2 antibody to IgG_1 antibody can cause false negatives. Smaller, but still elevated, ratios lead to prozones or reduced or atypical reactions (Plackett & Alton, 1975; McNaught *et al.* 1977). IgG_2 was probably the main cause of false negatives and atypical reactions in this case.

It follows from the definition of 'probable infection status' that the 82 apparent false negatives were given by sera which gave negative reactions in the CFTW but reactions of 500 ng or more in the RIA. Attempts were made to determine whether the RIA result was correct by using supplementary tests. Some but not all sera giving IgG_2-induced false negatives in the CFTW can be expected to be positive to the CFTC, which is less susceptible to IgG_2 interference. The SAT should also be positive in some cases but, because of its low sensitivity to IgG, not in every case. One or more of the CFTC, the SAT or, when repeated, the CFTW, were positive in 54 out of 76 sera. Of the remaining 22 sera, 17 were positive to the IHLT, a test which does not suffer from prozoning (Plackett *et al.* 1976).

The existence of atypical reactions leads to ambiguity (Table 2). One must distinguish here between reactions which are atypical, a concept of statistical rather than individual importance which was devised for the purposes of these studies, and the smaller number which are ambiguous. Ambiguity occurs because not all atypical reactions are caused by excess IgG_2 antibody, some being seen in the apparent absence of antibody. The stronger an atypical reaction, the more likely it is to represent a true positive, although no reaction is so weak that it can confidently be called a true negative.

Elevated ratios of IgG_2 to IgG_1 antibody probably lead to poor reproducibility. The reproducibility of the CFT for the majority of sera is satisfactory, as demonstrated with control sera. These control sera presumably contain an excess of IgG_1 antibody. However, 24 out of 76 sera giving apparent false negatives in the CFTW were positive when retested. This does not indicate that the percentage of false negatives has been overestimated, as an equal number of positives could have become negative on retesting.

It is suggested, then, that two groups of antibody-containing sera can be distinguished according to their behaviour in the CFTW. The larger group has high ratios of IgG_1 to IgG_2 antibody. These sera give positive results with good reproducibility. The smaller group has lower ratios of IgG_1 to IgG_2 antibody. These sera frequently give either atypical reactions or false negatives, and the reproducibility of these reactions is poor.

This study primarily involved the CFTW, which is preferred to the CFTC for routine use because it can be completed during the course of a working day. However, some information was also obtained about the CFTC, which is less subject than the CFTW to prozoning and IgG_2-induced false negatives (Plackett & Alton, 1975; McNaught *et al.* 1977).

The CFTC was positive in 46 out of 81 sera giving apparent false negatives in the CFTW. This indicates that the use of cold fixation would have reduced the

apparent false negative reactions from 12% to 5%. This calculation assumes, however, that no sera of infected animals were positive to the CFTW but negative to the CFTC: as the CFTC was not performed on every sample, this may not be so.

Like the CFTW, the CFTC gives some atypical reactions. This is to be expected if it, too, is subject to IgG_2-induced false negatives.

Routine diagnostic system

The routine diagnostic system used in Victoria was designed to overcome the problem of IgG_2-induced false negatives while retaining the convenience of the CFTW as a routine test. It is based on the fact that sera giving 'strong' RBPT reactions are usually from infected animals. Such sera, if negative to the CFTW, are retested by the CFTC and the SAT.

The routine diagnostic system gave 9% apparent false negatives, compared with the 12% given by the CFTW alone (Table 4*c*). There are two reasons why false negatives were still obtained. First, although most sera giving 'strong' RBPT reactions are from infected animals, the sera of many infected animals give 'weak' RBPT reactions (Chappel *et al.* 1978). Secondly, the CFTC and the SAT are not positive for all sera giving IgG_2-induced false negatives in the CFTW. The SAT is insensitive and the CFTC is itself subject to interference by IgG_2 antibody.

The SAT performed poorly as a supplementary test in this study. The routine diagnostic system gave 2% apparent false positive reactions (Table 4*a*). Most of these appeared to be due to the effect of residual vaccination antibody on the SAT. One can have no confidence in SAT reactions in the range 100–200 i.u./ml, and limited confidence in reactions in the range 200–400 i.u./ml. Furthermore, of 76 sera giving apparent false negative reactions in the CFTW, only one was positive to the SAT and negative to the CFTC.

Radioimmunoassay

Taking the minimum diagnostic value to be 200 ng, the RIA gave only seven (1%) false negative reactions. Of these, six were not reproducible, and were therefore technical errors or errors of identification or labelling, and not inherent in the test itself. The reproducibility of the RIA was shown to be satisfactory by Chappel *et al.* (1976) who studied for the purpose 166 sera drawn from the group studied for the present two papers.

If the minimum diagnostic value is taken to be 500 ng, the RIA gave 6% apparent false negatives, about the same as the CFTC appeared to give. This supports the suggestion of Chappel *et al.* (1978) that 200 ng is a more suitable minimum diagnostic reaction than 500 ng.

The use of the RIA, using a minimum diagnostic value of 200 ng, would in a test and slaughter situation result in the slaughter of more animals than the use of the CFTW. This is partly due to the apparent false negatives in the CFTW but it is also partly due to that group of sera which are negative to the CFTW, but give RIA reactions of 200–400 ng.

On the evidence presented, the RIA has some advantages over the CFT, and is worth considering as an alternative or supplementary diagnostic method. At

present, however, it is relatively laborious, and is less suitable than the CFTW for testing large numbers of samples. Field evaluation would also be necessary before the RIA could be adopted on a large scale.

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