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A RECOMMENDED METHOD FOR THE PREPARATION OF MONOSPECIFIC BRUCELLA SERA

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SYNOPSIS

A method is described in detail for the preparation of monospecific sera for typing brucella cultures. The importance of using entirely smooth cultures throughout the procedure is emphasized. A single intravenous injection of living brucellae in rabbits caused an active infection and the early appearance of high-titre antibodies. Sera obtained five to seven days after inoculation contained a higher proportion of specific antibodies than serum obtained ten days after inoculation. Trial absorptions of each serum sample were performed to determine the relative content of homologous and heterologous antibodies, so that the optimum proportions of absorbing cells to serum could be employed for the final absorption. In this manner sera were obtained which at a 1/160 dilution agglutinated the homologous antigen and at a 1/10 dilution did not agglutinate the heterologous antigen.

Monospecific sera were used in typing 180 brucella strains isolated in Great Britain and other countries, and over 3000 cultures recovered from experimental infections. With the exception of two strains of *Br. melitensis*, all cultures were agglutinated to titre with only one of the monospecific sera.

Monospecific sera are of great value in typing brucella cultures, but the preparation of such sera is considered to be a difficult procedure. Sera prepared in different laboratories may vary in specificity. A method for the preparation of monospecific typing sera was outlined in the second report of the Joint FAO/WHO Expert Committee on Brucellosis (1953).

Large volumes of monospecific sera have been prepared at the Central Veterinary Laboratory at Weybridge for distribution to laboratories throughout the world who wish to type brucella cultures or to compare their monospecific sera with the Weybridge preparations. Details of the methods developed at Weybridge are presented in the hope that more laboratories will employ serological typing as well as biochemical tests for species identification.

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Materials and Methods

Large rabbits with prominent ear-veins were selected for serum production. Before inoculation, serum from each rabbit was tested for brucella agglutinins, to eliminate the possibility of prior infection with brucellae or cross-reacting species. A single intravenous injection of 1 ml of a suspension containing 10^7 to 10^9 living organisms (grown on serum-dextrose slopes for 48 hours) was sufficient to establish active infection. Rabbits were bled from the ear-vein daily or at frequent intervals from the fifth day after inoculation. Serum was separated from the clot and stored at −20°C until required.

The cultures used in the infection of rabbits and in the production of antigens for agglutinin absorption were type strains selected by the Joint FAO/WHO Expert Committee on Brucellosis (1953). Smooth colonies of *Br. abortus* 544 and *Br. melitensis* 16M were selected and grown on serum-dextrose slopes for 48 hours. Heavy suspensions of growth from these slopes were used as inocula for Roux flasks of serum-dextrose or potato agar. Roux flasks were incubated in an inverted position to prevent the collection of water of condensation on the agar surface and thus diminishing the degree of dissociation. After three days' incubation all liquid was poured off before the growth was harvested. The bacteria were washed off with buffered saline (0.85% NaCl, pH 6.3) and filtered through glass wool to remove any agar particles. A sample of the bacterial suspension was streaked on glycerol-dextrose plates to check purity and dissociation. If more than 1% non-smooth colonies were observed the suspension was discarded. If the suspension was satisfactory it was heated for 1 hour at 60°C to kill the brucellae before centrifuging at a rate sufficient to sediment the cells. The supernatant fluid was discarded and the sediment was resuspended in 0.5% phenol saline to make the bacterial concentration approximately 0.05%. This concentrated suspension, stored at 4°C, was used for subsequent agglutinin-absorption tests. The actual volume of packed cells per ml of the concentrated suspension was determined by employing capillary centrifuge tubes and spinning for 70 minutes at 2700 revolutions per minute (r.p.m.) in a horizontal centrifuge.

For absorptions the desired volume of packed cells was obtained by pipetting the appropriate amount of the concentrated suspension into heavy-walled centrifuge tubes. After the cells had settled the supernatant fluid was discarded and measured quantities of undiluted serum were added and mixed with a pipette. An arbitrary relation of 1 part cells and 10 parts serum was selected for trial absorptions. (For example: for trial absorption of anti-*melitensis* serum one would pipette 2 ml of the concentrated suspension of *abortus* cells into centrifuge tubes. The packed cell volume would be 0.1 ml and to it 1 ml of anti-*melitensis* serum would be added.) Mixtures of cells and serum were held in a water-bath at 37°C with frequent mixing

for two hours before centrifuging and decanting the serum for agglutination tests.

The antigens for the agglutination tests were prepared from the concentrated suspensions by dilution with 0.5% phenol saline. In standardizing the *Br. abortus* antigen the appropriate dilution, determined by titration against the International Standard Anti-*Brucella abortus* Serum (Stableforth, 1954), was that which showed 50% agglutination with a 1/480 dilution of standard serum. The *Br. melitensis* antigen was then adjusted to the density of the *Br. abortus* antigen.

For the agglutination tests double dilutions of sera, beginning 1/5, were prepared in duplicate. Equal volumes of antigen (0.5 ml) were added; *abortus* antigen to one row of serum dilutions and *melitensis* antigen to the other row. For purposes of convenience the titres were recorded in the tables as the number of the last tube in which 50% agglutination was observed. Thus tube 1 represents a final serum dilution of 1/10 and tube 10 a dilution of 1/5120.

Trial absorptions of the serum obtained from each rabbit at each bleeding were performed in order to determine the best method to follow for the final absorption. Sera with similar agglutinin content were pooled, in a manner described later, for the final absorption. Eventually all serum pools showing a five-tube difference between the homologous and the heterologous agglutinin titre were mixed, Seitz-filtered, and stored at 4°C, after addition of phenol (final concentration 0.5%).

Experimental Results

Determination of optimal bleeding-time after infection of rabbits

Twenty rabbits were employed for the production of monospecific *melitensis* serum prepared for international distribution in 1954. A single intravenous injection of 10^7 living organisms of *Br. melitensis* 16M stimulated the production of high titre antibodies within six days. Rabbits were bled at varying intervals after inoculation (as recorded in Table 1) and serum aliquots from each rabbit at each bleeding-date were tested for agglutinins before and after absorption. Non-absorbed sera did not show a difference in titre greater than one tube with the homologous and heterologous antigens. High-titre serum was obtained from nearly all rabbits on the sixth day after infection. The titre had increased one or two tubes upon subsequent bleedings.

The results of trial absorptions recorded in Table 1 represent repeated tests with each sample. Agglutinins for the heterologous *abortus* antigen were removed by a single absorption from most of the sera obtained six days after infection, whereas one absorption was inadequate in removing all heterologous agglutinins from most sera taken later. The difference in

TABLE 1. AGGLUTININ TITRES * OF ANTI-*MELITENSIS* SERA, OBTAINED AT INTERVALS AFTER INOCULATION, BEFORE AND AFTER TRIAL ABSORPTION

Rabbit No.	Treatment of serum	Days after inoculation											
		6		9		11		16		23		53	
		A	M	A	M	A	M	A	M	A	M	A	M
9	Non-absorbed	7	8	8		9		9		9		7	
	Absorbed**	0	6	6	7	6	8	5	7	3	7	1	4
10	Non-absorbed	7	8	8		8		7		8		8	
	Absorbed	1	6	3	7	2	6	0	5	0	4	0	3
11	Non-absorbed	6	7	7		8		8		7		7	
	Absorbed	0	4	2	6	2	6	2	5	0	3	0	2
12	Non-absorbed	8	9	9		9		8		7		8	
	Absorbed	2	6	7	8	6	7	4	6	2	5	5	5
13	Non-absorbed	8	9	7		8		9		9		7	
	Absorbed	1	6	3	6	3	6	5	7	5	7	5	6
14	Non-absorbed	8	8	8		9		9		9		7	
	Absorbed	0	7	3	7	4	6	6	7	6	7	2	5
15	Non-absorbed	7	8	7		7		8		8		7	
	Absorbed	0	6	0	6	0	6	0	5	0	4	0	3
16	Non-absorbed	7	8	7		7		8		9		10	
	Absorbed	0	5	2	6	2	5	2	6	2	6	6	7
17	Non-absorbed	8	8	8		9		9		9		8	
	Absorbed	1	6	5	7	6	7	6	8	4	6	2	4
18	Non-absorbed	5	5	5		7		9		10		7	
	Absorbed	0	3	0	4	0	4	2	6	4	7	3	6
19	Non-absorbed	6	7	7		7		8		10			
	Absorbed	0	4	0	5	0	5	0	6	6	9		
20	Non-absorbed	6	7	7		7				8			
	Absorbed	0	4	2	5	0	4			0	4		
21	Non-absorbed	8	9	9		10		9		8		8	
	Absorbed	1	6	7	8	6	8	3	6	1	4	2	3
22	Non-absorbed	7	8	9	10	9		9		9		8	
	Absorbed	0	4	7	8	6	8	7	8	3	6	0	3
23	Non-absorbed	7	8	8		8		8		8		7	
	Absorbed	0	6	3	7	2	6	2	6	1	4	0	2
24	Non-absorbed	7	8	8		8		9	10	9		9	
	Absorbed	0	5	0	5	2	6	5	6	4	6	4	6
25	Non-absorbed	8	8	8		9		9		9		9	
	Absorbed	0	5	1	6	2	6	4	6	4	6	6	7
26	Non-absorbed	6	7	8		8		8		9		7	
	Absorbed	0	3	4	6	3	6	2	5	2	4	0	3
27	Non-absorbed	6	7	7		8		9		8		7	
	Absorbed	0	4	0	5	1	5	3	5	2	5	0	3
28	Non-absorbed	8	9	9		9		8		10		9	
	Absorbed	3	7	7	8	6	8	7	8	7	8	3	5
Average number of tubes difference (*melitensis*-*abortus* titre) following absorption		4.6		3.2		3.2		2.8		2.8		2.1	

* Recorded as number of last tube in which 50% agglutination was observed. Tube 1 represented a final serum dilution of 1/10, tube 2 represented 1/20, etc.

** 0.1 ml packed *abortus* cells + 1.0 ml serum held for two hours at 37°C

A = *Br. abortus* antigen; M = *Br. melitensis* antigen

titre of the homologous and heterologous agglutinins following absorption became decreasingly less as time went on. Thus, an average difference of 4.6 tubes was noted in the titres with *abortus* and *melitensis* antigens after absorption of sixth-day sera, whereas there was an average difference of only 2.8 tubes 16 days after infection.

Variation was observed among individual rabbits in their ability to produce specific sera. High-titre monospecific sera could be obtained with a single absorption from two rabbits (No. 15 and No. 19) from the sixth to the sixteenth day. Whereas other rabbits (for example, No. 9) did not produce sera which could be readily absorbed beyond the sixth day.

Similar results were obtained with the production of monospecific *abortus* serum in a small series recorded in Table 2.

TABLE 2. AGGLUTININ TITRES * OF ANTI-*ABORTUS* SERA, OBTAINED AT INTERVALS AFTER INOCULATION, BEFORE AND AFTER ABSORPTION

Rabbit No.	Treatment of serum	Days after inoculation															
		5		6		7		8		10		13		21		56	
		A	M	A	M	A	M	A	M	A	M	A	M	A	M	A	M
29	Non-absorbed	8	8	10		10		10		10		10		10		9	
	Absorbed**	6	0	9	5	9	6	9	7	9	8	8	7	7	6	6	5
30	Non-absorbed	6		9		9		10		10		10		10		10	
	Absorbed	5	0	7	1	7	3	9	6	9	8	9	8	9	8	8	7
31	Non-absorbed	9		10		10		10		10		10		10		10	
	Absorbed	7	0	7	1	7	3	9	5	9	8	9	8	8	6	10	8
32	Non-absorbed	9		9		10		10		10		9		9		10	
	Absorbed	6	0	6	0	7	1	9	4	8	6	7	6	6	4	7	5
Average number of tubes difference (*abortus*-*melitensis*) following absorption		6.0		5.5		4.2		3.5		1.2		1.0		1.5		1.5	

* Recorded as number of last tube in which 50 % agglutination was observed. Tube 1 represented a final serum dilution of 1/10, tube 2 represented 1/20, etc.

** 0.1 ml packed *melitensis* cells +1.0 ml serum held for two hours at 37°C
A = *Br. abortus* antigen; M = *Br. melitensis* antigen

Determination of optimum dose of absorbing cells

Statements on the preparation of monospecific sera usually mention the importance of adjusting the dose of absorbing organisms to the titre of the serum. It can be seen from the data in Tables 1 and 2 that the pre-absorption titre by itself gave little indication of the proportion of homologous to heterologous agglutinins in the serum. The time-interval between infection and bleeding and the individual idiosyncrasies of the rabbit are factors which necessitate trial absorptions. The information thus obtained

was used in determining the optimum absorbing dose for the remainder of the serum. Thus, a serum from which all heterologous agglutinins and most homologous agglutinins had been removed in the trial absorption using 1 ml of packed cells per ml of serum was absorbed with fewer cells—for example, a relation of 0.025 ml cells per ml serum. Whereas a serum which still contained heterologous and homologous agglutinins to a high titre would require 0.2 ml of cells for each ml of serum. Or, alternatively, such a serum might be absorbed twice: the first time with 0.1 ml cells per ml of serum, the second time with 0.025 cells per ml. In practice it was convenient to pool various aliquots of serum which upon trial absorptions had been shown to have similar specific agglutinin content. A suggested procedure is given below:

Pool	*Homologous titre * before absorption*	*Titres following trial absorptions with 0.1 ml cells per ml serum*		*Dose of cells per ml serum for final absorption (ml)*
		Homologous	*Heterologous*	
1	> 7	> 5	—	0.1
2	> 6	4	—	0.05
3	> 5	1-3	—	0.025
4	> 7	> 6	1-5	0.2
5	> 7	5	1-3	0.1: 1st absorption 0.025: 2nd absorption

* Number of last tube showing 50% agglutination

Each pool was tested after the final absorption, and only those giving an end-point in the fifth tube (1/160) or higher with the homologous antigen, and no reaction in the first tube with the heterologous antigen, were incorporated in the final product.

Influence of incubation time on agglutinin absorption

Lengthening the incubation time beyond two hours at 37°C did not increase the absorption of heterologous agglutinins, but it sometimes lowered the titre of the homologous agglutinins. Absorption overnight in the cold did not offer a better method than two hours in a water-bath at 37°C.

Results obtained with monospecific sera

The final preparations of monospecific sera were tested against a series of *Br. abortus*, *Br. suis* and *Br. melitensis* strains of known characteristics. When the results were found to check with those obtained with previous batches of sera, the new products were used for routine testing. An unknown culture was always standardized to the turbidity of Brown Tube 4 beforetesting against dilutions of monospecific sera.

A summary of the typing results obtained with brucella cultures received at Weybridge from January 1953 to March 1957 is given in Table 3. All isolations in Great Britain were from cattle, with the exception of two

TABLE 3. BRUCELLA CULTURES TYPED AT WEYBRIDGE FROM JANUARY 1953 TO MARCH 1957

Type	Growth characteristics				Agglutination with monospecific sera		Number of cultures from	
	CO_2-requiring	basic fuchsin	thionin	H_2S production on days 1 2 3 4	A	M	Great Britain	other countries
Br. abortus, strain 544	+	+	—	+ + ± ±	+160	—		
Br. suis, strain 1330	—	—	+	+ + + +	+160	—		
Br. melitensis, strain 16M	—	+	+	— — — —	—	+160		
Br. abortus	+	+	—	+ + ± ±	+160	—	50	4 (Sierra Leone, S. Rhodesia)
Br. abortus, aerobic	—	+	—	+ + + ±	+160	—	24	5 (Netherlands, France, Australia, Portugal, South Africa)
Br. abortus, dye-sensitive	+	—	—	+ + + +	+160	—	6	3 (USA, France, S. Rhodesia)
Br. abortus thionin-resistant	+	+	+	— + + +	+160	—		2 (Netherlands)
Br. suis	—	—	+	+ + + +	+160	—		4 (Germany, S. Viet Nam)
Br. suis, Danish type	—	—	+	— — — —	+160	—		7 (France, Germany)
Br. melitensis	—	+	+	— — — —	—	+160	33	12 (Europe, Africa, Afghanistan)
Br. melitensis, CO_2-requiring	+	+	+	— — — —	—	+160	1	
	+	+	+	+ + + +	—	+160	1	
Br. melitensis, H_2S-producing	—	+	+	+ + + +	—	+160	2	1 (Indonesia)
Br. abortus/ melitensis	+	+	—	+ + + +	—	+160	16	
Br. melitensis/ abortus	—	+	+	— — — —	+160	—		7 (Netherlands, Indonesia, Thailand)
Br. melitensis	—	+	+	— — — —	+80	+20		1 (Israel) *
Br. melitensis	—	+	+	— — — —	+20	+160		1 (Canada) **
Total cultures typed							133	47

* Isolated from sheep
** Isolated from human

typical CO_2-requiring *Br. abortus* cultures recovered from sheep. Cultures of the same type isolated from one herd are represented in the table only once, although several of the unusual types were repeatedly isolated from

infected herds—for example, *Br. abortus/melitensis* was recovered from 14 cows in one herd and CO_2-requiring *Br. melitensis* was recovered from seven cows in another herd.

A great number of the cultures did not conform to the classic characteristics of the three species. In the case of some cultures, which were sensitive to both dyes, species identification depended upon the serological characteristic. Cultures with the growth characteristics of *Br. abortus* and the serological properties of *Br. melitensis* were frequently isolated from cattle in Great Britain. Cultures with the reverse order of characteristics were received from other countries. With the exception of two cultures, however, all the cultures typed at Weybridge were agglutinated to titre with only one of the monospecific sera. The results obtained with the two exceptions are given in Table 3.

In addition to the cultures listed in Table 3, over 3000 cultures recovered from experimental infections of guinea-pigs, rabbits and goats have been typed. In all cases the culture recovered from the experimental animal gave the same typing result as the culture which was inoculated.

A rapid slide-test was employed for the serological identification of the cultures recovered from experimental infections. One drop of a 1/5 dilution of each of the monospecific sera was placed on a slide, a small portion of bacterial growth from a slope or a colony was suspended in each drop and the bacteria were agglutinated almost immediately by the corresponding serum. The 1/5 dilutions of sera were held at 4°C and used as required. This method was economical in time and material, and was suitable for this purpose. It could not, however, be depended upon to detect minor cross-reactions with atypical cultures.

Discussion and Conclusions

One intravenous injection of a large dose of living brucellae produces an active infection in rabbits and stimulates the production of agglutinins within a few days. Further inoculations are unnecessary. Landsteiner (1945) discussed the changes in immune sera during immunization and stated that there is a qualitative, as well as a quantitative, change in the combining capacity of antibodies upon continued presence of the antigen. The specificity of sera from early bleedings differed from that of sera taken later. Proom (1943) obtained the highest titre serum after a single injection; with further injections the specificity of the sera decreased and he was unable to remove the non-specific antibody by absorption.

For the production of monospecific sera it is advisable to use sera from the first bleeding that gives a 1/640 pre-absorption titre. In practice, however, it is not possible to predict when this will occur. Although we usually observed this titre in rabbits five and six days after infection, in a

later series sufficiently high titres were not obtained until the ninth day. If small quantities of blood were withdrawn and tested each day from the fifth day after infection, rabbits could be exsanguinated as soon as a titre of 1/640 was detected. Provided that no more than one day had elapsed between the time of appearance of high-titre antibodies and the time of final bleeding, the sera should contain a high proportion of specific antibodies.

The importance of using only smooth cultures for the inoculation of the rabbits, for the preparation of absorbing suspensions and for the agglutinating antigens cannot be over-emphasized. The type strains, 544 and 16M, cannot be depended upon to remain entirely smooth under the conditions of frequent sub-culture and mass growth involved in antigen production.

It is suggested that laboratories should check their monospecific sera against the Weybridge sera with representative cultures in their collections, and also should send samples of their sera to Weybridge for comparative tests.

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RÉSUMÉ

Les sérums monospécifiques sont d'une grande utilité pour le typage des *Brucella*, mais la préparation de ces sérums est considérée comme difficile. L'auteur décrit en détail dans cet article la technique appliquée au Central Veterinary Laboratory de Weybridge, Angleterre, avec l'espoir qu'un nombre croissant de laboratoires emploieront les méthodes sérologiques ainsi que les tests biochimiques de typage.

On utilise dans ce laboratoire les souches choisies par le Comité mixte FAO/OMS de la brucellose, c'est-à-dire des cultures lisses (ce caractère est important) de *Br. abortus* 544 et de *B. melitensis* 16M. Vingt lapins ont servi à la préparation du sérum anti-*melitensis*. Une seule injection intraveineuse de *Brucella* vivantes au lapin détermine une infection active et la formation d'anticorps à titre élevé. Cinq à sept jours après l'inoculation, les sérums contenaient, en général, un titre d'anticorps plus élevé qu'après 10 jours. On a déterminé la teneur relative du sérum en anticorps homologues et hétérologues de façon à établir, en fonction du sérum, la proportion optimum de cellules agglutinables à employer dans le test. On a obtenu par cette méthode des sérums agglutinant à 1/160 l'antigène homologue, qui n'agglutinaient pas, même à 1/10, les sérums hétérologues.

Des sérums monospécifiques ont été employés à Weybridge pour typer 180 souches de *Brucella* provenant de Grande-Bretagne et d'autres pays (prélevées sur des animaux malades, ou sur l'homme). Certaines cultures ayant les caractères biochimiques et tinctoriaux de *Br. abortus* avaient les caractères sérologiques de *Br. melitensis*, et vice-versa. Pourtant, toutes les cultures, sauf deux, n'étaient agglutinables, au titre requis, que par l'un des deux sérums spécifiques de type. Quelque 3000 cultures, provenant d'infections

expérimentales du cobaye, du lapin et de la chèvre ont été typées par les sérums monospécifiques. Toutes les souches récupérées étaient agglutinées par les mêmes sérums que lors de l'injection.

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