World Health Organization

WHO Information for Laboratory Diagnosis of New Influenza A (H1N1) Virus in Humans

All un-subtypable influenza A specimens are strongly recommended to be sent immediately to one of the five WHO collaborating Centres for influenza for diagnosis and further characterization.

21 May 2009

This document provides information on the diagnostics available as of the above date for the human influenza A (H1N1) A/California/4/2009-like viruses. Further diagnostic information will be updated when available.

Specimens

Upper respiratory tract specimens as recommended for seasonal influenza investigation are the most appropriate. Samples should be taken from the deep nostrils (nasal swab), nasopharynx (nasopharyngeal swab), Nasopharyngeal aspirate, throat or bronchial aspirate. It is not yet known which clinical specimen gives the best diagnostic yield. Appropriate precautions should be taken in collecting specimens since this may expose the collector to respiratory secretions from patients.

There is, as yet, no information on the diagnostic value of non-respiratory specimens, e.g., stool samples.

Acute and convalescent serum specimens should be used for the detection of rising antibody titres.

Laboratory tests

Molecular diagnostics

Molecular diagnostics are currently the method of choice for influenza A (H1N1) swine lineage (swl) virus (A/California/4/2009-like viruses).

The use of different target gene assays is more appropriate for correct identification of this virus. The following gene targets are important: type A influenza matrix gene; haemagglutinin gene specific for influenza A (H1N1)swl virus and haemagglutinin gene specific for seasonal influenza A H1/H3 and other subtypes.

The following protocols are currently available:
— influenza A type-specific conventional and realtime PCR (see Annexes 1 and 2); and

— CDC realtime RT-PCR (rRT-PCR) protocol for the detection and characterization of influenza A (H1N1) (version 2009).[1]

Sequence analyses of the type A influenza matrix gene PCR product using the primers in the WHO protocols (see Annex 1) will differentiate between M genes of swine-lineage and seasonal H1N1 viruses, however, additional analysis should be performed to confirm the origin of the virus.

Conventional RT-PCR assays are currently being evaluated. An update will be published when available.

Virus isolation and typing by haemagglutination inhibition or immunofluorescence:

Current protocols for virus isolation of seasonal influenza viruses using MDCK cells and egg inoculation can be used, although their sensitivity remains to be determined (see section on Biosafety below).

Turkey, chicken, guinea pig and human red blood cells will agglutinate with the influenza A (H1N1)swl virus.

Polyclonal antibodies specific for subtype H1 seasonal influenza viruses from the WHO influenza reagent kit will **not** react in the haemagglutination inhibition (HAI) test with the current influenza A (H1N1)virus.

Results obtained using the H1 monoclonal antibodies in the WHO kit should not be taken as conclusive and further verification is recommended.

Rapid tests or immunofluorescence:

The sensitivity and specificity of rapid-point-of-care or immunofluorescence tests designed for direct detection of influenza A viruses are currently unknown. An update will be published when available. It should be emphasized that these tests will not differentiate seasonal influenza from influenza A (H1N1)swl virus.

Serology

HAI and microneutralization tests using influenza A (H1N1)swl virus are expected to be able to detect antibody responses following infection.

Interpretation of laboratory results

- PCR — A sample is considered positive if results from tests using two different PCR targets (e.g. primers specific for universal M gene and swine H1 haemagglutinin gene) are positive but the PCR for human H1 + H3 is negative. If RT-PCR for multiple haemagglutinin (HA) targets (i.e. H1, H3, and H1-swine-lineage) give positive results in the same specimen, the possibility of PCR contamination should first be excluded by repeating PCR procedure using new RNA extract from the original specimen or RNA extract from another specimen. If repeated positive results for multiple HA targets are

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[1] http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html

obtained, this raises the possibility of co-infection, which should be confirmed by sequencing or virus culture. Annex 3 shows a flowchart for use in interpreting PCR results.

- CDC realtime PCR assays — Results should be interpreted as described in the CDC H1N1 real time assay manual. [1]

- A negative PCR result does not rule out that a person may be infected with influenza A (H1N1) virus. Results should be interpreted in conjunction with the available clinical and epidemiological information. Specimens from patients whose PCR results are negative but for whom there is a high suspicion of A (H1N1) infection should be further investigated and tested by other methods such as virus culture or serology, to rule out influenza A (H1N1)swl infection (see flowchart in Annex 3).

- Serology — A four-fold or greater rise in specific influenza A (H1N1)swl virus antibody titres indicates recent infection with the virus.

- Sequencing — At this stage, sequencing of at least one target product is essential for confirmation of conventional PCR.

- Virus isolation — Identification and typing of a cultured influenza virus can be carried out by PCR, indirect fluorescent antibody (IFA) testing using specific NP monoclonal antibodies, or HA and antigenic analysis (subtyping) by HAI using selected reference antisera.

Referral for confirmation and further characterization

Laboratories with no capacity for diagnosis of influenza A viruses are recommended to send representative specimens from suspect cases of influenza A (H1N1), according to case definition guidance by WHO, [2] to one of the WHO Collaborating Centres for influenza (WHOCCs).

Specimens with laboratory results indicative of influenza A that are untypeable ((i.e. negative for influenza A(H1) and A(H3)); are not confirmed according to the WHO criteria) should be forwarded to one of the WHOCCs for confirmation.

Laboratories with no virus isolation capacity (or which do not have the required biosafety containment level) should forward the specimens to any of the WHO CCs.

Standard influenza specimen storage, packaging and shipping practice, together with relevant IATA regulations, should be followed.

Biosafety

Diagnostic laboratory work on clinical specimens from patients who are suspected cases of influenza A (H1N1)swl virus infection should be conducted in BSL-2 containment conditions with the use of appropriate personal protective equipment (PPE). All clinical specimen manipulations should be done inside a certified biosafety cabinet (BSC). Please refer to the WHO *Laboratory biosafety manual*, 3rd edition.[3]

[2] http://www.who.int/csr/resources/publications/swineflu/interim_guidance/en/index.html
[3] http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Virus isolation currently requires higher biosafety containment measures. Please refer to the document *WHO Laboratory biorisk management for laboratories handling human specimens suspected or confirmed to contain influenza A (H1N1) causing the current international epidemics* for recommended guidance.[4]

Testing algorithms

The overall approach to influenza virus detection by RT-PCR should be considered in the context of the national situation, e.g., how many specimens can be handled (throughput), what gene sequence to target for RT-PCR, and whether to use concurrent or sequential testing for RT-PCR of M, NP and HA genes.

Good laboratory practices

Standard protocols for all procedures should be in place and reviewed regularly. Making sure that the recommended reagents are used and handled properly is critical, as reactions are complex and problems with a single reagent can have large effects on the results obtained.

Validation

All protocols should always be validated in each laboratory to ensure adequate specificity and sensitivity using the same controls that are employed in each run.

Quality assurance

Standard quality assurance protocols and good laboratory practices should be in place. Participation in the National Influenza Centres (NIC) evaluation exercises (external quality assessment programme) is highly recommended to confirm that laboratories are achieving an adequate level of sensitivity and specificity in their tests.

Training of personnel

Familiarity with protocols and experience in correct interpretation of results are cornerstones for successful execution of the diagnostic tests.

Facilities and handling areas

Specimen and reagent handling facilities (including cold chains) with appropriate separation for different steps of RT-PCR must be in place to prevent cross- contamination. Facilities and equipment should meet the appropriate biosafety level. RT-PCR should be performed in a space separate from that used for virus isolation techniques.

Equipment

Equipment should be used and maintained according to the manufacturer's recommendations.

[4] http://www.who.int/csr/resources/publications/swineflu/Laboratorybioriskmanagement.pdf

Annex 1:
Conventional RT-PCR analyses for the matrix gene of Influenza type A viruses

Conventional RT-PCR protocol [5]

The protocols and primers for conventional PCR and gel electrophoresis of products to detect influenza A viruses in specimens from humans are given below. These protocols have been shown to be widely effective for the identification of influenza type A viruses when used with the reagents and primers indicated. It is recommended that laboratories that have concerns about identifying currently circulating viruses contact one of the WHO reference laboratories[6] for diagnosis of influenza A/H5 infection or one of the WHO Collaborating Centres[7] for influenza for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp® Viral RNA Mini Kit (QIAGEN, Cat#52904. Other extraction kits can be used after proper evaluation）.
- OneStep RT-PCR Kit (QIAGEN, Cat#210212)
- RNase Inhibitor 20U/µl（Applied Biosystems, Cat# N8080119）
- RNase-free water
- Ethanol (96–100%)
- Microcentrifuge (adjustable up to 13 000 rpm)
- Adjustable pipettes（10, 20, 200, and 100 µl）
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes（0.2, 1.5 ml）
- Thermocycler (PCR machine)
- Primers sets
- Positive control (can be obtained upon request from a WHO Collaborating Centre in Centers for Disease Control and Prevention, Atlanta, USA)

Primers

Gene: **M**
Influenza type A viruses

Primers sequences

M30F2/08: 5`- ATGAGYCTTYTAACCGAGGTCGAAACG -3`
M264R3/08: 5`- TGGACAAANCGTCTACGCTGCAG -3`
Expected product size 244bp (reference: NIID[8])

[5] Protocol provided by Virology Division, Centre for Health Protection, Hong Kong SAR, China (WHO H5 Reference Laboratory).
[6] http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/
[7] http://www.who.int/csr/disease/influenza/collabcentres/en/
[8] Primers designed by Laboratory at National Institute of Infectious Diseases (NIID), Tokyo, Japan (WHO Collaborating Centre for Reference and Research on Influenza)

Procedure

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit, according to manufacturer's instructions.

2. Perform one step RT-PCR

   - Take out the reagents from its storage, thaw them at room temperature. After they are thawed out, keep them on ice.

   - Preparation of master mix (operate on ice)
   - Add the following to a microcentrifuge tube and mix gently by pipetting the master mix up and down ten times. (Note: To avoid localized differences in salt concentration. It is important to mix the solutions completely before use)

Reaction without Q-Solution

Water (molecular grade)	9.5 µl
5x QIAGEN RT-PCR buffer	5.0 µl
dNTP mix	1.0 µl
Forward primer (10 µmol/l)	1.5 µl
Reverse primer (10 µmol/l)	1.5 µl
Enzyme mix	1.0 µl
RNase inhibitor (20U/µl)	0.5 µl
Total volume	**20.0 µl**

- Dispense 20 µl of the master mix. to each PCR reaction tube.
- Add 5 µl sample RNA to the master mix. For control reactions, use 5 µl of RNase-free water for negative control, and 5 µl of appropriate viral RNA for positive control.
- Program the thermal cycler according to Thermal cycling conditions.
- Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached at 50 ˚C. Then place the PCR tubes in the thermal cycler.

Thermal cycling conditions

Reverse transcription	30 min, 50 °C
Initial PCR activation	15 min, 95 °C
<u>Three-step cycling</u>	
Denaturation	30 sec, 94 °C
Annealing	30 sec, 50 °C
Extension	1 min ,72 °C
No. of cycles	45
Final extension	10 min, 72 °C
Hold	4 °C ∞

3. Agarose gel electrophoresis of RT-PCR products

Prepare agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of marker under UV light. An example of the material required and the procedure is given below.

Materials required

- Agarose gel casting tray and electrophoresis chamber
- Power supply and electrode leads
- UV light box (λ = 302 nm)
- Camera and Polaroid® film or computer connected to the camera
- Adjustable pipettes
- 2% agarose gel in 1× TAE buffer
- 1× TAE buffer
- Ethidium bromide (10 mg/ml)
- 6x Gel loading buffer (GLB)
- Molecular weight marker

Procedure

A) Casting the agarose gel:

i) Place a gel-casting tray onto a gel-casting base. Insert a comb and level the base.
ii) Prepare 2% agarose by weighing out 4 g of agarose powder and dissolve it in 200ml 1× TAE buffer. Dissolve the agar by heating in microwave oven.
iii) Cool the melted agarose to about 60 °C, and then add 10 µl of ethidium bromide.
iv) Pour the melted agarose into the gel-casting tray.
v) Allow the gel to solidify at room temperature.
vi) Remove the comb from the frame.
vii) Place the tray into the electrophoresis chamber with the wells at the cathode side.
viii) Fill the buffer chamber with 1× TAE at a level that can cover the top of the gel.

B) Sample loading:

i) Add 5 µl of the gel loading buffer to each PCR tube.
ii) Load molecular weight marker to the first well of the agarose gel.
iii) Pipette 15 µl of the PCR product/GLB to the gel.
iv) Close lid on chamber and attach the electrodes. Run the gel at 100V for 30–35 min.
v) Visualize the presence of marker and PCR product bands with a UV light.
vi) Document gel picture by photographing it.

Interpretation of results

The size of PCR products obtained should be compared with the expected product size. If the test is run without a positive control, products must be confirmed by sequencing and comparison with available sequences.

Annex 2:
Realtime RT-PCR analyses for the matrix gene of Influenza type A viruses

Realtime RT PCR poses different challenges than conventional RT-PCR. In addition to the RT-PCR considerations described in Annex 1, specific considerations for realtime RT-PCR include:

- Ensuring appropriate equipment, software, and fluorescent-based reagents are used and handled correctly.
- Ensuring appropriate training of personnel for interpretation of results (experience in recognizing true positives, interpreting controls/Ct value and aberrant fluorescence, etc., is crucial).
- Validation in the laboratory and optimization of reactions are essential to making quantitative determinations.
- There is little likelihood of contamination when reactions are discarded after testing. However, many laboratories do further post-reaction analysis (e.g. restriction fragment length polymorphism using gels, sequencing, etc.) which can re-introduce contamination.

Realtime RT-PCR protocol 1 [9]

Extract viral RNA from clinical specimen as described in Annex 1: Conventional RT-PCR analyses.

Materials required

Reverse transcription

10x PCR buffer I with 15 mmol/l $MgCl_2$ (Applied Biosystems)
Random hexamer 50 µmol/l (Applied Biosystems)
MuLV Reverse Transcriptase 50 U/µl (Applied Biosystems)
RNase Inhibitor 20 U/µl (Applied Biosystems)

Realtime PCR

LightCycler® – FastStart™ DNA Master HybProbe kit (Roche)
Primers and probes mix: Add equal volume of the following components to prepare primers and probes mix for M gene

[9] Protocol provided by Virology Division, Centre for Health Protection, Hong Kong SAR, China, WHO H5 Reference Laboratory

Primers and probes

Influenza type A viruses

Primers sequences

FLUAM-1F: 5'-AAGACCAATCCTGTCACCTCTGA-3' (10 μmol/l)
FLUAM-2F: 5'-CATTGGGATCTTGCACTTGATATT-3' (10 μmol/l)
FLUAM-1R: 5'-CAA AGCGTCTACGCTGCAGTCC-3' (10 μmol/l)
FLUAM-2R: 5'-AAACCGTATTTAAGGCGACGATAA-3' (10 μmol/l)
FLUA-1P: 5'-(FAM)-TTTGTGTTCACGCTCACCGT-(TAMRA)-3' (5 μmol/l)
FLUA-2P: 5'-(FAM)-TGGATTCTTGATCGTCTTTTCTTCAAATGCA-(TAMRA)-3' (5 μmol/l)

The working primer and probe mix is prepared by mixing the above 6 reagents in equal volumes

Procedure

1. Perform RT step using the reagents shown in the following table and instructions 1-3 below it.

Reagent	Volume (μl) per reaction
10x PCR buffer I with 15 mmol/l $MgCl_2$	2
Extra 25 mmol/l $MgCl_2$	2.8
2.5 mmol/l dNTPs	8
Random hexamer 50 μmol/l	1
RNAase inhibitor 20U/μl	1
Reverse transcriptase 50 U/μl	1
Extracted RNA	4.2

i) Vortex and centrifuge the tube with the mixture briefly (~3 sec).
ii) Stand the tube at room temperature for 10 minutes and then incubate at 42 °C for at least 15 minutes.
iii) Incubate the tube at 95 °C for 5 minutes and then chill in ice.

2. Perform realtime PCR

i) Prepare “Hot Start” reaction mix by gently pipetting 60 μl of LightCycler-FastStart Reaction Mix HybProbe (vial 1b) into the LightCycler-FastStart Enzyme (vial 1a).
ii) For each test sample and positive and negative controls, prepare reagent mix with primers and probe mix as described in the following table:

Master Mix:

Reagent	Volume (μl)
PCR- grade H_2O	7.6
$MgCl_2$ (25 mmol/l)	2.4
Primers and probe mix	3
“Hot Start” reaction mix	2
Total volume	**15**

Each reaction:

Master Mix 15 µl
cDNA 5 µl

PCR temperature-cycling conditions:

Temperature (°C)	Time (minute:second)	No. of cycles
95	10:00	**1**
95	0:10	**} 50**
56	0:15	
72	0:10	
40	0:30	**1**

Real-time RT-PCR protocol 2[10]

Extract viral RNA from clinical specimen as described in Annex 1: Conventional RT-PCR analyses .

Materials required

- QIAGEN® QuantiTect®, Probe RT-PCR kit (#204443):
  - 2 x QuantiTect®, Probe RT-PCR Master Mix;
  - QuantiTect®, RT Mix;
  - RNase-free water.
- RNase Inhibitor (Applied Biosystems Cat# N808-0119)
- Primers
- TaqMan® MGB Probe
- Equipment: Chromo-4 Real-time PCR Detection system (BioRad), LigntCycler 2 (Roche) or LigntCycler 480 (Roche)

Real-time PCR

Real-time PCR is performed by One-step RT-PCR using TaqMan® probe

Primers and probes:

Gene: **Type A (M)**

Primers sequences

MP-39-67For 5'-CCMAGGTCGAAACGTAYGTTCTCTCTATC-3' (10 µmol/l)
MP-183-153Rev 5'-TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA-3' (10 µmol/l)

Probe sequences

[10] Protocol provided by National Institute of Infectious Diseases (NIID), Center for Influenza Virus Research, Tokyo, Japan (WHO Collaborating Centre for Reference and Research on Influenza).

MP-96-75ProbeAs 5' (FAM)-ATYTCGGCTTTGAGGGGGCCTG-(MGB)-3' (5 pmol/µl)

Reaction Mixture

Reagent	**Volume (µl)**
2x QuantiTect® Probe RT-PCR Master Mix	12.5
Forward Primer (10 µmol/l)	1.5
Reverse Primer (10 µmol/l)	1.5
TaqMan MGB Probe (5 pmol/µl)	0.5
QuantiTect® RT Mix	0.25
RNase-free water	3.75
Total	**20**

Procedure

1. Dispense 20 µl of the reaction mixture into each RT-PCR reaction plate.
2. Add 5 µl of the sample RNA to the reaction mixture. For control reactions, use 5 µl of RNase-free water for negative control and 5 µl of appropriate viral RNAs for positive control.
3. Program the thermal cycler as shown in the table below
4. Start the realtime RT-PCR program while the RT-PCR reaction plates are still ***on ice***.
5. ***Wait until the thermal cycler has reached at 50 °C*** then place the RT-PCR reaction plates in the thermal cycler.

RT-PCR temperature-cycling conditions: Chromo-4 Real-time PCR Detection system (BioRad)

Temperature (°C)	**Time (minute:second)**	**No. of cycles**
50	30:00	**1**
95	15:00	**1**
94	0:15	**} 45**
56	1:00	

RT-PCR temperature-cycling conditions: LightCycler 2 (Roche) and LightCycler 480 (Roche)

Temperature (°C)	**Time (minute:second)**	**No. of cycles**
50	30:00	**1**
95	15:00	**1**
94	0:15 (ramp rate 1.2 ° C/sec)	**} 45**
56	1:15 (ramp rate 1.2 ° C/sec) Data collection	

Annex 3:
PCR testing algorithm and results interpretation