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RESEARCH COMMUNICATION

A SHORT, RELIABLE, HIGHLY REPRODUCIBLE COMPLEMENT FIXATION TEST FOR THE SEROLOGICAL DIAGNOSIS OF CONTAGIOUS EQUINE METRITIS

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ABSTRACT

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A complement fixation test, using round-bottomed microtitration plates and an 8 channel microdiluter, based on that used for brucellosis by Herr, Huchzermeyer, Te Brugge, Williamson, Roos & Schiele, 1985, has been developed for use on the sera of horses to detect antibodies to the contagious equine metritis organism. The results with 2 known positive sera tested 116 times in 27 separate tests were reproducible for the most part within a twofold range. They seldom exceeded these limits and never exceeded a fourfold range. The test itself is capable of being carried out within 90 min. The test was slightly more sensitive when sera were inactivated in a hot air oven for 50 min at 58 °C, as compared to inactivation at 62 °C in a water-bath for 50 min. There were no false negative or false positive reactions and no anticomplementary activity in the sera tested.

INTRODUCTION

Various serological tests for contagious equine metritis (CEM) have been developed (Croxton-Smith, Benson & Dawson, 1978; Brewer, 1983; Sahu, Rommel, Fales, Hamdy, Swerczek, Youngquist & Bryans, 1983). Since the complement fixation test (CFT), described by Herr, Huchzermeyer, Te Brugge, Williamson, Roos & Schiele (1985), had proved very successful in various other disease conditions, this technique was investigated for the detection of CEM antibodies.

MATERIALS AND METHODS

Serological procedures

The method used was the same as that described by Herr *et al.* (1985). Known positive, freeze-dried antisera, labelled high titre or medium titre, were kindly supplied by the National Veterinary Services Laboratory (NVSL), Ames, Iowa, USA. The control negative sera were obtained locally from blood samples submitted for dourine testing but which had tested negative for CEM. Inactivation of both types of sera was done in a water-bath, initially for 50 min at 62 °C. This temperature was based on that used by Croxton-Smith *et al.* (1978), who used a temperature of 60 °C for 1 hour in a water-bath. Because *Brucella* and dourine test sera are routinely inactivated at 58 °C for 50 min in a hot air oven (Herr *et al.*, 1985), a series of tests was also carried out, using this inactivation procedure. The antigen was locally produced (as described below) and standardized against the NVSL positive sera.

Interpretation of serological end-points

The CFT end-point reactions were converted to South African units per mℓ (SAU/mℓ) (Table 1). A serum dilution is defined as the dilution factor with veronal buffer only added. This is not the same as the final dilution, in which the dilution factor is calculated after all reagents have been added (Table 1).

Antigen production

A freeze-dried culture[1] of the CEM organism was reconstituted with phosphate buffered saline (pH 6,4). It was plated out onto chocolate agar (Cruickshank, Duguid, Marmion & Swain, 1975), modified, as described below, and incubated for 7 days at 37 °C in air plus 6 to 10 % CO_2. The chocolate agar was prepared by the addition of 45 g of tryptose agar[2] to 900 mℓ of distilled water and autoclaving for 30 min at 121 °C. After cooling to 50 °C, 100 mℓ of defibrinated horse blood was added aseptically. The medium was then placed in a water-bath at 70 °C and stirred aseptically until it assumed a chocolate colour. It was then cooled to 50 °C, when 10 mℓ of Bio-X enrichment[3] was added before it was poured, under sterile conditions, into plates. Colonies were subcultured on the same medium at weekly intervals, until faster growing, larger colonies were obtained. Single colonies were then selected and inoculated onto the same medium in Mason tubes. After incubation at 37 °C in the atmosphere described above, the growth was harvested from the surface by scraping with a sterile, bent, glass Pasteur pipette.

The methods used in the further preparation of the antigen were a modification of standard procedures used for *Brucella abortus* CFT antigen production[4]. The final harvest was suspended in sterile saline (0,85 %) and checked for purity. Formalin was added to a final concentration of 0,2 % and vigorously stirred overnight. It was then left at room temperature for 3 days and finally checked for sterility. The product was passed through a coarse, sterile, plastic type air filter[5] to remove lumps and agar residue. The antigen was finally washed 3 times on a minitan[6] tangential flow filtration apparatus with 0,45 micron filters, using saline (0,85 %) to replace the formalinized saline. Finally, thiomersal[7] was added to a final concentration of 0,01 %.

The antigen was standardized by means of a chequerboard titration with the known positive sera. The dilution of antigen that gave the highest antibody titre was used in the test proper.

RESULTS

There were no anticomplementary reactions in any of the tests when either of the 58 °C or 62 °C inactivation procedures was used. The negative control serum always gave a negative result. The results obtained with the NVSL high titre sera, inactivated in a water-bath at 62 °C for 50 min, are shown in Fig. 1. In all cases the antibody titres fell within a 2,4-fold range (120–290 SAU/mℓ), and 98 % of the results within a twofold range (120–240 SAU/mℓ). The antibody titre most commonly seen was 172 SAU/mℓ (Fig. 1). The 2nd set of results (also shown in Fig. 1) were obtained when NVSL high titre serum

[1] Kindly supplied by J. G. Atherton Esq., Equine Research Station, P.O. Box 5, Balaton Lodge, Snailwell Rd., Newmarket, UK

[2] Biolab, 4 Bernard St., Colbyn, Pretoria 0083, RSA

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[3] Scott Laboratories, Fiskeville, Rhode Island 02823, USA

[4] Central Veterinary Laboratory, Weybridge, England, working document

[5] Serial 800/15, Filtamark, P.O. Box 27145, Benrose 2011, RSA

[6] Millipore Corporation, Bedford, Massachusetts 01730, USA

[7] BDH Chemicals Ltd, Poole, England

TABLE 1 The conversion of CFT end-point reactions to SAU/mℓ on a scale where 50 % haemolysis in a 1/220 serum dilution is equivalent to 1 000 SAU/mℓ

Serum dilution[a]	Final dilution[b]	End-point reading		SAU/mℓ [c]
		% haemolysis	Reaction	
1/4	1/20	75	1–+	15
		50	2–++	18
		25	3–+++	21
		0	4–++++	24
1/8	1/40	75	1–+	30
		50	2–++	36
		25	3–+++	43
		0	4–++++	49
1/16	1/80	75	1–+	60
		50	2–++	72
		25	3–+++	86
		0	4–++++	98
1/32	1/160	75	1–+	120
		50	2–++	145
		25	3–+++	172
		0	4–++++	196
1/64	1/320	75	1–+	240
		50	2–++	290
		25	3–+++	344
		0	4–++++	392
1/128	1/640	75	1–+	480
		50	2–++	581
		25	3–+++	688
		0	4–++++	784
1/220	1/1100	50	2–++	1000

[a] Serum dilution = dilution factor with veronal buffer only
[b] Final dilution = final dilution factor after all reagents are added
[c] SAU/mℓ = South African Units per millilitre

was used as well, but employing our routine method of inactivation in a hot air oven at 58 °C for 50 min. In this case, 344 SAU/mℓ was the antibody titre most commonly seen, and all the results fell within a twofold distribution (240–480 SAU/mℓ).

The results in Fig. 2 were obtained with NVSL medium titre sera, (a) inactivated in a water-bath for 50 min at 62 °C, and (b) inactivated for 50 min in a hot air oven at 58 °C. The medium titre sera showed the same titres as the high titre sera for each respective temperature range. From Fig. 2 it is clear that all the results fell within the twofold range for sera inactivated at 62 °C. Results for the medium titre sera, inactivated at 58 °C, showed 71 % within a twofold range (240–480 SAU/mℓ) and the rest within a fourfold range (172–688 SAU/mℓ).

To summarize, an average of 99 % of all the results fell within a twofold range at the 62 °C inactivation temperature, and 85,5 % of the results fell within a twofold range at the 58 °C inactivation temperature.

Discussion

It is clear from the results that, with the method described, the test is capable of repeatedly detecting antibody to contagious equine metritis organisms. Furthermore, since there were no false negative or false positive reactions, the method is an accurate and reliable test. At no stage were the results outside a fourfold dilution of each other for a particular serum, and the majority of the results fell within a twofold range of each other. This compares favourably with results reported for other disease conditions when the same test was used (Herr *et al.*, 1985).

The fact that inactivation of the sera at the lower temperature of 58 °C produced slightly higher titres suggests that the test was more sensitive when the sera were inactivated at 58 °C than at 62 °C. This could be explained in

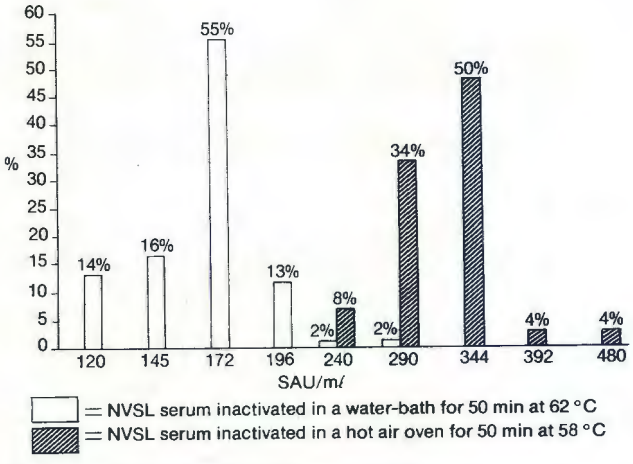

FIG. 1 The effect of inactivation procedure on the distribution of CFT titres for high titre sera

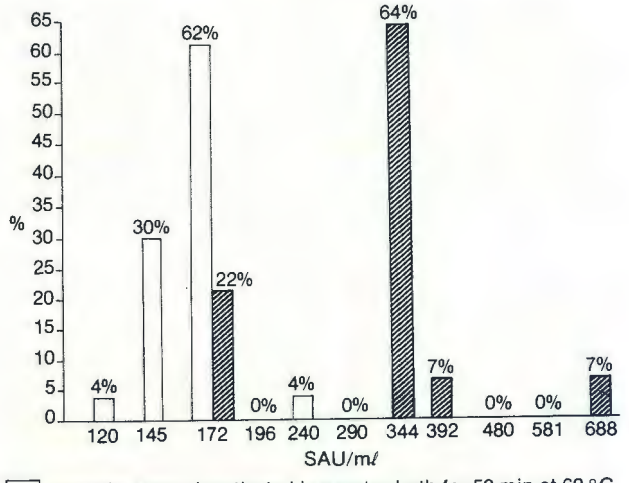

FIG. 2 The effect of inactivation procedure on the distribution of CFT titres for medium titre sera

the light of the work of Rice & Boyes (1970), who showed that cows, shedding *Brucella abortus* organisms in their milk, had a certain amount of heat-labile agglutinins in their sera which were destroyed when heated to 60 or 65 °C. Supporting this conclusion, Allan, Chappel, Williamson & McNaught (1976) found that, although Ig M is stable at 60 °C in buffer, it appeared to be partially labile in serum when heated to 60 °C. They also found that Ig M probably fixed complement twice as efficiently as Ig G_1 on a mass basis, which, on a molar basis, would make it about ten times as efficient. This implies that a small amount of Ig M could make a significant difference to the end result of the CFT. On the basis of these findings, the difference in sensitivity found between the 2 sets of sera done at different temperatures could be explained by the fact that a certain amount of Ig M, which may be present in the test sera in small quantities, is broken down at the higher temperature of 62 °C. This means that less complement will be bound as a result of there being less Ig M at the higher temperature, and consequently the test is less sensitive at the higher temperature range.

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