Comparison of the indirect fluorescent antibody test with agglutination, complement-fixation, and Coombs tests for *Brucella* antibody

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SYNOPSIS A comparison is made of a *Brucella* fluorescent antibody test with four standard tests on 342 normal, 86 diagnostic, and 41 known positive human sera. Where positive titres of accepted significance were obtained in standard tests, a titre of 1/10 or more was achieved in the fluorescence test.

The Standards Laboratory for Serological Reagents has been concerned with the assessment of antispecies globulin fluorescein conjugates for use in public health laboratories. Among the tests developed for this purpose was one using *Brucella* suspension and positive control serum. Between 1965 and 1968 a large number of sera were collected by the Public Health Laboratory Service Working Party on Brucellosis and 1,500 were to be examined in the Standards Laboratory for the presence of *Brucella* antibody by complement-fixation, agglutination, 2-mercapto-ethanol agglutination, and Coombs tests. The opportunity was taken to apply the indirect fluorescent antibody test to some of these at the same time so that its value could be compared with other tests. In addition a number of convalescent sera were available and these were subjected to all five tests.

This paper reports the procedures and the results obtained and comments on the comparative value of the indirect fluorescent antibody test.

Materials and Methods

SERA

The fluorescent antibody test was applied to 428 of the sera being examined for the Working Party. Of these sera, 342 were paired samples from

171 presumed normal adults living in various areas of England and Wales, and 86 were paired samples from 43 people who were ill and suspected of suffering from brucellosis. One of each pair of samples was collected before, and the other three to four weeks after, a brucellin skin test was performed. Neither of these types of sera was deliberately selected for the fluorescent antibody test.

Sera with an agglutination titre of 1/160 or more, obtained from patients diagnosed as suffering from brucellosis, are sent to the Standards Laboratory from laboratories in England and Wales for the preparation of the positive control serum for the *Brucella* agglutination test. These sera are refrigerated at 4°C until pooled and freeze dried. The five tests were applied to 41 of these convalescent sera which were in store at the time this work was undertaken.

ANTIGEN

The Standards Laboratory *Brucella abortus* concentrated suspension, of an opacity equivalent to $8,000 \times 10^6$ *Escherichia coli* per millilitre, was used for the agglutination tests. The same suspension was diluted 1/60 with veronal buffer for the complement-fixation test and diluted 1/8 with phosphate-buffered saline, pH 7.2, for the fluorescent antibody test.

SEROLOGICAL REAGENTS

For the Coombs test the Coombs reagent (AHG) supplied by Burroughs Wellcome & Company was used at a dilution of 1/16, giving a final dilution of 1/160 in the test. For the fluorescence tests two batches of an antihuman globulin (Caprine) F.I.T.C. conjugate prepared by Baltimore Biological Laboratories were used at a dilution of 1/20. This dilution had been found satisfactory in the fluorescent treponemal antibody-200 test (US PHS, 1964) and in the *Brucella* test developed for assessment of indirect fluorescent conjugates in the Standards Laboratory. The serum used as a control in the fluorescence tests was the current batch of positive control serum for agglutination tests. This serum has an agglutination titre of 1/640. In the fluorescence test it was used at a dilution of 1/10. A negative serum was used at the same dilution for the negative control.

METHODS

All sera were first made up as a master dilution of 1/10 in saline. Further dilutions were made in the diluent appropriate for each test. Tests 1 to 4 were begun on the same day. For convenience the fluorescence test was performed on the original 1/10 dilution on the following day. This dilution was stored at 4°C between tests. Comparative tests showed that no alteration in titre resulted from such storage.

Agglutination test (AGGL)

The technique used is described in Memorandum No. 4 of the Public Health Laboratory Service (1961). The water bath incubation period used was extended to 48 hours. In 1 ml amounts of 0.4% phenol saline doubling dilutions of serum were made beginning with a dilution of 1/20 using 50.8 mm × 12.7 mm (2 in. × ½ in.) tubes. To each tube 0.05 ml of concentrated antigen was added and after shaking the test was incubated in a water bath at 37°C. Readings from +++ to ((±)) were made, taking into account the aggregation of deposit, clearing of supernatant, and the size of the aggregates after agitation of the tube. The titre of the serum was recorded as the last tube showing + or agglutination readily visible to the naked eye after gentle shaking. If the tubes are not shaken this aggregation may be missed, particularly at low dilutions of opaque sera which do not readily show clearing of the supernatant.

2-Mercaptoethanol test (2-ME)

The same technique was used for this agglutination test but the phenol saline diluent was replaced by 0.05 M 2-mercaptoethanol in saline.

Coombs test

For reasons of economy of labour and materials a third agglutination test was not set up for this

test. It therefore varied from the usual Coombs test for brucellosis in that the antigen was weaker and it was performed after 48 rather than 24 hours' incubation. All tubes found negative the standard agglutination test after 48 hours' incubation in a water bath at 37°C were centrifuged and, after discarding the supernatant, the deposit was vigorously resuspended in 0.5 ml saline. This centrifugation and resuspension was repeated twice. A final centrifugation was followed by resuspension in 0.5 ml of 0.4% phenol saline. Then 0.05 ml Coombs reagent was added to each tube. The tubes were incubated at 37°C for 24 hours. The titre was taken as the last tube showing clearing.

Complement-fixation test (CFT)

The technique used was that described by Bradstreet and Taylor (1962). This technique used overnight fixation and a dose of 3 HD₅₀ complement. The microtechnique was employed for some of the tests in which 0.025 ml volume of reagents were used in a WHO Perspex plate covered by a tight-fitting plastic lid to prevent evaporation.

Fluorescent antibody test (FA)

Three well spaced circles, 3.0 mm in diameter were marked with a diamond on clean thin slides. Each circle was filled with antigen from a 3 mm loop. After drying in air these smears were fixed by passing the slide rapidly through a flame. These slides were used for the indirect fluorescent antibody test. Doubling dilutions from 1/10 to 1/320 were made of the human serum to be tested and 0.025 ml of each of the six dilutions was transferred to the six smears on a pair of slides using the complement-fixation test microtechnique dropper. The slides were incubated in a closed moist box at 37°C for 30 min, and then washed vigorously in phosphate-buffered saline at pH 7.2 for 10 minutes. The slides were dried in air and 0.01 ml of a 1/20 dilution of antihuman globulin fluorescein conjugate was applied to each antigen-serum spot and the slides were again incubated in a closed moist box for 30 min at 37°C. They were then washed in the phosphate buffer for 10 min, dried and the smears mounted with polar-fluor B under a long cover slip. Fluorescence was read using the equipment described by Lidwell, Taylor, Clark, and Heimer (1967) and degrees from +++ to negative were recorded. A reading of ++ was given when the fluorescent outer rim of the bacillus could just be distinguished from the non-fluorescent inner body. This was taken as the titre of the human serum. Positive and negative control sera were used with each batch of tests to standardize the readings from day to day. This full fluorescence test was performed only on sera showing + or more with a preliminary screening test using the human serum at 1/10. The technique

for the screening test was otherwise as described above.

Results

These are presented in Tables I to V. Only 18 of the 342 sera from normal people showed a positive fluorescence titre of 1/10. These people came from the Preston, Truro, or Wolverhampton areas where exposure to brucellosis might be expected. Ten of these fluorescence-positive sera were pairs from five individuals obtained before and after a brucellin skin test. The other eight were all the second specimens obtained after a skin test. The complement-fixation and 2-mercaptoethanol agglutination reactions were positive in 14 and 16 respectively of these 18 specimens, and an agglutination or Coombs titre of 1/80 or over was recorded in 10 and 16 respectively (Table I).

Titre	No. of Sera Positive in Each Test at Titre Shown				
	FA	CFT	AGGL	2-ME	Coombs ¹
<i>Results for 18 sera positive by fluorescence test</i>					
1/10	18	—	—	—	—
1/20	3	14	16	16	18
1/40	2	10	15	13	18
1/80	—	5	10	6	16
1/160	—	4	6	3	14
1/320	—	4	3	2	9
1/640	—	1	2	—	4
1/1280	—	—	2	—	2
Total positive from 18 specimens	18	14	16	16	18
<i>Results for 324 sera negative by fluorescence test</i>					
1/20	—	17	71	20	83
1/40	—	—	34	4	55
1/80	—	—	6	—	25
1/160	—	—	2	—	4
1/320	—	—	—	—	2
1/640	—	—	—	—	—
Total positive from 110 specimens	—	17	71	20	83

Table I Results of tests on 342 sera from normal individuals participating in a brucellin skin test survey

¹In the three tests where the Coombs procedure did not produce a positive reaction the titre recorded is the same as the agglutination test result.

Among the 324 specimens with a negative fluorescence reaction, 214 were negative in all other tests; 110 showed a positive in some other test. Most of these were positive only at the lowest titres. Only 13 had a positive reaction in three tests and two in all four (Table I).

Table II shows the results of tests in 86 sera from 43 cases in which the possibility of brucellosis was considered sufficiently likely for a diagnostic brucellin skin test to be performed by the clinician. Thirteen specimens had a positive fluorescence reaction. Ten of these were paired sera from five patients showing clear

Titre	No. of Sera Positive in Each Test at Titre Shown				
	FA	CFT	AGGL	2-ME	Coombs ¹
<i>13 sera positive by fluorescence test</i>					
1/10	13	—	—	—	—
1/20	6	13	13	12	13
1/40	4	8	13	11	13
1/80	3	4	12	6	13
1/160	—	4	8	4	13
1/320	—	4	4	4	11
1/640	—	1	1	1	6
1/1280	—	—	—	—	—
Total positive from 13 sera	13	13	13	12	13
<i>73 sera negative by fluorescence test</i>					
1/20	—	—	12	—	20
1/40	—	—	3	—	8
1/80	—	—	—	—	3
1/160	—	—	—	—	3
1/320	—	—	—	—	—
Total positive from 20 sera	—	—	12	—	20

Table II. Results of tests on 86 sera from patients with a diagnosis of possible brucellosis

¹In two tests where the Coombs procedure did not produce a positive reaction the titre recorded is the same as the agglutination test result.

positive reactions in all the standard and fluorescence tests at the time that the diagnostic skin test was requested. Three patients had a positive fluorescence reaction in the post-brucellin specimen only, and in these patients the standard tests gave an equivocal result in the serum obtained before the skin test. One had an agglutination titre of 1/40 and a Coombs titre of 1/160; the complement-fixation and 2-mercaptoethanol tests were negative at 1/20. The other two sera gave negative reactions except for a Coombs titre of 1/20 and 1/40 respectively.

In Table II, among 73 sera with a negative fluorescence test, 53 were negative in all other tests. No sera gave positive 2-mercaptoethanol or complement-fixation tests. Three sera gave Coombs titres of 1/160 combined with an agglutination titre of 1/20 or 1/40. One of these was the first specimen of a pair, taken before the skin test. The second specimen of the pair, taken after the skin test, appears among the fluorescent-positive sera. The remaining 17 sera gave positive titres of less than 1/80 in Coombs or agglutination tests, seven of them in both tests.

In Table III a full comparison has been made of the results of all the tests on each serum specimen which had been sent to us because it had a positive agglutination titre of 1/160 or more. The first few sera have a very low titre in all tests. It is possible that these had deteriorated during storage. Three of these specimens happened to be a very small quantity. These results are arranged in order of fluorescence titre and are set out in full for each individual serum to allow detailed study.

A small reproducibility test was performed by accident. Specimens 1 to 7 were found, after testing, to be one specimen distributed into seven

Specimen No.	Reciprocals of Titres for Each Test				
	FA	AGGL	2-ME	Coombs ¹	CFT
24	10	40	40	<20	<20
44	10	160	20	160	<20
48	10	40	40	40	<20
39	10	40	20	160	<20
34	10	<20	<20	80	<20
36	10	20	<20	—	<20
54	10	160	40	320	<20
47	10	160	80	160	20
28	10	160	20	640	20
45	10	40	40	320	40
39965	10	80	40	160	40
33	10	80	80	—	40
42	10	2,560	80	5,120	20
25	10	640	20	1,280	40
49	10	160	320	640	160
30	20	640	80	1,280	40
22	20	160	40	640	80
13	20	160	80	320	160
26	20	1,280	40	2,560	160
14	20	160	160	1,280	320
35	20	320	80	640	80
8	40	320	320	2,560	80
31	40	320	80	640	160
40	40	320	160	1,280	320
27	40	160	320	—	640
17	80	320	160	640	80
43	80	1,280	160	5,120	160
41	80	320	320	1,280	320
20	80	320	1,280	>2,560	640
52	80	640	80	1,280	640
38	80	320	320	640	640
11	80	320	640	1,280	1,280
57	80	320	160	2,560	>1,280
23	80	640	640	10,240	>1,280
Taylor	80	2,560	640	—	>1,280
1-7 ²	160	320	640	1,280	640
50	160	640	160	2,560	320
9	160	160	160	1,280	640
51	320	1,280	320	—	160
29	320	640	640	1,280	640
46	320	1,280	2,560	—	1,280

Table III Forty-one sera sent for pooling as standard *Brucella*-positive control serum

¹In three tests where the Coombs procedure did not produce a positive reaction the titre recorded is the same as the agglutination test result.

²This specimen was received in seven different bottles and the contents of each bottle were examined separately. The results are given in detail in Table IV; the titre given in this table is the modal titre from Table IV.

	FA	AGGL	2-ME	Coombs	CFT
1	1/80	1/320	1/640	1/1,280	1/320
2	1/160	1/640	1/640	1/1,280	1/320
3	1/80	1/320	1/320	1/1,280	1/640
4	1/160	1/320	1/640	1/1,280	1/640
5	1/80	1/320	1/640	1/1,280	1/640
6	1/160	1/640	1/320	1/2,560	1/640
7	1/160	1/320	1/320	1/1,280	1/640

Table IV Results on seven samples of a single specimen all tested separately in the belief that they were seven different sera

serum bottles and not, as was thought at the time of the test, one specimen from each of seven different people. Sera 1 to 6 were examined on one day and specimen 7 four weeks later. The results of these tests are in Table IV which shows excellent reproducibility in all tests.

In Table V the results of the four tests on each of the 41 sera appearing in Table III are compared. For each fluorescence titre the titre for each of the other tests has been plotted separately.

Discussion

The comparative value of the conventional tests for the detection of *Brucella* antibody is being considered, using much larger numbers of specimens, by the Public Health Laboratory Service Working Party on Brucellosis and will not be discussed in this paper. The value of the fluorescent antibody test and its relation to the other tests is discussed.

The results in Table III as plotted in Table I suggest that the relationship between fluorescent antibody titre and 2-mercaptoethanol agglutination titre has the best rectilinear fit with the complement-fixation titre a close second. There is a suggestion of a curvilinear relationship between fluorescent antibody titre and complement-fixation titre which is much more marked with the Coombs titres. This may be influenced by the practice of recording very high titres as 'greater than' some titre where no further dilutions were included in the tests and the exact endpoint was not pursued by further tests. The agglutination titre shows the widest scatter and the least suggestion of a close relationship.

The tests usually considered to indicate the IgG level are the complement-fixation test and 2-mercaptoethanol agglutination, whereas standard agglutination and Coombs tests are also influenced by IgM or IgA levels. The results with the convalescent sera suggest that the fluorescent antibody reaction in this series indicates the IgG level. Specimens nos. 25 and 42, and to a lesser extent no. 26, in Table III are of particular interest because the fluorescent antibody, complement-fixation, and 2-mercaptoethanol agglutination titres are all low whereas the standard agglutination and Coombs titres are higher.

The conjugates used in these tests are made for routine diagnostic use and would be expected to contain predominantly antihuman IgG. Conjugates of antihuman globulin sera specific for IgG, IgM, and IgA might be useful for the detailed study of the chronic type of case described by Kerr, Coghlan, Payne, and Robertson (1966), and Kerr, Payne, Robertson, and Coombs (1967), where IgG and IgA antibodies were shown to be present but little IgM.

If a specimen arrives sufficiently early in the day a result can be obtained by the fluorescent antibody test within two hours, but continuous attention is required during this period. Complement-fixation and agglutination tests require less working time. A complement-fixation test result can be obtained in 18 to 24 hours, and a provisional agglutination reading can be made 24 hours although 48 hours are required for the development of the full titre.

It is possible that examination of the patient's serum at a dilution lower than 1/10 would provide a positive result earlier in the disease. It was not possible to assess this point in this series because few of the sera were obtained at an early stage of

Fluorescent Antibody Reciprocals of Titre	Numbers of Sera Positive at Titre								
	< 20	20	40	80	160	320	640	1,280	> 1,280
Complement fixation									
10	7	3	4	—	1	—	—	—	—
20	—	—	1	2	2	1	—	—	—
40	—	—	—	1	1	1	1	—	—
80	—	—	—	1	1	1	3	1	3
160	—	—	—	—	—	1	2	—	—
320	—	—	—	—	1	—	1	1	—
2-Mercaptoethanol agglutination									
10	3	4	4	3	—	1	—	—	—
20	—	—	2	3	1	—	—	—	—
40	—	—	—	1	1	2	—	—	—
80	—	—	—	1	3	2	3	1	—
160	—	—	—	—	2	—	1	—	—
320	—	—	—	—	—	1	1	—	1
Agglutination									
10	1	1	4	2	5	—	1	—	1
20	—	—	—	—	3	1	1	1	—
40	—	—	—	—	1	3	—	—	—
80	—	—	—	—	—	6	2	1	1
160	—	—	—	—	1	1	1	—	—
320	—	—	—	—	—	—	1	2	—
Coombs¹									
10	1	—	—	1	4	2	2	1	1
20	—	—	—	—	—	1	2	2	1
40	—	—	—	—	—	—	1	1	1
80	—	—	—	—	—	—	2	3	4
160	—	—	—	—	—	—	—	2	1
320	—	—	—	—	—	—	—	1	—

Table V Numbers of sera positive in each test at a given titre from Table III charted against the fluorescence titre

¹Six sera were not tested.

the illness and only a master dilution of 1/10 was available for the fluorescence test. The three first specimens of pairs in which the second specimen was positive in Table II might have given a positive result at a dilution below 1/10 but the result might be as difficult to interpret as the positive Coombs tests of 1/20 and 1/40. Similarly a positive fluorescent antibody test at 1/5 might have provided additional positive information in the case in which only an agglutination titre of 1/40 and Coombs titre of 1/160 were present. However, at a serum dilution of 1/5, many more fluorescent antibody tests may also be positive in symptom-free people who have low levels of antibody in the other tests. As shown in the study of farmers and veterinarians by Foley and Corridan (1968), and in the work of Brodie and Gordon (1967) on blood donors in the Orkney Islands, the threshold titre of diagnostic significance is difficult to assess in all tests.

The fluorescent antibody test in this series, when positive, gives a good indication that other tests will be positive. This is in agreement with the results of Biegeleisen, Bradshaw, and Moody (1962). It appears to have no particular advantage over other tests except speed. If, however, large numbers of sera are to be tested in a survey screening by the fluorescent antibody technique might be invaluable. At a dilution of serum of 1/10 no false positives were found. A screening dilution of 1/5 or 1/8 might be worth investigating.

In Table I it will be seen that the final fluorescence titre of 15 out of 18 sera is 1/10 and in the complement-fixation test and 2-mercaptoethanol agglutination tests nine and 10 sera respectively of the same 18 have final titres of 1/20 or 1/40. In Tables II and III it is clear that the titre in the complement-fixation and 2-mercaptoethanol tests is often two- or four-fold above the titre in the fluorescence test at this level. In Table I there are 17 positive complement-fixation tests and 20 2-mercaptoethanol tests positive at 1/20. Among these as many as half might be positive by fluorescence test at 1/5, that is, 10 in 324 or approximately 3% of 'normal' individuals. This gives some indication of the possible false positive rate by screening at 1/5.

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