

## The use of EDTA in the microtitration serum agglutination test in bovine brucellosis

A. D. POTTS

Onderstepoort Veterinary Institute, Onderstepoort, 0110 South Africa

### ABSTRACT

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The use of EDTA in the microtitration serum agglutination test reduced the incidence of low-titre agglutinations in sera with titres < 30 international units per ml in the complement fixation test, while not affecting agglutinations in sera with titres  $\geq$  30 international units per ml in the complement fixation test.

### INTRODUCTION

The microtitration serum agglutination test (MSAT) has been shown to be of value as a 2nd screening test (Herr, Te Brugge & Guiney 1982). At the Onderstepoort Veterinary Institute, where the rose bengal test (RBT) is used as a screening test and the complement fixation test (CFT) as the confirmatory test, the MSAT serves as a check on both the RBT and the CFT. Sera delayed in transit tend to give more false negative reactions in the RBT and the MSAT is of value in this situation (Herr *et al.* 1982). The MSAT also functions as a check on human error in both the CFT and RBT.

One of the drawbacks of the MSAT is the occurrence of low-titre [mostly titres of 21 international units (IU) ml<sup>-1</sup>] agglutination reactions. These reactions may not be indicative of infection and can often be regarded as non-specific reactions.

These low titres have a certain nuisance value and result in greater time being spent in reading microtitre plates and recording the results.

EDTA<sup>1</sup> has been shown to be of value in causing a reduction in non-specific titres in the serum agglutination test (Nielsen, Samagh, Speckmann & Stems-horn 1979; Nielsen, Stilwell, Stems-horn & Duncan 1981; Garin, Trap & Gaumont 1985; Nowlan & De Geus 1985; MacMillan & Cockrem 1985). Thus, an EDTA-modified MSAT was carried out on sera submitted to the Onderstepoort Veterinary Institute for routine brucellosis testing to determine the effect of EDTA on low-titre reactions in the MSAT.

### MATERIALS AND METHODS

#### Sera

A total of 1 379 sera received for routine testing by the Onderstepoort Veterinary Institute during the period December 1991 to March 1992 were subjected to an EDTA-modified MSAT. All sera were initially subjected to the RBT, the normal MSAT, and the CFT. The methods for these tests were those of Herr 1982 and Herr *et al.* 1982.

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<sup>1</sup> Ethylenediaminetetra-acetic acid (disodium salt) Na<sub>2</sub>C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>8</sub>·2H<sub>2</sub>O M.W. 372,252

At the Onderstepoort Veterinary Institute, MSAT results are not used to decide on the status of an animal, but all sera with titres of 84 IU ml<sup>-1</sup> or higher are subjected to the CFT to account for possible errors in the RBT.

### Test method

#### SAT in microtitre plates

The method followed was that of Herr, Williamson, Prigge & Van Wyk (1986), modified for use with a hand-held 8-channel pipette and a 96-well dispenser. Briefly, 75 µl of phenol saline diluent was added to all the wells of a U-bottom microtitre plate using a 96-well dispenser<sup>2</sup>. Twenty-five µl volumes of sera were diluted serially from row 1–4 using an 8-channel pipette<sup>3</sup>. This produced serum dilutions of 1/4, 1/16, 1/64 and 1/256. This procedure was repeated for rows 5–8 and rows 9–12 of the microtitre plate. Thus, 24 sera were tested per microtitre plate. Seventy-five µl of antigen<sup>4</sup> was added to all the wells using the 96-well dispenser. The plates were then incubated at 37 °C for 90 min on a shaker. After incubation the plates were centrifuged at 300 g for 1 min and read over a black background with a diffuse light source coming from below and to the side. Titres were recorded in IU ml<sup>-1</sup>.

#### EDTA-modified SAT

EDTA<sup>5</sup> was added to the phenol saline diluent at a concentration of 10 mmol l<sup>-1</sup> and the test carried out as above. In another series of tests EDTA was added to the antigen at a concentration of 10 mmol l<sup>-1</sup> in the diluent (phenol saline) used to dilute the antigen (1/10). Possible differences of adding EDTA to the diluent compared with adding EDTA to the antigen were checked for (N = 355).

The EDTA-modified MSAT was carried out some time after the normal MSAT. To check whether this had any effect on the titre a normal test was carried out at the same time as the EDTA-modified test and compared to the first MSAT (N = 147).

The addition of EDTA results in a decrease in the pH. To check if this lowering of pH had any influence on the results, a comparison was made between tests where the pH of the diluent was adjusted to 7,2 using NaOH and tests where the pH of the diluent was not adjusted (pH = ± 4,3) (N = 266).

<sup>2</sup> 96PW, SLT Labinstruments, A-5082 Grodig, Salzburg, Austria

<sup>3</sup> Dynatech, Daux Rd, Billingshurst, Sussex, England

<sup>4</sup> *Brucella abortus* SAT antigen, Onderstepoort Veterinary Institute, Onderstepoort, 0110 South Africa

<sup>5</sup> Protea Lab Services, Box 5598, Johannesburg, 2000 South Africa

## RESULTS

### Initial tests

The results from the normal test carried out at the same time as the EDTA-modified test did not differ significantly from the results of the first MSAT ( $\chi^2 = 9,3$  P < 0,1 N = 147). Thus, the results of the first MSAT were used in all comparisons.

No significant difference in results was found when EDTA was added to the antigen solution compared with EDTA added to the diluent ( $\chi^2 = 6,6$  P < 0,5 N = 355). Thus, subsequent tests were carried out with EDTA added to the diluent. No significant effect was found as a result of differences in the pH of the diluent ( $\chi^2 = 9,5$  P < 0,1 N = 166).

### Sera with CFT titres < 30 IU ml<sup>-1</sup>

Of the 1 379 sera tested, 1 056 had CFT titres < 30 IU ml<sup>-1</sup>. The incorporation of EDTA into the MSAT caused a significant reduction in titre ( $\chi^2 = 954$  P < 0,001 N = 1056). The biggest reduction was from 21 IU ml<sup>-1</sup> to zero (Table 1). Overall, 61 % (644/1056) of titres were reduced to zero, 19 % (200/1056) showed a reduction in titre, 12 % (126/1056) remained the same and 8 % (86/1056) of titres increased. There was a decrease in titre from ≥ 84 IU ml<sup>-1</sup> to < 84 IU ml<sup>-1</sup> in 8 % (89/1056) of sera and an increase in titre from < 84 IU ml<sup>-1</sup> to ≥ 84 IU ml<sup>-1</sup> in 4 % (43/1056) of sera (Table 1).

### Sera with CFT titres ≥ 30 IU ml<sup>-1</sup>

Of the 1 379 sera tested, 323 sera had CFT titres ≥ 30 IU ml<sup>-1</sup>. The use of EDTA in the MSAT had no significant effect on the level of titre ( $\chi^2 = 3,8$  P < 0,5 N = 323). Twenty-two per cent (72/323) of titres remained the same, 32 % (103/323) of the titres decreased [7 % (23/323) decreased from ≥ 84 IU ml<sup>-1</sup> to < 84 IU ml<sup>-1</sup>], and 46 % (148/323) of the titres increased [5 % (15/323) increased from < 84 IU ml<sup>-1</sup> to ≥ 84 IU ml<sup>-1</sup>] (Table 2).

TABLE 1 Titres obtained in the normal MSAT and the EDTA-modified MSAT in sera with CFT titres < 30 IU ml<sup>-1</sup>

Titre in normal MSAT (IU ml <sup>-1</sup> )	Titre in EDTA-modified MSAT (IU ml <sup>-1</sup> )									
	0	21	54	64	74	84	215	256	338	Total
0										0
21	378	55	13	5	1	7				459
54	138	67	33	10	2	8				258
64	71	27	22	5	2	11	2			140
74	28	16	13	3	4	9	2	3	1	79
84	28	9	10	8	17	26	3	3	2	106
215	1	1		2	3	2	3	2		14
Total	644	175	91	33	29	63	10	8	3	1 056

TABLE 2 Titres obtained in the normal MSAT and the EDTA-modified MSAT in sera with CFT titres  $\geq 30$  IU ml $^{-1}$ 

Titre in normal MSAT (IU ml $^{-1}$ )	Titre in EDTA-modified MSAT (IU ml $^{-1}$ )														
	0	21	54	64	74	84	215	256	297	338	860	1024	1188	1352	Total
0	1		2		2		1								6
21	1			1	1										3
54	4	3	7	2	5	4		1		1					27
64	2	2	5		3	3									15
74	1		3		4	2	1		1	1					13
84	2		3	4	10	14	4	5	2	2		1			47
215	1				1	5	3	6	4	6		1			27
256						2	1	1		2	1				7
297			1			2	2	2	3	3		1		2	16
338					1	4	6	4	3	18	4	4	1	5	50
860								2		1	4	6	3	11	27
1024								1		2	1	1	4	14	23
1188						2				3	2	8	3	25	43
1352											1	4	2	12	19
Total	12	5	21	7	27	38	18	22	13	39	13	26	13	69	323

## DISCUSSION

It is evident from the results obtained that the use of EDTA in the MSAT reduced the incidence of low-titre agglutinations in sera with CFT titres  $< 30$  IU ml $^{-1}$ , while not affecting agglutinations in sera with CFT titres  $\geq 30$  IU ml $^{-1}$ . There were a small number of sera which deviated from this pattern. Twelve percent (126/1056) of sera with CFT titres  $< 30$  IU ml $^{-1}$  were unchanged and it can be speculated that these titres were due to cross-reactions as has been reported elsewhere (Anon 1986). The increase in titre in 8 % (86/1056) of sera with CFT titres  $< 30$  IU ml $^{-1}$  could be due to variations in reagents used; variations in test procedure, or experimental error (volume inaccuracies, etc.). The increase in titre in 46 % (148/323) of sera with CFT titres  $\geq 30$  IU ml $^{-1}$  implies that EDTA *per se* is having an effect, a condition found by others (Garin *et al.* 1985). The reason for this is not known.

At the Onderstepoort Veterinary Institute sera negative in the RBT and with titres of 84 IU ml $^{-1}$  or higher in the MSAT are subjected to the CFT to account for possible errors in the RBT. The fact that some sera are reduced below 84 IU ml $^{-1}$  in the EDTA-modified MSAT may result in sera with CFT titres  $\geq 30$  IU ml $^{-1}$ , but negative in the RBT being missed. However, a survey of all test results for the period December 1991 to March 1992 revealed that sera negative in the RBT and with a titre of 84 IU ml $^{-1}$  or higher in the MSAT accounted for only 0.12 % (121/97 524) of the total. Bearing in mind that 7 % of sera with CFT titres  $\geq 30$  IU ml $^{-1}$  are reduced below 84 IU ml $^{-1}$  in the EDTA-modified MSAT it would mean that only 0.008 % of sera would be affected.

It is difficult to make comparisons with other studies as conditions vary, but MacMillan & Cockrem

(1985) found that in the case of sera with CFT titres  $< 20$  IU ml $^{-1}$ , the titre dropped below 100 IU ml $^{-1}$  in the EDTA-modified test in 64 % of samples. In the present study, applying a threshold of 84 IU ml $^{-1}$  instead of 100 IU ml $^{-1}$ , it was found that 66 % (79/119) of sera with CFT titres  $< 30$  IU ml $^{-1}$  were reduced below 84 IU ml $^{-1}$  in the EDTA-modified MSAT. MacMillan & Cockrem (1985) also found that in the case of sera with CFT titres  $\geq 20$  IU ml $^{-1}$ , 5 % of samples fell below 100 IU ml $^{-1}$  in the EDTA-modified MSAT. In the present study, 7 % (23/323) of sera with CFT titres  $\geq 30$  IU ml $^{-1}$  fell below 84 IU ml $^{-1}$ . Thus, it would seem that the effects of EDTA in the two cases are comparable.

Previous studies suggest that 70–80 % of agglutination reactions in CFT-negative sera are EDTA-labile (Anon 1986). This is comparable to the 61 % (644/1056) found in the present study. The remainder of sera are postulated to contain a mixture of EDTA-labile and EDTA-stable agglutinins (Garin *et al.* 1985).

The simplicity of adding 10 mmol l $^{-1}$  EDTA to the diluent and the benefit of greater specificity in agglutination reactions makes the EDTA-modified MSAT a worthwhile alternative to the normal MSAT in bovine brucellosis diagnostics.

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