

Reproducibility of a Neuraminidase Inhibition Test Employed to Measure Anti-Influenza Neuraminidase Antibody

A. V. HENNESSY AND F. M. DAVENPORT

Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104

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The standard deviation was determined for 146 replications of the neuraminidase inhibition test for antineuraminidase antibody employing two ferret antisera and the recombinant viruses X-15 and X-15(HK). The standard deviation found was 0.612 log₂ and is a quantitative estimate of the reproducibility of the test.

In recent years, enzyme inhibition methods have been extensively employed in studies on the antigenic composition of influenza virus neuraminidases (3, 4). However, since there are no published reports concerning the reproducibility of antineuraminidase antibody titer values, selection of titer differences that reflect true antigenic differences has remained an arbitrary decision. The purpose of the present investigation was to provide objective criteria for discriminating between the viral enzymes of different strains on the basis of different enzyme inhibition titer values. To this end, replicate titrations of antineuraminidase antibody were carried out using convalescent ferret sera and viral recombinants. Mean titers and standard deviations of the means were then calculated. The results comprise the substance of this report.

Virus. The X-7, X-15, and X-15(HK) recombinant viruses were kindly supplied by Edwin Kilbourne. The X-7 has the hemagglutinin of NWS and the neuraminidase of A₂/RI/5+/57. X-15 is a recombinant containing the hemagglutinin of Equi-1 and the enzyme of A₂/RI/5+/57; X-15(HK) has the hemagglutinin of Equi-1 and the neuraminidase of A₂/HK/16/68. The A₂/HK/16/68 strain was obtained through the courtesy of Franklin Top, Jr., from the Walter Reed Army Research Center.

Antisera. The X-7 ferret antiserum was a hyperimmune serum obtained 2 weeks after reinfection; the ferret antiserum against A₂/HK/16/68 virus was a 3-week-post-primary infection specimen. The sera were stored at 4 C.

Neuraminidase inhibition test. The tests were performed using a method described by Kendal, Madeley, and Allan (2) using Cohn serum fraction IV-4 as neuraminidase substrate. Freshly prepared 0.25-ml samples of a mixture containing 2 mg of substrate and a standard virus suspension possessing 7 to 14 units of enzyme activity in 0.1 M phosphate buffer, pH 6.0, were added to equal volumes of serial fourfold serum dilutions (1/20 to 1/1,280). After overnight incubation at 37, free sialic acid was determined by the method of Aminoff (1) using synthetic *N*-acetylneuraminic acid as a sialic acid standard. A unit of neuraminidase is defined as that amount of enzyme causing the release of 1 μg of sialic acid from 2 mg of substrate in 0.5 ml of reaction mixture at pH 6.0 after overnight incubation in the absence of serum. The anti-enzyme titer of a serum was taken as the reciprocal of the final serum dilution inhibiting 50% of the enzyme activity found in control tests not containing serum. The spectrophotometer used was model B Beckman.

Antiserum from a ferret convalescent from X-7 infection and from a ferret infected with A₂/HK/16/68 were tested repeatedly on different days. X-15 and X-15(HK) were the strains used as enzyme sources in the tests. By employing these recombinant viruses, the possibility that hemagglutination-inhibiting antibodies might contribute to the titer values found is circumvented. Table 1 shows the mean titers, the difference from the mean of each single determination, the squares of the difference, and the standard deviation found when the X-7 antiserum was tested with X-15

TABLE 1. Distribution of antineuraminidase antibody titers found on repeated testing of ferret antisera

Test	$\Delta-\bar{\Delta}^a$	$\Delta-\bar{\Delta}^2$	$\Delta-\bar{\Delta}$	$\Delta-\bar{\Delta}^2$	$\Delta-\bar{\Delta}$	$\Delta-\bar{\Delta}^2$	$\Delta-\bar{\Delta}$	$\Delta-\bar{\Delta}^2$	$\Delta-\bar{\Delta}$	$\Delta-\bar{\Delta}^{2b}$
X-7 ferret tested with X-15, mean titer = 10.013 ^c	+0.916	0.839	+0.375	0.141	+0.331	0.110	-0.106	0.011	+0.275	0.076
	+0.631	0.398	+0.631	0.398	+1.116	1.245	-0.265	0.070	+0.064	0.004
	+0.418	0.175	+0.037	0.001	+1.309	1.713	-0.199	0.040	-0.136	0.018
	+0.631	0.398	+0.364	0.132	-0.884	0.781	+0.192	0.037	+0.064	0.004
	+0.240	0.058	+0.489	0.239	+0.263	0.069	+0.331	0.110	-0.183	0.033
	+0.216	0.047	-0.884	0.781	+0.909	0.826	-0.765	0.585	+0.216	0.047
	-0.562	0.316	+0.142	0.020	-0.501	0.251	+0.192	0.037	-0.443	0.196
	+0.418	0.175	+0.064	0.004	-0.936	0.876	-0.019	0.0004	-0.168	0.028
	-0.265	0.070	-0.823	0.677	-0.168	0.028	-0.691	0.477	-0.019	0.0004
	-0.199	0.040	-1.936	3.748	+0.557	0.310	-1.232	1.518	-0.019	0.0004
	X-7 ferret tested with X-15(HK), mean titer = 8.292 ^d	+1.522	2.316	+0.489	0.239	+0.159	0.025	-0.648	0.420	+0.489
+0.066		0.004	-0.063	0.004	-1.883	3.546	-0.800	0.640	+0.422	0.178
+0.837		0.701	-0.063	0.004	-0.326	0.106	-0.385	0.148	-1.270	1.613
+0.880		0.774	+0.117	0.014	+1.239	1.535	-0.194	0.038	+0.730	0.533
+0.674		0.454	-0.578	0.334	+0.012	0.0001	+0.436	0.190	+0.030	0.001
+0.730		0.533	+0.352	0.124	+0.352	0.124	+0.785	0.616	-0.163	0.027
-2.270		5.153	-0.722	0.521	+0.436	0.190	+0.278	0.077	-0.163	0.027
+0.984		0.968	-1.385	1.918	-0.511	0.261	-0.112	0.013		
-0.761		0.579	+0.030	0.001	+0.456	0.208	+0.489	0.239		
-0.326		0.106	-0.270	0.073	-0.385	0.148	+0.259	0.067		
HK ferret tested with X-15(HK), mean titer = 10.248 ^e		+0.907	0.823	+0.264	0.070	+0.244	0.060	+0.414	0.171	+0.151
	+0.396	0.157	-0.500	0.250	-0.467	0.218	-0.145	0.021	-0.311	0.097
	+0.517	0.267	+0.868	0.753	+0.161	0.026	-0.282	0.080	+0.224	0.050
	+0.396	0.157	+0.203	0.041	-0.119	0.014	-0.341	0.116	-0.254	0.065
	-0.068	0.005	+0.203	0.041	-0.403	0.162	-0.171	0.029	-0.254	0.065
	+0.322	0.104	+0.224	0.050	+0.162	0.026	-0.341	0.116	-0.926	0.857
	+0.074	0.005	-1.678	2.816	-0.371	0.138	-0.226	0.051	+0.203	0.041
	+0.283	0.080	+0.151	0.023	-0.226	0.051	+0.396	0.157	+0.322	0.104
	-0.282	0.080	-0.226	0.051	+1.074	1.153	-0.403	0.162	+0.028	0.001
	+0.028	0.001	+0.244	0.060	-0.226	0.051	-0.226	0.051		

^a Difference from the mean.

^b Square of difference from the mean.

^c Sum of squares = 18.152; sigma = 0.609.

^d Sum of squares = 26.0291; sigma = 0.752.

^e Sum of squares = 9.9900; sigma = 0.456.

and X-15(HK) and A₂/HK/16/68 serum was tested with X-15(HK). Fifty separate neuraminidase inhibition titrations of the X-7 serum with X-15 virus yielded a mean titer of 10.013 log₂ and a standard deviation of 0.609 log₂. A mean titer of 8.292 log₂ was found in 47 measurements of the same antiserum against X-15(HK). The standard deviation was 0.752 log₂. Forty-nine replicate titrations of the A₂/HK/16/68 antiserum against X-15(HK) gave a mean titer of 10.248 log₂ and a standard deviation of 0.456 log₂.

To obtain the best estimate of the average reproducibility of the neuraminidase inhibition test, the sum of squares of the differences from the mean of the three distributions were combined and a standard deviation of 0.612 log₂ was found for 146 replications. Two standard deviations is 1.224 log₂; thus, differences of 2.3-fold or greater may be considered significant.

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