J. Hyg., Camb. (1983), **90**, 33–39
Printed in Great Britain

Comparison of standard tube and microagglutination techniques for determining *Brucella* antibodies

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(*Received* 6 *April* 1981; *accepted* 8 *July* 1982)

SUMMARY

A microagglutination method for determining the agglutinating and 'blocking' antibodies to *Brucella abortus* is described. A collection of sera from healthy blood donors in two rural areas of New Zealand were tested by the microagglutination methods and the standard methods in tube. The results are compared and show that where discrepancies occur, these are due to the microagglutination methods being more sensitive. It is concluded that these are suitable methods for screening populations.

INTRODUCTION

Brucella antibody levels are most commonly determined by tube agglutination methods. Of these the standard agglutination test (SAT) has been extensively used and a number of commercial reagents are available. In some laboratories the Coombs or antihuman globulin test (AHGT) is subsequently performed as an extension of the SAT for the detection of 'incomplete' or 'blocking' antibodies.

Microagglutination methods have been introduced recently for a number of test systems because they can be performed more rapidly and employ less serum and antigen (Brown *et al.* 1980, 1981).

A microagglutination test has been developed by C. P. Beaton (pers. comm.) for the SAT and AHGT. This has been used by Gilbert *et al.* (1980) to survey workers employed in three abattoirs in Victoria, Australia. This micro-method was designed such that the AHGT can be performed subsequent to the SAT unlike the method of Brown *et al.* (1981).

Since 1974 there has been established a New Zealand National Serum Bank (NSB) at the National Health Institute (NHI). This consists to date of approximately 10000 sera taken by the New Zealand Blood Transfusion Service from various parts of New Zealand. Two surveys of antibody levels to *Brucella*, *Leptospira* and *Toxoplasma* have been published to date (Metcalfe *et al.* 1979, 1981). Due to the large number of sera being processed a micro-method appeared an attractive alternative. In this report the microagglutination method of C. P. Beaton for performing the *Brucella* SAT and AHGT is compared with the accepted tube technique, in order to assess its suitability to determine *Brucella* agglutinating antibody levels of populations.

MATERIALS AND METHODS

The sera

The human serum specimens used for the comparison of the tube and microagglutination methods were provided by the NSB. The NSB is a collection of sera held at the NHI which have been obtained from various systematic multipurpose serological surveys performed by the Institute. The serum specimens are collected in cooperation with the New Zealand Blood Transfusion Service which tests each specimen for hepatitis B surface antigen (HBsAg). Only HBsAg negative specimens are accepted into the NSB. Each person from whom a serum sample was obtained, completes a questionnaire recording personal and medical information.

Each serum sample is divided into six 0·5 ml portions. Four of these 0·5 ml aliquots are freeze dried, sealed under nitrogen and placed in the dark at room temperature for long term storage. The remaining two aliquots are stored at −90 °C until used for the various serological tests required by the survey being undertaken. It was from one of these frozen aliquots that serum was taken for the present study of comparing the tube and microagglutination methods.

The areas within New Zealand chosen for the multipurpose serological surveys are such that within a four year period of operation, all the major urban areas and four rural districts are sampled. The sera used in this study were collected in Timaru (South Island) and Manawatu (North Island) in 1980. These rural areas were chosen for this study because earlier surveys had shown (Metcalfe *et al.* 1979, 1981) that a large number of reactors is more likely to be found in rural than in urban areas.

The tube agglutination test

The tube agglutination methods for the SAT and AHGT were based on those described by Robertson *et al.* (1980).

Serum specimens were diluted 1:10 in 0·85 w/v NaCl in distilled water (physiological saline, PS) and subsequently diluted in PS in a doubling dilution series out to 1:640 using 1 ml volumes. All dilutions were performed with a 1 ml Clay Adams sellectapette in polypropylene test tubes, 12 × 76 mm. To each dilution was added 0·05 ml of Wellcome (stained) *Brucella abortus* antigen (batch K7716-1). Tubes were shaken manually and incubated for 18 h in a 37 °C incubator. As the Wellcome antigen preparation contains 0·25 % formalin and 0·01 % thiomersal and since incubation was only 18 h, it was considered unnecessary to use phenol saline as preservative. The agglutination was determined visually and the lowest dilution showing 50 % agglutination was considered the SAT titre of the serum. All tubes showing partial or no agglutination were centrifuged at 600 ***g*** (MSE Major) for 15 min, the supernatant decanted and 0·5 ml PS added. The pellet was resuspended by mechanical agitation (Whirlimixer, Fisons) and centrifuged again. This procedure was repeated twice. Antihuman globulin reagent (Behring Institut) was diluted according to the manufacturer's instructions and 0·5 ml was added to the final pellet which was resuspended as above. The tubes were incubated at 37 °C for 18 h. The agglutination was determined visually and the lowest dilution giving 50 % agglutination was considered as the AHGT titre. Sera which gave titres greater than 1:640 for either test were retested at higher dilutions so as to obtain a 50 % end point titre.

The microagglutination method

Rigid V bottomed microtitration plates (Cooke Dynatech Corp) were marked off with one row of eight wells assigned to each specimen, allowing 12 specimens to be tested on each plate. A 1:5 dilution of each serum specimen was made by adding 0·1 ml of serum to 0·4 ml of PS in a small test tube. After mixing, 0·05 ml of the 1:5 dilution of serum was added to the first well of each row of the microtitration plate. A subsequent series of doubling dilutions in PS, starting from the first well, was performed by means of an automatic diluter dispenser (Dynatech Corp, Dynatitre). An equal amount (0·05 ml) of the haematoxylin stained brucella antigen solution consisting of 0·4 ml Commonwealth Serum Laboratories (CSL) *Brucella abortus* Milk Ring Test Antigen (haematoxylin stained), 0·5 ml bovine serum albumin solution (10% w/v in distilled water) and 24·1 ml PS was added to each well using the automatic diluter dispenser machine (Dynatitre, Dynatech Corp). The serum dilutions were doubled by the addition of antigen so that the final dilution in the first well of each specimen being tested was 1:20 as in the tube method.

After sealing with tape to prevent evaporation, the contents of the plates were mixed on a horizontal shaker (Microshaker II Dynatech Corp), for 15 s then incubated for 18 h in a 37 °C incubator. The plates were read after removing the tape and placing each plate at 60° inclination from the horizontal for 90 s before scoring the result. The absence of agglutination was indicated by a running button of blue-stained bacteria. Agglutination was indicated by a shield of stained bacteria covering the bottom of the well or by a diminished discrete non-running button of blue-stained bacteria remaining in the centre of the well. The lowest dilution to show an agglutination pattern without a running button being present was considered as the microagglutination equivalent of the SAT titre. The plates were centrifuged at 600 ***g*** for 15 min and the supernatant manually 'flicked' out. Additional washing steps were not described by C. P. Beaton. When three washing steps were performed as in the tube method no difference in result was observed. Therefore the single washing step was maintained. As in the tube method, 0·05 ml of the same antihuman globulin preparation was added to each well. The plates were covered, agitated and incubated as above. The microagglutination AHGT titre was determined as for the microagglutination titre described above.

Controls

The primary control serum for the SAT was bovine serum obtained from the Central Veterinary Laboratory, Weybridge, Surrey, England. This is known as the second W.H.O. International Standard Serum for *Brucella abortus*. It contains 1000 international units of agglutinating activity per ml and has been in use to standardize the SAT throughout the world for many years (W.H.O., 1979). The antibodies of this standard have been shown to consist almost entirely of immuno-globulin G. This serum which is supplied in freeze-dried form was reconstituted and used according to the instructions provided with it. Due to the limited supply of the international standard, it is recommended by W.H.O. for each laboratory to prepare its own standards and to calibrate them against the international standards.

The AHGT is also used to assess antibody levels to *Brucella* in humans. Therefore the only appropriate controls for this procedure are human standard antisera. Twelve human control sera were obtained from patients sent to the laboratory for the determination of *Brucella* antibody levels. They were chosen because they encompass a wide range of titres from negative to high positive in both SAT and AHGT and a sufficient amount was available to use throughout the study as well as being used as routine laboratory standards (Table 4). These standards had also been tested by the microagglutination method in Melbourne by C. P. Beaton and comparable results to ours were obtained.

Table 1. *Distribution of tube SAT and microagglutination titres*

		Tube SAT titre							
		< 20	20	40	80	160	320	640	Total
Microagglutination titre	640								0
	320	3	1	3	2	1			10
	160	11	15	7	4				37
	80	47	27	18	5				97
	40	109	13	2					124
	20	47							47
	< 20	150							150
	Total	367	56	30	11	1	0	0	465

RESULTS

Table 1 gives a comparison of the tube SAT and the same test done by the microagglutination method. This shows that the microagglutination method appears more sensitive. Of the 367 sera giving a negative result by tube SAT, 150 (40·9 %) gave the same result by microagglutination. The remaining 217 (59·1 %) sera which were negative by tube for the SAT were positive by the microagglutination method with 14 (3·8 %) having titres of 1:160 or above. The tube and microagglutination titres agreed within ±one dilution step with 43 (43·8 %) of the 98 sera giving a titre of 1:20 or above by the tube SAT. There were 55 (56·1 %) serum specimens whose microagglutination and tube titres did not agree within ±one dilution step. This disagreement was entirely due to the microagglutination titres being two dilutions or more above the tube titres.

In Table 2 the comparison of the tube and microagglutination AHGT is presented. Again the microagglutination method appears more sensitive but to a lesser extent than shown in Table 1.

Of the 291 sera giving a negative result by the tube AHGT, 117 (40·2 %) gave the same result by the microagglutination method. The remaining 174 (59·8 %) tube AHGT negative sera were positive by microagglutination AHGT, with only one serum giving a titre above 1:160. There were also 174 sera giving a titre of 1:20 or above for the AHGT by both methods, for which agreement within ±one dilution step occurred with 113 (64·9 %). There were 62 (35·6 %) sera whose microagglutination and tube AHGT titres did not agree to within ±one dilution

Table 2. *Distribution of tube AHGT and microagglutination AHGT titres*

Microagglutination AHGT titres	Tube AHGT titres: < 20	20	40	80	160	320	640	1280	2560	Total
2560									1	1
1280										0
640									1	1
320	1	1		4	4	1				11
160		4	15	17	4		1			41
80	29	34	46	22	7	1				139
40	84	6	1							91
20	60	3	1							64
< 20	117									117
Total	291	48	63	43	15	2	1	0	2	465

Table 3. *Comparison of microagglutination titres with the tube AHGT titres*

Microagglutination titres	Tube AHGT titres: < 20	20	40	80	160	320	640	1280	2560	Total
2560										0
1280										0
640										0
320		1		4	3	1			1	10
160	2	3	13	15	3				1	37
80	16	19	33	22	5	1				97
40	81	21	16	2	4		1			124
20	43	3	1							47
< 20	149	1								150
Total	291	48	63	43	15	2	1	0	2	465

Table 4. *Comparison of control sera*

Serum control no.	Tube method: SAT	AHGT	Microagglutination method: Microagg. SAT	Microagg. AHGT
1	40	40	80	80
2	< 20	40	80	80
3	40	40	80	80
4	< 20	< 20	< 20	< 20
5	< 20	< 20	< 20	< 20
6	< 20	< 20	< 20	< 20
7	< 20	2560	640	1280
8	160	640	320	640
9	80	80	160	160
10	40	80	80	80
11	80	160	160	160
12	1280	2560	2560	2560

step. This discrepancy was predominantly due to the microagglutination titres being higher than the tube titres. Only three sera gave microagglutination AHGT titres two dilutions below the tube titres.

A comparison of the four test results showed that of sera which were tube SAT negative but tube AHGT positive, all but one were positive by the microagglutination equivalent of the SAT. These titres and the tube AHGT titres were therefore compared (Table 3).

The reproducibility of the microagglutination method was studied with the sera listed in Table 4 and this showed that allowing for the usually accepted variation of ± one dilution step, this test was 100 % reproducible, both within and between assays. This included monitoring changes in antigen batches over a number of months.

DISCUSSION

In a study of the microagglutination method for *Brucella* SAT by Brown *et al.* 1981, they found for sera with titres below 1:160 by both methods a 98 % agreement to within ± one dilution step. For sera with titres above 1:160 a 72 % agreement was observed. In the study reported here, 235 sera (56 %) with titres below 1:160 showed agreement to within ± one dilution step. Out of 47 sera with titres of 1:160 or greater, five (10·6 %) gave results within ± one dilution. However where a difference of greater than ± one dilution occurred, the titres obtained by the microagglutination method were in the majority of cases (97·4 %), higher than by the tube method.

Of the 367 sera (Table 1) giving a negative result by tube SAT, 291 (Table 2) also gave a negative result by tube AHGT. Of the remaining 76 sera, which were tube SAT negative but AHGT positive, all but one were positive by the microagglutination equivalent of the SAT. This suggested that there may be a correlation between the tube AHGT results and these microagglutination results. This comparison is given in Table 3. Of these 291 sera giving a negative result by tube AHGT, Table 3 shows that 149 were also negative for the SAT equivalent by the microagglutination method and 142 were positive. Only one serum out of the 465 sera studied, which was negative by the tube SAT but gave a titre of 1:20 by the tube AHGT method, would have been missed if the microagglutination equivalent SAT had been the only test used; however it was detected by the microagglutination AHGT. Although there may be discrepancies between the titres obtained by the two tests in both methods, it is noteworthy that the microagglutination methods did not give any false negative results.

However false positive results occurred in 217 of the 367 sera (Table 1) which were negative by tube SAT and in 174 of the 291 sera (Table 2), which were negative by tube SAT and AHGT, although only 15 of these false positive sera gave titres of 1:160 or greater. In conclusion it is considered that as a screening procedure, the microagglutination method is adequate for detecting *Brucella* agglutinating antibody levels of survey populations, since it is demonstrated here that it detects all those sera which by the tube method give a positive SAT or AHGT.

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