SENTINEL LABORATORY GUIDELINES FOR SUSPECTED AGENTS OF BIOTERRORISM AND EMERGING INFECTIOUS DISEASES

Avian Influenza A H5N1

American Society for Microbiology
Credits: Avian Influenza A H5N1

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I. General Information

A. Special Instructions
Do NOT perform culture on specimens if avian influenza A H5N1 is suspected, unless performed under Biosafety Level 3 (BSL-3) laboratory conditions. Very few laboratories have the capability to operate under BSL-3 conditions. These instructions for culturing influenza A H5N1 may change if the epidemiological and clinical characteristics of the disease, and pathogenesis of the virus change.

Sentinel laboratories may perform rapid influenza antigen tests and direct fluorescent antibody staining on respiratory specimens from suspected avian influenza A H5N1 cases, but only under BSL-2 conditions in a Class II biological safety cabinet. However, influenza A H5N1-specific reverse-transcriptase (RT)-PCR, available at Laboratory Response Network (LRN) Reference and other limited laboratories, is the preferred method because of its high sensitivity.

The next influenza pandemic will likely result in a dramatic increase in testing orders and the temporary deterioration of production and distribution systems. As such, laboratorians should plan for shortages of basic laboratory supplies.

The U.S. Department of Health and Human Services has prepared an influenza pandemic plan that outlines testing and biosafety requirements (www.hhs.gov/pandemicflu/plan/).

B. Description of Organism
Influenza A virus is a member of the family Orthomyxoviridae. Influenza viruses are enveloped, with a segmented, single-stranded RNA genome. This family also contains influenza B and C viruses. Point mutations in the envelope protein hemagglutinin (H), referred to as antigenic drift, result in the emergence of new strains of influenza A and B viruses and the resultant annual outbreaks and epidemics. Subtyping of influenza A virus is based on antigenic characteristics of two envelope proteins, H and neuraminidase (N). New influenza A virus subtypes emerge as the result of reassortment of H and N sequences from two different subtypes, referred to as antigenic shift. These new subtypes are responsible for influenza pandemics. There are currently 16 recognized H subtypes and 9 recognized N subtypes. While virtually all combinations of influenza A subtypes naturally infect waterfowl and shorebirds, certain subtypes infect poultry and mammalian species. Subtypes H1N1, H3N2, H2N2 and H1N2 have circulated, or are currently circulating widely, among humans. Subtype H5N1, causing highly pathogenic avian influenza, was identified in 1996 in southern China. Influenza A H5N1 is significant, though not unique, in its ability to cross normal species barriers and directly infect humans. Avian subtypes H9N2 and H7N7 are also known to cause infection in humans, so public health influenza surveillance programs monitor for emergence of any novel strains in humans. However, the wide geographical distribution of H5N1 in avian species, and the number and severity of human infections are unprecedented. If, or when, the virus reassorts to a strain transmitted readily among humans, and unless there is a dramatic
decrease in the pathogenicity of the resulting virus, the result will likely be an influenza pandemic with mortality rates not seen since the 1918 pandemic.

C. Epidemiology

The classical epidemiologic cycle of influenza A virus includes wild waterfowl and shorebirds, which are naturally infected; domestic waterfowl and poultry, which acquire virus from wild birds; pigs, which serve as “mixing vessels” for both avian and mammalian adapted strains; and humans, who are susceptible to the reassorted viruses. Reassortment can also occur during human-to-human transmission. Influenza A virus also infects maritime mammals, including seals and whales, dogs, and horses. The H5N1 virus has bypassed this epidemiologic cycle, crossed normal species barriers, and is capable of being transmitted directly from poultry to humans. First identified as a cause of highly pathogenic avian influenza in southern China in 1996, the virus has since spread to Southeast Asia, Middle East, Eastern and Western Europe, and Africa. H5N1 has been found in domestic fowl and a variety of migratory and resident wild bird species. Avian influenza strains infect the intestinal tract and are shed at high titers in feces. Transmission rates are high among birds congregating at bodies of water. The presence of H5N1 in a number of migratory bird species has resulted in its rapid spread among continents. In addition to birds, the virus has also been found in several mammalian species. Felines have become infected as a result of consumption of infected dead birds. Human infections caused by H5N1 were first identified in 1997. Human H5N1 infections are the result of exposure to high viral titers in infected birds or feces. There is evidence of human-to-human transmission, yet secondary cases are very limited due to avian host specificity of H5N1. As of September 10, 2008, nearly 400 confirmed human cases of avian influenza H5N1 have been reported to the World Heath Organization (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_09_10/en/index.html), and the mortality rate has exceeded 60%. Human cases have been reported from Azerbaijan, Bangladesh, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Lao People’s Democratic Republic, Myanmar, Nigeria, Pakistan, Thailand, Turkey, and Viet Nam. Cases from Indonesia and Viet Nam represent over 60% of the total reported cases.

Influenza pandemics result from the emergence of antigenically distinct subtypes of influenza A virus. During the twentieth century, influenza pandemics occurred in 1918, 1957 and 1968. The subtypes causing these pandemics all had avian origins, and adapted to high transmissibility among humans. Pathogenicity among the pandemic virus strains varied.

Influenza virus is considered a Category C biothreat agent. Agents in this category are emerging, readily available, and easily disseminated. In addition, they are capable of causing high morbidity and mortality rates http://www.bt.cdc.gov/agent/agentlist-category.asp
D. Clinical Features
Influenza caused by H5N1 shares features with those caused by the Spanish influenza pandemic of 1918. Morbidity and mortality are severe in previously healthy, young and middle-aged persons. The innate immune response is in part responsible for pathogenesis, causing fluid accumulation in the lungs. While influenza caused by H5N1 is notable for its aggressive course and high mortality rates, evidence indicates that mild disease and asymptomatic infections occur. Symptomatic cases are characterized by high fever, cough, and lower respiratory tract symptoms (shortness of breath, pulmonary infiltrates) in virtually 100% of patients. Diarrhea occurs more frequently than with influenza caused by human-adapted subtypes. The frequency of pneumonia and diarrhea distinguish avian from seasonal influenza. Over 50% of reported H5N1 influenza cases were fatal. Death is primarily due to respiratory or multi-organ failure. Unlike human-adapted subtypes, H5N1 is found in relatively high titers in lower respiratory tract specimens, throat swabs and stool. Although it is not known if infections with a pandemic strain will have frequently demonstrable viremia, the current H5N1 virus has been isolated from serum. The Centers for Disease Control and Prevention (CDC) has developed a case definition and risk assessment for human influenza caused by influenza A H5N1 (http://www2a.cdc.gov/han/archivesys/ViewMsgV.asp?AlertNum=00246). When there is a suspect case, clinicians and infection control practitioners should work closely with state health department epidemiologists to perform a case risk assessment.

E. Treatment and Prevention
Recent isolates of influenza A H5N1 show varying resistance to the adamantanes (amantadine and rimantadine): clade 1 viruses are resistant, while the majority of clade 2 viruses are sensitive. The neuraminidase inhibitors, oseltamivir and zanamivir, are active against influenza A H5N1. However, the emergence of high-level resistance to oseltamivir during oseltamivir treatment has been demonstrated in some patients with influenza A H5N1 infections. These two patients had detectable virus at the end of a full course of treatment. An important component of avian influenza A H5N1 pandemic preparedness programs is the stockpiling of adequate supplies of neuraminidase inhibitors. Clinical trials and development efforts are currently ongoing for both inactivated and attenuated influenza A H5N1 vaccines.

A number of scientific agencies and organizations including the Food and Drug Administration (FDA, http://www.fda.gov/cber/flu/fluhealthcare.htm) and the CDC (http://www.cdc.gov/flu/professionals/acip/specificpopulations.htm#HCP), recommend annual influenza vaccinations for health care personnel because of their increased risk of contracting and transmitting influenza. Sentinel laboratory personnel are strongly advised to adhere to these recommendations. While vaccination against seasonal influenza provides little or no protection against influenza A H5N1, it may provide protection against other emerging influenza strains. Safe and immunogenic H5 vaccines have been developed, but are not sold commercially. An H5 vaccine has been purchased by the U.S. federal government for
inclusion within the Strategic National Stockpile (http://www.cdc.gov/flu/avian/gen-info/vaccines.htm).

II. Procedures

A. General
Sentinel laboratories should NOT inoculate specimens suspected of containing influenza A H5N1 virus into cell culture. Only laboratories capable of performing culture under BSL 3 conditions with enhancements should perform culture to evaluate a suspected influenza H5N1 case. If these criteria are met, and culture is performed, consultation with CDC and the state public health laboratory is recommended.

The use of rapid antigen tests for influenza is increasing in laboratories, point of care locations, and in physicians’ offices. These tests are among the least reliable for diagnosis of influenza, and should not be used to rule out avian influenza in a suspect case, especially during the current pre-pandemic phase.

Although influenza A H5N1 is the agent of highest concern at this time, it is important to note that a pandemic could occur from other novel strains of influenza. For this reason, testing for H5N1 virus alone is not recommended and any unusual influenza viruses that cannot be subtyped should be referred to the CDC.

B. Precautions
Culture diagnosis of suspected avian influenza A H5N1 requires enhanced BSL-3 laboratory conditions. Enhancements include use of respirators, decontamination of all waste (solid and liquid), and showering of personnel before exiting. Molecular and rapid antigen testing can be performed on respiratory specimens under standard BSL-2 conditions in a Class II biological safety cabinet. A complete description of enhanced BSL-3 requirements is available at (www.hhs.gov/pandemicflu/plan/).

C. Specimens

**Respiratory specimens.** Throat swabs and lower respiratory samples such as bronchoalveolar lavage and tracheal aspirates are the preferred specimens for detection of influenza A H5N1 virus. Nasal swabs and aspirates are acceptable, but may contain lower titers than throat swabs. This is an important distinction between H5N1 and seasonal, human adapted influenza A subtypes.

Collection of a nasopharyngeal swab and a throat swab from the same patient (can be submitted in the same tube of viral transport medium) would provide optimal specimens for both human adapted and avian influenza strains.

**Rectal specimens.** In contrast to seasonal human influenza, diarrhea is a common symptom of H5N1 infections. Influenza A H5N1 viral RNA has been detected in rectal swabs by RT-PCR. However, current CDC testing recommendations
(www.hhs.gov/pandemicflu/plan/) do not include rectal/stool specimens. Additionally, rectal specimens are inappropriate for human-adapted influenza strains.

**Serum.** Influenza A H5N1-specific antibody can be detected in serum by the microneutralization assay. Paired specimens, the first collected during acute illness and the second collected 2-4 weeks later, are required for definitive diagnosis. Sentinel laboratories should contact their local or state public health laboratory, or the CDC for information on serologic testing for influenza A H5N1.

**Specimen collection and handling**
Detection of influenza A H5N1 is more likely from specimens collected within the first three days of illness onset. If possible, serial specimens should be collected over several days from the same patient to increase clinical sensitivity.

Dacron or rayon tipped swabs should be used for specimen collection, as other materials may inhibit RT-PCR. Rapid antigen detection kits provide or specify swab types. Swabs placed in viral transport medium are generally suitable for RT-PCR testing. Specific specimen requirements are provided by the testing laboratory.

The collection of lower respiratory specimens generates aerosols, and requires infection control precautions for influenza A H5N1, including the use of gloves, gown, eye protection, and a respirator rated at least N-95.

Specimens should be stored at refrigerated temperatures, unless specified otherwise by test procedures. For virus isolation, specimens should be stored at refrigerated temperatures no longer than 2 days, or frozen at $\leq 70^\circ$, and shipped on dry ice. Follow current regulations for packaging and shipping hazardous materials. (ASM Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases/Packing and Shipping Infectious Substances can be downloaded at http://www.asm.org/ASM/files/LeftMarginHeaderList/DOWNLOADFILENAME/00000001202/PackingandShipping1-08.pdf).

The Sentinel laboratory should contact the nearest designated LRN Reference laboratory prior to shipping specimens.

**Rejection criteria**
Rejection criteria include lack of patient identification on the specimen, incomplete documentation on the requisition form, and improper specimen type or handling. Exceptions may be made, at the discretion of the laboratory director, out of public health or medical necessity. Due to the potential for false positive results and public health panic, specimens may also be rejected for H5N1-specific testing if the patient does not meet clinical case or risk assessment criteria.
D. Testing

Rapid antigen tests
Because rapid influenza antigen tests provide a result in 30 minutes or less, they significantly impact patient treatment and management. These tests are widely used for diagnosis of influenza in central, point-of-care, and physician office laboratories. Several rapid antigen tests are commercially available, some of which are able to distinguish between influenza A and B types. Some of these tests are CLIA waived. Rapid antigen tests are less sensitive than culture or RT-PCR. Manufacturer claimed sensitivity ranges from 40 to 100% and specificity from 52-100% compared to viral culture. Because of the varied specificity, the positive predictive value of rapid influenza tests is often reduced when disease prevalence is low. Therefore, positive results outside of the influenza “season” should be interpreted with caution, and confirmed by additional tests.

While rapid antigen capture assays may detect avian influenza subtypes, including H5N1, currently available tests are not capable of distinguishing specific influenza A subtypes. Recent evidence indicates that currently available rapid antigen tests are extremely insensitive for H5N1, and should not be used to rule out avian influenza in a suspect case, especially during the current pre-pandemic phase. Rapid antigen testing can be performed on respiratory specimens from suspected avian influenza cases under standard BSL-2 conditions in a Class II biological safety cabinet.

Fluorescent antibody staining of antigens
The staining of influenza antigens with fluorescent antibody is an additional rapid test. When performed directly on cells from respiratory specimens, this method can provide results in less than an hour. Availability of fluorescent antibody staining is restricted to laboratories with immunofluorescent microscopes and trained technologists able to accurately interpret fluorescent staining patterns. Since this is not a point-of-care test, the factors with the most significant impact on turn around time of test results are specimen transport to the testing laboratory, and batching of specimens. Fluorescently-labeled antibodies specific for influenza A and B viruses are available. Some commercially available influenza antibodies are provided in pools with antibodies to other common respiratory viruses. Fluorescent antibody staining is generally considered to be slightly more sensitive than rapid antigen tests. Specificity is high, but depends on well trained, experienced technologists.

Fluorescent antibody staining reagents specific for influenza A virus will detect avian influenza A H5N1. When this guideline was written there was no H5N1-specific fluorescent antibody reagent commercially available. Fluorescent antibody staining can be performed on respiratory specimens from suspected avian influenza cases under BSL 2 conditions in a Class II biological safety cabinet.

Nucleic acid amplification
Nucleic acid amplification methods such as RT-PCR and nucleic acid sequence-based amplification (NASBA) are becoming more commonly used for detection of
influenza virus and other respiratory viruses. Using real-time, fluorescent detection of amplified product, laboratories are able to perform molecular tests in less than 3 hours. These are consistently the most sensitive methods for detection of influenza virus, including H5N1. High specificity requires judicious selection of primers and probes, optimization of amplification conditions, and interpretation of results. Continuous adherence to laboratory protocol is essential to avoid false positives due to carry-over contamination. Currently, there are two commercially available FDA cleared tests that detect influenza viruses by nucleic acid amplification. Kits marketed in the U.S. have performance claims for H1 and H3 subtypes of influenza A virus. A third FDA cleared test detects and differentiates influenza A, B, H1, H3, and H5. This test is manufactured by the CDC and distributed only to LRN Reference laboratories. In addition to LRN Reference laboratories, some commercial and hospital laboratories may offer “home-brewed” nucleic acid amplification testing for influenza A H5N1. Unlike the FDA cleared tests, these assays are developed and validated in-house by each laboratory. As such, the performance characteristics of the tests may vary between laboratories.

RT-PCR testing only for the H5N1 subtype is not recommended. Specimens from suspect cases should be tested for both influenza A and B, and currently circulating influenza A subtypes in addition to H5N1.

Initial specimen processing, including addition of lysis buffer can be performed within a biological safety cabinet in a BSL 2 laboratory. Some specimen lysis buffers do not inactivate viruses. If lysis buffer that is known to inactivate the virus is used, further processing can be performed outside the biological safety cabinet.

Culture
Culture provides highly specific laboratory diagnosis of influenza, but requires fresh, refrigerated specimens for optimal sensitivity. Specimens in viral transport medium must be kept at 2-8°C and processed within 48 hours to avoid excessive decrease in viral titer. With proper specimen handling, culture is more sensitive than antigen detection methods. Historically, culture methods have been considered too slow to impact patient management. Incubation for at least 5 days is generally required to detect influenza virus in tube cultures. Tubes are generally held for 14 days prior to reporting a negative result. Influenza virus is detected in tube cultures by the presence of cytopathic effect (CPE), adsorption or agglutination of red blood cells, and fluorescently-labeled antibodies specific for influenza A and B viruses. Spin-amplified shell vial cultures have reduced the time to detection to 1-3 days. However, this is considered by many to be too slow to impact patient treatment or isolation decisions. Culture is important for detecting influenza infection missed by rapid testing, confirmation of non-culture results when disease prevalence is low, and to obtain isolates for characterization and surveillance.

Influenza A H5N1 will grow in cell lines commonly used for isolation of human-adapted influenza virus, including primary monkey kidney, Madin Darby canine kidney, A549, and others. Sentinel laboratories must be cognizant that H5N1 and
other highly pathogenic novel subtypes can be cultivated unknowingly from unrecognized human cases of avian influenza. Do not perform culture on specimens if avian influenza A H5N1 is suspected, unless performed under enhanced BSL 3 laboratory conditions. Contact the LRN Reference laboratory for instructions on handling and shipping of specimens from suspected avian influenza cases.

Instructions for sentinel laboratories that set up viral cultures under BSL2 conditions on a specimen or patient later determined to be positive for influenza A H5N1:

- Consult with the public health laboratory or CDC. These laboratories may request specimens or cultures.
- Isolate specimens, cell culture vessels and supplies potentially contaminated with the influenza A H5N1 virus. Sterilize by autoclaving, and discard.
- Disinfect work area.
- Monitor potentially exposed staff for symptoms. Public health or medical experts may recommend quarantine and prophylactic treatment.
- Highly pathogenic avian influenza viruses, including H5N1 are select agents regulated by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA). If live H5N1 virus is isolated from a clinical specimen, CDC or APHIS must be notified, and the agent must be transferred or destroyed. Documentation of transfer or destruction must be maintained for three years.

Serology

Serologic test methods to detect influenza virus-specific antibodies are available, and generally performed in reference and public health laboratories. These methods include indirect fluorescent antibody (IFA), complement fixation (CF), hemagglutination inhibition (HI) and neutralization. HI and neutralization are often used with specific antigens to identify influenza virus type- and subtype-specific antibody titers. The diagnostic utility of serology is limited by the need, generally, to collect both acute and convalescent sera to identify either seroconversion or a four-fold rise in antibody titer. As such, serologic methods that detect IgG responses have relatively little impact on patient management. IFA and other tests that detect IgM antibodies can detect acute infection, but sensitivity is reduced because serum IgM levels are often low due to repeated exposure to vaccine or circulating virus.

E. Interpretation/Reporting/Action

When a patient presents with suspected avian influenza, communication with the public health department is essential.

Specimens from suspected avian influenza cases should be referred to the local or state public health laboratory, or another LRN Reference laboratory. A list of emergency contacts for State Public Health Laboratories can be downloaded at http://www.aphl.org/aphlprograms/ep/Documents/EPrep110707_SPHL_contact_list.pdf. CDC has provided all state public health laboratories with RT-PCR protocols for molecular detection of influenza A and B viruses, and molecular subtyping for H1,
H3 and H5. Contact the LRN Reference laboratory for instructions on handling and shipping of specimens from suspected avian influenza cases.

Thoroughly document the referral of specimens to the LRN Reference laboratory, as well the results reported by the laboratory. Follow institutional protocols for reporting positive test results obtained by an outside referral laboratory.

Positive influenza A H5N1 test results should be confirmed at the CDC.
III. References


