

## Recommended laboratory tests to identify avian influenza A virus in specimens from humans

### General information

Highly pathogenic avian influenza (HPAI) caused by certain subtypes of influenza A virus in animal populations, particularly chickens, poses a continuing global human public health risk. Direct human infection by an avian influenza A(H5N1) virus was first recognized during the 1997 outbreak in Hong Kong Special Administrative Region of China. Subsequently, human infections with avian strains of the H9 and H7 subtypes have been further documented. The current outbreak in humans of avian A(H5N1) and the apparent endemicity of this subtype in the poultry in southeast Asia require increased attention to the need for rapid diagnostic capacity for non-typical influenza infections. Laboratory identification of human influenza A virus infections is commonly carried out by direct antigen detection, isolation in cell culture, or detection of influenza-specific RNA by reverse transcriptase–polymerase chain reaction.

These recommendations are intended for laboratories receiving requests to test specimens from patients with an influenza-like illness, in cases where there is clinical or epidemiological evidence of influenza A infection. The laboratory procedures outlined below do not contain all the information required to perform the tests: further details should be obtained from the references cited, the technical working group (see Annex ) or from a WHO H5 Reference Laboratory<sup>1</sup>.

The optimal specimen for influenza A virus detection is a nasopharyngeal aspirate obtained within 3 days of the onset of symptoms, although nasopharyngeal swabs and other specimens can also be used ([http://www.who.int/csr/disease/avian\\_influenza/guidelines/humanspecimens/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/humanspecimens/en/index.html)).

All manipulation of specimens and diagnostic testing should be carried out following standard biosafety guidelines([http://www.who.int/csr/disease/avian\\_influenza/guidelines/handlingspecimens/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/index.html) [http://www.who.int/csr/resources/publications/biosafety/WHO\\_CDS\\_CSR\\_LYO\\_2004\\_11/en/](http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/)).

The strategy for initial laboratory testing of each specimen should be to diagnose influenza A virus infection rapidly and exclude other common viral respiratory infections. Results should ideally be available within 24 hours.

### Procedures for influenza diagnosis

Assays available for the diagnosis of influenza A virus infections include:

1. **Rapid antigen detection.** Results can be obtained in 15–30 minutes.
  - *Near-patient tests for influenza.* These tests are commercially available (Nicholson, Wood & Zambon, 2003).
  - *Immunofluorescence assay.* A widely used, sensitive method for diagnosis of influenza A and B virus infections and five other clinically important respiratory viruses (Lennette & Schmidt, 1979).
  - *Enzyme immunoassay.* For influenza A nucleoprotein (NP).

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<sup>1</sup> A list of WHO H5 Reference Laboratories can be found at:  
[http://www.who.int/csr/disease/avian\\_influenza/guidelines/referencelabs/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/index.html)

2. **Virus culture.** Provides results in 2–10 days. Both shell-vial and standard cell-culture methods may be used to detect clinically important respiratory viruses. Positive influenza cultures may or may not exhibit cytopathic effects but virus identification by immunofluorescence of cell cultures or haemagglutination-inhibition (HI) assay of cell culture medium (supernatant) is required.
3. **Polymerase chain reaction and Real-time PCR assays.** Primer sets specific for the haemagglutinin (HA) gene of currently circulating influenza A/H1, A/H3 and B viruses are becoming more widely used. Results can be available within a few hours from either clinical swabs or infected cell cultures. Additionally several WHO Collaborating Centres are developing PCR and RT-PCR reagents for non-typical avian/human influenza strains (Fouchier et al., 2000; Lee & Suarez, 2004).

Any specimen with a positive result using the above approaches for influenza A virus and suspected of avian influenza infection should be further tested and verified by a designated WHO H5 Reference Laboratory.<sup>1</sup> Laboratories that lack the capacity to perform specific influenza A subtype identification procedures are requested to:

1. Forward specimens or virus isolates to a National Influenza Centre (<http://www.who.int/csr/disease/influenza/centres/en/index.html>) or to a WHO H5 Reference Laboratory<sup>1</sup> for further identification or characterization.
2. Inform the WHO Office in the country or WHO Regional Office or WHO HQ Global Influenza Programme ([whoinfluenza@who.int](mailto:whoinfluenza@who.int)) that specimens or virus isolates are being forwarded to other laboratories for further identification or further characterization.

## Identification of avian influenza A subtypes

### *Immunofluorescence assay*

Immunofluorescence assay (IFA) can be used for the detection of virus in either clinical specimens or cell cultures. Clinical specimens, obtained as soon as possible after the onset of symptoms, are preferable as the number of infected cells present decreases during the course of infection. Performing IFA on inoculated cell cultures is preferable as it allows for the amplification of any virus present.

Materials required:

- WHO Influenza Reagent Kit for the Identification of Influenza A/H5 Virus (1997–98, 2003 or 2004 version). The reagents in this kit for the immunofluorescence assay include:
  - influenza type A/H5-specific monoclonal antibody pool
  - influenza A type-specific and influenza B type-specific monoclonal antibody pools
  - influenza A/H1 and an A/H3 subtype specific monoclonal antibodies
- Anti-mouse IgG FITC conjugate
- Microscope slides
- Cover slips, 24 x 60 mm
- Mountant
- Acetone
- Immunofluorescence microscope.

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[http://www.who.int/csr/disease/avian\\_influenza/guidelines/referencelabs/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/index.html)

## Procedure

This test should be performed in accordance with the instructions included in the WHO Influenza Reagent Kit. Epithelial cells are washed free of contaminating mucus by centrifugation, fixed, and stained with specific monoclonal antibodies. Infected respiratory epithelial cells in a clinical specimen are very labile and easily damaged; they should therefore be kept cold on ice during processing and not centrifuged at more than 500g. Control slides with influenza A/H3- and H1-infected cells (and, when available, H5-infected cells) and uninfected cells should be included to allow appropriate control of monoclonal antibodies and conjugate and to assist with interpretation of specific staining.

## Interpretation of results

Specific staining should be an intense intracellular apple-green fluorescence. Nuclear and/or cytoplasmic fluorescence may be observed. It is important to ensure that cell density is adequate. One or more intact cells showing specific intracellular fluorescence can be accepted as a positive result.

Because commercially available monoclonal antibodies for the subtyping of influenza A/H1 have been shown to cross-react with influenza A/H5 subtypes, including current (2004) strains, confirmatory testing should be carried out using the monoclonal antibody provided in the WHO kit.

## Virus culture

Virus isolation is a sensitive technique with the advantage that virus is available both for identification and for further antigenic and genetic characterization, drug susceptibility testing, and vaccine preparation. MDCK cells are the preferred cell line for culturing influenza viruses. Identification of an unknown influenza virus can be carried out by IFA using specific monoclonal antibodies (see above) or, alternatively, by haemagglutination (HA) and antigenic analysis (subtyping) by haemagglutination-inhibition (HAI) using selected reference antisera.

Unlike other influenza A strains, influenza A/H5 will also grow in other common cell lines such as Hep-2 and RD cells. Standard biosafety precautions should be taken when handling specimens and cell cultures suspected of containing highly pathogenic avian influenza A.

***GOLDEN RULE. Clinical specimens from humans and from swine or birds should never be processed in the same laboratory.***

## Materials required

- Madin-Darby Canine Kidney cells (MDCK), ATCC CCL34
- WHO Influenza Reagent Kit for the Identification of Influenza A/H5 Virus. Reagents for identification of A/H5 in cell culture include:
  - influenza A/H5 control antigen: inactivated virus
  - goat serum to A/Tern/South Africa/61/ H5
  - chicken pooled serum to A/Goose/Hong Kong/437-4/99
- WHO Influenza Reagent Kit (Annually distributed)
  - A (H1N1) and A (H3N2) reference antigens and reference antisera
- Receptor-destroying enzyme (RDE).
- Red blood cells ( chicken, turkey, human type O, or guinea-pig red blood cells) in Alsever's solution.

## Procedure

1. Standard laboratory cell-culture procedures should be followed for the propagation of cell cultures, the inoculation of specimens, and the harvesting of infected cells for IFA or culture supernatant for HA and HAI testing (Lennette & Schmidt, 1979; WHO, 2002). Standard laboratory biosafety

guidelines should be followed when manipulating amplified virus ([http://www.who.int/csr/disease/avian\\_influenza/guidelines/handlingspecimens/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/index.html)).

2. Standard HA and HAI procedures should be followed, with the inclusion of all recommended controls. Specific details relating to the reference sera and antigens are included in the *WHO manual on animal influenza diagnosis and surveillance* (WHO, 2002).

### **Interpretation of results**

The highest dilution of virus that causes complete haemagglutination is considered to be the HA titration end-point. The HAI end-point is the last dilution of antiserum that completely inhibits haemagglutination. The titre is expressed as the reciprocal of the last dilution.

Identification of the field isolate is carried out by comparing the results of the unknown isolate with those of the antigen control. An isolate is identified as a specific influenza A subtype if the subtype-specific HAI titre is four-fold or greater than the titre obtained with the other antiserum.

Nonspecific agglutinins may be present in sera and may result in false-negative reactions; alternatively, some isolates may be highly sensitive to non-specific inhibitors in sera, resulting in false-positive reactions.

### ***Polymerase chain reaction***

Polymerase chain reaction (PCR) is a powerful technique for the identification of influenza virus genomes. The influenza virus genome is single-stranded RNA, and a DNA copy (cDNA) must be synthesised first using a reverse transcriptase (RT) polymerase. The procedure for amplifying the RNA genome (RT-PCR) requires a pair of oligonucleotide primers. These primer pairs are designed on the basis of the known HA sequence of influenza A subtypes and of N1 and will specifically amplify RNA of only one subtype. DNAs generated by using subtype-specific primers can be further analysed by molecular genetic techniques such as sequencing. A WHO technical working group is currently evaluating the standard and real time primers now being widely used for diagnostics of H5N1. In the past two years, the genetic diversification of the H5N1 viruses has made recommendation of specific primer sets more and more difficult. Additionally, the increase in capacity to perform real time PCR has brought this technology to more and more labs. The protocol and recommended primers below are for standard PCR where products are evaluated by gel electrophoresis and should be considered as effective for the identification of so-called Clade 1 and 3 viruses. The H5 primer set may also be effective for other H5N1 clades (there are now as many as 10 of these based on publicly available sequences), however it is recommended that laboratories wishing to have a full set of primers for all clades contact one of the members of the WHO Expert Committee on AI PCR (see Annex ) or a member of the WHO H5 reference laboratories<sup>1</sup> for assistance in identifying the optimal primers for their region. Other primer sets in routine use for H5 (particularly Clade 1,2,3) may be obtained from the publication by Spackman et. al. 2002. The range of positivity for the recommended N1 primers is likewise under review and investigators should not consider these primers as reactive across all recent H5N1 strains.

### **Materials required**

- QIAamp Viral RNA Mini Kit or equivalent extraction kit
- QIAGEN OneStep RT-PCR kit
- RNase inhibitor (ABI) 20U/  $\mu$ l
- Sterile microcentrifuge tubes, 0.2, 0.5 and 1.5 ml

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- **Primer sets**  
 HA gene primers for Clade 1 and 3 H5 HA amplification (modified from Yuen et al. 1998):  
 H5-1: GCC ATT CCA CAA CAT ACA CCC  
 H5-3: CTC CCC TGC TCA TTG CTA TG  
 Expected product size: 219 bp  
  
 HA gene primers for H9 amplification:  
 H9-426: GAA TCC AGA TCT TTC CAG AC  
 H9-808R: CCA TAC CAT GGG GCA ATT AG  
 Expected product size: 383 bp  
  
 NA gene primers for N1 amplification (Wright et al. 1995):  
 N1-1: TTG CTT GGT CGG CAA GTG C  
 N1-2: CCA GTC CAC CCA TTT GGA TCC  
 Expected product size: 616bp

- Positive control (Obtained upon request from a WHO H5 Reference Laboratory <sup>1</sup>)
- Adjustable pipettes, 10, 20 and 100  $\mu$ l
- Disposable filter tips
- Microcentrifuge, adjustable to 13 000 rpm
- Vortex mixer
- Thermocycler
- Agarose gel casting tray, electrophoresis chamber and power supply
- UV-light box or hand-held UV light (302 nm)

## Procedure

1. Extract viral RNA from clinical specimen with QIAamp viral RNA mini kit or equivalent extraction kit according to manufacturer's instructions.
2. One step RT-PCR

### H5 or N1

Prepare master mixture for RT-PCR as below:

5x QIAGEN RT-PCR buffer	10 $\mu$ l
dNTP mix	2 $\mu$ l
5x Q-solution	10 $\mu$ l
Forward primer (5 $\mu$ M)	6 $\mu$ l
Reverse primer (5 $\mu$ M)	6 $\mu$ l
Enzyme mix	2 $\mu$ l
RNase inhibitor (20U/ $\mu$ l)	0.5 $\mu$ l
Water (Molecular grade)	9 $\mu$ l
<b>Total</b>	<b>45 <math>\mu</math>l</b>

Add 5  $\mu$ l viral RNA

### H9

Prepare master mixture for RT-PCR as below:

5x QIAGEN RT-PCR buffer	10 $\mu$ l
dNTP mix	2 $\mu$ l

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Forward primer (5 uM)	6µl
Reverse primer (5 uM)	6µl
Enzyme mix	2µl
RNase inhibitor (20U/ul)	0.5µl
Water (Molecular grade)	19µl
<b>Total</b>	<b>45µl</b>

Add 5 µl viral RNA

### 3. RT-PCR reaction for H5, N1, H9

Set the follow PCR conditions:

Reverse transcription	30 min	50 °C
Initial PCR activation	15 min	95 °C
3-step cycling		
Denaturation	30 sec	94 °C
Annealing	30 sec	55 °C
Extension	30 sec	72 °C
Number of cycles	40	
Final extension	2 min	72 °C

### 4. Agarose gel electrophoresis of PCR product.

5. Prepare agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of maker and PCR product bands under UV light.

### Interpretation of results

The expected size of PCR products for influenza A/H5 is 219 bp, for A/H9 is 383 bp, and for N1 is 616 bp. If the test is run without a positive control, products should be confirmed by sequencing and comparison with sequences in deposited databases. The absence of the correct PCR products (i.e. a negative result) does not rule out the presence of influenza virus. Results should be interpreted together with the available clinical and epidemiological information. Specimens from patients with a high probability of infection with influenza A/H5 or H9 should be tested by other methods (IFA, virus culture or serology) to rule out influenza A (A/H5 or H9) infection.

### Laboratory confirmation

All laboratory results for influenza A/H5, H7 or H9 during Interpandemic and Pandemic Alert periods of the WHO Global Influenza Preparedness Plan

([http://www.who.int/csr/resources/publications/influenza/WHO\\_CDS\\_CSR\\_GIP\\_2005\\_5/en/index.html](http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_5/en/index.html))

should be confirmed by a WHO H5 Reference Laboratory<sup>1</sup> or by a WHO recommended laboratory.

Influenza A/H5, H7 or H9 -positive materials, including human specimens, RNA extracts from human specimens, and influenza A/H5, H7 or H9 virus in cell-culture fluid or egg allantoic fluid, should be forwarded to a WHO H5 Reference Laboratory<sup>1</sup> or a WHO recommended laboratory. Communication and publication of analysis results should be according to the WHO Guidance for the timely sharing of influenza viruses/specimens with potential to cause human influenza pandemics

([http://www.who.int/csr/disease/avian\\_influenza/guidelines/Guidance\\_sharing\\_viruses\\_specimens/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/Guidance_sharing_viruses_specimens/en/index.html)).

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## Serological identification of influenza A/H5 infection

Serological tests available for the measurement of influenza A-specific antibody include the haemagglutination inhibition test, the enzyme immunoassay, and the virus neutralization tests. The microneutralization assay is the recommended test for the measurement of highly pathogenic avian influenza A specific antibody. Because this test requires the use of live virus, its use for the detection of highly pathogenic avian influenza A specific antibody is restricted to those laboratories with Biosafety Level 3 containment facilities.

## References

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## Annex

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