Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1973, **48**, 199-202

Influenzavirus neuraminidase and neuraminidase-inhibition test procedures

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The adequate characterization of type A influenzaviruses requires the identification not only of the haemagglutinin but also of the neuraminidase. The neuraminidase-inhibition test, as performed in the two WHO international reference centres for influenza, is described and its use in other laboratories—particularly those collaborating in the WHO programme—is recommended.

In a memorandum [7] proposing a revised system of nomenclature for influenzaviruses, it was recommended that the neuraminidase antigens of the type A influenzaviruses be divided into subtypes based on the neuraminidase-inhibition (NI) test. NI tests representing the contributions of a number of investigators are currently in use. In general, methods for the assay of neuraminidase activity are based on the methods described by Warren (1959) and modified by Aminoff (1961). The procedures for the inhibition of enzyme activity by antibody are largely derived from those described by Ada et al. (1963) and by Webster & Laver (1967).

Because the NI test has evolved gradually, the current method is not reported in its entirety anywhere in the literature. This has precluded standardized test conditions and laboratory procedures. The present article describes the NI test as performed by the WHO World Influenza Centre, London, England, and the WHO International Influenza Center for the Americas, Atlanta, Ga., USA, and recommended to the laboratories collaborating in the WHO influenza programme.

Instructions for performing the NI test are presented in two parts: (1) the assay of neuraminidase activity; and (2) the NI test itself. The principal steps involved in the assay of neuraminidase are: (*a*) the release of free *N*-acetyl neuraminic acid (5-acetamido-3,5-dideoxy-*N*-acetyl-D-glycero-D-galacto-nonulosonic acid) from the fetuin substrate by the action of neuraminidase; (*b*) the conversion of *N*-acetyl neuraminic acid to β-formyl pyruvic acid by periodate oxidation; (*c*) the formation of chromophore by thiobarbituric acid; and (*d*) the extraction of chromophore into organic solvent for spectrophotometric analysis. By this method the potency of the neuraminidase is determined and the standard dose for use in the NI test is selected. The principal steps involved in the NI test are: (*a*) incubation of the standard neuraminidase dose with serial dilutions of normal and test sera; (*b*) determination of the inhibitory effect of serum on neuraminidase activity; and (*c*) calculation of the NI titre.

ASSAY OF NEURAMINIDASE ACTIVITY

(1) Make serial 2-fold dilutions of virus in 0.15M saline. For allantoic fluids a suitable dilution series would be 1 : 4 to 1 : 128. Higher dilutions will be required for virus concentrates. Reference virus neuraminidase and unknown test strains should be assayed in the same test.

(2) Transfer 0.05 ml of each virus dilution into each of a series of test tubes (approximately 16 × 100 mm).

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[7] *Bull. Wld Hlth Org.*, 1971, **45**, 119-124.

Include duplicate blank tubes containing 0.05 ml of saline in place of the virus.[1]

(3) To each tube (including the blanks) add 0.05 ml of saline and 0.1 ml of fetuin containing pH 5.9 buffer. (These can be mixed and added as 0.15 ml of mixture.) Mix well and allow to stand at 37°C for 18 hours.

(4) Cool the tubes to 20°C and add 0.1 ml of periodate reagent (see Annex 1) to each tube; mix thoroughly and allow to stand at 20°C for exactly 20 minutes.

(5) Add 1.0 ml of arsenite reagent. A brown colour will form as a result of the release of iodine. Shake until the brown colour disappears. (If desired, the test can be stopped at this stage and the mixture maintained at 4°C.)

(6) Add 2.5 ml of thiobarbituric acid reagent and mix thoroughly.

(7) Immediately place the tubes in a boiling water bath and let them remain there for 15 minutes. The red colour that develops indicates neuraminidase activity.

(8) Cool the tubes to room temperature in ice water and add 4.0 ml of butanol reagent. Mix well with a mechanical mixer (Vortex or Whirlmixer, if available) or vigorously shake by hand. This procedure extracts the colour, which passes into the organic (butanol) phase.

(9) Centrifuge the tubes for 2–5 min at 500–1 000 rev/min. The butanol layer must be free of turbidity. Carefully pipette the upper (butanol) phase into a colorimeter tube.

(10) Read the absorbance at a wavelength of 549 nm in a spectrophotometer using cells with a 1-cm lightpath. The fetuin blanks are used to equilibrate the machine, and tests are read against one fetuin blank.[2] Determine the virus concentrations giving an absorbance of 0.45–0.85.

[1] Since free *N*-acetyl neuraminic acid is often found in low dilutions (1 : 4 to 1 : 8) of infected allantoic fluids and may interfere in the neuraminidase test, controls should be included in which test virus fluids are processed in the absence of fetuin. Free *N*-acetyl neuraminic acid may be removed from test fluids by centrifugation and resuspension of the virus.

[2] Alternatively, the machine may be equilibrated and the test read against water. The absorbance of the fetuin blank is then subtracted from the final absorbance.

NEURAMINIDASE-INHIBITION (NI) TEST

To identify the neuraminidase antigen of virus isolates, specific reference antisera should be used. Antisera should be prepared against isolated neuraminidase antigens or against virus recombinants (antigenic hybrids) possessing haemagglutinin antigens that are unrelated to the antigens of the test isolates. The NI titre of reference sera with the unknown viruses is compared with the titre of the sera with the appropriate reference viruses.

(1) Use test viruses at appropriate dilutions (i.e., those giving an absorbance (λ = 549 nm) of 0.45–0.85 after 18 hours' incubation with fetuin).

(2) Mix in test tubes 0.05 ml of virus dilution with 0.05 ml of serum in 0.5 $\log_{10}$ dilutions.[3] Control and blank tubes should be included. The essential elements of the NI test for the antigenic characterization of unknown isolates are summarized in Annex 2.

(3) The neutralization reaction is carried out at 37°C for 1 h.

(4) Now add 0.1 ml of fetuin containing the phosphate buffer, pH 5.9; shake the tubes and incubate them at 37°C for 18 hours in a water bath.

(5) Remove the tubes from the water bath and assay for released *N*-acetyl neuraminic acid by adding the reagents as previously described for the assay, beginning with step 4.

(6) The NI titre of an antiserum is defined as the dilution of serum giving 50% inhibition of neuraminidase activity. Plot absorbance against the serum dilution on linear graph paper. The NI endpoint is read from the graph as the serum dilution that gives a 2-fold reduction in absorbance compared with the appropriate control tube (virus + normal serum dilution).

An alternative method of calculating NI titres is to divide the absorbance of the virus + test serum dilution by that of the virus + control serum at the same dilution and multiply by 100 to obtain the activity as a percentage. The percentage activity is plotted against the serum dilution factor. The NI titre is calculated as the dilution of antiserum that reduces neuraminidase activity by 50%. An example of this method of calculation is shown in Annex 3.

[3] A 0.5 $\log_{10}$ dilution step is 1 : 3.16 but an approximation may be made by using 1 : 3.2.

RÉSUMÉ

TECHNIQUES DE L'ÉPREUVE D'ACTIVITÉ ET DE L'ÉPREUVE D'INHIBITION DE LA NEURAMINIDASE DES VIRUS GRIPPAUX

Pour caractériser de façon correcte les virus grippaux de type A, il est indispensable d'identifier non seulement leur hémagglutinine mais aussi leur neuraminidase. Le présent article décrit la technique de l'épreuve d'inhibition de la neuraminidase telle qu'elle est pratiquée au Centre mondial OMS de la grippe, National Institute for Medical Research, Londres (Angleterre) et au Centre international OMS de la grippe pour les Amériques, Center for Disease Control, Atlanta, Ga. (Etats-Unis d'Amérique). Il est recommandé que cette technique soit adoptée par les laboratoires qui participent au programme OMS relatif à la grippe.

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Annex 1

WORKING REAGENTS FOR NEURAMINIDASE ASSAY

Use analytical grade chemicals or their equivalent.

Phosphate buffer

(*a*) 0.4M disodium hydrogen orthophosphate
(*b*) 0.4M sodium dihydrogen orthophosphate

Mix 19 ml of solution (*a*) with 81 ml of solution (*b*) to give 0.4M buffer with pH 5.9; adjust the pH if necessary. The addition of the acidic buffer (*b*) lowers the pH. Add calcium chloride hexahydrate to give a final concentration of 6mM (Dimmock, 1971).

Fetuin

Reconstitute with sterile water to give a final concentration of 48 g/litre. Dilute 1 : 2 with phosphate buffer containing 6mM Ca^{++}.

Periodate reagent

Dissolve 4.28 g of sodium periodate ($NaIO_4$) in 38 ml of distilled water. Add 62 ml of syrupy orthophosphoric acid, and mix well. Store in a glass-stoppered brown bottle.

Arsenite reagent

Dissolve 10 g of sodium arsenite ($NaAsO_2$) and 7.1 g of anhydrous sodium sulfate in 100 ml of distilled water. Add 0.3 ml of concentrated sulfuric acid.

Thiobarbituric acid reagent [1]

Dissolve 1.2 g of thiobarbituric acid and 14.2 g of anhydrous sodium sulfate in 200 ml of distilled water by heating in a boiling water bath.

Butanol

Add 5 ml of concentrated hydrochloric acid to 100 ml of butanol.

[1] Make up fresh each week.

Annex 2

SUMMARY OF ESSENTIAL ELEMENTS OF THE NEURAMINIDASE-INHIBITION TEST FOR ANTIGENIC CHARACTERIZATION OF UNKNOWN TYPE A INFLUENZAVIRUS ISOLATES

Reaction	Reference anti-neuraminidase serum [a] (ml)	Normal rabbit serum [b] (ml)	Fetuin (ml)	Virus (ml)	Saline (ml)
reference virus [c] (test)	0.05		0.1	0.05	
unknown virus (test)	0 05		0.1	0 05	
reference virus (control)		0.05	0.1	0.05	
unknown virus (control)		0.05	0 1	0 05	
fetuin control (duplicate blanks)			0.1		0.1

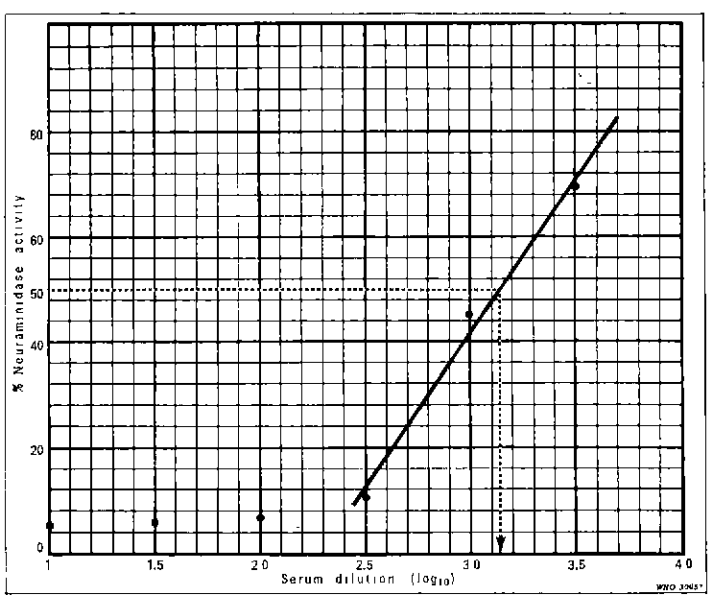
[a] 0.5 $\log_{10}$ dilutions of **1 : 10** to endpoint—i.e., 1 : 10, 1 : 32, 1 : 100.
[b] 0.5 $\log_{10}$ dilutions of **1 : 10**, up to **1 : 1** 000.
[c] Reference reagents are available in limited quantities through the two WHO influenza centres.

Annex 3

CALCULATION OF NI TITRE

Step 1 : Determination of neuraminidase activity.

Serum dilution ($\log_{10}$)	Absorbance		Neuraminidase activity (%)
	Virus + normal rabbit serum	Virus + immune rabbit serum	
1.0	0.862	0.046	5
1 5	0.864	0 052	6
2.0	0.902	0.060	7
2 5	0 676	0.074	11
3.0	0.622	0.282	45
3 5	0.604	0.422	70
4.0	0.612	0.592	97



Step 2: Neuraminidase activity is plotted against serum dilution.

Step 3: The dilution of antiserum that reduces neuraminidase activity by 50% is read from the graph as 3.15. The serum NI titre is the antilog$_{10}$ of this figure, i.e., 1 420.