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A SINGLE DILUTION ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE QUANTITATIVE DETECTION OF ANTIBODIES TO AFRICAN HORSESICKNESS VIRUS

R. WILLIAMS, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

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A single dilution enzyme-linked immunosorbent assay (ELISA), utilizing a reference serum with a known, predetermined end-titre was used to compute antibody titres of serum samples against African horsesickness virus. Compared to the complement fixation test it was found to be highly reproducible and sensitive. The assay also reduced the effect of between-test variations on test results and proved superior to an existing single-dilution ELISA method.

INTRODUCTION

The enzyme-linked imunosorbent assay (ELISA) has already been shown to have great potential as a quantitative serological tool in the detection of antibodies against several virus infections (Blomberg, Nilsson & Anderson, 1983). It has been proved that the sensitivity and specificity of the ELISA are superior to those of the complement fixation test (CFT) (Collins, Bulla, Riegel & Butcher, 1984; Hübschle, Lorenz & Matheka, 1981), which is commonly used as the routine quantitative serological test for African horsesickness virus (AHSV).

In this study, the reproducibility of these 2 assays was compared by repeatedly testing a series of consecutive weekly serum samples from a horse experimentally infected with AHSV and then calculating the standard deviation of each sample for both the assays. To evaluate the sensitivity of the assays, a series of consecutive daily samples from the same horse was tested to determine whether the assays were able to discriminate between small daily increases or decreases in the antibody titre.

According to Benjamin (1979), there is a number of problems associated with ELISA that prevents realistic comparison of test results from different laboratories. These include variable antigen preparations, difficulties in absorbing standard amounts of antigen to the solid phase and leaching off of antigen during the test procedure, all these differences leading to between-test variations. Consequently, a new single dilution, end-titre method, the reference serum (RS) method, was used in an attempt to minimize the effect of between-test variations on ELISA titres.

To evaluate this method, it was first compared to an existing single dilution ELISA method by using the same raw optical density (OD) data of the first trial to calculate the end-titres. For this, the double regression (DR) method (Snyder, Marquardt & Russek, 1983), which appears to be superior to other existing single dilution methods, was used. Secondly, 2 tests were performed on the series of weekly samples in which 50 % and 200 % of the standard antigen concentration, respectively, were used. The results were then compared with those of the standard assay to determine whether the RS method was able to neutralize the variations.

MATERIALS AND METHODS

Production and purification of ELISA antigen

Virulent AHSV type 1 was used to infect monolayers of VERO cells* in roller bottles at a multiplicity of infection of 2×10^6 plaque-forming units (PFU). Once a cytopathic effect of 80 % was achieved, usually 4 days after infection, the cells and medium were collected and purified according to a Triton/Sephadex method (Huismans, Van der Walt, Cloete & Erasmus, 1986). The purified virus antigen was transferred to glass ampoules in volumes of 0,5 mℓ each, freeze-dried, and stored at 4 °C until use.

Antisera

Three horses, all susceptible to AHSV and kept in insect-free stables, were experimentally infected with AHSV type 1. Two horses died within 8 days after infection. Seven days post-infection, the 3rd horse developed a temperature reaction which lasted for 7 days, after which the horse recovered completely. Serum samples of this horse were collected daily for 30 days and then weekly up to 12 weeks post-infection.

A known, positive horse serum against AHSV was used as the reference serum, while the negative prebled sera of all 3 horses were pooled to serve as the negative control.

ELISA procedure

The procedure was based on a combination of methods by Hübschle *et al.* (1981) and Voller, Bidwell & Bartlett (1976). The signal/noise ratio was used as a criterion to determine the optimum conditions for each variable by using positive and negative sera in cross titrations.

Purified AHSV antigen was diluted in TEN buffer (pH 9,6) to a concentration of approximately 300 μg protein per mℓ. Purified bluetongue virus antigen, prepared in exactly the same manner as the AHSV antigen and diluted to the same concentration, served as a negative control antigen. A 96-well, flat-bottomed, microtitration plate* was coated with 100 $\mu\ell$ per well of the diluted antigen and left overnight at 4 °C. The following day the plate was washed 3 times with a phosphate buffer solution, consisting of 50 mℓ of a 0,5 M phosphate buffer, diluted in 5 000 mℓ of distilled water with 1 % Tween 20 added to it, and kept at 37 °C (pH minimum 7,2).

After drying, the plate was blocked by 100 $\mu\ell$ per well of a blocking solution, consisting of 10 % normal bovine serum (NBS), 1 % Tween 20 and 1 % casein hydrolysate in phosphate buffer saline (PBS).

After incubation for 1 h at 37 °C the plate was washed once and tapped dry. The serum samples as well as the positive reference and negative control sera were diluted 1:100 in the same blocking solution as before, and 100 $\mu\ell$ was added to each well.

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* Flow Laboratories, P.O. Box 17, Second Avenue, Industrial Estate, Irvine, Ayrshire KA12 8NB

* Linbro/Titertek, Flow Laboratories, Hamden, Connecticut, USA

TABLE 1 Summary of end-titre results of the CF, RS-ELISA and DR-ELISA tests, each performed 5 times on a series of consecutive weekly sera from a horse infected with AHSV

Weeks after infection	Test No.					Average	Standard deviation
	1	2	3	4	5		
CF titre (reciprocal)							
0	0	0	0	0	0	0	— (—)*
1	16	16	8	8	6	11	5 (45%)
2	32	24	32	24	24	27	4 (16%)
3	2 048	1 024	2 048	1 024	718	1 372	629 (46%)
4	2 048	1 536	2 048	1 536	1 536	1 741	280 (16%)
5	3 072	4 096	3 072	3 072	3 072	3 277	458 (14%)
6	1 024	1 536	1 024	968	718	1 054	298 (28%)
7	192	256	192	256	128	205	54 (26%)
8	64	64	96	128	48	80	32 (40%)
9	48	32	64	64	32	48	16 (33%)
10	24	24	48	32	32	32	10 (31%)
11	16	12	24	32	32	23	9 (39%)
12	12	12	24	32	16	19	9 (45%)
							Mean: 30%
RS-ELISA titre (reciprocal)							
0	0	0	0	0	0	0	0 (—)*
1	0	0	0	0	0	0	0 (—)
2	1 420	1 090	1 330	970	780	1 118	261 (23%)
3	7 940	7 740	7 030	9 030	7 190	7 786	791 (10%)
4	13 230	13 270	12 470	14 210	12 070	13 050	825 (6%)
5	13 570	12 840	13 600	15 720	14 000	13 946	1 076 (8%)
6	15 290	14 760	15 060	16 280	15 190	15 316	575 (4%)
7	15 200	14 830	14 800	15 170	14 150	14 830	423 (3%)
8	14 920	14 300	13 930	13 100	14 590	14 168	700 (5%)
9	14 060	13 500	13 070	12 830	13 110	13 314	481 (4%)
10	13 170	12 840	12 270	12 140	13 560	12 796	598 (5%)
11	12 860	12 580	11 350	12 970	12 670	12 486	653 (5%)
12	13 480	12 140	12 410	12 000	12 740	12 554	590 (5%)
							Mean: 7%
DR-ELISA titre (reciprocal)							
0	0	0	0	0	0	0	0 (—)*
1	0	0	0	0	0	0	0 (—)
2	130	60	40	20	10	52	48 (92%)
3	7 140	5 690	2 020	3 300	1 650	3 960	2 379 (60%)
4	23 300	19 820	7 620	9 420	5 480	13 128	7 919 (60%)
5	24 700	18 360	9 320	11 920	7 720	14 404	7 042 (49%)
6	32 590	25 360	11 800	12 910	9 320	18 396	10 074 (55%)
7	32 130	25 620	11 320	10 970	7 910	17 590	10 639 (60%)
8	30 790	23 550	9 850	7 810	8 500	16 100	10 450 (65%)
9	26 830	20 630	8 500	7 440	6 630	14 006	9 167 (65%)
10	23 050	18 360	7 340	6 540	7 160	12 490	7 686 (62%)
11	21 820	17 500	6 120	7 620	6 120	11 836	7 329 (62%)
12	24 320	16 970	7 530	6 370	6 200	12 278	8 084 (66%)
							Mean: 63%

* Standard deviation as % of average titre

After 1 h incubation at 37 °C, the plate was washed 3 times as before and tapped dry. A peroxidase-conjugated rabbit anti-horse IgG* (heavy and light chain specific) was diluted 1:3 000 in the same blocking solution as before, and 50 $\mu\ell$ was added to each well. After another incubation period of 1 h at 37 °C, the plate was washed 3 times and tapped dry.

O-phenylene diamine-2HCl (OPD) was used as a substrate in a concentration of 1 mg per mℓ of substrate buffer, consisting of 7,74 g of citric acid, 17,3 g of sodium dihydrogen phosphate and 1 litre of distilled water (pH 5,5–6,0). This was freshly made up with 0,8 $\mu\ell$ of hydrogen-peroxide per mℓ buffer added just before use. One hundred $\mu\ell$ of this substrate solution was added to each well. After 40 min, the reaction was stopped with 10 $\mu\ell$ per well of a 2 N sulphuric acid solution.

A "Bio-Tek EL 307C" microplate reader** was used to photospectrometrically determine the OD at a wavelength of 405 nm.

ELISA titre

The RS method for the transformation of absorbance values into a single figure representing the antibody titre depends on using a reference serum with a known, predetermined end-titre to calculate the titres of test samples on a proportional basis. The reference serum was serially diluted and repeatedly tested with the ELISA to determine an average end-titre. This was ultimately established as 1:14 000. Using this as the standard of reference, end-titres of all samples in a specific test were calculated according to the following formula:

[(OD serum sample − OD neg. control)/(OD reference serum − OD neg. control)] × 14 000

* Cappel Laboratories, Cooper Diagnostics, P.O. Box 37, West Chester, P.A. 19380, USA

** Bio-Tek Instruments, Highland Park, Box 998, Winooski, VT 05404-0995, USA

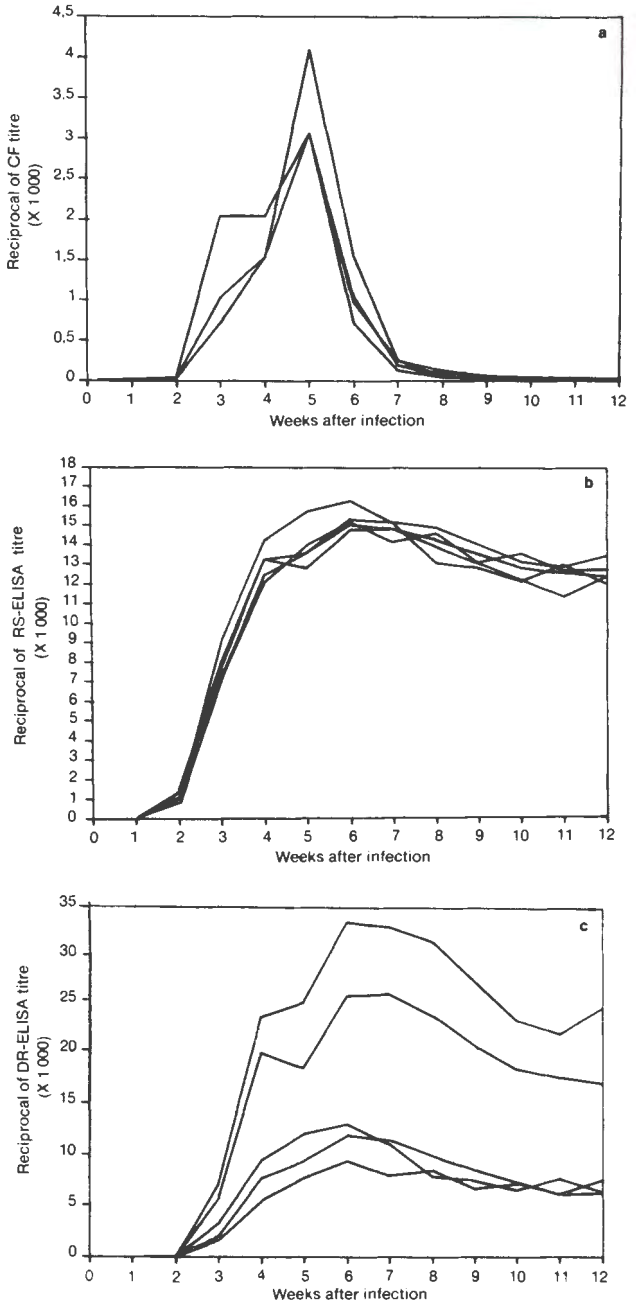

FIG. 1 End-titre results of the CF (a), RS-ELISA (b) and DR-ELISA (c) tests, each performed 5 times on a series of consecutive weekly sera from a horse infected with AHSV

This implies that, irrespective of the OD value of the reference serum, the end-titre of this serum will always remain at 1:14 000, and the end-titres of the serum samples will be fixed at a level directly proportional to that of the reference serum, based on the ratio between their corrected OD values.

The calculation of end-titres was done by microcomputer, using a "Lotus 1-2-3" programme.

Complement fixation test

A 6 volume CF test, comprising 1 volume of serum, 1 volume of antigen, 2 volumes of complement and 2 volumes of sensitized sheep red blood cells, was used. The antigen, complement and haemolysin were diluted to contain 2 units each. The sucrose-acetone extracted antigen was prepared according to the method of Clarke & Casals (1958).

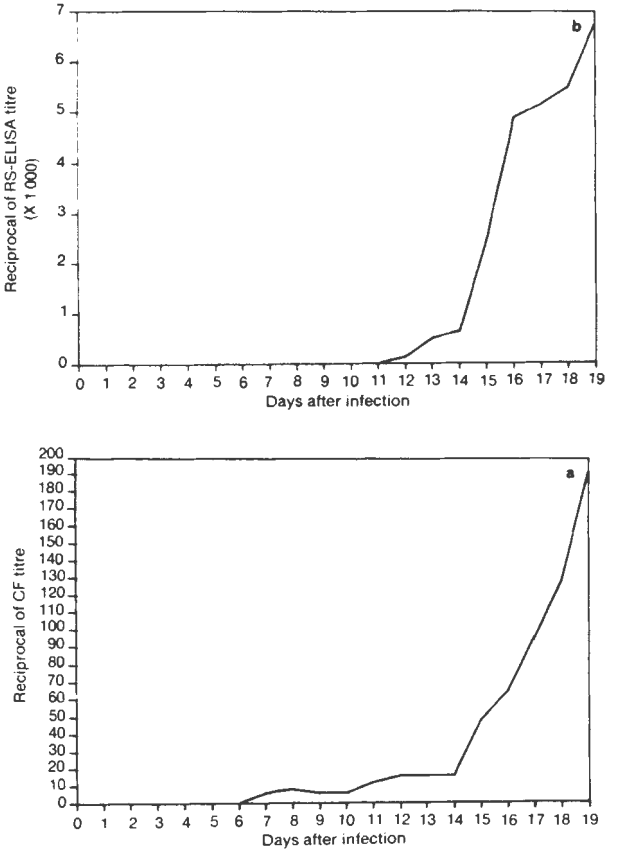

FIG. 2 End-titre results of the CF (a) and RS-ELISA (b) tests, performed on a series of consecutive daily sera from a horse infected with AHSV

TABLE 2 End-titre results of the CF and RS-ELISA tests performed on a series of consecutive daily sera from a horse infected with AHSV

Days after infection	CF titre (reciprocal)	RS-ELISA titre (reciprocal)
0	—	—
1	—	—
2	—	—
3	—	—
4	—	—
5	—	—
6	—	—
7	6	—
8	8	—
9	6	—
10	6	—
11	12	—
12	16	140
13	16	490
14	16	640
15	48	2 470
16	64	4 870
17	96	5 160
18	128	5 510
19	192	6 780

RESULTS

The end-titre results of the CF and ELISA tests, performed in 5 separate trials on different days on 13 consecutive weekly serum samples of a horse infected with AHSV, are graphically presented in Fig. 1. The same OD data of the ELISA test were used to calculate the end-titres by both the reference serum (RS) and the double regression (DR) methods.

Substantial differences that were found in corresponding OD values in the 5 different ELISA trails (data not shown) confirmed the relatively poor between-test

reproducibility of the ELISA mentioned by Benjamin (1979). These differences were reflected by the DR-ELISA (Fig. 1c) but not by the RS-ELISA (Fig. 1b).

Table 1 is a summary of the same test results with the standard deviation of the end-titres calculated for each sample. For a proper comparison of the repeatability of the different assays, it was necessary to express the standard deviation as a percentage of the average titre. When comparing the CF and RS-ELISA on this basis, it is conspicuous that in both assays the higher titres tend to deviate less than the lower ones, thus favouring the RS-ELISA, while the CF test was further adversely effected by the fact that two-fold dilutions were used.

The CF and ELISA test results for the series of consecutive daily serum samples from Day 0– Day 19 post-infection are shown in Table 2 and graphically displayed in Fig. 2. Using the CF test, antibodies could be detected as soon as 7 days after infection, while the ELISA showed the first positive reaction only after 12 days. Both assays showed up a daily rising antibody titre, although the CF test did not do so at the low titre levels.

Table 3 presents the end-titre results of 3 RS-ELISA tests with different antigen concentrations, i.e. standard, 50 % and 200 % of the standard. In the case of the latter 2 tests, the deviation from the standard was expressed as a percentage of the standard with the greatest deviation again occurring at the lower titres.

TABLE 3 Comparison of end-titre results of three RS-ELISA tests with different antigen concentrations, performed on a series of consecutive weekly sera from a horse infected with AHSV.

Weeks after infection	RS-ELISA titre (reciprocal)		
	Standard [Ag]	50% [Ag]	200% [Ag]
0	0	0 (—)*	0 (—)*
1	0	0 (—)	0 (—)
2	1 118	2 450 (121%)	1 910 (71%)
3	7 786	2 750 (65%)	2 450 (68%)
4	13 050	15 560 (19%)	14 820 (14%)
5	13 946	16 150 (16%)	16 910 (21%)
6	15 316	16 510 (8%)	17 910 (17%)
7	14 830	16 270 (10%)	18 000 (21%)
8	14 168	15 970 (13%)	16 730 (18%)
9	13 314	15 320 (15%)	16 090 (21%)
10	12 796	14 900 (16%)	13 820 (8%)
11	12 486	13 820 (11%)	14 090 (13%)
12	12 554	13 460 (6%)	13 640 (8%)
	Range ...	6–121%	8–71%
	Average ...	27%	25%

* % deviation from the standard

DISCUSSION

The purpose of this study was to establish if the ELISA could be used as a reliable alternative to or in conjunction with the CF test for the detection and quantification of antibodies to AHSV. Although the repeatability of the actual OD values was rather poor (data not shown), the RS-ELISA test was able to eliminate these variations to a large extent (Fig. 1b) and ultimately produce test results that compared more than favourably with the CF test (Table 1).

It is important to note the high ELISA titres after 12 weeks in comparison with the very low CF titres in the same period. This points to the main difference between the 2 assays and indicates a specific need which ELISA could fulfil in a serological profile for AHSV.

Antibodies could be detected by the CF test 5 days before the ELISA (Table 2). This is ascribed to the fact that, because of the use of anti-IgG conjugate, the ELISA measures only IgG as opposed to the CF test which measures both IgG and IgM, the latter showing up a few days earlier. It would therefore be unrealistic to compare the sensitivity of the 2 assays on this basis, but the fact that the ELISA registered small daily changes in the antibody titres is an indication that its sensitivity is in no way inferior to that of the CF test.

The fact that the RS-ELISA was able to accommodate a significant variation in the antigen concentration by yielding test results within reasonably acceptable limits (Table 3), suggests that it was relatively unaffected by between-test variations. This is supported by the high reproducibility obtained for the RS-ELISA, in spite of significant variations in the OD values, as opposed to the DR-ELISA which was adversely affected by the same variations (Table 1). Before the assay could be considered insensitive to between-test variations, it would be necessary, however, to evaluate all other test-variables in the same manner. Once it was confirmed, comparison of test results between different laboratories with anticipated differences in test procedures could prove to be feasible with the RS-ELISA, provided all laboratories use the same reference serum with the same end-titre.

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