Influenza - Neuraminidase Inhibition Test

This protocol is a copy of the standard operating procedure used by the OIE/FAO international reference laboratory for AI at the Animal and Plant Health Agency. If you have any technical queries please contact aiwrl@apha.gov.uk

Contents

1. INTRODUCTION

1.1 Purpose/Scope of this Protocol

1.1.1 To perform a neuraminidase inhibition (NI) test to determine the N type of avian and mammalian influenza isolates.

1.2 Background information

1.2.1 NI is a technique for characterising the neuraminidase of influenza viruses. Neuraminidase is identified by the inhibition of enzyme activity by antisera prepared against antigen from reference strains.

1.2.2 Viral neuraminidase acts upon the fetuin substrate to release quantities of free N-acetyl neuraminic acid (5-acetamido-3, 5-dideoxy-N-acetyl-D-galacto-nonulosonic acid). The free N-acetyl neuraminic acid is then converted to β-formyl pyruvic acid by periodate oxidation. Thiobarbituric acid is added next to form a chromophore, boiled to release the colour and can be read.

In the presence of its homologous antiserum, the viral N-acetyl neuraminic acid is bound and therefore the reaction won't progress and no colour will be released.

1.2.3 Isolates requiring testing are samples of Chorioallantoic fluid that have been confirmed by HI testing to be Influenza viruses. The titre of these isolates ranges between 2^4 and 2^{12}.

1.2.4 Mixed populations of viruses may cause unexpected results.

2. SAFETY

2.1 It is your laboratory's responsibility to ensure all work described in this protocol is conducted to a high safety standard.

2.2 ⚠ Areas within this procedure which refer to Safety Critical activities are denoted in the paragraph number column with the ⚠ sign to highlight these areas to users.

2.3 Avian influenza is categorised as SAPO Group 4 and ACDP 2/3, and should be worked with at relevant Containment Level. Refer to risk assessments when working with this pathogen and Influenza Virus risk matrix.

2.4 ⚠ Due to the toxicity of the chemicals used (HCl: corrosive and inhalation hazard, Sulfuric acid: corrosive, Sodium Arsenite: carcinogenic poison, Sodium Periodate: corrosive and inhalation hazard, TBA: irritant), a fume cupboard/ safety cabinet must be used when handling them, including the preparation of the solutions.

This precaution measure is even more important when boiling the tube contents on day 2 at the end of the test.

3. MATERIALS

3.1 Documentation and software

3.1.1 Follow datasheets for storage and use of antigens and antisera.

3.2 Chemicals and reagents

3.2.1 Antiserum to cover all 9 neuraminidase subtypes and negative serum (SPF).

3.2.2 0.1M Phosphate Buffered Saline pH 5.9

Dilute 3.05g Na_2HPO_4 in 250mls DI water
Dilute 6g NaH_2PO_4 in 500mls DI water
Mix Na_2HPO_4 into NaH_2PO_4 slowly to get pH 5.9
Store at 2-8ºC for up to 6 months from date of manufacture

3.2.3 ⚠ 0.025M Sodium Periodate in 0.125N H_2SO_4

Add 1.75 ml Sulphuric Acid to 500 ml DI water.
Dilute 2.67 g Sodium Periodate into the mix and stir until dissolved.

Store at 2-8ºC for up to 6 months from date of manufacture

3.2.4 ⚠ 2% Sodium Arsenite in 0.5 N HCL

Dilute 7.625 ml of HCl specific gravity 1.18 in 500 ml of DI water.
Add 10g of Sodium Arsenite to the solution and mix until dissolved.
Store at room temperature for up to 5 years from manufacture

3.2.5 0.1M Thiobarbituric acid pH 9.0 (TBA)

14.4g Thiobarbituric acid in 800mls of DI Water
5g NaOH into 125ml DI water
Add NaOH solution until TBA has dissolved (TBA dissolves in pH 9.0 Solution) and pH meter reads pH 9.0
Make up to 1 litre with DI water.
Store at 2-8ºC for up to 6 months from date of manufacture

3.2.6 Standardised Fetuin

Dilute 1g Fetuin from Fetal bovine serum (0.1% w/v) into 1litre DI water.

Aliquot into 4ml glass bijou tubes.

Make up solution and aliquot and store below -70ºC for up to 2 years

3.3 Animals/Micro-organisms/Cells

3.3.1 Virus isolate to type.

3.4 Equipment

3.4.1 Glass tubes suitable for boiling – socket size 14/23, length 150mm, diameter, capacity 19ml

3.4.2 Stoppers to fit glass tubes

3.4.3 Racks suitable for holding glass tubes

3.4.4 Pipettes capable of delivering the volumes stated in this SOP

3.4.5 Waterbath capable of being maintained at boiling temperature.

3.4.6 Incubator capable of being maintained at 37ºC ± 2ºC

3.4.7 Pipette tips

3.4.8 Refrigerator – capable of being maintained at 2-8ºC

3.4.9 Freezer -70ºC or lower

3.4.10 Laboratory glassware and consumables

3.4.11 pH meter

3.4.12 Calibrated timer

4. PROCEDURE/METHOD

4.1 Test Reliability

4.1.1 Process of assuring ongoing test reliability:

4.1.2 **Avian Influenza Virus :** IQC for this test is neat positive antisera. EQA schemes should be carried out twice yearly. Additional testing should be carried out on known inactivated antigens for validation and fitness for purpose testing.

Quality control antigens are supplied to QAU by Avian Virology, APHA Weybridge.
An annual EU proficiency test is also carried out for Avian Influenza Virus

and Paramyxoviruses. Material is supplied by the EU/OIE/FAO Reference Laboratory at APHA Weybridge.

Swine influenza : The test sample acts as a positive control in each test to ensure that neuraminidase activity is present and a negative serum control is included in each test to check reagents are fit for purpose. All staff performing the test are fully trained.

An IQA panel, supplied by SI Reference Laboratory, is performed twice yearly to demonstrate both test assurance and individual competency.

Uncertainty of measurement UM005 supports these tests.

Because of the subjective nature of this test all results are checked by a second operator to assure test reliability.

4.2 Neuraminidase Inhibition test – Day 1

4.2.1 Select antisera to cover all nine neuraminidase subtypes and negative serum control. The antisera used should not have the same H type as the isolate being typed.

4.2.2 Dilute antisera 1/10 in 0.1M phosphate buffer pH 5.9 from stock reagents containing 0.1% Azide or from lyophilised ampoules. These can be diluted in bulk and stored at 2-8ºC for up to 6 months

4.2.3 ⚠ Dilute avian influenza virus 1/20 in 0.1M phosphate buffer pH 5.9
Dilute swine influenza virus 1/3 in 0.1M phosphate buffer pH 5.9

4.2.4 Ensure reagents are added to the bottom of the tube and not down the sides. Add 100µl of diluted N1 subtype antisera to tube 1, 100µl of diluted N2 subtype antisera to tube 2. This is repeated for all diluted antisera to N9 subtype into tube 9. Tube 10 will contain the diluted negative antiserum.

4.2.5 Add 100µl of 0.1M PBS pH 5.9 to a tube labelled V (virus – positive control) and 200µl to a tube labelled B (blank).

4.2.6 Add 100µl of diluted virus to all tubes except the blank (B). Gently shake tubes to mix.

4.2.7 Incubate for 20 minutes at room temperature (18-30ºC).

4.2.8 Add 300µl of the substrate Fetuin to all tubes and shake gently to mix.

4.2.9 Place stoppers in tubes and incubate at 37ºC overnight or a minimum of 12 hours up to 24 hours.

4.3 Neuraminidase Inhibition Test – Day 2

4.3.1 Remove tubes from incubator and complete all following steps in fume cupboard/safety cabinet.

Remove lids from tubes.

4.3.2 ⚠ Add 200µl of 0.025M Sodium Periodate to each tube, shake tubes to mix and incubate for 30 minutes at 37°C in an incubator. Towards end of incubation period switch on waterbath to reach boiling temperature.

4.3.3 ⚠ Add 200µl of 2% Sodium Arsenite to each tube and shake tubes to mix. The liquid in the tubes will turn brown at this stage. Shake tubes gently until dark brown colour fades before 4.3.5. At this stage the tubes can be stored in the refrigerator for up to 24 hours.

4.3.4 Add 2ml TBA to each tube, gently shaking tubes to mix.

4.3.5 ⚠ Visually check water is boiling. Place all the tubes per virus into the waterbath and boil until pink colour develops in the virus control for a maximum of seven minutes.

4.3.6 Read test results and record.

If you are lone working please take a picture of the tubes for a second reader to confirm at a later date.

N.B – the second reader does not have to be trained in the Neuraminidase test, they are just signing to confirm colour change.

4.4 Reverse Neuraminidase Inhibition Test- Day 1

4.4.1 Select antigens to cover all nine neuraminidase subtypes. The antigens used should not have the same H type as the isolate being typed.

4.4.2 Dilute antigens 1/20 in 0.1M phosphate buffer pH 5.9.

4.4.3 Ensure reagents are added to the bottom of the tube and not down the sides. Add 100 µl of diluted N1 subtype antigen to tube 1, 100 µl of diluted N2 subtype antigen to tube 2. This is repeated for all diluted antigens to N9 into tube 9. Prepare 4 rows of tubes 1-9 with antigens.

4.4.4 Dilute the antiserum we want to test 1/10 in 0.1M PBS pH 5.9 and add 100µl of the diluted antiserum to the first row of tubes 1 to 9. Gently shake tubes to mix.

4.4.5 <u>Controls:</u>

Blank: Add 200 µl PBS into a tube labelled B for blank. No antigen or

antiserum will be added to this tube.

Negative controls: Add 100 µl of diluted SPF antiserum to another row of tubes 1 to 9.

Virus blanks: Add 100 µl of 0.1M PBS 5.9 to another row of tubes 1 to 9.

Positive controls: In the last row of tubes, add 100 µl of diluted antiserum N1 to tube 1 with N1 antigen, add 100 µl of diluted antiserum N2 to tube 2 with N2 antigen. This is repeated for all diluted antisera to tube 9.

4.4.6 Gently shake all tubes to mix.

4.4.7 Follow from section 4.2.7 of this procedure. The next steps must be followed in the same way as if it was a Neuraminidase Inhibition Test.

4.4.8 Read test results and record.

If you are lone working please take a picture of the tubes for a second reader to confirm at a later date.

N.B – the second reader does not have to trained in the Neuraminidase test, they are just signing to confirm colour change.

5. RESULTS

5.1 Results for the Neuraminidase Inhibition Test:

The liquid in the negative control (SPF) tube should be pink.

The liquid in the tube labelled V (see 4.2.5) should be pink.

The liquid in the tube labelled B (see 4.2.6) should be clear.

The liquid in tubes 1-9 where the test antigen was added, the tube containing the homologous N subtype antiserum should be clear showing inhibition and the rest of the tubes should be pink. This indicates the neuraminidase subtype of the isolate.

5.2 If no neuraminidase activity is seen, repeat steps 4.2.1 to 4.3.7 but dilute the virus 1/10 with 0.1M PBS pH 5.9 for avian influenza viruses and use virus neat for swine influenza viruses.

5.3 Results for Reverse Neuraminidase Inhibition Test:

The liquid in the tube labelled Blank should be clear.

The row of Negative Controls should be pink.

The row of Virus Blanks should be pink.

The row of Positive Controls should be clear because of the binding of each antigen with its homologous antiserum.

In the row of tubes where the test antiserum was added, the tube containing the homologous N subtype antigen should be clear showing inhibition and the rest of the tubes should be pink.

5.4 Records results on worksheet. P = pink, C = clear and PP = Pale Pink for tubes with greatly reduced colour.

6. REFERENCES

6.1 Webster, R.G., and C. H. Campbell. An inhibition test for identifying the neuraminidase antigen on influenza viruses. Avian Dis. 16:1057 – 1066. 1972.