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ANTIGENIC RELATIONSHIP OF *BRUCELLA OVIS* TO *BRUCELLA ABORTUS* AND *BRUCELLA MELITENSIS* USING THE COMPLEMENT FIXATION TEST

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ABSTRACT

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The CF test was used to investigate the serological relationship of *Br. ovis* to *Br. abortus* and *Br. melitensis*. A definite antigenic relationship between *Br. ovis* and *Br. abortus* could be demonstrated. Definitive results were obtained by absorbing sera with *Br. ovis*, *Br. melitensis* and *Br. abortus* before testing.

INTRODUCTION

The aetiological agent of the most important cause of infectious epididymitis in rams was first isolated by Buddle & Boyes (1953) in New Zealand and by Simmons & Hall (1953) in Australia. Buddle (1956) described the organism and named it *Brucella ovis*. The Subcommittee on the Taxonomy of *Brucella* of the International Committee on Bacteriological Nomenclature was not satisfied that the organism was correctly classified as a *Brucella* (according to Diaz, Jones & Wilson, 1967). The main objection was apparently that no serological relationship to smooth *Brucella* was revealed by agglutination tests (Meyer & Cameron, 1956). More recent studies have shown that there are definite antigenic similarities between *Br. ovis* and *Brucella melitensis* (Hughes, 1892), especially when *Br. melitensis* in the rough phase is used (Diaz, Jones & Wilson, 1967). There seems little doubt, therefore, that the organism will now be accepted as a member of the genus *Brucella*. The question remaining is probably whether it is classed as a biotype of *Br. melitensis* or whether it is accepted as a separate species.

In our experience the most valuable serological test for the detection of *Br. ovis* antibodies in sheep is the complement fixation (CF) test. The object of the present study was to use the CF test to investigate the serological relationship between strains of *Br. ovis*, isolated in South Africa and in other countries, to smooth phase *Br. melitensis* and *Br. abortus* (Schmidt & Weiss, 1901). A further object was to discover whether there were antigenic differences between various South African strains of *Br. ovis*.

MATERIALS AND METHODS

Strains:

The *Brucella* strains used and details of their origin are listed in Table 1.

Cultivation of organisms and preparation of cell suspensions

Br. ovis strains were grown in the liquid medium described by Lawrence & Jones (1961) in large Roux flasks under a 15 per cent carbon dioxide atmosphere. The flasks were shaken on a shaking machine during incubation at 37 °C for 72 hours. Cultures were then tested for purity, bulked, killed in a waterbath at 60 °C for 1 hour, washed twice in 0.85 per cent saline containing 0.5 per cent phenol, suspended in a small amount of phenol saline and used immediately for antigen production or stored at 4 °C until required for serum absorption tests. Similar washed suspensions of *Br. abortus* and *Br. melitensis* were prepared from cultures grown on potato agar medium in Roux flasks.

TABLE 1. — *Strains used in the investigation*

Strain	Species	Source
21422	*Br. ovis*	Onderstepoort isolate
6010	*Br. ovis*	Onderstepoort isolate
19703	*Br. ovis*	Onderstepoort isolate
6323	*Br. ovis*	Dr. Buddle, Animal Research Station, Wallaceville, New Zealand
22855	*Br. ovis*	Onderstepoort isolate
6324	*Br. ovis*	Dr. Buddle, Animal Research Station, Wallaceville, New Zealand
16060	*Br. ovis*	Onderstepoort isolate
6504	*Br. ovis*	Onderstepoort isolate
292	*Br. ovis*	Dr. Buddle, Animal Research Station, Wallaceville, New Zealand
4154	*Br. ovis*	Dr. Buddle, Animal Research Station, Wallaceville, New Zealand
15641	*Br. ovis*	Onderstepoort isolate
6218	*Br. ovis*	Mr. Simmons, Animal Research Institute, Yeerongpilly, Queensland, Australia
16058	*Br. ovis*	Onderstepoort isolate
19957	*Br. ovis*	Onderstepoort isolate
16073	*Br. ovis*	Onderstepoort isolate
Q28E	*Br. ovis*	Mr. Simmons, Animal Research Institute, Yeerongpilly, Queensland, Australia
16M	*Br. melitensis*	Central Vet. Laboratory, Weybridge, England
99	*Br. abortus*	Central Vet. Laboratory, Weybridge, England
544	*Br. abortus*	Central Vet. Laboratory, Weybridge, England

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Antigens

Soluble antigens were made by heating suspensions containing 10 to 20 per cent cells in a boiling water bath for 10 minutes. Sufficient merthiolate was added to produce a dilution of 1 in 10,000 and the suspension was held at room temperature for three weeks. Each suspension was then centrifuged at 12000 *g* for 30 minutes and the clear supernatant fluid was stored at 4°C until required for use as antigen.

Antigens were titrated against standard serum to determine the optimum dilution at which they should be used in the CF test.

Rabbit antisera

All strains were grown on tryptose blood agar slopes. Well-grown 24 hour cultures, two tubes from each strain, were harvested in 9 ml of normal saline and the suspension mixed with an equal amount of a mixture containing nine parts of Bayol F* to one part of Arlacel A** and shaken until a stable emulsion was formed. Rabbits were injected subcutaneously with 2 to 3 ml of the emulsion weekly for three weeks. The rabbits were bled weekly and the sera tested until high CF titres were obtained, usually 1 to 3 weeks after the last injection. A large amount of blood was then collected and 1 ml amounts of serum freeze-dried in ampoules were stored at —10°C until used.

CF and serum absorption tests

CF tests were done according to the method described in the appendix. Standard positive *Br. abortus* and *Br. ovis* sera were tested with every batch of tests done. End titres of the standard sera remained constant throughout the experiment. All sera were tested against all the different antigens.

Sera were absorbed with cells prepared from Strains 6010 (*Br. ovis*), 99 (*Br. abortus*) and 16M (*Br. melitensis*). The serum absorptions were carried out as follows: To 1 ml of packed cells 7 ml of serum and 1 ml of undiluted guinea pig complement was added. The cells were well mixed with the serum and incubated at 37°C for 2 hours during which time thorough mixing was repeated every 30 minutes. The serum mixture was then centrifuged for 1 hour (2500 *g*). The clear supernatant serum was withdrawn and again added to 1 ml of packed cells and incubated as before for a further 2 hours. The serum mixture was then placed in a refrigerator overnight. The following day it was centrifuged and the clear, absorbed serum was withdrawn and inactivated at 56°C for 30 minutes. The absorbed sera were tested against all the antigens used in the experiment by the normal CF methods. Sera to which complement was added and then inactivated, but which had not been subjected to absorption, were used as controls.

Results

Details of the results are given in Tables 2 to 5. In Table 2 the results of CF tests on unabsorbed sera are given, while those of sera absorbed with *Br. ovis* (Strain 6010), *Br. abortus* (Strain 99) and *Br. melitensis* (Strain 16M) respectively are shown in Tables 3 to 5.

Absorption of sera with *Br. ovis* (Strain 6010) cells removed virtually all anti-*Br. ovis* antibodies from the sera (compare Tables 2 and 3). The two Australian strains, 6218 and Q28E, appeared to contain antigenic factors not shared by the New Zealand and South African strains. After absorption with Strain 6010, antisera against Strains 6218 and Q28E had extremely low titres against the South African and New Zealand antigens but still showed considerable titres against their homologous antigens.

The absorption of anti-*Br. ovis* sera with *Br. abortus* (Strain 99) cells did cause a slight drop in the titres against *Br. ovis* antigens (compare Tables 2 and 4). The Walsh test (Siegel, 1956) was used to test the hypothesis that the titres of unabsorbed sera and sera absorbed with *Br. abortus* Strain 99 were equal. The hypothesis of equality was rejected for all strains, indicating that *Br. ovis* and *Br. abortus* do contain some similar antigenic factors.

The absorption of anti-*Br. ovis* sera with *Br. melitensis* (Strain 16M), caused minimal drops in titres against *Br. ovis* antigens (compare Tables 2 and 5). In this case the hypothesis of equality was only rejected in the case of Strains 6010, 19703, 6218 and 16073. It is, therefore, concluded that there is little antigenic relationship between soluble *Br. ovis* and *Br. melitensis* antigens prepared in the manner used in these experiments. However, other *Br. melitensis* strains, and different methods of antigen preparation or serological tests other than CF tests might give different results.

Discussion

From the results obtained it is clear that *Br. ovis* as it occurs in nature in the rough phase is antigenically distinct from *Br. abortus* and *Br. melitensis*, although some evidence of antigenic similarities, especially between *Br. abortus* and *Br. ovis*, is provided. These findings do therefore confirm the view that *Br. ovis* is correctly classified in the genus *Brucella*. From a practical point of view, it is apparent that the differences between *Br. ovis* and the other members of the genus are sufficiently distinct to allow a differential diagnosis to be made on serological tests alone, if sera submitted for diagnosis are tested to end titre with *Br. ovis* and *Br. abortus* antigens. This distinction is especially important when serological surveys are being done with a view to determine the distribution of various types of infection.

There was some indication of antigenic differences within the *Br. ovis* group. The two Australian strains showed what appeared to be slight differences from the other strains but further more detailed studies have failed to confirm this finding (Worthington, 1968, unpublished data). Any strain which reveals favourable cultural characteristics could therefore be used as an antigen strain for routine tests. The Onderstepoort Strain 6010 which has been used

(*) Esso Petroleum Co., 36 Queen Ann's Gate, London, S.W. 1

(**) Mannide Mono-oleate; Atlas Power Co., Wilmington, Delaware

TABLE 2. — *CF titres of unabsorbed anti-*Brucella *sera*

Antigen Strain		Antisera Strain: *Br. ovis* 21422	6010	19703	6323	22855	6324	16060	6504	292	4154	15641	6218	16058	19957	16073	Q28E	*Br.mel.* 16M	*Br.ab.* 544
21422	*Br. ovis*	128	128	512	16	256	256	128	256	128	128	256	128	1024	128	1024	128	—	2
6010	,,	256	512	512	32	512	256	256	1024	512	256	256	256	1024	64	512	256	2	4
19703	,,	512	512	512	32	1024	128	256	512	256	128	256	64	1024	64	512	128	—	2
6323	,,	512	256	2048	64	512	512	256	512	256	128	128	128	1024	64	1024	256	—	4
22855	,,	512	512	512	32	2048	512	64	1024	256	64	128	128	1024	128	256	64	—	4
6324	,,	256	512	2048	32	1024	512	64	1024	256	128	128	256	512	64	512	256	—	4
16060	,,	256	32	512	32	512	128	256	256	64	256	64	64	256	16	512	32	—	2
6504	,,	128	256	2048	32	2048	512	256	1024	128	128	256	128	1024	64	1024	64	—	4
292	,,	256	64	256	64	1024	256	128	512	64	64	64	128	512	128	512	64	—	4
4154	,,	64	32	512	32	512	256	128	256	128	256	16	64	256	64	512	64	—	8
15641	,,	256	64	512	64	512	128	256	128	128	128	128	64	256	32	512	128	—	4
6218	,,	128	64	512	32	256	256	256	256	128	256	64	256	512	64	512	64	—	4
16058	,,	512	128	512	32	256	128	128	512	256	128	64	128	256	64	1024	128	—	4
19957	,,	256	128	512	32	1024	256	256	512	256	128	64	256	1024	256	512	256	—	8
16073	,,	512	256	1024	64	1024	512	128	512	256	256	64	256	2048	128	1024	128	—	16
Q28E	,,	64	64	1024	64	2048	512	256	256	512	512	64	256	1024	128	2048	256	—	32
16M.	*Br. mel.*	—	—	2	—	—	—	—	—	—	—	—	—	2	—	—	—	128	256
99	*Br. ab.*	—	—	2	—	—	—	2	—	—	2	—	—	2	—	—	—	128	512

TABLE 3. — *CF titres of anti-*Brucella *sera absorbed with* Br. ovis (*Strain* 6010)

Antigen Strain		Antisera Strain: *Br. ovis* 21422	6010	19703	6323	22855	6324	16060	6504	292	4154	15641	6218	16058	19957	16073	Q28E	*Br.mel.* 16M	*Br.ab.* 544
21422	*Br. ovis*	2	—	2	—	2	4	—	—	2	2	4	—	2	4	2	2	—	2
6010	,,	2	2	2	2	2	4	2	—	2	2	2	2	4	2	4	2	—	2
19703	,,	2	2	2	—	2	2	2	—	2	2	4	2	4	2	4	2	—	2
6323	,,	2	2	—	2	2	8	2	—	2	2	2	—	2	2	2	4	—	2
22855	,,	2	4	2	2	2	4	4	2	2	2	2	2	2	2	2	2	—	2
6324	,,	2	2	2	2	2	2	2	—	2	2	2	—	2	2	2	2	—	2
16060	,,	2	2	2	2	2	2	2	—	2	2	2	—	2	2	2	2	—	2
6504	,,	2	—	2	—	—	2	2	—	2	2	2	2	2	2	2	—	—	2
292	,,	2	2	2	—	—	2	2	—	—	2	2	—	4	—	4	2	—	2
4154	,,	4	—	—	4	—	2	2	—	—	2	2	4	2	2	2	2	—	4
15641	,,	—	—	—	4	—	2	—	—	2	2	2	2	4	2	2	—	—	2
6218	,,	4	—	—	—	—	2	2	—	2	—	2	64	4	2	2	8	—	2
16058	,,	2	2	2	—	2	2	2	—	2	2	2	2	4	2	4	4	—	2
19957	,,	—	2	2	2	2	2	2	—	2	2	2	4	8	—	—	2	—	2
16073	,,	—	2	2	—	—	2	2	—	2	2	2	2	8	2	4	4	—	2
Q28E	,,	2	2	2	2	—	—	2	4	2	2	2	128	4	4	2	64	—	8
16M	*Br. mel.*	2	2	—	—	—	2	2	—	2	2	2	2	4	2	2	2	128	256
99	*Br. ab.*	—	2	2	—	—	2	2	—	2	2	2	2	2	—	2	2	64	256

TABLE 4. — *CF titres of anti-*Brucella *sera absorbed with* Br. abortus (*Strain* 99)

Antigen Strain		Antisera Strain																	
		Br. ovis																*Br. mel.*	*Br.ab.*
		21422	6010	19703	6323	22855	6324	16060	6504	292	4154	15641	6218	16058	19957	16073	Q28E	16M	544
21422	*Br. ovis*	32	32	64	8	256	256	32	32	128	64	64	64	512	64	256	32	—	2
6010	,,	32	32	64	16	512	128	256	64	128	128	8	128	1024	32	256	32	—	2
19703	,,	16	32	128	8	512	128	16	32	128	64	16	32	256	32	512	32	—	2
6323	,,	16	64	128	16	512	512	64	64	64	128	32	64	256	64	512	64	—	2
22855	,,	64	64	256	8	512	256	32	64	128	128	64	128	512	32	512	32	—	2
6324	,,	64	64	256	16	512	256	16	64	64	64	16	128	256	32	512	32	—	4
16060	,,	32	16	128	4	128	64	8	16	32	64	4	32	256	8	256	32	—	2
6504	,,	64	32	128	8	512	256	16	64	128	64	8	128	256	8	256	32	—	2
292	,,	32	4	256	16	256	32	16	64	64	128	8	64	128	64	256	64	—	4
4154	,,	64	16	128	16	128	64	16	32	16	64	8	64	128	32	256	32	—	4
15641	,,	32	4	64	16	16	32	4	4	4	16	4	32	32	32	512	64	—	2
6218	,,	64	16	64	8	128	128	16	64	32	64	4	128	128	32	256	64	—	2
16058	,,	64	8	128	32	256	64	32	64	64	64	2	32	256	128	256	128	—	2
19957	,,	64	32	256	32	1024	256	32	32	64	128	8	64	256	16	512	64	—	2
16073	,,	64	16	256	64	512	256	32	16	64	256	8	256	256	64	1024	128	—	8
Q28E	,,	32	32	512	32	1024	256	16	64	256	256	16	256	128	128	1024	128	—	8
16M	*Br. mel.*	2	2	2	4	—	—	—	—	2	2	2	—	2	2	2	2	—	4
99	*Br. ab.*	2	—	2	8	—	—	—	—	2	2	—	—	2	4	2	2	—	2

TABLE 5. — *CF titres of anti-*Brucella *sera absorbed with* Br. melitensis (*Strain* 16*M*)

Antigen Strain		Antisera Strain																	
		Br. ovis																*Br.mel.*	*Br.ab.*
		21422	6010	19703	6323	22855	6324	16060	6504	292	4154	15641	6218	16058	19957	16073	Q28E	16M	544
21422	*Br. ovis*	128	64	256	16	256	256	32	256	128	128	64	64	512	128	256	64	—	4
6010	,,	512	128	256	16	512	256	256	512	128	256	128	128	2048	64	256	256	—	8
19703	,,	128	256	256	32	512	128	128	64	128	128	128	32	512	64	512	64	—	8
6323	,,	128	256	256	32	1024	512	128	512	128	128	256	128	256	64	512	64	—	4
22855	,,	512	256	512	32	1024	512	512	2048	512	128	64	128	256	64	512	64	—	8
6324	,,	512	128	512	32	1024	512	128	64	256	128	64	128	512	64	512	32	—	4
16060	,,	128	64	256	16	256	128	64	128	64	64	64	32	512	16	128	64	—	4
6504	,,	128	256	1024	32	512	512	64	256	256	64	64	128	512	32	256	32	—	8
292	,,	128	64	256	16	256	256	64	256	128	128	32	64	256	64	512	128	—	8
4154	,,	64	32	256	16	256	256	64	512	64	128	16	64	128	64	256	32	—	8
15641	,,	256	64	256	64	256	128	256	128	128	128	64	64	64	64	512	64	—	4
6218	,,	64	32	256	16	256	256	64	128	64	128	16	128	256	64	256	128	—	8
16058	,,	256	64	128	16	256	128	64	128	64	128	32	64	256	128	512	128	—	4
19957	,,	256	128	256	64	1024	256	64	128	256	128	64	64	512	64	512	128	—	4
16073	,,	256	64	512	128	512	512	128	512	64	256	32	256	512	64	512	128	—	32
Q28E	,,	256	128	256	64	2048	512	256	2048	256	512	64	256	512	128	1024	128	—	16
16M	*Br. mel.*	2	2	2	8	2	—	2	2	2	2	2	—	2	2	2	2	—	8
99	*Br ab.*	2	—	2	8	—	—	2	2	2	2	2	—	2	2	2	2	—	32

for a number of years in this laboratory is suitable for routine antigen production.

No studies have been undertaken with rough *Br. abortus* and *Br. melitensis* strains as difficulties were experienced in preparing suitable antisera and antigens from them. Further studies using rough strains are, however, indicated because the findings of Diaz, Jones & Wilson (1967), showed a close relationship between *Br. ovis* and rough *Br. melitensis*.

Summary

The serological relationship of 16 strains of *Br. ovis*, originating from South Africa, New Zealand and Australia, and standard strains of *Br. melitensis* (16M) and *Br. abortus* (Strains 99 and 544) were investigated using the CF test. Antisera against each strain were prepared. Unabsorbed antisera and antisera absorbed with *Br. ovis*, *Br. melitensis* and *Br. abortus* were tested against soluble antigens prepared from all strains used in the investigation. It was demonstrated that *Br. ovis* is antigenically related to *Br. abortus* but there appears to be little relationship between *Br. ovis* and *Br. melitensis*. The classification of *Br. ovis* within the genus *Brucella* seems to be justified.

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APPENDIX

The Complement Fixation Test

I. *Reagents*

A. *Veronal buffer:* Is made up according to the method of Kabat & Mayer (1961).

B. *Complement:*

1. Twenty to 50 male guinea-pigs are starved overnight.
2. About 20 ml of blood is drawn from each by cardiac puncture.
3. The blood is allowed to clot, and the clot is loosened with a sterile needle.
4. After a maximum of 4 hours room temperature, the tubes are centrifuged at 1020 *g* for 30 min.
5. The serum is carefully collected without contaminating with red blood cells, and is re-centrifuged if necessary.
6. The serum is distributed into ampoules in 1.0 ml amounts and freeze-dried. The preparation of the serum for freeze-drying should be completed in as short a time as possible.
7. The freeze-dried complement is stored at −18°C until used, and each batch titrated individually.

C. *Amboceptor:* Amboceptor is made according to the method of Kabat & Mayer (1961).

D. *Red blood cell suspension:* 1. Negative sheep blood is aseptically collected into an equal volume of sterile Alsever's solution (Kabat & Mayer 1961) and stored at 4°C for 1 week.

2. The blood is suitable for use for a period up to 2 months.
3. Fifteen ml sheep blood in Alsever's solution is centrifuged for 5 min at 1650 *g* and the supernatant fluid is discarded.
4. The red blood cells are washed three times in veronal buffer.
5. A suspension containing 3.0 per cent packed red blood cells, is made up for use in the test.

Blood from different sheep should not be mixed. Sheep should not be bled more than once in 8 weeks.

E. *Haemolytic system:* The haemolytic system is prepared by mixing equal parts of 3 per cent red blood cell suspension and amboceptor. The suspension is incubated for 10 min at 37°C and stored at 4°C until used.

F. *Antigens:* For antigen preparation see article.

II. *The complement-amboceptor titration*

A block titration of amboceptor and complement is done as follows:—

1. Increasing dilutions of complement and amboceptor are used as shown in the example given below. Any other suitable dilutions may be used depending on the end points of the particular reagents being used.
2. Into each tube 0.2 ml amboceptor, 0.2 ml complement, 0.2 ml red blood cell suspension and 0.4 ml of veronal buffer is pipetted.
3. The tubes are incubated at 37°C for 60 min.
4. The percentage haemolysis in each tube is read as described under the test IV.
5. Two typical sets of results are given in Table 1 (App.).
6. The highest complement dilution showing complete haemolysis at the lowest amboceptor dilution is noted, e.g. in titration A it is 1 in 40 and in B, 1 in 50. For the actual test one dilution lower than this is used, i.e. in case A, 1 in 30 and in B, 1 in 40. The highest dilution of amboceptor showing complete haemolysis of red blood cells at this dilution of complement is defined as 1 unit, e.g. in case A, 1 in 10,000 and in B, 1 in 900. In the test 2 units of amboceptor are used

III. *The antigen titration*

1. In titrating a batch of antigen, the antigen is tested in various dilutions against a known positive serum, usually the standard positive serum.
2. The test is performed in exactly the same way as described in IV except that the serum is titrated to a titre of 1 in 512.
3. A typical example of an antigen titration is given in Table 2 (App.).
4. The highest dilution of serum in which complete inhibition of haemolysis occurs is noted — in the above example 1 in 64. The highest dilution of antigen that still gives this end titre is used in the test — 1 in 90 in the above example.

IV. *The test*

1. Blood is collected in boric acid preservative (3 per cent boric acid in normal saline), approximately nine parts of blood are collected in one part of preservative. The blood is allowed to clot and the serum decanted into a clean bottle.

Table 1 (App.)

A.

Comp.	Amboceptor								
	1/400	1/500	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/10,000	1/20,000
1/10	—	—	—	—	—	—	—	—	—
1/20	—	—	—	—	—	—	—	—	—
1/30	—	—	—	—	—	—	—	—	2
1/40	—	—	—	—	—	—	—	—	2
1/50	2	—	—	—	—	—	—	—	2
1/60	2	2	2	—	—	—	2	2	2
1/70	2	2	2	—	—	2	2	2	2
1/80	2	2	2	2	—	2	2	2	2
1/90	2	3	3	3	3	3	3	3	3
1/100	4	4	4	4	4	4	4	4	4

B.

Comp.	Amboceptor										
	1/200	1/300	1/400	1/500	1/600	1/700	1/800	1/900	1/1,000	1/2,000	1/3,000
1/10	—	—	—	—	—	—	—	—	—	—	T
1/20	—	—	—	—	—	—	—	—	—	T	1
1/30	—	—	—	—	—	—	—	—	—	1	2
1/40	—	—	—	—	—	—	—	—	T	1	2
1/50	—	—	—	—	T	T	T	1	1	2	2
1/60	T	T	T	T	T	T	T	1	1	2	2
1/70	T	T	1	1	1	1	1	2	2	3	4
1/80	1	1	1	1	1	1	2	2	2	3	4
1/90	2	2	2	2	2	2	2	2	3	3	4
1/100	2	2	2	2	3	3	3	4	4	4	4

TABLE 2 (APP.)

Antigen dilutions	Serum dilutions									
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	None*
1/10	4	4	4	4	4	4	2	—	—	—
1/20	4	4	4	4	4	4	2	—	—	—
1/30	4	4	4	4	4	4	2	—	—	—
1/40	4	4	4	4	4	4	1	—	—	—
1/50	4	4	4	4	4	4	T	—	—	—
1/60	4	4	4	4	4	4	—	—	—	—
1/70	4	4	4	4	4	4	—	—	—	—
1/80	4	4	4	4	4	4	—	—	—	—
1/90	4	4	4	4	4	4	—	—	—	—
1/100	4	4	4	4	3	2	—	—	—	—
None*	—	—	—	—	—	—	—	—	—	—

* Reagent replaced by veronal buffer

TABLE 3 (APP.)

Reagents	Tube No.		
	1	2	3
Veronal buffer (ml)	0.6	0.4	0.2
Complement (ml)		0.2	0.2
Antigen (ml)			0.2
Haemolytic system (ml)	0.4	0.4	0.4

2. The serum is inactivated at 56°C for 30 min.
3. Antigen, complement and amboceptor are used at the optimum strength as determined in the titrations.
4. Before each day's testing, a control test of the reagents is set up as shown in Table 3 (App.).

   Tests are incubated at 37°C for 15 min.

   If all reagents are satisfactory there should be no haemolysis in Tube 1 and 100% haemolysis in Tubes 2 and 3.
5. The test is set up as shown in Table 4 (App.).

   Re-incubate at 37°C for 30 min
6. With each batch of sera tested a standard positiv anti-*Br. abortus* serum and an anti-*Br. ovis* serum are included. The end titres of these sera should remain constant.
7. The reactions in each tube are read with the aid of a suitable lighting box as follows: 0, 1, 2, 3, 4, represent 100 per cent, 75 per cent, 50 per cent, 25 per cent and no haemolysis respectively. Tubes should be spun to see whether any unhaemolysed red blood cells have settled to the bottom of the tube.
8. Anti-complementary activity of the individual sera is controlled in Tubes 1 and 2. Any inhibition of haemolysis in these tubes renders the serum unsuitable for testing.
9. The end point is the highest dilution showing 50 per cent, or more, inhibition of haemolysis.

REFERENCES

KABAT, E. A. & MAYER, M. M., 1961.Experimental Immunochemistry, 2nd ed. Springfield: Charles C. Thomas.

TABLE 4 (APP.)

Reagents	Tube No.					
	1	2	3	4	5	6
Veronal buffer (ml)	0.2	0.2	0.2	0.2	0.2	0.2
Serum (ml)	0.2	Twofold serial dilutions	0.2	Twofold serial dilutions		
		——>		——————>		
Veronal buffer (ml)	0.2	0.2	—	—	—	—
Antigen (ml)	—	—	0.2	0.2	0.2	0.2
Complement (ml)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate at 37°C for 60 min						
Haemolytic system (ml)	0.4	0.4	0.4	0.4	0.4	0.4