EVALUATION OF AN IMMUNODIFFUSION TEST FOR THE DETECTION OF ANTIBODIES TO *BRUCELLA ABORTUS* IN BOVINE SERUM

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CONSIDERABLE attention has been paid to the selection of diagnostic procedures for differentiation of antibodies resulting from vaccination of cattle with *Brucella abortus*, strain 19, from those resulting from natural infection (Yuskovets, 1956; Anderson *et al.*, 1964; Rose and Amerault, 1964; Schimmel and Erler, 1967). Relatively less attention has been given to the problems in diagnosis arising from the use of *Br. abortus* strain-45/20 adjuvant vaccine. This vaccine is weakly agglutinogenic but gives rise to significant titres of antibodies active in the complement fixation (CF) and antiglobulin tests. Although, in most cases, the antibody titres in all of these tests decline to insignificant levels within 1 yr of vaccination, some animals show persistent titres sufficiently high to cause difficulties in the interpretation of diagnostic tests. Usually in these cases the CF test shows inconclusive or weakly positive reactions and the titres in the antiglobulin test are also raised.

These problems are accentuated in herds in which strain 19 and strain 45/20 vaccines have been used. In such cases, agglutinins active in the serum agglutination (SA) and Rose Bengal plate (RBP) tests may also persist at significant levels for prolonged periods.

Clearly, to resolve this situation, a supplementary test is needed that would enable features characteristic of either the vaccinated or the naturally infected state to be identified. A procedure of potential value in this situation is the immunodiffusion test. This has the advantage of enabling the reaction of specific antigenic components with their respective antibodies to be identified. Thus, antibodies to components specific either to the infected or the vaccinated state could, hypothetically, be identified.

In the present study, a comparison of an immunodiffusion test with standard diagnostic tests was made on serum samples from unvaccinated cattle, from cattle vaccinated with strain 19 or strain 45/20, and from cattle known to be infected with virulent *Br. abortus* strains.

MATERIALS AND METHODS

Brucella abortus strains. The three strains used were from the culture collection maintained at this laboratory; they were a living smooth vaccine strain—strain 19 (Buck, 1930), a rough vaccine strain—strain 45/20 (McEwen and Roberts, 1936) and a smooth virulent FAO/WHO *Br. abortus* biotype-1 reference strain originally isolated by McEwen—strain 544.

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Serum samples. Except when stated otherwise, the 312 bovine serum samples were those submitted for routine testing in connexion with the Brucellosis Incentives and Eradication Schemes of the Ministry of Agriculture, Fisheries and Food. Apart from the collection of approximately equal numbers of samples from cattle vaccinated with strain 19 or strain 45/20 and from unvaccinated cattle, sera were taken at random. In most instances, vaccination with strain 19 had been made 1–5 yr, and with strain 45/20 (two doses) 1–2 yr, before the serum samples were collected. Some of the animals in these groups would have been exposed to natural infection with *Br. abortus*. Fifteen serum samples were obtained from cattle that had been experimentally vaccinated with strain 19, experimentally infected with *Br. abortus*, or shown by cultural methods to be naturally infected.

If not tested immediately after collection, sera were stored at -20°C until required.

Antigen preparations. Diethyl ether-water extracts of strain 544 were prepared according to Ribi, Milner and Perrine (1959) and concentrated by counter-dialysis against polyethylene glycol 20M to a dry weight concentration of c. 10 mg per ml. Before use in immunodiffusion tests, each batch was diffused against a collection of sera from cattle known to be infected with *Br. abortus* to ensure that the antigen concentrations were optimal for precipitation. Sodium dodecyl sulphate (SDS) extract of strain 45/20 was prepared according to Sterne, Trim and Broughton (1971).

Serological tests. The SA, CF and RBP tests were performed as described by Morgan *et al.* (1971). Immunodiffusion tests were done according to Corbel and Cullen (1970) but with a diethyl ether-water extract of strain 544 or a SDS extract of strain 45/20 as antigen and a diffusion medium made 1.0M in respect of NaCl. Diffusion was allowed to proceed for up to 7 days at room temperature. Antibody reacting with the smooth lipopolysaccharide antigen of *Br. abortus* could be differentiated from antibodies reacting with sub-surface antigens by its characteristic precipitation arc formed very close to the antigen well on diffusion against a diethyl ether-water extract of strain 544. The position of this arc, which corresponded to the antigenic component identified as the lipopolysaccharide agglutinin by Diaz *et al.* (1967, 1968), never varied. Other precipitation lines were assumed to correspond to sub-surface or intracellular antigens.

RESULTS

All the bovine sera included in this study were examined by the standard diagnostic tests, i.e., the SA, CF and RBP tests. Without reference to the results of these tests, the sera were then examined by the immunodiffusion technique. In all cases, sera were tested against ether-water extracts of strain 544, and some sera were also tested against SDS extracts of strain 45/20.

The results of the standard tests showed that the sera from unvaccinated animals, and from animals vaccinated with strain 19 or strain 45/20 could be divided into three categories, namely, those with high titres in all tests, those giving insignificant reactions to all tests and those giving inconclusive reactions to the tests. Included in the latter group were sera showing an elevated titre in a single test, as well as those giving inconclusive reactions to more than one test.

Serum samples from unvaccinated cattle

As shown in table I, most of the sera from unvaccinated animals did not give any reaction to the standard tests or to the immunodiffusion test with ether-water- and SDS-extracted antigens. One serum (no. 10) did, however, precipitate with sub-surface components of *Br. abortus* although negative in the other tests. Sera (nos. 11, 12, 85, 87, 88) that gave high titres in the standard

tests also invariably precipitated with both sub-surface and lipopolysaccharide antigens of *Br. abortus*; these reactions were presumably the result of natural infection. A few sera (nos. 1, 89, 90, 91, 92) gave low titres in the SA test and two (nos. 86 and 89) gave a weak reaction in the CF test; none of these reacted in the immunodiffusion test.

TABLE I

Results of standard tests and immunodiffusion tests on sera from unvaccinated cattle

Sample number	Result of			Number of precipitation lines given in immunodiffusion test with		
	serum agglutination test*	complement fixation test*	Rose Bengal plate test†	ether-water extract of strain 544 against		sodium dodecyl sulphate extract of strain 45/20
				sub-surface antigens	lipopoly-saccharide antigen	
1	1/10	-/2	—	0	0	0
2-9	-/10	-/2	—	0	0	0
10	-/10	-/2	—	2	0	0
11	1/160	>4/20	++	>4	1	0
12	3/80	>4/20	++	>1	1	0
13-84	-/10	-/2	—	0	0	NT
85	4/320	>4/20	++	>1	1	NT
86	-/10	4/4	—	0	0	NT
87	3/640	>4/20	++	>3	1	NT
88	3/640	>4/20	++	>1	1	NT
89	3/10	2/10	+	0	0	NT
90-91	2/10	-/2	—	0	0	NT
92	2/20	-/2	—	0	0	NT

* Results are expressed as degree of reaction/dilution of serum (Morgan *et al.*).

† ++ = Strongly positive reaction; + = positive reaction; — = negative reaction.

NT = Not tested.

Serum samples from cattle vaccinated with strain 19 living vaccine

The results obtained showed (table II) a trend similar to that found in unvaccinated animals. Sera that gave negative or insignificant reactions in the standard tests were for the most part also negative in the immunodiffusion test. Sera from animals with appreciable but diagnostically insignificant titres in the CF and SA tests (e.g., nos. 101 and 102) in some cases precipitated with ether-water extracts of *Br. abortus*. In most of these the reaction was confined to the sub-surface antigens. As in the case of unvaccinated animals, a few sera (e.g., nos. 99 and 100) had weak precipitating activity but no titre in the quantitative tests. Sera with high titres in the standard tests also gave strong reactions in immunodiffusion tests with an ether-water extract of strain 544. Antibodies to both sub-surface and lipopolysaccharide antigens were almost invariably detected in such sera, and it is probable that these animals were naturally infected. The results obtained with sera from experimentally

TABLE II

Results of standard tests and immunodiffusion tests on sera from cattle vaccinated with strain 19

Sample number	Result of			Number of precipitation lines given in immunodiffusion test with		
	serum agglutination test*	complement fixation test*	Rose Bengal plate test†	ether-water extract of strain 544 against		sodium dodecyl sulphate extract of strain 45/20
				sub-surface antigens	lipopoly-saccharide antigen	
93-98	-/10	-/2	-	0	0	0
99-100	-/10	-/2	-	1	0	0
101	1/20	4/4	+	1	0	0
102	3/40	4/2	-	1	0	0
103-104	3/20	-/2	-	0	0	0
105	3/10	2/4	-	0	0	0
106	4/10	2/10	+	0	0	0
107	1/20	4/4	-	1	0	0
108	3/40	>4/20	++	0	1	0
109	3/20	2/10	+	0	0	0
110	3/20	4/2	-	0	0	0
111	2/80	>4/20	++	>2	1	1
112	4/20	2/10	++	1	0	0
113-114	2/10	-/2	-	0	0	0
115	2/160	>4/20	++	>1	1	0
116	4/40	>4/20	++	>1	1	0
117	4/160	>4/20	++	1	1	1
118	1/10	-/2	-	0	0	NT
119-151	-/10	-/2	-	0	0	NT
152-153	3/10	-/2	-	0	0	NT
154	4/160	>4/20	++	>1	1	NT
155	3/160	4/20	++	>1	1	NT
156	3/40	-/2	-	0	0	NT
157	3/20	4/4	++	0	0	NT
158	2/10	-/2	+	0	0	NT
159-160	3/20	-/2	-	0	0	NT
161	2/10	-/2	-	0	0	NT
162	3/640	>4/20	++	3	1	NT
163	2/80	4/4	+	0	0	0
164	4/160	4/200	++	0	1	0
165	3/160	4/40	++	>1	1	0
166	2/640	4/40	++	>1	1	0
167	3/40	4/80	++	>1	1	0
168	4/20	4/4	++	>1	0	0
169	1/40	-/2	-	0	0	0
170	2/20	2/2	-	1	0	0

* Results are expressed as degree of reaction/dilution of serum (Morgan *et al.*).

† ++ = Strongly positive reaction; + = positive reaction; - = negative reaction.

NT = Not tested.

Samples nos. 163-170 were from four animals vaccinated experimentally with strain 19. The intervals between vaccination and sampling were 7 days (nos. 163 and 164), 28 days (nos. 165 and 166), 122 days (nos. 167 and 168) and >2 yr (nos. 169 and 170).

TABLE III

Results of standard tests and immunodiffusion tests on sera from cattle vaccinated with strain 45/20

Sample number	Result of			Number of precipitation lines given in immunodiffusion test with		
	serum agglutination test*	complement fixation test*	Rose Bengal plate test†	ether-water extract of strain 544 against		sodium dodecyl sulphate extract of strain 45/20
				sub-surface antigens	lipopoly-saccharide antigen	
171	1/160	>4/20	++	>1	1	1
172	3/20	4/2	—	1	0	0
173	2/10	4/4	—	1	0	0
174	3/10	-/2	—	0	0	0
175	4/320	>4/20	++	>1	1	1
176-177	2/10	-/2	—	1	0	0
178-191	2/10	-/2	—	0	0	0
192-193	2/20	2/4	—	1	0	0
194	2/20	-/2	—	1	0	0
195	1/20	-/2	—	1	0	0
196-197	2/20	4/2	—	1	0	0
198	3/320	>4/20	++	>1	1	1
199-227	1/10	-/2	—	1	0	0
228	3/10	-/2	—	1	1	0
229	3/10	4/4	—	1	0	0
230	2/10	-/2	—	1	1	0
231	3/20	4/4	—	0	0	0
232	2/10	4/2	—	0	0	0
233	3/10	2/2	—	0	0	0
234-237	3/10	-/2	—	1	0	0
238	3/10	4/10	+	1	0	0
239-240	3/20	-/2	—	2	0	0
241	2/40	-/2	—	1	0	0
242	2/80	-/2	—	3	0	0
243	1/40	4/4	—	>2	0	0
244	1/40	-/2	—	>2	0	0
245	2/40	2/2	—	1	1	0
246	1/40	-/2	—	3	1	0
247	2/640	>4/20	++	>2	1	1
248	3/320	>4/20	++	>2	1	1
249	1/640	>4/20	++	4	1	1
250-263	1/10	-/2	—	0	0	0
264‡	3/80	-/2	—	2	0	0
265	2/640	>4/20	++	>1	1	NT
266-283	-/10	-/2	—	0	0	NT
284	4/2560	>4/20	++	>4	1	NT
285	4/640	>4/20	++	>2	1	NT
286‡	3/10	2/10	+	0	0	NT
287‡	3/160	>4/20	++	>1	1	NT
288‡	3/20	4/10	+	1	1	NT
289‡	3/80	4/10	++	0	0	NT
290‡	3/320	>4/20	++	>2	1	NT
291	2/640	>4/20	++	>3	1	NT
292	4/160	>4/20	++	>1	1	NT
293	3/20	-/2	—	0	0	NT
294	2/20	-/2	—	0	0	NT
295	1/10	-/2	—	0	0	NT
296	1/320	>4/20	++	0	1	NT
297	3/40	-/2	—	>1	0	NT
298-305	2/10	-/2	—	0	0	NT

* Results are expressed as degree of reaction/dilution of serum (Morgan *et al.*).

† ++ = Strongly positive reaction; + = positive reaction; — = negative reaction.

‡ Also vaccinated with strain 19.

NT = Not tested.

vaccinated cattle (nos. 163–170) indicated that precipitins for both the lipopolysaccharide agglutinin and the intracellular antigens became detectable in the weeks following vaccination. The precipitins for the lipopolysaccharide agglutinin declined the most rapidly; the presence of precipitins to this antigen in the sera of cattle vaccinated more than 1 yr earlier is probably indicative of infection.

Only two sera (nos. 111 and 117) precipitated with the SDS extract of strain 45/20. Both had moderately high titres in the conventional tests.

TABLE IV
Results of standard tests and immunodiffusion tests on sera from cattle infected with virulent strains of Brucella abortus

Sample number	Result of			Number of precipitation lines given in immunodiffusion test with		
	serum agglutination test*	complement fixation test*	Rose Bengal plate test†	ether-water extract of strain 544 against		sodium dodecyl sulphate extract of strain 45/20
				sub-surface antigens	lipopolysaccharide antigen	
306‡	4/2560	4/4000	++	4	1	1
307‡	2/1280	4/2000	++	4	1	1
308‡	2/640	4/800	++	3	1	1
309§	2/160	4/10	++	3	1	1
310§	3/320	4/80	++	3	1	0
311§	4/320	4/80	++	2	1	0
312§	1/160	4/80	++	3	1	0

* Results are expressed as degree of reaction/dilution of serum (Morgan *et al.*).

† ++ = Strongly positive reaction; + = positive reaction; – = negative reaction.

‡ From three cattle shown by cultural methods to have a natural *Br. abortus* biotype 1 infection of at least 2 years' duration.

§ From four cattle infected 28 days previously with c. 10^{10} *Br. abortus* strain 544 by the subcutaneous route.

*Sera samples from cattle vaccinated with strain 45/20
killed adjuvant vaccine*

Sera from cattle vaccinated with strain 45/20 differed from those in the other categories in that a larger proportion contained appreciable titres of antibodies active in one or more of the standard tests (table III). In most instances the sera reacted in the CF and SA tests to low titre, but a proportion gave titres high enough to indicate natural infection. On examination by immunodiffusion against ether-water extract of strain 544, most of the sera with appreciable but low titres gave precipitin reactions with one to two sub-surface antigens; a small number also reacted with the lipopolysaccharide antigen (e.g., no. 230). Sera from cattle vaccinated with strain 45/20 that gave high titres in the standard tests usually reacted in the immunodiffusion test with

both sub-surface and lipopolysaccharide antigens. Sera from animals vaccinated between 6 mth and 1 yr previously (e.g., nos. 173, 229, 238 and 286) with significant titres in the CF test but relatively low titres in the SA test showed precipitating activity directed towards the sub-surface antigens. A few sera (e.g., nos. 228 and 246) without diagnostically significant titres contained precipitins for both sub-surface antigens and the lipopolysaccharide agglutinin. As these sera were from herds with a history of infection, the animals were possibly in the initial stages of infection.

Most sera from cattle vaccinated with strain 45/20 did not precipitate with SDS extracts of strain 45/20. Those that did (e.g., nos. 175 and 198) had titres in the conventional tests consistent with infection and also reacted with the lipopolysaccharide agglutinin and the sub-surface antigens.

Serum samples from cattle infected with virulent Br. abortus strains

Control experiments performed with sera from cattle known to have been infected either naturally or experimentally with virulent *Br. abortus* strains produced results consistent with the observations made on the other sera. Thus all sera in this group (table IV) had moderate or high titres in the conventional tests and precipitins for both the lipopolysaccharide agglutinin and the sub-surface antigens of strain 544. The precipitins for both types of antigen apparently persisted indefinitely, since the naturally infected animals were known to have infections of more than 2 years' duration.

Only four of the seven animals studied had precipitins for SDS extracts of strain 45/20.

DISCUSSION

In cattle vaccinated with the strain-45/20 adjuvant vaccine, the differentiation of residual antibodies to *Br. abortus* from antibodies resulting from active infection may pose peculiar difficulties. When these animals have residual antibody, the CF titre is usually raised to significant levels but the RBP and the SA tests may give marginal or negative reactions. The antiglobulin test is of no assistance in this situation because titres are raised both in actively infected and vaccinated animals. Evidence I have presented (Corbel, 1972) suggests that positive CF reactions, in the absence of antibody detectable in the SA and RBP tests, may result from the persistence of antibodies to non-agglutininogenic antigens in cattle vaccinated with strain 45/20. The content of smooth *Brucella*-specific agglutinin present in strain 45/20 is low. As far as is known, all *Br. abortus* strains capable of infecting cattle are of the smooth type and thus contain the agglutininogenic lipopolysaccharide described by Diaz *et al.* (1968). Cattle infected with virulent, smooth strains of brucellae are therefore likely to develop antibodies to the agglutinin. This is supported by observations of the very high titres of agglutinins present in the sera of cattle in the early and intermediate stages of *Br. abortus* infection. However, the agglutinin levels decline in the chronic phase of the disease, probably because of shift in the pattern of immunoglobulin response (Anderson *et al.*, 1964; Rice, Tailyour and Cochrane, 1966). Nevertheless, precipitins persist for very long periods,

possibly for the duration of infection (Corbel, to be published), suggesting that differences in immunodiffusion patterns might be used to distinguish between the antibody responses to 45/20 vaccine and active infection.

This is supported by the results of the present study. Precipitins to the smooth lipopolysaccharide agglutinin were mainly confined to sera that gave high titres in one or more of the standard diagnostic tests, usually the CF test. These sera usually also contained antibodies to the sub-surface antigens of *Br. abortus*. The majority of sera from unvaccinated animals gave no reactions in either the standard tests or the immunodiffusion test, although a minority, presumably from naturally infected cattle, gave reactions in the immunodiffusion test and also marked reactions in the other tests. Most of the samples taken from cattle more than 1 yr after vaccination with strain 19 showed very little activity in either the immunodiffusion test or standard tests. Sera giving reactions in the immunodiffusion test also had titres in the other tests that were suggestive of infection.

Many sera from cattle vaccinated with strain 45/20 showed detectable activity in one or more of the standard tests. Most of them reacted in the immunodiffusion test with sub-surface antigens of *Br. abortus*, but a number of sera also precipitated with the lipopolysaccharide antigen. It is conceivable that these precipitins to lipopolysaccharide antigen might have resulted from persistence of antibody to the low content of agglutinin in the 45/20 vaccine, but it is more likely that, in most instances at least, natural infection with virulent *Br. abortus* was responsible. In most of the sera precipitating with lipopolysaccharide, the titres of the SA and CF tests were raised, but this was not invariably the case.

The immunodiffusion test with an ether-water extract of a smooth strain of *Br. abortus* may therefore assist in the identification of antibodies specific to infection with smooth strains of *Br. abortus* in sera that give equivocal results in the standard tests.

Precipitins reacting with SDS extracts of strain 45/20 were detected in the sera of some, but not all, animals with marked reactions to the standard tests, irrespective of their previous vaccination history. Thus tests with this material could not be used for the specific detection of antibodies resulting from vaccination with strain 45/20.

Yersinia enterocolitica serotype IX evokes precipitins to the lipopolysaccharide agglutinin of *Br. abortus* (Corbel and Cullen, 1970). However, this is unlikely to be a source of confusion, because further studies have shown that this organism provokes a transient serological response, and the agglutinin titres to *Br. abortus* are always much higher than the titres of the complement-fixing or precipitating antibodies (Corbel, to be published).

SUMMARY

Examination of bovine serum samples submitted from the field in connexion with the Brucellosis Eradication Scheme showed the value of immunodiffusion tests made with a diethyl ether-water extract of a smooth virulent strain of *Brucella abortus*.

Sera from cattle vaccinated with the killed strain-45/20 adjuvant vaccine frequently gave appreciable reactions when examined by standard tests, namely the serum agglutination, complement fixation and Rose Bengal plate tests. When these reactions were weak, the immunodiffusion test usually gave one or more precipitation lines, but these were formed only against sub-surface antigens. When these reactions were strong enough to suggest natural infection, a line against the smooth surface lipopolysaccharide antigen was present in addition. Infrequently, a similar line occurred in the absence of significant reactions to the standard tests.

Sera from cattle vaccinated with living strain 19 less frequently gave appreciable reactions in the standard tests. When the reactions were weak, immunoprecipitation lines were often formed against the sub-surface antigens but seldom against the lipopolysaccharide; when they were stronger, a line against the lipopolysaccharide was usually formed in addition.

Sera from unvaccinated cattle usually gave negative results in all tests, but several that gave marked reactions in the standard tests also gave precipitation lines with the sub-surface antigens and the lipopolysaccharide antigen. One sample gave two lines with sub-surface antigens, but was otherwise negative.

In immunodiffusion tests with sodium dodecyl sulphate extracts of the rough strain 45/20 a single precipitation arc was formed with some but not all of the sera that gave marked reactions in the standard tests.

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REFERENCES

- ANDERSON, R. K., JENNESS, R., BRUMFIELD, HELENE P., AND GOUGH, PATRICIA 1964. *Brucella*-agglutinating antibodies: relation of mercaptoethanol stability to complement fixation. *Science, N.Y.*, **143**, 1334.
- BUCK, J. M. 1930. Studies of vaccination during calfhood to prevent bovine infectious abortion. *J. Agric. Res.*, **41**, 667.
- CORBEL, M. J. 1972. Characterisation of antibodies active in the Rose Bengal plate test. *Vet. Rec.*, **90**, 484.
- CORBEL, M. J., AND CULLEN, G. A. 1970. Differentiation of the serological response to *Yersinia enterocolitica* and *Brucella abortus* in cattle. *J. Hyg., Camb.*, **68**, 519.
- DIAZ, R., JONES, LOIS M., LEONG, D., AND WILSON, J. B. 1967. Differences between *Brucella* antigens involved in indirect hemagglutination tests with normal and tanned red blood cells. *J. Bact.*, **94**, 499.
- DIAZ, R., JONES, LOIS M., LEONG, D., AND WILSON, J. B. 1968. Surface antigens of smooth brucellae. *J. Bact.*, **96**, 893.
- MCEWEN, A. D., AND ROBERTS, R. S. 1936. Bovine contagious abortion. The use of guinea-pigs in immunisation studies. *J. Comp. Path. Ther.*, **49**, 97.
- MORGAN, W. J. B., MACKINNON, D. J., GILL, K. P. W., GOWER, S. G. M., AND NORRIS, P. I. W. 1971. Standard laboratory techniques for the diagnosis of brucellosis. Ministry of Agriculture, Fisheries and Food.
- RIBI, E., MILNER, K. C., AND PERRINE, T. D. 1959. Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. Methods for separation and some biologic activities. *J. Immun.*, **82**, 75.
- RICE, C. E., TAILYOUR, J., AND COCHRANE, D. 1966. Ultracentrifugal studies of sera from cattle vaccinated or naturally infected with *Brucella abortus*. *Canad. J. Comp. Med.*, **30**, 270.

- ROSE, J. E., AND AMERAULT, T. E. 1964. Electrophoretic and ultracentrifugation studies on serum from pregnant heifers after exposure to virulent *Brucella abortus*. *Amer. J. Vet. Res.*, **25**, 998.
- SCHIMMEL, D., AND ERLER, W. 1967. Characterization of agglutinins of cattle serum against *Brucella abortus*. *Amer. J. Vet. Res.*, **28**, 1919.
- STERNE, M., TRIM, GLADYS, AND BROUGHTON, E. S. 1971. Immunisation of laboratory animals and cattle with non-agglutinogenic extracts of *Brucella abortus* strain 45/20. *J. Med. Microbiol.*, **4**, 185.
- YUSKOVETS, M. K. 1956. Eradication de la brucellose en U.R.S.S. *Bull. Off. int. epizoot.*, **46**, 366.