A TEST WITH BRUCELLA ANTIGEN AND ANTISERA FOR ASSESSING THE TITRE OF FLUORESCEIN CONJUGATES OF ANTI-HUMAN-GLOBULIN

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FLUORESCEIN conjugates of anti-human-globulin are used in a variety of laboratories for many purposes. Users of these conjugates often wish to explore new fields, where not only is the titre of the conjugate in their particular lighting system unknown but the behaviour of antigen and antibody in the fluorescence test may also be unknown. The titre of the conjugate may vary when it is used in different antigen-antibody systems, even when the same lighting system is employed, but this variation is not usually very great. It is therefore useful to have a simple antigen-antibody system for assessment of the titre of the conjugate in the apparatus of the individual laboratory, before one goes on to use an unknown antigen-antibody system.

Anti-nuclear factor (Johnson, Beutner and Holborow, 1967), various rickettsia and virus systems (Brighton, 1969) and fluorescent treponemal antibody (FTA) – 200 (U.S. Public Health Service, 1964) tests have all been used for preliminary assessment of the anti-human-globulin-conjugate titre because the workers have long experience in these fields. However, not all laboratories have facilities for cutting sections for the anti-nuclear factor test. Virus antigens have no precise shape, and choosing the right time to stain after infection of cells is a problem unless the seed and cells are in constant use. The FTA test is suitable, but the antigen and control sera are difficult to prepare and supply outside venereology laboratories.

Bacterial systems are useful for measurements because the antigen has a precise shape. With bacteria of sufficient size an end-point can be recognised between “maximum” and “no fluorescence” where a brightly fluorescent outline can just be distinguished from a faintly fluorescent centre due to the greater thickness of the wall seen at the edge of the organism. This is the “peripheral staining” of somatic antigens described by Thomason, Cherry and Edwards (1959).

Bacilli without envelope antigens are the most suitable, because antigens such as the K antigens of *Escherichia coli* vary according to growth medium and are easily dispersed into the suspending fluid by heat. Dr C. E. D. Taylor and Mr G. Heimer tell us that they have been successful in assessing anti-rabbit-globulin-conjugates by means of *Shigella sonnei* and Sonne antiserum prepared in rabbits.

A bacterial system for the assessment of anti-human-globulin-conjugate titres is difficult to find owing to the lack of supplies of suitable human anti-

Received 24 Apr. 1969; accepted 1 July 1969.

bacteria sera of high titre and of negative control sera. However, many clinical cases of *Brucella abortus* still occur in the United Kingdom each year (Brodie and Gordon, 1967; Dickie, 1967), and the Standards Laboratory for Serological Reagents collects positive sera to provide a freeze-dried standard serum for control of the antigen in the agglutination test. Organisms of the *Brucella* group have a suitable shape and no envelope antigen. The high incidence of laboratory infection associated with the preparation and use of live cultures of *Br. abortus* prohibits the use of this form of the antigen, but the agglutinable antigens commercially available are satisfactory for the procedure.

Studies have been made in the Standards Laboratory on the use of this *Brucella* system for comparing conjugates and for making preliminary titre assessments. A chessboard or block titration is used similar to the anti-nuclear factor test of Beutner, Holborow and Johnson (1967). It is suggested that laboratories might like to use this test for these purposes under the conditions of lighting and work that they will use for their projected system.

MATERIALS AND METHODS

Chessboard studies of conjugates were made with Standards' *Br. abortus* suspension batch 187, made in November 1966. This antigen had been used extensively in fluorescence work over a period of 2 yr. Comparative studies of antigens were also made with eight suspensions, three made in the Standards Laboratory, three made by Burroughs Wellcome & Co., two made by the Central Veterinary Laboratory, Weybridge, and one from a source outside the United Kingdom. Each antigen was diluted to give an opacity equivalent to 1000×10^6 *Escherichia coli* per ml.

The lyophilised *Br. abortus* antiserum used was one batch of pooled sera from recently diagnosed clinical cases of *Brucella* infection. This is issued by the Standards Laboratory as a positive control serum, with the *Br. abortus* suspension. Six different commercial anti-human-globulin-conjugates were tested and also an experimental conjugate made in this Laboratory.

Test 1: assessment of titre of anti-human-globulin-conjugate

Three circles 3 mm in diameter were etched with a diamond on each slide. A drop of the diluted *Br. abortus* antigen was placed in each circle with a 3-mm loop. The drops were allowed to dry in air, and were then fixed by passing the slides very rapidly through a flame. Doubling dilutions of the human *Brucella* antiserum were made from 1 in 5 to 1 in 80. In the tests for which little conjugate was available the first dilution was 1 in 10. With a complement-fixation micropipette delivering 0.025 ml, a drop of diluted serum was delivered on to each circle. Two slides (bearing six circles) were used for each dilution of serum. The slides were incubated in a moist chamber at 37°C for 30 min.

The slides were removed and the serum rinsed off each with phosphate buffered saline, pH 7.2, before they were placed in a slide tank of the same buffer for 10 min. The buffer was changed at the end of 5 min.

The slides were drained dry. Doubling dilutions of conjugate were made in the buffer from 1 in 4 to 1 in 128. Drops of conjugate were placed on the slides in such a way that each pair of slides treated with the same dilution of serum bore six drops of conjugate at the six different dilutions. The slides were then incubated and washed as before. They were then drained dry, mounted in polafuor B and read immediately.

The fluorescence was scored thus: +++ maximum fluorescence; ++ fluorescence bright enough to distinguish the cell outline (50 per cent. end-point); + definite fluorescence; - autofluorescence only. The lamp used was that described by Lidwell *et al.* (1967).

TABLE I

Assessment of anti-human-globulin-conjugates against Brucella abortus antigen 187

Intensity of fluorescence of smears of <i>Brucella abortus</i> 187 treated first with human <i>Brucella</i> antiserum							
diluted 1 in	and then with fluorescent conjugate						
	labelled	diluted 1 in					
		4	8	16	32	64	128
80 40 20 10 5	A	++ +++ +++ +++ +++	+ ++ +++ +++ +++	+ + ++ ++ ++	- + ++ ++ ++	- + + + +	- - - + -
80 40 20 10 5	B	++ ++ +++ +++ +++	++ ++ +++ +++ +++	++ ++ +++ +++ +++	+ + ++ ++ ++	- + ++ ++ ++	- - + + +
80 40 20 10 5	C	++ +++ +++ +++ +++	++ ++ +++ +++ +++	+ ++ +++ +++ +++	+ + ++ ++ ++	+ + ++ ++ +	- + + + +
80 40 20 10 5	D	++ ++ +++ +++ +++	++ ++ +++ +++ +++	+ ++ ++ ++ ++	+ + + ++ +	+ + + ++ +	+ + + + -
80 40 20 10	E		+ + + ++	- + + ++	- + + +	- + + +	- - - +
80 40 20 10	F	+ + ++ ++	+ + ++ ++	- + + +	- + + +	- - + +	- - - -
80 40 20 10 5	G	++ +++ +++ +++ +++	++ +++ +++ +++ +++	+ ++ +++ +++ +++	+ ++ +++ +++ +++	+ + ++ ++ ++	+ + ++ ++ ++

Test 2: comparison of eight antigens

Further tests were performed with the same staining technique but with a different antigen for each pair of slides. These were treated with the standard human *Brucella* antiserum at 1 in 20 dilution only and subsequently with the range of conjugate from 1 in 4 to 1 in 128 as before.

Readings of single slides were made and recorded without the observer knowing which strength of serum or which antigen was used. The middle spot on the slide was read and recorded first and then the lateral ones. The observer would always know that the three spots were in order of descending potency of conjugate from left to right but not which set of three strengths of conjugate occurred on that slide. On one day the chessboard of the conjugate and the test on the eight antigens were performed together and all read at once, without the reader knowing whether the slide belonged to one or other test until the result was recorded.

TABLE II

Comparison of eight antigens in tests with Brucella standard serum diluted 1 in 20

Antigen no.	Intensity of fluorescence of smears of each antigen treated with human <i>Brucella</i> standard serum diluted 1 in 20 and then with fluorescent anti-human-globulin-conjugate											
	A, diluted 1 in						B, diluted 1 in					
	4	8	16	32	64	128	4	8	16	32	64	128
1	+++	+++	++	+	+	—	+++	+++	++	++	+	+
2	+++	++	++	++	+	—	+++	+++	+++	++	+	+
3	+++	+++	++	++	+	+	+++	+++	+++	++	++	+
4	+++	++	+	+	—	—	+++	+++	+++	++	+	—
5	++	++	+	+	—	—	+++	++	++	++	+	+
5a	...	...	...	...	...	...	...	...	...	...	...	...
6	++	++	+	+	—	—	+++	++	++	+	+	—
7	+++	+++	++	+	+	—	+++	+++	++	++	+	+
8	+++	++	++	++	+	—	+++	+++	+++	++	+	+

Test 3: reproducibility of readings

A reproducibility test, the purpose of which was to assess the accuracy of the reading of the 50 per cent. end-point, was performed independently of the above tests. Smears of *Br. abortus* agglutinable antigen batch 187 were made as in test 1. Two sets of twelve such smears were made on any one day and all treated with the human *Brucella* antiserum at a dilution of 1 in 20. To stain the first set of twelve smears 12 dilutions of conjugates were picked at random from all the dilutions of conjugates used in test 2. For the second set the same conjugates were used, but in an order different from the first. The orders and dilutions were known only to one worker who prepared, stained and coded the smears. The other, who had made all the readings in tests 1 and 2, read and recorded the results. The results were decoded only when the sets were complete. Nine such comparisons were made.

RESULTS

Test 1: assessment of titre of anti-human-globulin-conjugate

In table I the chessboard titration of each conjugate is given; this charts the degree of fluorescence at a given dilution of human *Brucella* antiserum and anti-human-globulin-conjugate. The titre at which the conjugate can be used is assessed from the 50 per cent. end-point, where a reading of ++ forms a vertical column on the chessboard to the right of the last column of maximum fluorescence. The conjugate is then used at twice the strength at this end-point.

It was not possible to examine all conjugates over the complete range and conjugates E and F gave no pattern from which a 50 per cent. end-point could be determined.

No identification of the conjugates is given because both satisfactory and unsatisfactory conjugates were deliberately used to show a range of performance. The conjugate showing the highest titre, G, is an early high-titred but unsatisfactory conjugate made in this Laboratory, with a high fluorescein-protein ratio which was found to give considerable non-specific staining in tissue-culture work. The conjugate showing a very low titre, E, had been a satisfactory conjugate, but had been stored under poor conditions for some months.

Test 2: comparison of antigens

The purpose of test 2 is to compare 8 examples of antigens in common use in the agglutination test to assess their adequacy as antigens in the fluorescence

Intensity of fluorescence of smears of each antigen treated with human <i>Brucella</i> standard serum diluted 1 in 20 and then with fluorescent anti-human-globulin conjugate																	
C, diluted 1 in						F, diluted 1 in						G, diluted 1 in					
4	8	16	32	64	128	4	8	16	32	64	128	4	8	16	32	64	128
+++	+++	+++	++	+	+	++	+	+	+	+	—	+++	+++	+++	++	+	+
+++	+++	+++	++	+	+	+++	+	+	+	+	—	+++	+++	+++	++	+	+
+++	+++	+++	++	++	—	+++	++	+	+	+	—	+++	+++	+++	++	++	+
+++	+++	+++	++	+	—	+++	+	+	+	+	—	+++	+++	+++	++	++	+
+++	++	++	++	+	+	+++	...	...	...	...	...	+++	+++	+++	++	...	...
+++	++	++	...	...	+	+++	+	+	+	+	—	+++	+++	+++	++	+	+
+++	+++	++	++	+	+	+++	+	+	+	+	—	+++	+++	+++	++	+	+
+++	+++	+++	++	+	+	+++	+	+	+	+	—	+++	+++	+++	++	++	+

test. In table II the fluorescence reading is given for each of the antigens 1–8 in a straight-line titration of conjugate, against the human *Brucella* antiserum at 1 in 20 throughout. This dilution was chosen as it gives the upper limit of maximum fluorescence and therefore might best reveal variations in adequacy of antigens. Only very small quantities of conjugates D and E were available, and the antigen comparison was omitted with these. The results indicate that the antigens are similar to each other in potency, with the exception of antigens 5 and 6 which give lower readings. The same pattern of results is given with each different conjugate. These two antigens were made in 1962 and might be expected to have deteriorated slightly. Another antigen made in 1968 from the same source as 5 appears as 5a in tests F and G. It is equivalent in behaviour to the others. No attempt was made to obtain a more recent antigen from the source of no. 6 as this was not likely to be used in the United Kingdom.

Test 3: reproducibility of readings

The purpose of this test was to assess the accuracy of a ++ reading on which the assessment of the conjugate titre is based. In this test the readings are made in complete isolation from each other, which is a more exacting test

of judgement than that in a normal titration when each reading tends to be related to the stronger and weaker readings on either side.

Results of the reproducibility test given in table III showed disagreement of

TABLE III

Test 3: reproducibility of fluorescence readings on comparable smears

Smear group	Reading of intensity of fluorescence of paired smears (A and B) and difference (B-A) in comparison											
	1			2			3			4		
	A	B	(B-A)*	A	B	(B-A)	A	B	(B-A)	A	B	(B-A)
1	+	+	0	++	++	0	++	++	0	++	+	-1
2	+	+	0	+	++	1	+	+	0	+	+	0
3	++	++	0	++	+	0	+++	++	-1	++	++	0
4	+	+	0	+	+	0	+	+	0	-	-	0
5	++	++	0	++	+	-1	++	++	0	+	++	1
6	++	+++	1	++	+	-1	++	++	0	-	-	0
7	++	++	0	+	++	1	++	+	-1	+	+	0
8	+++	++	0	+++	+++	0	+	+	0	+	+	0
9	++	+++	1	++	++	0	++	++	0	++	++	0
10	+	+	0	++	+++	1	++	+++	1	-	-	0
11	++	++	0	++	++	0	++	++	0	+	++	1
12	++	++	0	++	++	0	+++	+++	0	-	-	0

1 unit of intensity of fluorescence (e.g., + instead of ++, or +++ instead of ++) between pairs of readings in from one to seven of the set of twelve pairs with an average of 3.2 disagreements. Disagreement was never more than 1 unit, was as often up as down in the second set of readings, and did not occur more often in any particular position in the series of slides, so that any effect of "getting the eye in" or fatigue was discounted. The readings were assigned a numeral value between 0 and 3. A t-test was done on the difference between replicate readings of 108 smears. No significant difference was found.

DISCUSSION

The chessboard technique described is useful for assessing the likely range of titre of a conjugate, especially when a new lighting or antigen-antibody system is being developed. This test gives no indication of non-specific staining of the conjugate due either to free fluorescein or to conjugated antibodies to human serum proteins other than gamma-globulin.

In order to make studies comparable from time to time and between different laboratories it is evidently desirable to have a stable preparation of a conjugate available to laboratories as a reference reagent. It is expected that a British Research Standard for conjugate will soon become available.

Anti-rabbit-globulin-conjugates can be assessed with the same technique if the human antiserum is replaced with a *Br. abortus* antiserum of known titre made in rabbits. There is no special virtue in the *Brucella* organism except in the availability of the antigen and standard serum. Standards and Burroughs Wellcome "O" agglutinable suspensions of the *Salmonella* group have been studied and shown to be satisfactory for anti-human-globulin-conjugate assessments with high-titred homologous positive human sera. Where a laboratory is

more easily able to obtain antisera of sufficiently high titre this system can be used. These *Salmonella* suspensions have also been used here for the assessment of anti-horse-globulin-conjugate titres. The "provisional standard" *Salmonella typhi* and *Salmonella paratyphi B* antisera made in horses by Felix (1950) were used as antisera in the horse system.

Reading of intensity of fluorescence of paired smears (A and B) and difference (B-A) in comparison														
5			6			7			8			9		
A	B	(B-A)	A	B	(B-A)	A	B	(B-A)	A	B	(B-A)	A	B	(B-A)
+++	+++	0	+++	+++	0	++	+	-1	+	+	0	+++	++	0
+++	++	0	+++	+++	0	++	++	0	++	++	0	+++	+++	0
++	++	0	+++	+++	0	+	++	1	+	+	0	++	++	0
+	+	0	+++	+++	0	+	+	0	++	++	0	++	++	0
++	+	-1	++	++	0	-	+	0	++	++	0	++	++	-1
+++	+++	-1	+++	+++	0	+	+	1	+++	+++	0	+++	+++	-1
++	++	0	++	++	0	++	+++	1	+++	++	-1	+++	++	-1
+++	++	0	++	++	1	+	++	1	++	+	0	++	+	0
+++	+++	0	+++	+++	0	++	+	-1	+++	++	0	+++	++	0
++	++	0	+++	+++	0	+	+	0	+++	++	-1	++	+	0
++	+	0	+++	++	-1	-	-	0	++	+	-1	++	++	0
+	+	0	+++	+++	0	+	++	1	+	+	0	++	++	0

* If reading B is larger than reading A, (B-A) is called 1; if it is less, (B-A) is called -1.

SUMMARY

A simple technique is described in which common laboratory reagents are used to determine the titre of anti-human-globulin-conjugates for use with a given lighting system.

I wish to thank Mrs J. R. Diamond of the Epidemiological Research Laboratory for the statistical analysis and Mrs D. Marriner and Miss Teresa Scott for capable secretarial and technical assistance.

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