

Detection of complement fixation by enzyme linked immunosorbant assay (COMPELISA)

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SUMMARY A method is described by which complement fixation is detected with an enzyme linked immunosorbant assay (ELISA) technique. The method obviates the need for sensitised sheep red blood cells as an indicator of complement fixation and the titration of complement is not critical. The dose response curve has the advantage of being steep and the test result is read photometrically. As test serum and complement do not react together serum anticomplementary effects are eliminated. The ELISA complement fixation test (COMPELISA) was more sensitive than the conventional CFT for detecting brucella antibodies.

The complement fixation test (CFT) is widely used for detecting antibodies to many bacterial, viral, and other antigens. A specific amount of complement of known activity is incubated with antigen and a possible source of antibody such as a patient's serum. If an immune reaction takes place some or all of the complement may be fixed on to the antigen-antibody complex. The residual complement is assayed by its ability to haemolyse sensitised sheep red blood cells and the antibody titre can be expressed as that dilution of serum which leaves sufficient complement free to haemolyse 50% of the red cells.

The ELISA CFT (COMPELISA) estimates the amount of complement fixed directly with a specific peroxidase conjugated goat anti-guinea-pig C3 serum. A similar technique, termed CELISA, was described by Tandon *et al*³ in the serological investigation of Chagas' disease.

Material and methods

REAGENTS

Antigens

These are lipopolysaccharides extracted from freeze-dried *Brucella abortus* (Ipswich strain) and *B melitensis* (Bangalore strain) cells by treatment with ethylene-diaminetetra-acetate (EDTA) at 37°C.¹

Complement

Guinea-pig serum preserved by Richardson's method² and freeze-dried (Don Whitley).

Peroxidase conjugated goat anti-guinea-pig C3 serum
This reagent is prepared by Cappel Labs Inc, Cochranville, PA, USA and is supplied by Dynatec Laboratories Ltd, Billingshurst, England.

Substrate

The substrate was prepared by dissolving 40 mg *o*-phenylenediamine in 100 ml phosphate-citrate buffer, pH 5, and adding, immediately before use, 200 µl of 30 vol H₂O₂.

Sera

Human sera were diluted 1/40 in phosphate-buffered saline containing 0.05% Tween 20 (PBST) and inactivated by heating at 56°C for 30 min.

Solid phase support

LINBRO flat-bottomed 96 well microtitration plate (Flow Labs 76-381-04).

METHOD

The COMPELISA utilises the ELISA technique as described by Voller *et al*.⁴ The antigens were diluted in carbonate-bicarbonate coating buffer, pH 9.6, and distributed in 200 µl volumes into the wells of the plate which was then kept at 4°C overnight. The plate was washed three times in PBST with three minute contact between washes.

The test sera were dispensed into the wells in 200 µl volumes. The plate was covered and incubated for one hour at room temperature in a moist chamber. It was then washed as before and 200 µl of guinea-pig complement, suitably diluted in PBST, were distributed into the wells. The plate was incubated at room temperature for one hour and then washed

three times. Anti-guinea-pig C3 conjugate in 200 µl volumes was added to each well and the plate incubated at room temperature for 2 h. After washing, substrate was added to each well in 200 µl volumes and the plate incubated at room temperature in the dark for 30 min. Finally 50 µl of 2 M H₂SO₄ was added to each well and the intensity of the resultant colour was measured in a Dynatech MiniELISA Reader at a wavelength of 490 nm.

Conjugate concentration (Table 1)

A chessboard titration was set up as follows. Preserved guinea-pig serum was diluted 1/8 with distilled water to obtain a 1/10 isotonic solution. Further tenfold dilutions from 1/10² to 1/10⁵ were prepared in coating buffer and a row of eight wells was coated with each dilution. As negative controls two further rows were added, one exposed to buffer only and the other coated with a 1/40 dilution of human serum. After washing, the coated wells were allowed to react with 200 µl volumes of twofold dilutions of conjugate, 1/500 to 1/32 000, and the ELISA completed as described above.

Antigen concentration

The optimum antigen concentration was determined with a chessboard titration against positive serum followed by the addition of excess complement and optimally diluted conjugate. The dilution of antigen found to give maximum absorbance with the highest dilution of serum was considered to be the optimum.

Complement concentration

The optimum concentration of complement was determined with a chessboard titration in which antigen and conjugate were used at their optimum concentrations. The solid phase antigen was exposed to dilutions of positive and negative human serum and, after washing, varying dilutions of complement were added. The weakest concentration of complement giving maximum absorbance with the positive serum and showing no reaction with the negative serum was chosen as the optimum concentration. In practice, although wasteful, an excess of complement can be added and this does not significantly affect the absorbance.

Table 1 Absorbances of anti-guinea-pig C3 conjugate reactions with guinea-pig serum adsorbed on to plastic microtitration plate

Guinea-pig serum. Reciprocal dilutions	Anti-guinea-pig C3 conjugate: reciprocal dilutions							
	500	1000	2000	4000	8000	16 000	32 000	Control
10 ²	101	92	66	62	49	35	23	0
10 ³	145	120	107	86	57	44	22	0
10 ⁴	112	101	86	68	50	37	19	0
10 ⁵	35	18	14	15	9	8	4	0
Neg control	14	3	6	2	1	3	0	0
Human serum control	66	23	23	18	17	11	6	0

Table 2 Comparison of COMPELISA absorbances of two specimens at different times and temperatures of incubation

Reciprocal serum dilution	Serum A		Serum B	
	4°C for 18 h	RT for 1 h	4°C for 18 h	RT for 1 h
40	174	194	196	196
80	98	112	125	190
160	30	10	27	35
320	8	0	5	3
640	0	0	0	0

RT = room temperature.

Time and temperature of incubation (Table 2)

Two combinations of time and temperature of incubation were compared by titrating two positive sera at 4°C for 18 h and at room temperature for one hour.

Controls

All COMPELISA tests must include a negative control for each serum in which complement is replaced with PBST. Each batch of tests should include a control prepared from pooled negative serum and positive serum controls should be serially diluted over a suitable range with 20% differences between dilutions. Sera should initially be screened at a dilution of 1/40 in duplicate and if the absorbance readings differ from their mean by more than 10% of the mean the test should be repeated assuming that at least one of the readings is in the positive range.

Results

The results of the antigen and complement titrations showed their optimum concentrations to be 1/200 and 1/100 respectively. Table 1 contains the results of the conjugate titration. The optimum dilution was taken as 1/1000 because the lower dilution of 1/500, while giving slightly higher absorbances, also showed a threefold increase in absorbance with the human serum negative control. It is important that the anti-guinea-pig C3 conjugate should have minimum reaction with human immunoglobulin, the con-

centration of which, in this negative control, is much higher than would be encountered in a routine positive test where only the human antibrucella immunoglobulin is retained.

Table 2 contains the results of a comparison between two time/temperature combinations. It can be seen that with both sera the differences are small, with higher absorbances at low serum dilution resulting from incubation at room temperature for one hour rather than at 4°C overnight.

Table 3 compares the CFT results of 20 sera with their COMPELISA absorbances. A high correlation is evident though this can be seen more clearly in Table 4. An interesting feature is the large reduction in absorbance which often results from a twofold dilution of serum.

Table 4 shows the distribution of the results of 130 serum specimens, including those contained in Table 3, into specified categories of COMPELISA and

Table 4 Combined distribution of COMPELISA results and CFT results of 130 human sera tested against brucella antigen

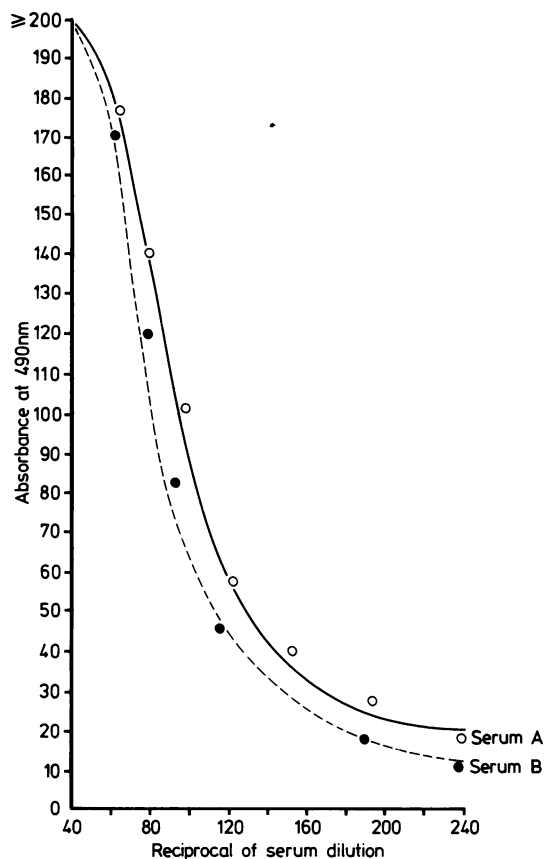
Reciprocal CFT titre	COMPELISA absorbances			
	0-19	20-39	40-100	≥101
< 10	79	1	1	
10	10	2	4	1
20			3	6
40			1	2
80				8
160				6
320				3
640				3

CFT. Of those 81 sera which had a CFT titre < 1/10 one had a COMPELISA absorbance greater than 20 and one had an absorbance >40. Seventeen sera had a CFT titre of 1/10. Four of these had absorbances of 40 to 100 and one had an absorbance of >100. Of the 12 sera which had CFT titres of 1/20 or 1/40, four had

Table 3 Comparison of conventional CFT and COMPELISA: results of 20 sera

Specimen No	Antigen	CFT titre (recip)	COMPELISA Absorbance at a dilution of:				
			1/40	1/80	1/160	1/320	1/640
1	Ab	320	>200	>200	>200	199	65
	Mel	320	>200	>200	>200	145	44
2	Ab	10	0	0	0	0	0
	Mel	> 10	1	0	0	1	0
3	Ab	10	1	0	0	0	0
	Mel	> 10	3	1	1	0	0
4	Ab	> 10	0	0	0	0	0
	Mel	> 10	0	0	0	0	0
5	Ab	> 10	18	4	1	2	0
	Mel	> 10	9	2	1	0	1
6	Ab	> 10	11	4	1	0	0
	Mel	> 10	7	3	2	0	0
7	Ab	80	165	95	26	13	1
	Mel	10	13	3	1	1	0
8	Ab	10	17	6	3	0	0
	Mel	10	10	4	2	1	1
9	Ab	> 10	4	1	0	0	0
	Mel	> 10	4	1	1	0	0
10	Ab	40	97	37	16	6	1
	Mel	20	81	26	7	2	2
11	Ab	> 10	10	2	3	0	0
	Mel	> 10	10	4	4	1	1
12	Ab	160	102	35	5	7	1
	Mel	10	10	4	2	1	0
13	Ab	320	>200	>200	>200	192	68
	Mel	160	>200	>200	>200	156	57
14	Ab	80	>200	155	47	8	1
	Mel	80	17	4	0	0	0
15	Ab	80	>200	128	22	1	0
	Mel	10	6	1	0	0	0
16	Ab	80	>200	182	42	5	1
	Mel	10	29	6	0	0	1
17	Ab	160	>200	175	17	2	0
	Mel	640	>200	>200	>200	199	50
18	Ab	80	>200	55	6	1	0
	Mel	160	>200	>200	114	6	3
19	Ab	80	154	22	2	0	0
	Mel	80	>200	135	16	0	1
20	Ab	2560	198	198	197	188	117
	Mel	640	>200	>200	190	180	62

Ab = abortus.
Mel = melitensis.



Graph of COMPELISA absorbance of serum reaction with brucella antigen

absorbances of 40 to 100 and eight had absorbances of >100.

All 20 sera which had CFT titres >1/80 had COMPELISA absorbances >100.

The Figure shows two graphs generated by plotting the COMPELISA absorbances of serum dilutions prepared at 20% intervals. Features of interest are the near linearity of the graphs between absorbances of 50 and 150 (correlation coefficient = 0.97), and the steepness of the graphs in this region, resulting in large differences in absorbance for small differences in serum dilution.

Discussion

The COMPELISA has several advantages over the conventional CFT. It does not require sheep red blood cells and the titration of complement is not so critical. Comparative tests showed there to be no advantage in incorporating veronal in the complement diluent. The assay can take advantage of

the automation already designed for ELISA and the end-point of the test, a colour reaction, can be quantified photometrically.

As the results in Table 2 show, the time/temperature combinations of 18 hours at 4°C and one hour at room temperature gave similar results and the choice is one of convenience.

Whatever the animal species of the serum being examined the COMPELISA requires only the anti-guinea-pig C3 conjugate. This also applies when human sera are being examined together with control sera which have been raised in, for example, rabbits. In contrast the ELISA needs different and expensive species-specific antiserum conjugates according to the source of the antibody to be detected.

In trying to interpret the shape of the graphs in the Figure it is necessary to take into account the fact that the response of the photometer is not linear over the entire absorbance range of 0–200. However, an absorbance of 100 appears to lie in the centre of the most sensitive linear region of response and if results are to be reported as titres the appropriate dilution would be that which, by interpolation, gives this absorbance.

Screening test sera at a dilution of 1/40 would enable those specimens with results which fall in the normal range, as determined by each laboratory, to be excluded from further investigation. Sera giving a positive result may require further dilution.

In the COMPELISA patient's serum and complement are not combined in the presence of antigen as in the CFT and therefore serum anticomplementary effects are eliminated.

The overall results as shown in Table 4 indicate that the COMPELISA is the more sensitive of the two tests for while none of the sera which had COMPELISA absorbances of 0–19 had CFT titres greater than 1/10, 41% (7/17) of those sera which had CFT titres of 1/10 gave COMPELISA absorbances greater than 19.

References

- Leive L, Shovlin VK, Mergenhan SE. Physical, chemical and immunological properties of lipopolysaccharide released from *E coli* by ethylenediaminetetra-acetate. *J Biol Chem* 1968;**243**:6384–91.
- Richardson GM. The preservation of liquid complement serum. *Lancet* 1941;ii:696.
- Tandon A, Zahner H, Lammler G. CELISA (Complement Enzyme Linked Immunosorbant Assay) a new method for the estimation of complement fixing antibodies: its use for Chagas' disease. *Tropenmed Parasitol* 1979;**30**:189–93.
- Voller A, Bidwell DE, Bartlett A. *The enzyme linked immunosorbant assay (ELISA)*. Micro Systems Ltd, Summerfield House, Vale, Guernsey, 1979.

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