AN ENZYME-LABELLED IMMUNOSORBENT ASSAY FOR *BRUCELLA ABORTUS* ANTIBODIES

J. T. MAGEE

Bacteriology Department, The Hallamshire Hospital, Glossop Road, Sheffield S10 2JF

CLINICAL diagnosis of human brucellosis is often difficult, especially in chronic and subclinical infections, in which the symptoms may be relatively non-specific. The diagnosis must often be made on the basis of laboratory tests; and, because brucellosis is a possible diagnosis in patients with pyrexia of undetermined origin, the help of the laboratory is often sought.

Brucella abortus is isolated from only a minority of infected patients (Wilson and Merrifield, 1951) and most of these have an acute infection. Most commonly, therefore, the diagnosis is made by serological methods particularly in the chronic form of the disease. Several tests are usually employed in attempts to detect and distinguish acute, chronic and past infections. These methods include the direct and mercaptoethanol agglutination tests, a complement-fixation test and an anti-human-globulin (Coombs) test (Kerr *et al.*, 1968). The results of these tests are not always easy to interpret because none is specific for a single immunoglobulin class, and several tests are required for what is often merely a screening procedure.

Parratt *et al.* (1977) introduced a radioimmunoassay (RIA) to measure *Br. abortus*-specific antibodies of the IgG, IgM or IgA class. This specificity for antibody class simplifies interpretation and the test can demonstrate small amounts of antibody. RIA would be an adequate substitute for the current range of tests but its use is restricted by the availability of facilities for handling and counting ¹²⁵I. A mixed reverse passive antiglobulin haemagglutination technique for brucella serology has been reported by Coombs *et al.* (1978); this, too, is antibody-class specific and may be more suited to a routine diagnostic laboratory than RIA.

Enzyme-labelled immunosorbent assay (ELISA) is comparable in sensitivity to RIA and has been found useful in many areas of serology (Voller, Bartlett and Bidwell, 1978). ELISA techniques for detecting *Br. abortus* antibody in cattle (Saunders *et al.*, 1977) and in rabbit sera (Carlsson, Hurvell and Lindberg, 1976) have been described. In both methods, culture of the organism and extraction of the antigen were necessary. The present investigation was undertaken to study the application of ELISA in brucella serology with a more convenient antigen.

MATERIALS AND METHODS

Sera

A total of 112 sera were tested by ELISA and by established methods; 12 sera were known to contain antibodies and of these three were from patients with acute brucellosis, two from patients with chronic brucellosis, three from patients with past brucella infections, and four gave weak positive results in the conventional tests. The remaining 100 sera were submitted to the laboratory for other studies; 80 were sent for glandular fever and toxoplasmosis tests, mostly from outpatients with chronic symptoms, and 20 were sent for gentamicin assay from patients who were in hospital and receiving a broad range of medication for severe infections caused by gram-negative bacteria.

Received 18 April 1979; accepted 26 June 1979.

ELISA for Br. abortus-specific antibody

The initial stages of the assays for specific IgG, IgM and IgA were identical, and all three assays could be done concurrently on the same microtitration plate.

Adsorption of antigen. A concentrated suspension of killed *Br. abortus* cells (Standards Laboratory, Central Public Health Laboratory, Colindale, London) was diluted 100-fold in a coating buffer (pH 9.6) consisting of Na_2CO_3 1.59 g, NaHCO_3 2.93 g and NaN_3 0.2 g dissolved in 1 litre of sterile distilled water; 0.3 ml portions were distributed in the wells of a microtitration plate (M129 B, Dynatech Ltd). The plates were then covered and left overnight at room temperature, or stored for up to 1 week at 4°C. All reagents were allowed to equilibrate to room temperature before use.

Washing procedure. The wells were emptied and washed three times with 0.3-ml volumes of Dulbecco A Solution (Oxoid) containing 0.05% of Tween 20 (PBST) to remove excess antigen. Each wash was allowed to stand for 3 min., and great care was taken to ensure adequate aspiration of the solution.

Addition of sera. The test sera were diluted 100-fold in PBST + bovine serum albumin 1% w/v, (Cohn Fraction V, Armour) and 0.3 ml of the dilution was put into the antigen-coated wells. Unbound antibody was removed by the standard washing procedure after incubation for 1 h at room temperature.

Assay for specific IgG. Goat anti-human-IgG antibody conjugated with alkaline phosphatase (Don Whitley Scientific) was diluted 500-fold in PBST, 0.3 ml of the dilution was added to the appropriate wells, and the plate was incubated for 1 h at room temperature. Unbound conjugate was removed by the standard washing procedure. Retained enzyme activity was estimated by the addition of 0.3 ml of a solution of p-nitro-phenyl phosphate (1 mg/ml) in diethanolamine buffer (Voller, Bidwell and Bartlett, 1976) to each well and incubation at room temperature for 1 h. The enzyme reaction was stopped by the addition of 0.02 ml of 2M NaOH to each well and gentle shaking of the plate.

Assay for specific IgM. This was done in the same way as the IgG assay but with an IgM-specific alkaline phosphatase conjugate (Don Whitley Scientific).

Assay for specific IgA. Anti-human-IgA/horseradish peroxidase conjugate (Mercia Brocades Ltd) was diluted 500-fold in PBST and 0.3 ml was put into the wells. After incubation for 1 h at room temperature, unbound conjugate was removed by washing and 0.3 ml of substrate was added. The substrate was a modification of the unbuffered solution of Voller *et al.* (1976); it contained 80 mg of 5-amino-salicylic acid (Aldrich Ltd) dissolved in 100 ml of 0.02M phosphate buffer at pH 6.0. Immediately before use 1 μl /ml of 20-volume H_2O_2 was added to the substrate. After incubation for 1 h at room temperature the enzyme reaction was stopped by the addition of 0.02 ml of 2M NaOH.

Spectrophotometric readings

In all three assays the absorption at 410 nm of the well contents was measured in a Cecil CE 393 spectrophotometer equipped with a Micro-drain Cuvette (Hellma Ltd). The spectrophotometer zero was set with an appropriate blank control in which serum was replaced by diluent. The reading was converted to arbitrary units (AU) by multiplying the absorbance by the serum-dilution factor. When the absorbance was greater than 1.00, serial tenfold dilutions of the serum were retested, and the lowest dilution that gave a reading of < 1.00 was used in the calculation for the AU value. Although the same units were used to express the levels of the three immunoglobulin fractions, an AU of IgG cannot be assumed to be equivalent to an AU of IgM or IgA. AU values given are the mean of three separate determinations.

Minor adjustments to the concentration of conjugate were occasionally required to correct for batch-to-batch variations in potency.

Conventional serology

Direct agglutination, complement fixation and Coombs tests were performed as described by

Kerr *et al.* (1968). Sera that gave negative results in ELISA, direct agglutination, and complement-fixation tests were screened by the method of Singh (1969), and only sera positive in this test were screened by the more specific Coombs test (Anderson, 1970).

RESULTS

Of the 112 sera tested, 15 gave positive results by either ELISA or conventional serology. The results obtained with these sera are given in the table. The remaining 97 sera gave non-significant levels of specific IgG by ELISA and negative results by the other methods; all were from patients without clinical evidence of brucellosis.

ELISA for Br. abortus-specific IgG

The control group of 100 sera showed specific IgG levels forming a normal distribution with a mean of 3.7 AU and a standard deviation (SD) of 4.0 AU. Three sera from this group gave aberrant results of > 12.0 AU (mean + 2 SD). These were the sera numbered 10, 14 and 15 in the table. All sera giving positive results in conventional tests showed IgG levels of ≥ 12.0 AU. All sera from patients with active brucellosis gave specific IgG levels ≥ 100 AU. These strongly positive sera often gave a prozone effect at the 10^2 dilution; this was variable for the same serum tested on different occasions and occurred on some occasion with all the strongly positive sera, but never decreased the absorbance at 410 nm to less than 0.80 at the 10^2 dilution.

ELISA for Br. abortus-specific IgM and IgA

A random sample of 25 sera from the control group and the 15 sera that gave positive results for specific IgG were assayed for specific IgM and IgA. The control group gave a mean IgM level of 5.2 AU (SD 3.1 AU). All sera in this group and 12 from the IgG-positive series gave assay results of < 12.0 AU of IgM. These were considered to be not significant and are recorded as NS in the table. Similar results were obtained in the assay for specific IgA. The control group gave a mean level of 2.1 AU of IgA (SD 5.0 AU) and the 12 IgG-positive sera that showed non-significant levels in the test for IgM also gave non-significant levels of IgA (table).

Results from established methods

Only one serum from the control group gave positive results in the conventional tests; this was serum no. 10 in the table.

DISCUSSION

During this study it was possible to examine only a small number of sera from patients whose clinical presentation or occupational history suggested present or past infection with *Brucella abortus*. Nevertheless, the results suggest that the ELISA method may be of significant value in the diagnosis of brucellosis. In fact, no serum gave positive results from established methods and negative results from ELISA, indicating that ELISA would be an adequate substitute for conventional techniques. An analysis of the data in the table by the ranking method of Kendall (1970, cited by Swinscow, 1976) showed strong correlations between the ELISA IgG results and the CFT titre ($t=0.7361$; $p<0.5\%$), and between ELISA IgG and the Coombs-test titre ($t=0.6173$; $p<1\%$). Parratt *et al.* (1977) also noted good correlations between brucella-specific IgG levels measured by RIA and CFT and Coombs-test titres. These correlations were expected because IgG is the reactive component of most positive sera in CFT and the Coombs test.

The results of the present studies indicate that the sera giving positive ELISA for specific IgG

TABLE
Results of serological tests on all sera showing anti-*Brucella abortus* antibody

Serum no.	Clinical details of patient	Results obtained in					ELISA test for		
		direct agglutination test (titre)	complement-fixation test (titre)	Coombs test (titre)	IgG (AU)	IgM (AU)	IgG (AU)	IgM (AU)	IgA (AU)
1	Acute brucellosis	320	512	3280	7200	22	27		
2	Acute brucellosis	5120	2048	80 000	5900	66	38		
3	Acute brucellosis	5120	128	10 240	730	1040	13		
4	Chronic brucellosis in a slaughterhouseman	40	40	1280	740	NS	NS		
5	? Chronic brucellosis in a butcher	160	8	320	240	NS	NS		
6	Brucellosis 2 years previously	40	16	256	72	NS	NS		
7	Livestock owner	<20	<4	320	57	NS	NS		
8	Brucellosis 30 years previously	<20	8	<40	56	NS	NS		
9	Stomach carcinoma	<20	4	<40	49	NS	NS		
10	Farmworker	<20	4	80	42	NS	NS		
11	? Autoimmune disease	80	<4	320	26	NS	NS		
12	Brucellosis 8 years previously	80	16	40	55	NS	NS		
13	Butcher living on farm	80	<4	<40	44	NS	NS		
14	Gram-negative septicaemia	<20	<4	<40	18	NS	NS		
15	Gram-negative septicaemia	<20	<4	<40	18	NS	NS		

AU = Arbitrary units (see *Materials and methods*).

NS = results were not significantly different from the control group ($p \geq 5\%$).

fall into three groups which are illustrated by: (i) sera from three patients with acute brucellosis (nos. 1, 2 and 3) in which high levels (> 500 AU) of specific IgG accompanied by significant amounts of IgM and IgA were detected; (ii) sera from two patients with chronic brucellosis (nos. 4 and 5) in which moderately high levels (> 100 AU) of specific IgG were present, but significant levels of IgM and IgA were not detected; (iii) sera from 10 patients (nos. 6–15) in which 12–100 AU of specific IgG were found; none of these showed significant levels of IgA or IgM, and no patient in this group was diagnosed clinically as suffering from a current *Br. abortus* infection. Two of these sera (nos. 14 and 15) gave extremely low IgG levels in ELISA and antibody was not detected by any other method.

Specific IgG > 100 AU would thus seem to represent current infection although this limit may need to be revised because it is based on a small number of positive sera; specific IgG levels of 12–100 AU probably represent residual antibody. The serum that gave the lowest ELISA IgG level that was considered to be clinically significant (240 AU) was from a butcher suffering from night sweats; paired sera showed a twofold rise in CFT and Coombs-test titres during two weeks. These results were thought to represent either chronic brucellosis or possibly be a result of contact with the S19 vaccine strain of *Br. abortus*, but the high ELISA IgG level is compatible only with very recent, i.e., < 2 years', infection, or active chronic brucellosis.

The presence of specific IgM indicates acute brucellosis and is usually inferred from the direct-agglutination titre supported by a mercaptoethanol agglutination titre. The direct-agglutination test alone is not specific for IgM (Heremans, Vaerman and Vaerman, 1963; Wilkinson, 1966; Parratt *et al.*, 1977). Six sera gave positive agglutination titres and negative ELISA for specific IgM in this study. The detection of specific IgM by a single test is therefore a major advantage of both RIA and ELISA and is relevant to many infectious diseases in which serological tests are important in the diagnosis of primary acute infection. From the present studies it appears that levels of *Br. abortus*-specific IgM of > 15 AU indicate acute infection. The serum that gave the highest IgM level was taken for initial diagnosis of brucellosis, whereas the two sera with lower levels were taken after a diagnosis had been made and treatment started.

IgA may contribute to positive reactions in the direct-agglutination and Coombs tests (Heremans *et al.*, 1963; Wilkinson, 1966; Kerr *et al.*, 1968) but no conventional test is specific for IgA, and its significance in the diagnosis of brucellosis is difficult to ascertain. Kerr *et al.* (1968) suggested that it may be a skin-sensitising immunoglobulin associated with rashes. Wilkinson (1966) noted that the circulatory IgA response was late, prolonged and sustained in human and bovine acute brucellosis, and this was supported by the present study, but there is insufficient evidence to assign any diagnostic significance to the presence of circulating *Br. abortus*-specific IgA at present.

The current investigation shows that ELISA is more sensitive than the conventional methods used in the study of brucellosis, and interpretation of results is improved because the active immunoglobulin classes are identified. Moreover, it is rapid, requires little standardisation of reagents, is not labour-intensive and is suited to mass screening and automation. Chessum and Denmark (1978) commented upon irregularities in antigen binding to microtitration plates. This problem was not encountered with the three batches of plates used in this study, and Denmark and Chessum (1978) commented that more recent batches of plates showed considerable improvement in this respect. Immunoglobulin-class specificity of the conjugates has been assumed to be that stated by the manufacturer, and the results support that assumption. The most difficult technical problem was the presentation of the ELISA results. The expression of a result as a titre seems inappropriate, because each ELISA well yields a quantifiable result, and end points were difficult to read. The use of statistical significance levels, as suggested by Voller *et al.* (1978), encourages accumulation of large control (negative) groups as a firm basis for the recognition of abnormal levels of specific immunoglobulin. In this study statistical limits have been given, but positive sera gave such high levels of IgG that it was impractical to cite probability levels for them. The AU system was a compromise and was not entirely satisfactory but it was more convenient than any other system tried.

SUMMARY

An enzyme-linked immunosorbent assay (ELISA) for *Brucella abortus* antibody was compared with direct agglutination, complement fixation and Coombs tests in studies of 112 sera. Of these, 15 gave positive results by ELISA tests which included the 13 sera that gave positive results in the other tests. The assay aided recognition of four groups: (i) sera with very high levels of specific IgG and significant levels of IgM and IgA from patients with acute brucellosis; (ii) sera with high levels of IgG only from patients with chronic brucellosis; (iii) sera with low levels of IgG only, representing residual antibody; (iv) sera with little or no brucella-specific antibody.

ELISA was antibody-class specific, and the results were more readily interpreted than conventional serological data. Moreover, ELISA was more sensitive, more rapid and simpler than the battery of agglutination, Coombs and complement-fixation tests commonly used.

I am grateful to Dr J. Colquhoun and Dr D. Harris for their encouragement and for helpful discussions and comments, and to Mr P. Booth who performed the conventional serological tests.

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