SWINE FLU
EMERGING THREAT

VIEW POINT

Laboratory Diagnosis of Novel H1NI Virus

N.M.Kaore*, S.N.Kaore, P. Sharma, V.K.Yadav, R.Sharma

This novel influenza virus A /H1NI contains a combination of human, swine and avian virus genes. One the most notable thing about this novel Influenza A/H1N1 virus is high efficiency of human to human transmission which has culminated into alarming spread across the globe in very short time resulting in pandemic (1,2). This calls for good laboratory services for rapid diagnosis and treatment.

Influenza-like-illness (ILI) is defined as fever (temperature of 100°F [37.8°C] or greater) and a cough and/or a sore throat in the absence of a known cause other than influenza.Case definition for the reporting of pandemic (H1N1) 2009 virus infections in humans:-The following case definition should be used to report confirmed cases of pandemic (H1N1) 2009 virus infection to WHO. An individual with laboratory ***confirmed*** pandemic (H1N1) 2009 virus infection by one or more of the following tests-*Polymerase Chain Reaction (PCR); Viral Culture; 4-fold rise in pandemic (H1N1) 2009 virus specific neutralizing antibodies*.

A ***probable case*** of novel influenza A (H1N1) virus infection is defined as a person with an influenza-like-illness who is positive for influenza A, but negative for human H1 and H3 by influenza RT - PCR. A ***suspected case*** of novel influenza A (H1N1) virus infection is defined as a person who does not meet the confirmed or probable case definition, and is not novel H1N1 test negative, and is/has: a previously healthy person < 65 years hospitalized for ILI or ILI & resides in a state without confirmed cases, but has traveled to a state or country where there are one or more confirmed or probable cases or ILI & has an epidemiologic link in the past 7 days to a confirmed case or probable case (3,4).

Testing for Novel influenza A (H1N1) Virus

Sample Collection :The duration of shedding with novel influenza A (H1N1) virus is unknown. Therefore, until data are available, the estimated duration of viral shedding is based upon seasonal influenza virus infection. Infected persons are assumed to be shedding virus and potentially infectious from the day prior to illness onset until resolution of fever. Infected persons should be assumed to be contagious up to 7 days from illness onset. Some persons who are infected might potentially shed virus and be contagious for longer periods (e.g. young infants, immunosuppressed, and immunocompromised persons) (4). 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 (5).

Laboratory Tests:- (6,7) A definitive diagnosis of seasonal human influenza A or B is made by isolating the virus or detecting it by properly validated antigen detection or nucleic acid testing methods. A presumptive diagnosis can be made by a validated rapid antigen or "point-of-care" test.

1) Molecular Diagnostics:-

Molecular diagnosis using real time reverse transcriptase Polymerase Chain Reaction (rRT-PCR) is currently the method of choice for diagnosis of influenza A (H1N1) swine lineage. In India this facility is available at National Institute of Virology (NIV), Pune and The National Institute of Communicable Diseases (NICD), New Delhi.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 (M gene); haemagglutinin gene specific for influenza A (H1N1) swl virus and haemagglutinin gene specific for seasonal influenza A H1/H3 and other subtypes (6,7).

The following protocols are currently available:-

A) Influenza A -specific conventional & realtime PCR.

B) CDC realtime RT-PCR (rRT-PCR) protocol for the detection and characterization of influenza A (H1N1) (version 2009).Sequence analysis of the type A influenza matrix gene PCR product using the primers in the WHO protocols will differentiate between M genes of swine-lineage and seasonal H1N1 viruses, however, additional analysis should be performed to confirm the origin.

From the Department of *Microbiology and Pharmacology, PCMS,Bhanpur Bhopal (MP), India
Correspondence to : Dr Navinchandra M. Kaore, Senior MIG-C/4, Pcms Campus, PCMS, Bhanpur, Bhopal -462037(MP)-India

Table 1. Collection, Labeling, Packaging and Transport of Specimens (5)

Specimens	Test / specimen collection	Timing of specimen collection
Respiratory aspirates (nasopharyngeal, tracheal)	Direct detection and culture: Aspirate respiratory secretions into viral transport media ("T/M") like • Viral transport medium • Hanks balanced salt solution • Tryptose-phosphate broth • Sucrose-phosphate broth • Cell culture medium • Veal infusion broth	As soon as possible after onset of illness
Respiratory swabs (nasopharyngeal, throat & nasal)	Culture: Vigorously swab mucous membrane, especially inflamed areas, with swabs with synthetic tips made of polyester or Dacron and with aluminium or plastic shafts. Place into viral transport media ("T/M") and break off shaft.Cotton tips with wooden shafts or calcium alginate swabs are not recommended.	As soon as possible after onset of illness
Paired sera	Viral titre: Collect 5 ml of blood sample in a plain container without anticoagulants.	Acute and convalescent samples 10-14 days apart.
Specimen labeling	Specimen container must be labeled with 2 unique patient identifiers matching the information on the request form.	
Specimen packaging	***Triple packaging system should be used for transport:*** -Primary container containing the specimen must be watertight, leak-proof, and properly and securely capped or screwed. - Secondary leak-proof container should be used to protect the primary container. Request form must be placed outside the secondary container, using a separate plastic bag. - Tertiary container (transport box) should have adequate strength for its capacity and intended use which can be cleansed and disinfected. It should bear the biohazard warning label.	
	Specimens should be sent to laboratory as soon as possible. If delay is unavoidable, keep at 4°C for up to 72 hours. They should be kept at 4°C and upright during transport to minimize the possibility of spillage.	

2) Virus Isolation and Typing by Haemagglutination Inhibition or Immunofluorescence

The traditional method of influenza virus recovery from clinical specimens is by cell culture. Virus isolation is usually more sensitive than the rapid culture and antigen detection assays, it recovers novel or highly divergent strains missed by other tests, it provides an isolate for subsequent characterization and consideration as potential vaccine strains, and it allows the simultaneous recovery of other respiratory viruses if an appropriate range of cell lines is used.Influenza virus replication within cell culture, often using Madin Darby Canine Kidney cells (MDCK), is detected by observing the cytopathic effect, generally manifested by 5th day. Virus is readily isolated (BSL-3 facilities are recommended) from the nose and throat of humans with infection as well as blood and rectal swabs.Virus isolation in the allantoic and amniotic cavities of 10-11 day-old embryonated chicken eggs is labour intensive and not commonly performed by diagnostic laboratories. However, it yields higher viral titres, and remains vital for vaccine production but requires biosafety level 2 and above.Initial typing of influenza virus isolates is most rapidly and conveniently accomplished by immunofluorescence using commercially available type-specific monoclonal antibodies, and should be performed as soon as possible after isolation. The reference virus subtyping method is the haemagglutination inhibition assay using specific antisera, a technique that characterizes both antigenic drift and shift. Subtyping using reverse transcription polymerase chain reaction (RT-PCR) with primers specific for various human and avian influenza strains can be performed on virus isolates or directly on clinical specimens. DNA microarrays have been used to detect type & subtype-specific amplification sequences (6,7).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 (6,7).

3) Serology

Serology can be used where specimens for virus isolation or antigen detection are negative, inadequate, or unavailable. It also provides useful, if delayed, surveillance data. Serological diagnosis of influenza is retrospective, as it requires acute and convalescent serum samples. Furthermore, it generally does not provide information on the antigenic composition of circulating strains.As influenza virus infection generally represents reinfection, most people have some pre-existing immunity. Thus, the detection of influenza-specific IgG or total antibodies on

a single serum specimen is not diagnostic of recent infection. The definitive serological diagnosis of acute influenza requires the demonstration of increasing antibody titres on paired acute and convalescent serum samples.HAI (Haemagglutination Inhibition) and microneutralization tests using influenza A (H1N1) swl virus are expected to be able to detect antibody responses following infection. A fourfold rise in antibody titer is taken as positive (6,7).

4) Rapid Tests or Immunofluorescence

Immunofluorescence or enzyme immunoassay using commercial type-specific monoclonal antibodies directed against conserved influenza antigens are the most common rapid assays performed directly on clinical specimens.The availability of the neuraminidase inhibitors has added impetus to the development of simple, rapid antigen detection assays, sometimes called point-of-care tests. Typically, these tests produce a visual result on an immunochromatographic strip using influenza A or B nucleoprotein-specific monoclonal antibodies within about 15 minutes of adding an extracted specimen. The sensitivity and specificity of rapid-point-of-care or immunofluorescence tests designed for direct detection of influenza A viruses are currently unknown. It should be emphasized that these tests will not differentiate seasonal influenza from (H1N1) swl virus (6,7).

Interpretation of Laboratory Results (6)

A) 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 obtained, this raises the possibility of co-infection, which should be confirmed by sequencing or virus culture.-CDC realtime PCR assays - Results should be interpreted as described in the CDC H1N1 real time assay manual.(http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html)-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 (H1N1)infection.

B) Serology:-A 4 fold or greater rise in specific H1N1virus antibody titres indicates recent infection.

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

D) 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.

E) Rapid Diagnostic Tests:-Preliminary data from the CDC suggest that rapid influenza diagnostic tests have a low overall sensitivity for novel influenza A (H1N1), MMWR reports. The tests were generally more sensitive for seasonal flu (range, 60% to 83%) than for novel influenza A/H1N1 (8).

Referral for Confirmation and Further Characterization:- Specimens should be forwarded to the WHO Collaborating Centers for influenza (WHOCCs) if a)laboratories with no capacity for diagnosis of influenza A viruses or virus isolation capacity (or which do not have the required biosafety containment level-2) b)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] (6).

References

1) Novel Swine Origin Influenza A (H1N1) Virus Investigation Team. Emergence of novel swine origin influenza A in humans. *N Eng J Med* 2009; 360:2505-15
2) World Health Organization WHO- Influenza A/ H1N1 - update 63, 30th August 2009. Available at:http://www.searo.who.int/LinkFiles/Influenza_A(H1N1)_map_H1N1_30Aug.pdf.Accessed on 6th September, 2009
3) World Health Organization WHO- Influenza A/ H1N1 - Human infection with pandemic (H1N1) 2009 virus: updated interim WHO guidance on global surveillance, 10 July 2009. Available at: http://www.who.int/csr/disease/swineflu/guidance/surveillance/WHO_case_definition_swine_flu_2009_04_29.pdf. Accessed on 6th September, 2009
4) Center for Disease Control (CDC),USA, Interim Guidance on Case Definitions to be Used For Investigations of Novel Influenza A (H1N1) Cases.Available at: http://www..cdc.gov/h1n1/flu/casedef.htm. Accessed on 6th September, 2009
5) World Health Organization,Geneva, Geneva,2004, Laboratory biosafety manual,Third edition.Available at: http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/. Accessed on 6th September, 2009
6) World Health organization (WHO), 21 May 2009, WHO Information for Laboratory Diagnosis of New Influenza A (H1N1)Virus in Humans.Available at: http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf Accessed on 6th September, 2009
7) Dwyer DE, Smith DW,Catton MG, Barr IG. Laboratory diagnosis of human seasonal and pandemic influenza virus infection. *Med J Australia* 2006; 185 (10 Suppl): S48-S53.
8) CDC: H1N1 Update: Rapid Diagnostic Tests Have Low Overall Sensitivity for Flu,August 7,2009.Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5830a2.htm.Accessed on 6th Septr, 2009