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SENSITIVITY AND ERROR LIMITS OF TWO TESTS FOR DISTEMPER VIRUS NEUTRALIZING ANTIBODY: A CONVENTIONAL NEUTRALIZATION TEST AND THE "SINGLE DILUTION-PROBIT TEST"

By

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Tests for detection of distemper virus (DV) neutralizing antibody have often been conducted under varying conditions (7). The interpretation and comparison of the results of these tests is difficult. Furthermore, the sensitivity and error limits of the different test procedures have not been systematically studied. In a previous study (11), we applied "the single dilution-probit test" to the estimation of the minimal DV dose required to confer immunity in ferrets and mink. The purpose of the present investigations was to compare the sensitivity and error limits of a "conventional" neutralization test, presently employed in many laboratories, with the same parameters of the single dilution test.

The conventional neutralization test was conducted by the technique described by *Baker et al.* (1) in which serial serum

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dilutions are tested against a constant virus dose. In the single dilution test, the degree of virus neutralization was estimated by testing a single virus-serum mixture and the results analysed by the probit method. The kinetics of the antibody response in ferrets, which had received a single subcutaneous injection of DV, was followed and compared by the two test procedures.

MATERIALS AND METHODS

Sera. Normal sera were obtained by cardiac puncture of 4-month-old ferrets from a commercial source. The ferrets were tested for the presence of neutralizing antibodies to DV on arrival and these tests were negative.

Immune ferret sera were collected from 5-month-old ferrets which had been injected subcutaneously with 1 ml stock virus containing 15,000 EID_{50} of DV. Sera were collected just prior to inoculation and at 2, 4, 6, 8, 10, 12, 14, 18, 22 and 31 days after inoculation. All sera were stored at —20°C and incubated at 56°C for 30 minutes prior to use.

Virus. The 65th to 70th passage of the Wisconsin FXNO strain of DV was used. The virus was propagated in White Leghorn eggs supplied by a single hatchery. Virus stocks were prepared by inoculating 7-day-old embryonated eggs on the chorioallantoic membrane (CAM) with 0.2 ml virus (200—400 EID_{50}) using the stab method (5). After further incubation of 7 days at 37°C, the CAMs were harvested and ground in a glass grinder with diluent to obtain a $10^{-0.3}$ suspension (V/V) of infected membranes. The diluent was phosphate buffer of pH 7.4 which contained 5 % glycerin and 50 μg dihydrostreptomycin sulphate, 200 i.u. penicillin G. potassium and 100 μg neomycin/ml. The tissue suspension was clarified by low speed centrifugation and the virus stocks were stored in 2 ml quantities in screw-cap vials at —60°C. Each vial was used only once.

Antibody titration.

Conventional neutralization tests. Sera to be used in conventional neutralization tests were diluted in 5-fold increments, and the dilutions were divided into three sets of tubes which were stoppered and frozen at —20°C until tested.

Aliquots of undiluted sera or dilutions of sera and virus (200—300 EID_{50}) were mixed in screw-cap tubes kept in an ice-

bath. Four to five serum dilutions were tested. The tubes were stoppered, shaken and placed in a 25°C water-bath for 16 hours. Following incubation the virus-serum mixtures were again transferred to an ice-bath and inoculated into embryonated eggs, using 5, 8 or 10 CAMs per dilution. After an additional 7 days' incubation, the CAMs were examined in a Quebec colony counter. Two or more greyish-white opaque pocks were necessary to consider a membrane infected with DV.

Single dilution tests. In these tests, aliquots of undiluted serum and virus were mixed in screw-cap tubes kept in an ice-bath. The tubes were shaken and placed in a 25°C water-bath for 16 hours. Following incubation the virus-serum mixtures were transferred to an ice-bath and inoculated into 5, 15 or 25 embryonated eggs. After 7 days further incubation, the CAMs were examined for DV lesions. The virus dose (∞ 30 EID_{50}) in this test represented a 1:8 dilution of the dose (200—300 EID_{50}) employed in the conventional test. In preliminary studies, the former virus dose, diluted 1:2 in buffer, had been found to evoke about 80 % infectivity.

Collected sera were tested 3 times in both tests at 2 week intervals. In each conventional test, the results were recorded for 5, 8 and 10 CAMs/virus-serum mixture (Table 1). In the single dilution test, the virus-serum mixture was injected into 25 CAMs and the results obtained with 5, 15 and 25 CAMs recorded each time (Table 2). Geometric mean serum and virus titers as well as mean probit values were computed for the two test procedures (Tables 1 and 2).

Table 1. Geometric mean serum titers in three conventional neutralization tests.

Number of tests	CAMs per test	Days postinoculation							Test dose of virus (EID_{50})
		8	10	12	14	18	22	31	
		Mean serum titers							
3	5×5	1.92*	2.83	2.86	2.71	3.91	3.84	3.45	115
3	8×5	1.97	2.85	2.84	2.66	3.96	3.83	3.45	148
3	10×5	1.92	2.81	2.81	2.70	3.95	3.80	3.45	135

* Serum titers are expressed as the negative $\log_{10}$ of the serum dilution which protected 50 % of infected embryos.

Table 2. Mean probit values for virus-serum mixtures in three "single dilution tests".

Number of tests	CAMs per test	Days postinoculation					Mean probit values for virus-buffer controls	Percentage of infected CAMs in virus-buffer controls	Test dose of virus (EID_{50})
		0	4	6	8	10			
		Mean probit values for virus-serum mixtures							
3	5	5.3	4.6	3.7	3.7	3.4	5.9	82	15
3	15	5.0	4.3	3.9	3.2	2.9	5.6	72	19
3	25	5.5	4.3	4.0	3.0	2.7	5.5	69	17

Virus titration.

Concurrent virus titrations were conducted for each conventional neutralization test. The virus dilution used in the neutralization test was further diluted in six to seven 2-fold increments in phosphate buffer. These virus dilutions were assayed in embryonated eggs by the stab method, using 8—10 CAMs per dilution.

RESULTS

Sensitivity of the two tests for detection of early antibody.

No antibody response was detectable with the conventional neutralization test during the first 6 days post-immunization (Fig. 1). Thereafter, the mean serum titers rose rapidly to a peak of $10^{2.8}$ $\log_{10}$ on days 10 to 12. The antibody response curve had a biphasic appearance reaching a second, higher antibody peak on day 18 with a mean serum titer of $10^{3.95}$.

The degree of neutralization in the single dilution test was measured by the difference between the $\log_{10}$ virus dilution, reflected from the probit value of any other day than 0, from the virus dilution reflected from the probit value of day 0. The results, summarized in Fig. 2 and Table 5, show a gradual reduction in the incidence of infected (positive) CAMs expressed in probits, from day 0 to day 10. When the mean probit values were based on the recording of 75 CAMs (Table 2), the virus control as well as the preinoculation serum-virus mixture evoked 69 % infectivity. In contrast, when the 4 day serum was tested, only 24 % infectivity (4.3 probits) was observed and when subsequently collected sera were assayed the infectivity declined

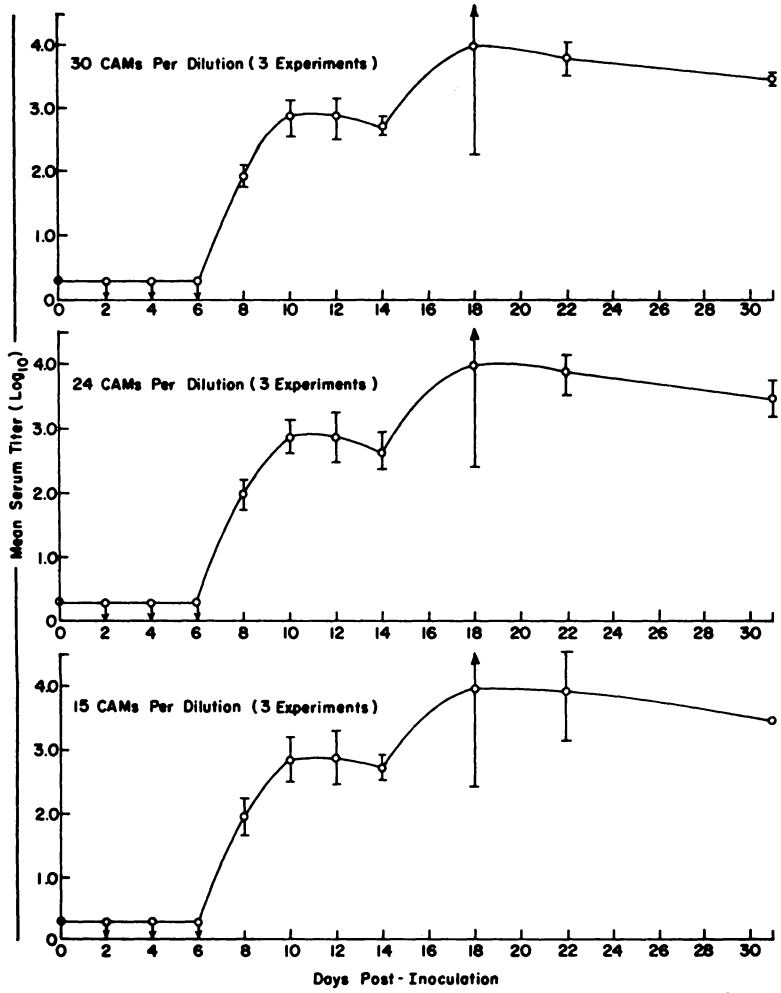


Fig. 1. A comparison of the 95 % confidence intervals of mean serum titers from three conventional neutralization tests employing increasing numbers of chorioallantoic membranes. o mean serum titer; I, 95 % confidence interval.

steadily. The recorded infectivities were 16 % for the 6 day serum, 2 % for the 8 day serum and only 1 % for the 10 day serum.

Error limits in the two tests for antibody.

The standard error and 95 % confidence interval (CI) of the mean serum titers and mean probit values for each day post-

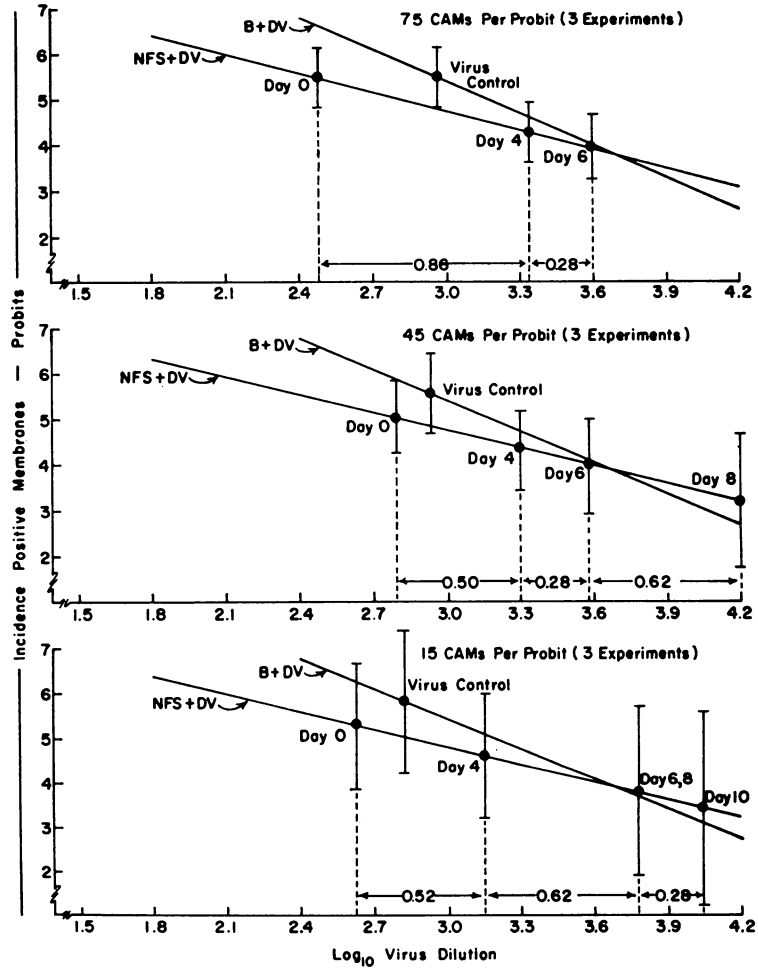


Fig. 2. A comparison of the 95 % confidence intervals of mean probit values from three "probit" neutralization tests employing increasing numbers of chorioallantoic membranes. • mean probit value; I, 95 % confidence interval; NFS, normal ferret serum; DV, distemper virus; B, buffer solution.

immunization were calculated. The results are tabulated in Tables 3 and 4. It may be seen in Table 3 that, up to day 14, the standard error and 95 % CI of the mean serum titers decreased as more CAMs were used per dilution. The error limits associated with the mean serum titers on day 18, representing the approximate peak of the second antibody rise, were quite large. As expected,

Table 3. Variation among serum and virus titers of the conventional neutralization test.

Number of tests	CAMs per test	Days postinoculation							Virus control
		8	10	12	14	18	22	31	
	Standard error (SE) of the mean serum titer								SE
3	5×5	0.07*	0.08	0.09	0.03	0.34	0.16	0	0.11
3	8×5	0.05	0.06	0.09	0.07	0.38	0.08	0.06	0.10
3	10×5	0.04	0.06	0.07	0.06	0.40	0.06	0.02	0.09
	95 % confidence interval (CI) of the mean serum titer								CI
3	5×5	0.6	0.7	0.8	0.2	3.0	1.3	0.0	0.9
3	8×5	0.4	0.5	0.7	0.5	3.3	0.7	0.5	0.9
3	10×5	0.3	0.6	0.6	0.5	3.4	0.6	0.2	0.7

* Serum titers are expressed as the negative $\log_{10}$ of the serum dilution which protected 50 % of infected embryos.

Table 4. Variation among probit values of the "single dilution test".

Number of tests	CAMs per test	Days postinoculation					Virus control
		0	4	6	8	10	
	Standard error (SE) of the mean probit value						SE
3	5	0.33	0.33	0.45	0.45	0.51	0.37
3	15	0.19	0.21	0.24	0.34	0.44	0.20
3	25	0.15	0.16	0.17	0.30	0.41	0.15
	95 % confidence interval (CI) of the mean probit value						CI
3	5	2.9	3.8	3.9	3.9	4.3	3.2
3	15	1.6	1.8	2.0	3.0	3.8	1.7
3	25	1.3	1.3	1.5	2.5	3.5	1.3

the standard error of the mean probit values similarly decreased as more CAMs were employed (Table 4).

The reference probit regression lines used in the single dilution test were based on titration data with mixtures of normal ferret serum-DV and phosphate buffer-DV (8). The probit values obtained with mixtures of post-immunization ferret sera and DV were superimposed on these reference regression lines. The slopes (regression coefficients) of the two reference regression lines were significantly different (Fig. 2). The flat slope of the normal

Table 5. The neutralizing activity of post-immunization sera estimated by probit analysis of data from "single dilution tests".

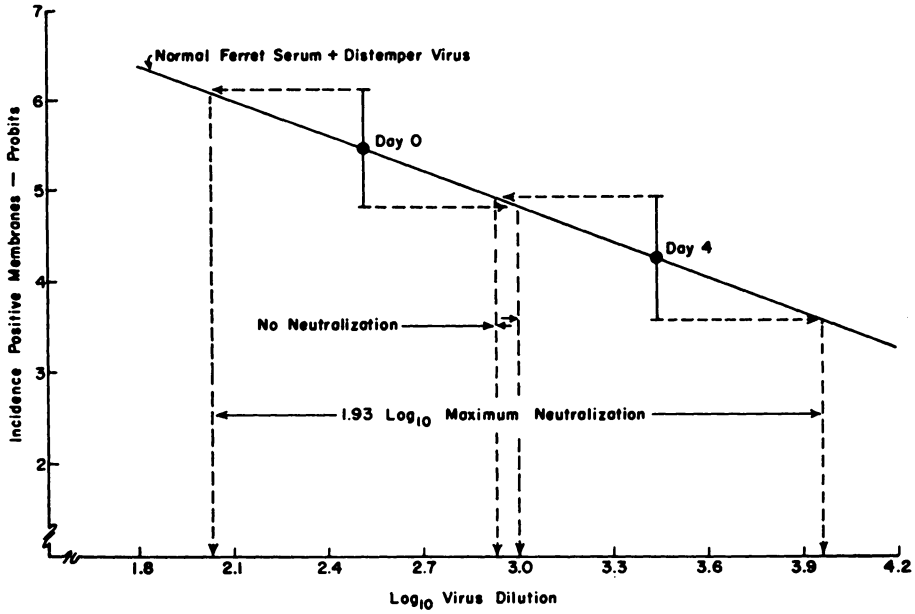
Number of tests	CAMs per test	Days postinoculation			
		4	6	8	10
3	5	0.52*	1.1	1.1	1.4
3	15	0.50	0.78	1.4	>1.4
3	25	0.86	1.1	>1.4	>1.4

* Reduction in $\log_{10}$ virus infectivity (virus dilution corresponding to the probit value of the day indicated minus virus dilution corresponding to the probit value of day 0).

ferret serum-DV line is a disadvantage as a reflection of the 95 % CI of a recorded probit value on the abscissa covers a large range of virus dilutions and consequently the error associated with the measured neutralization becomes large.

It appeared that the 95 % CI of the mean serum titers was of less magnitude than the corresponding intervals of the mean probit values. In the conventional test, the serum titers ranged from 0 to 4.5 $\log_{10}$ units. The two largest CI recorded in this test (3.4 and 1.3 $\log_{10}$), on days 18 and 22 respectively, did not cover this serum titer range. In the single dilution test the response range covering 5 to 95 % infected membranes corresponded to about 2.4 $\log_{10}$ virus dilutions when the reference probit regression line constructed for normal ferret serum and DV was used. Thus, the largest CI observed in this test (4.3 probits) exceeded the virus dilution range covered in the test.

To illustrate the degree of the variation of neutralization measured in the single dilution test, the effect of the 95 % CI of two mean probit values was reflected on the amount of neutralization (Fig. 3). Ninety-five per cent of the time the true probit value for day 0 (75 CAMs) extended from 4.8 to 6.1 probits and for day 4 from 3.6 to 4.9 probits. When the probit value for day 0 occurred at its maximal limit (6.1) and for day 4 at its minimal limit (3.6), the estimated virus neutralization would amount to 1.9 $\log_{10}$. However, if the probit value representing day 0 was at its minimal limit (4.8) and the value for day 4 at its maximal value (4.9), no neutralization would be demonstrable. When fewer CAMs were used, the CI was larger.



F i g. 3. The error in the measurement of neutralization by the "single dilution-probit test". The effect of 95 % confidence intervals of two mean probit values (days 0 and 4) as reflected on the amount of neutralization observed. • mean probit value; I, 95 % confidence interval.

DISCUSSION

The conventional *in ovo* neutralization test for antibody to DV was found to be superior to the single dilution test for quantitative studies but less sensitive for the detection of minimal quantities of antibody. In the conventional test, no antibody to DV was detectable until 8 days post-immunization (Fig. 1), while in the single dilution-probit test an increase in virus neutralization was observed on the 4th day (Tables 2 and 5). This is somewhat surprising in as much as the probit test in the present system (normal ferret serum-DV) could be expected to be insensitive for the detection of antibody as the slope of the reference probit-regression line is rather flat. However, the smaller virus dose (8 times less than in the conventional test) employed in the single dilution test favored its sensitivity. Although the error terms in the single dilution test were too large for quantitative studies the test, employing a small number of test animals, could

be useful as a screening test when a large number of sera are to be tested for the presence of antibody.

In a single dilution test, the reference probit-regression line must be constructed from titration data obtained with virus and normal serum from the species to be tested for the production of antibodies. This is necessary as the slopes of probit-regression lines obtained with normal sera from different species and DV differ slightly; the sera containing varying amounts of heat-stable inhibitors of viral infectivity (8). The fact that a homologous reference probit-regression line is desirable can present a problem in case one wants to study the antibody response of animals susceptible to DV infection as it is difficult to obtain antibody-free sera from such animals.

Several investigators have studied the antibody rise in dogs following exposure to DV (2, 4, 6, 9, 10, 13). However, probably because the kinetic curves were based on too few points, none of the described responses agreed with the pattern of the antibody rise in ferrets in this study. The response in ferrets was distinctly biphasic with an early antibody peak on day 10 and a late, about 12-fold higher, peak on day 18. These response curves resemble the kinetic curves for the formation of neutralizing antibody to poliovirus and the antihaemagglutinin response to influenza virus in rabbits (3, 12). In these latter responses, the early antibody peak represented γ-M-globulin antibody.

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SUMMARY

The sensitivity and error limits of a conventional *in ovo* neutralization test for antibody to distemper virus were compared with the same parameters of a single dilution-probit test for neutralizing antibody.

With the former test, antibody was demonstrable in ferrets 8 days following a single subcutaneous injection of virus while probit analysis of data from the single dilution test revealed the appearance of antibody as early as 4 days post-immunization. The error terms of the single dilution test were relatively large as compared to those of the conventional test for antibody.

Distinctly biphasic antibody responses were observed in ferrets following a single subcutaneous injection of distemper virus.

ZUSAMMENFASSUNG

Empfindlichkeit und Sicherheitsgrenzen bei zwei Testen für Hundestaupevirus neutralisierende Antikörper: Ein konventioneller Neutralisationstest und der s.g. „einzige Verdünnungs"-probit Test.

Die Empfindlichkeit samt Standardfehlern und Sicherheitsgrenzen bei einem konventionellen *in ovo* Neutralisationstest für Antikörper gegen Hundestaupevirus wurden mit ensprechenden Eigenschaften bei einem s.g. „einzigen Verdünnungs"-probit Test für neutralisierende Antikörper verglichen.

Mit den konventionellen Testen konnten Antikörper bei Fretten 8 Tage nach einer subkutanen Virusinjektion entdeckt werden. Probit Analyse von Resultaten von „einzigen Verdünnungs" Testen enthüllte dagegen das Auftreten von Antikörpern bereits 4 Tage nach Immunisierung. Der Standardfehler in „einzigen Verdünnungs" Testen war relativ gross verglichen mit dem in den konventionellen Testen für Antikörper.

Deutlich bifasische Antikörperantworten wurden bei Fretten nach einer einzigen subkutanen Injektion von Hundestaupevirus erhalten.

SAMMANFATTNING

Känslighet och trygghetsgränser hos två tester för valpsjukevirus neutraliserande antikroppar: En konventionell neutralisationstest och den s.k. „enda spädning"-probit testen.

Känsligheten samt standardfel och trygghetsgränser hos en konventionell *in ovo* neutralisationstest för antikroppar mot valpsjukevirus jämfördes med motsvarande egenskaper hos en s.k. „enda spädning"-probit test för neutraliserande antikroppar.

Med den konventionella testen kunde antikroppar hos fretter upptäckas 8 dagar efter en subkutan virusinjektion. Probit analys av resultat från „enda spädning" testen avslöjade däremot uppträdandet av antikroppar redan 4 dagar efter immunisering. Standardfelet i „enda spädning" testen var relativt stort jämfört med det i den konventionella testen för antikroppar.

Tydligt bifasiska antikroppssvar erhölls hos fretter efter en enda subkutan injektion av valpsjukevirus.

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