# Guidelines for Avian Influenza Viruses

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## Purpose

The Division of Agricultural Select Agents and Toxins (DASAT) prepared this document to assist individuals and entities develop policies and implement procedures for working safely with avian influenza viruses
(AIV) in the laboratory. The guidelines provide a basic understanding of AIV as well as guidance to meet the requirements of Title 9, *Code of Federal Regulations* (CFR), Parts 121 (Possession, Use, and Transfer of Select Agents and Toxins) and 122 (Importation and Transportation of Controlled Organisms and Vectors).

## Introduction

AIV is one the most significant viruses of concern to the poultry industry in the United States and around the world. The main focus of the U.S. Department of Agriculture (USDA) is the domestic poultry population comprised of chickens and turkeys; they make up the largest percentage of the U.S. commercial poultry industry. However, under the broader definition, domestic poultry also includes ducks, quail, pheasants, and pigeons.

Avian influenza (AI) is an infectious disease of birds caused by type A strains of the influenza virus. Type B and C influenza viruses are not known to infect poultry or do not cause disease in poultry. AI is a highly contagious disease and some strains can cause high mortality in poultry. Influenza A virus in the natural environment is generally spread by ingestion or inhalation. The virus is found in high concentrations in saliva, nasal secretions, and feces. AIV can remain viable for long periods in tissues, feces, and water, especially at low temperatures. Virus-laden feces and respiratory secretions present on fomites such as equipment, clothing, flies, and contaminated feed and water are effective means of transmitting the virus. AIV is also transmitted by airborne dissemination. However, AIV is among the easiest viruses to inactivate using disinfectants or heat treatment.

The highly pathogenic form of the disease is systemic and may be characterized clinically by severe depression, ocular and nasal discharges, snicking (a hacking sound made to clear the throat), decrease in egg production, nervous system changes, edema of the head, tissue necrosis, sudden death, and high mortality. Morbidity and mortality associated with outbreaks of this highly pathogenic form may reach 90–100 percent within 1–2 weeks in susceptible poultry. Illness associated with the low pathogenic form of the disease is usually mild unless complicated by concurrent bacterial or viral infections, and environmental stressors.

Depending on the size of an outbreak, the measures taken to control and eradicate the virus, and the speed to implement control and eradication strategies, trading partners may implement regional or country-wide restrictions, resulting in significant economic losses in the poultry industry and increased costs to consumers. The magnitude and duration of these events will ultimately determine the overall impact to the economy. Outbreaks in the United States have occurred in 1924, 1983, 2004, and 2015. The 2015 United States outbreak incurred an estimated cost of $1.6 billion in lost birds and $3.3 billion in economy-wide losses. In addition, the USDA committed $500 million to address emergency efforts to block the disease and paid out $190 million for indemnity payments (8).

## Categorizing Influenza A and Nomenclature

Influenza A viruses are divided into subtypes and are identified on the basis of two surface glycoproteins or antigens: hemagglutinin (HA or H) and neuraminidase (NA or N). There are eighteen known HA subtypes (H1 to H18) and eleven known NA subtypes (N1 to N11); each virus has one HA and one NA protein on the surface. For example, an "H5N1 virus" designates an influenza A subtype that has an HA 5 surface protein and an NA 1 surface protein. The nomenclature of AIVs is based on a standardized format: Type/species/location/virus identification/year of isolation (HxNx), representing the influenza type, the host origin, the place of isolation, the strain number, and the influenza A subtype. For example, A/Ck/TX/309402/04 (H5N2) represents an H5N2 influenza A virus that was isolated from a chicken in Texas in 2004 and assigned strain number 309402.

AIV strains are further classified as low or highly pathogenic on the basis of specific molecular determinants of the HA protein and the biological behavior of the virus using *in-vitro* and *in-vivo* tests. Most AIVs are associated with mild disease in poultry and are termed low pathogenic avian influenza viruses (LPAIVs). By contrast, AIVs that are associated with severe illness and high mortality in poultry are termed highly pathogenic avian influenza viruses (HPAIVs). To date, all outbreaks of the highly pathogenic form of the disease have been caused by influenza A viruses of the subtypes H5 or H7. However, it is important to emphasize that the majority of H5 and H7 subtypes isolated from birds have been LPAIVs.

All known influenza A viruses circulate in their natural bird hosts, wild aquatic birds (e.g., ducks, geese, and swans) and shorebirds (e.g., gulls and terns), except subtypes H17N10 and H18N11 which have only been found in bats. Traditionally, HPAIVs have not been observed in reservoir hosts. However, there is evidence to suggest that some migratory waterfowl can now carry the H5N1 virus in its highly pathogenic form in many parts of the world (11, 12). It remains unclear whether wild migrating birds are an established reservoir for H5N1 in its highly pathogenic form. Based on previous investigations of HPAI outbreaks, it is generally recognized that most HPAIVs evolve after transmission of LPAIV H5 and H7 subtypes from a wild reservoir host to a poultry host.

The virus adapts to poultry hosts and mutates to a HPAIV through multiple replication cycles and/or bird transmissions. Low pathogenic H5, H7, and H9 subtypes have adapted to and circulate in domestic poultry (15). HPAIV field isolates are often utilized in laboratories, and are subject to select agent regulation pursuant to 9 CFR Part 121. The pathotype of an AIV cannot be determined simply by its subtype. For example, some H5N1 subtypes are highly pathogenic (i.e., the Asian H5N1 lineage) and some are low pathogenic (i.e., the North American H5N1 lineage).

The ability of LPAIV H5 and H7 subtypes to mutate into highly pathogenic forms and result in outbreaks of HPAI will often result in the immediate restrictions on trade in poultry and poultry products. Additionally, outbreaks of LPAIV H5 or H7 subtypes in poultry may result in trade restrictions because of the capacity of those subtypes to adapt and mutate to HPAIV in poultry. Therefore, the World Organisation for Animal Health (OIE) classifies all H5 and H7 subtypes of AIV as notifiable AIVs (22) and requires they be reported. However, only HPAIVs are regulated as select agents in the United States; movement of all LPAIVs are controlled by the USDA APHIS veterinary permitting program.

## Defining Highly Pathogenic Avian Influenza Virus

HPAI or virulent AI may be described as a highly contagious viral infection and/or disease of many avian species including poultry, wild and exotic birds, ratites, shorebirds, and migratory waterfowl caused by influenza A strains of the virus. Simply put, it refers to poultry infected with virulent strains of influenza A virus. The defined and internationally recognized criteria used for classifying an AIV as low or highly pathogenic is based on the *in-vitro* and *in-vivo* biological characteristics of the virus. More specifically, it is based on an intravenous pathogenicity index in chickens and the amino acid sequence at the cleavage site of the HA protein (6, 22).

The HA protein of AIV is responsible for viral attachment and entry into cells. The amino acid patterns of the HA protein cleavage site are uniquely different between LPAIVs and HPAIVs. While HA is not the only determinant of virulence in poultry, it is certainly the driver. Other gene segments and factors have been identified as virulence factors in poultry, so virulence in poultry is multigenic (3, 15, 16). However, the HA is the protein we understand most and is a prerequisite for the virus' high pathogenicity in poultry. Replication requires that the HA be cleaved into two subunits in order to infect host cells. If there is no cleavage, then there will be no infection, no replication, and no disease.

The cleavage site of the HA protein of most AIVs is comprised of only one to two basic amino acids at specific positions. Typsin-like proteases expressed at the surface of cells lining the respiratory and gastrointestinal tracts recognize this motif. Therefore, virus replication is restricted to these tissue types and causes only mild disease or no clinical sign. These viruses are classified as LPAIV. By contrast, AIVs that express multiple basic amino acids (i.e., arginine and lysine) at the cleavage site are recognized by a wide range of proteases that are distributed ubiquitously and results in virus replication in many tissues and causes systemic disease. These viruses are classified as HPAIV. This difference in the ability of certain proteases to cleave AIVs is just one piece of information to help make the distinction between HPAIV and LPAIV (1, 6).

Field isolates are traditionally tested to differentiate HPAIVs from LPAIVs because of the reporting requirement at the State, national, and international levels.

## Understanding Influenza A Reassortant Viruses

Influenza A virus is an enveloped, negative-sense, single stranded ribonucleic acid (RNA) virus with a segmented genome that codes for ten conserved proteins. Each gene segment encodes one or two proteins. Some influenza A viruses may encode an additional protein, PB1-F2 (9). The eight gene segments of influenza A virus encode the ten proteins listed in blue in Table 1 below. The PB2, PB1, PA, NP, M, and NS genes are often referred to as the internal genes of AIV. Although the M gene encodes for the surface exposed protein M2, it is still referred to as an internal gene.

**Table 1. Influenza A Gene Segments**

| RNA Gene Segment | Encoded Protein(s) |
|---|---|
| 1 | HA–hemagglutinin |
| 2 | NA–neuraminidase |
| 3 | M1 + M2–matrix proteins |
| 4 | NS1 + NS2–nonstructural proteins |
| 5 | NP–nucleocapsid protein |
| 6 | PB1 (+/–PB1–F2)–polymerase protein |
| 7 | PB2–polymerase protein |
| 8 | PA–polymerase protein |

The segmented genome of influenza A virus facilitates genetic reassortment when two influenza A viruses infect the same cell. This provides another means by which HPAIVs may arise. Advancements in biotechnology have led to methods for generating experimental reassortants (i.e., created by using reverse genetics systems) and permits the strategic creation of a large number of influenza A virus reassortants for study in the laboratory (4). Theoretically, 254 viral progeny (excluding the parental genomic constructs) could be created from the 16 RNA segments contributed from two different parent influenza viruses. Progeny viruses that inherit RNA segments from at least two different parent influenza A viruses are known as reassortants.

It is not well understood why virulence is restricted to just H5 and H7 subtypes. The biological behavior of a reassortant influenza virus is often unpredictable, particularly with reassortants composed of avian–avian gene segments or avian–mammalian gene segments. There have been rare exceptions where field isolates have not conformed to the multibasic cleavage site correlating with virulence and vice versa (7, 13). It appears that the composition of certain gene segments and sequences work together better than others. Thus, there is frequently a need to document the genotypic and phenotypic characteristics of reassortant viruses using a set of established criteria to validate biological behavior. APHIS regulates reassortant influenza under 9 CFR §121.3(c).

# Regulating

## Avian Influenza Virus

APHIS regulates, as select agents, certain influenza reassortant viruses based on their construct until demonstrated to be sufficiently attenuated pursuant to 9 CFR §121.3(e). Under 9 CFR § 121.3(b), APHIS regulates as select agents all AIVs that have been subtyped and classified as highly pathogenic. AIVs subtyped and classified as low pathogenic, including H5 and H7 subtypes, APHIS regulates as controlled organisms under 9 CFR Part 122.2.

## Experimental Reassortant Influenza Viruses

The influenza A virus infects a variety of species, including humans. The H1 and H3 subtypes cause significant morbidity and mortality in humans (21). The outbreak of highly pathogenic H5N1 AI in Hong Kong in 1997, East and Southeast Asia in 2003, and its subsequent spread throughout Asia, Europe, and Africa significantly impacted agricultural trade in poultry and poultry products; the resulting infection in humans also posed a considerable public health threat (9, 10, and 17). While the highly pathogenic H5N1 viruses do not spread efficiently among humans, infection has resulted in high human mortality and morbidity (9, 10). The Asian H5N1 strains continue to circulate in avian species and occasionally transmit and infect humans (10).

As a result of these developments, there has been concern over the potential emergence of a pandemic virus associated with highly pathogenic H5N1 or a variant. Early in the 21st century, research intensified on preparedness and focused on understanding the genetic determinants of pathogenicity in humans, the molecular determinants that might contribute to efficient human transmission, and development of vaccine candidates.

APHIS regulates AIVs and experimental reassortant influenza viruses which meet the internationally recognized definition of HPAIV – as defined in the OIE Terrestrial Animal Health Code – as select agents, under 9 CFR §121.3(c). The use of reverse genetic approaches to create experimental reassortant influenza viruses allows for the potential generation of thousands of influenza reassortants, across a broad virulence range. The criteria or data points used to determine the virulence of reassortant viruses in poultry are outlined in Table 2 below. The preferred cell line for the plaque characterization assays is the chicken embryo fibroblast cell line; however, other suitable cell lines are acceptable, e.g., Madin–Darby canine kidney cell line (1, 6).

**Table 2. Data Required for Classifying Avian Influenza Reassortant Viruses**

| Parameter | Method | Outcome Specification |
|---|---|---|
| Source of all genes in construct; description of modification | Reference source material for viruses, plasmids, etc. | Description of gene composition of recombinant/attenuated strain |
| Complete nucleotide sequence analysis of the entire HA gene and analysis of the amino acid motif at the HA cleavage site | Standard laboratory methods | Confirmation of expected sequence for attenuated strain; demonstration that only the LPAI cleavage site is present. |
| Pathogenicity testing in chickens | As described in the current OIE Manual of Standards for Diagnostic Tests and Vaccines | Confirmation of low pathogenic phenotype in chickens |
| Plaque characterization on chicken embryo fibroblast (CEF) cells (or other suitable cell line) without trypsin | Test duplicate dilutions of strain in CEF cells with and without trypsin | Demonstration of inability to form clearly defined plaques in the absence of trypsin |
| Plaque characterization on CEF cells (or other suitable cell line) with trypsin | Determine plaque formatting units/ml of representative product | Demonstration of ability to form viral plaques in the presence of trypsin |

DASAT will accept reassortant virus exclusion requests absent of live bird lethality testing data for avian influenza constructs in which:

- The backbone strain is a PR8 or similar human vaccine strain previously **demonstrated to have limited to no replication in poultry**, regardless of the HA gene; and
- The H5/H7 cleavage site has been altered from highly pathogenic avian influenza (HPAI) to low pathogenic avian influenza (LPAI); and
- Viruses used for gene inserts have been well characterized as LPAI prior to use in reverse genetically derived processes.

In lieu of the live bird lethality testing, DASAT will require all reverse genetically derived H5/ H7 viruses with PR8 or similar human vaccine strain backbone to:

- Confirm by genetic sequencing that the amino acid sequence at the HA cleavage site is compatible with only LPAI viruses; and
- Demonstrate functionality as a LPAI virus either by cell culture system with/without exogenous trypsin, *or in vivo* test in chickens by the intravenous pathogenicity index (IVPI) per World Organisation for Animal Health (OIE) standards.

Because the virulence of HPAIV is multigenic and the predominant virulence determinant of HPAIV in poultry is the multibasic cleavage site of HA, APHIS provides reasonable deductions regarding the regulation of reassortant influenza viruses to assist in reducing time and financial burden on laboratories. The outline below may be used to determine whether or not it is necessary to provide the data listed in Table 2 to validate the attenuation of a reassortant virus.

1. **Avian-mammalian reassortants (HPAIV-HA):** When a reassortant is composed of RNA segments from a mammalian influenza virus and AIV, and the HA RNA segment is contributed by a HPAIV, the reassortant virus is regulated as a select agent unless it has been excluded pursuant to 9 CFR §121.3(e).

2. **Avian-avian reassortants:** When a reassortant is composed of RNA segments from at least two AIVs, and at least one parental virus is a highly pathogenic AIV, the reassortant virus is regulated as a select agent if the HA RNA segment is contributed by an HPAIV unless it has been excluded pursuant to 9 CFR §121.3(e).

3. **Avian-mammalian reassortants (Mammalian-HA):** When a reassortant is composed of a mammalian HA RNA segment and the NA RNA segment and/or internal RNA segments originate from a highly pathogenic AIV, the reassortant virus is not regulated as a select agent but movement is controlled as an organism pursuant to 9 CFR Part 122.2.

4. **Influenza A virus reassortants with synthetic HA (HPAIV cleavage site):** When a reassortant is composed of an AI A virus HA RNA segment of any subtype that has a change within the cleavage site compatible with an HPAIV, and the other seven gene segments of any influenza A virus, the reassortant virus is regulated as a select agent unless it has been excluded pursuant to 9 CFR §121.3(e).

5. **Avian influenza-other virus constructs:** When a reassortant virus is constructed by assembling an RNA segment (usually HA or NA) or RNA segments from a highly pathogenic AIV, short of all eight segments, with nucleic acid(s) from a different virus, the construct is not regulated as a select agent but movement is controlled as an organism pursuant to 9 CFR Part 122.2.

Per 9 CFR §121.3(e)(2), if an excluded attenuated strain is subjected to manipulation that restores or enhances its virulence, the resulting select agent will be subject to the requirements of the regulations. APHIS does not consider mutants created from excluded reassortant viruses to be altered sufficiently to revert to virulent, provided the established motif at the HA cleavage site has not been changed. While some reassortants may not be subject to select agent regulations, they are still subject to regulation pursuant to 9 CFR Part 122.

Unlike field strains of AIV for which the nomenclature has been standardized, there is no standard format for naming reassortants. However, most reassortants bear some reference to the parent viruses. Thus, the nomenclature of reassortants will vary from laboratory to laboratory and according to individual laboratory protocol.

## Nucleic Acids

### Fully Intact Genome

The segmented genome of AIV is of negative polarity or negative-sense. Unlike genomes of positive polarity, the AIV genome cannot be directly or immediately translated by host cells into proteins, and is not considered infectious. Therefore, APHIS does not regulate the fully intact genome of HPAIV as a select agent, but under 9 CFR Part 122.2. However, the method by which nucleic acid is extracted from a system must ensure that no viable virus will cross-contaminate the extracted RNA preparation.

### Nucleic Acids

APHIS does not regulate individual RNA segments from an HPAIV genome as select agents, because they are not considered infectious. However, the method by which nucleic acid is extracted from a system must ensure that no viable virus will cross-contaminate preparations. APHIS regulates movement of such RNA segments, as well as intermediaries carrying HPAIV nucleic acid used to express individual proteins or generate an influenza A virus – including viral complementary deoxyribonucleic acid (cDNA) (plasmids), (-) viral RNA, (+) mRNA, and the expressed viral proteins – under 9 CFR Part 122.2.

# Introduction to Biocontainment Provisions

Biocontainment may be described as processes or methods to safely manage infectious materials or agents. These methods reduce the risk of exposure to personnel and unintentional release in the environment. From an agricultural perspective, maintaining proper biocontainment is the primary concern while working with AIV in the laboratory. Laboratory containment of AIV must be sufficient to mitigate the risk of exposure to the environment and ultimately to poultry. The potential economic impact of HPAI on domestic and international trade, the poultry industry, and ultimately the consumer could be significant. Facilities and practices must meet standards that will reduce the probability of an unintentional release that could lead to an outbreak.

All laboratories are not built the same, and the scope of work conducted in each laboratory varies. Therefore, determining the appropriate exclusion criteria should begin with a robust risk assessment for the type and scope of work to be undertaken in a laboratory. To paraphrase 9 CFR §121.12(a), a registered entity is required to develop and implement a written biosafety plan that is commensurate with the risk of the select agent. No containment system is perfect; however, there is no substitute for proper and thorough training in operations and procedures to reduce unintentional releases.

This guideline document is not intended to focus on how to design a facility for proper biocontainment or to address all of the generally accepted requirements of basic laboratory practices. Those standards can be found elsewhere (18, 19). Instead, this document is intended to match the scope of work conducted in a laboratory with minimal additional provisions or exclusion criteria to mitigate risks while working with AIV. If an institution has determined that it is unable to meet one or more of the mitigating factors discussed below, and can provide a risk assessment to justify an alternative, DASAT will consider the proposal at the applicant's request.

## Biosafety Level 4 (BSL-4) and Animal Biosafety Level 4 (ABSL-4)

Laboratory work with HPAIV conducted in BSL-4 and ABSL-4 laboratories do not require additional provisions. Meeting the BSL-4 and ABSL-4 criteria are sufficient for appropriate biocontainment.

## Biosafety Level 3 Agriculture (BSL-3Ag)

Animals that are housed loosely on the floor or in open caging systems and have been infected with HPAIV must be contained in a BSL-3Ag facility. However, under most circumstances the BSL-3Ag facility is reserved for large animal species, e.g., adult swine, in which the use of primary biocontainment housing would not be practical. The animal room is an airtight barrier serving as primary containment for the animal. In this situation, the additional provision is:

**Personnel quarantine policy:** As an added precaution, APHIS requires the implementation of a written restriction policy for laboratory staff, as well as visitors. The restriction prevents laboratory staff and visitors from having contact with susceptible avian species for a minimum of 5 days after last engaging in work with the virus. The prohibition includes avian wildlife, pet birds, backyard poultry, fair birds, commercial poultry operations, and zoos. The policy must be read and signed by staff to ensure compliance.

## Biosafety Level 3 (BSL-3)

In-vitro laboratory work with HPAIV can be diverse and laboratories engaging in such activities may include clinical, diagnostic, teaching, and research facilities. HPAIV may be aerosolized, amplified, or propagated in eggs, cell culture, or tissue culture. HPAIV may also be used in procedures with known concentrated virus preparations. All of these activities must be conducted in BSL-3 laboratories with the following provisions:

**Air handling:** APHIS mandates the exhaust system include a high efficiency particulate air (HEPA) filtration of the laboratory air. The exhaust system should have a sealed ductwork system from the containment barrier to the filter. Supply HEPA filtration is not necessary if directional airflow is maintained inward through entry doors. However, the supply and exhaust air handling systems must be interlocked. Ideally, there should be independent air supply and exhaust systems. APHIS does not require independent air handling systems; however, they must be isolated from other areas.

**Showers:** A gown-in, shower-out procedure is used to enforce a change of street clothing, to reduce the risk of fomite transmission of this highly contagious agent. Ideally, personal showers should be located at the containment/non- containment interface.

**Decontamination of laboratory liquid effluents:** Liquid effluents originating from laboratories should be collected locally and chemically disinfected or heat treated, or collected and processed in a

central effluent decontamination system before being released into the local sanitary system. The decontamination of shower and toilet effluents is not a requirement, provided appropriate practices and procedures are in place for primary containment.

**Protective clothing:** Change of clothing prior to entering the laboratory is important. The attire donned for gowning should include the following: 1) disposable hood or head cover; 2) protective eyewear (e.g., safety glasses); 3) respiratory protection; 4) disposable double gloves; 5) disposable Tyvek gown or coveralls; and 6) disposable shoe covers.

**Personnel quarantine policy:** APHIS requires entities to implement a written policy for visitors as well as their staff. The restriction prevents laboratory staff and visitors from having contact with susceptible avian species for a minimum of 5 days after last working with the virus. The prohibition includes avian wildlife, pet birds, backyard poultry, fair birds, commercial poultry operations, and zoos. The policy must be read and signed by staff to ensure compliance.

There is no need for a proximity restriction to avian species located outside of the facility because exhaust air from the laboratory must be HEPA-filtered. Also, this policy does not apply to susceptible avian species housed within the same facility, provided appropriate practices and procedures are in place to prevent cross-contamination.

## Animal Biosafety Level 3 (ABSL-3)

Animal species used in the laboratory to study HPAIV may range from small (e.g., mice) to large (e.g., swine), which necessitates special attention to concerns for containing higher viral loads, aerosols, primary containment caging, and animal husbandry practices. This work can be conducted in Animal Biosafety Level 3 (ABSL-3) laboratories with additional provisions.

The influenza viruses have an innate ability to reassort. The following is an example of a high consequence situation: when two or more experiments are conducted at the same time with HPAIV and the 1918 influenza virus (a U.S. Department of Health and Human Services select agent), there is the possibility of naturally generating a reassortant influenza virus that is both deadly and efficiently transmitted from person-to-person. These experiments must be separated temporally, meaning not conducted simultaneously in the same laboratory room; and/or spatially. If such experiments are conducted simultaneously in the same facility, they must be conducted in separate laboratory rooms and the airspace must not be shared (i.e., independent air systems).

The additional provisions are:

**Air handling:** APHIS mandates the HEPA filtration of laboratory air is on the exhaust system. The exhaust system should have a sealed ductwork system from the containment barrier to the filter. Supply HEPA filtration is not necessary if directional airflow is maintained inward through entry doors. However, the supply and exhaust air handling systems must be interlocked. Ideally, there should be independent air supply and exhaust systems. It is not a requirement that air handling systems are independent; however, they must be isolated from other areas.

**Special caging:** Animals infected with HPAIV must be placed in appropriate biocontainment units for animal housing. Containment at the cage level may be achieved in several ways depending on preference and animal size. For example, primary biocontainment housing may be a containment

cage or rack system, flexible film isolator, or glove box. Caging must be ventilated and the exhaust air HEPA filtered in all instances. Static micro-isolators are not effective in achieving the desired effect of preventing air leakage into the laboratory space.

**Showers:** A gown-in, shower-out procedure is implemented to enforce a change of street clothes, which reduces the risk of fomite transmission. Ideally, personal showers should be located at the containment/non- containment interface.

**Decontamination of laboratory liquid effluents:** Liquid effluents originating from laboratories should be collected locally and chemically disinfected or heat treated, or collected and processed in a central effluent decontamination system before being released into the local sanitary system. The decontamination of shower and toilet effluents is not a requirement, provided appropriate practices and procedures are in place for primary containment.

**Decontamination of solid animal wastes:** All animal tissues, carcasses, and bedding originating from the animal room must be decontaminated by an effective and validated method (e.g., use of a tissue autoclave) before leaving the containment barrier. There should be an appropriate final method of disposal (e.g., incineration).

**Protective clothing:** Change of clothing prior to entering the laboratory is important. The attire donned for gowning should include the following: 1) disposable hood or head cover; 2) protective eyewear (e.g., safety glasses); 3) respiratory protection; 4) disposable double gloves; 5) disposable Tyvek gown or coveralls; and 6) disposable shoe covers.

**Personnel quarantine policy:** APHIS requires entities to implement a written policy for visitors as well as their staff. The restriction prevents laboratory staff and visitors from having contact with susceptible avian species for a minimum of 5 days after last working with the virus. The prohibition includes avian wildlife, pet birds, backyard poultry, fair birds, commercial poultry operations, and zoos. The policy must be read and signed by staff to ensure compliance.

There is no need for a proximity restriction to avian species located outside of the facility because exhaust air from the laboratory must be HEPA-filtered. Also, this policy does not apply to susceptible avian species housed within the same facility, provided appropriate practices and procedures are in place to prevent cross-contamination.

## Biosafety Level 2 (BSL-2) and Animal Biosafety Level 2 (ABSL-2)

*In-vitro* and *in-vivo* laboratory work with H5 and H7 subtypes of LPAIV as well as reassortant AIVs that are not categorized as select agents may be conducted in BSL-2 and ABSL-2 laboratories with the enhancements outlined below. *In-vitro* and *in-vivo* work with all other LPAIV subtypes may be conducted in laboratories meeting BSL-2 and ABSL-2 criteria.

Many laboratories (e.g., veterinary diagnostic laboratories) conduct routine screening surveillance on samples collected from wild birds as well as domestic poultry. Laboratories are usually surveying for early detection of highly pathogenic H5N1 in migrating wild birds and detection of AIV that may be circulating in domestic poultry. We consider the specimens collected domestically or from regions around the world that are declared free of HPAIV to be low risk materials. *In-vitro* testing of unknown samples for diagnostic screening (e.g., commercial enzyme-linked immunosorbent assay

[ELISA] kits, commercial antigen capture kits, and agar- gel immunodiffusion tests [AGID]), may be conducted in laboratories meeting BSL-2 criteria.

More sophisticated or specific testing using reverse-transcription polymerase chain reaction or real-time polymerase chain reaction may also be conducted in laboratories meeting BSL-2 criteria. However, any additional work following the detection of H5 or H7 antigen must be done at a higher biocontainment level: BSL-2 with enhancements or BSL-3. Following confirmation of H5 or H7 HPAIV nucleic acid, all additional work must be performed at BSL-3 with provisions.

By contrast, specimens collected in regions around the world that are known to be affected with HPAIV, and especially where it is endemic (e.g., Bangladesh, China, Egypt, India, Indonesia, and Vietnam), are considered high risk materials. As a precaution, APHIS assumes such materials contain HPAIV until otherwise demonstrated. Diagnostic work with such specimens begins at containment level 3 with provisions (as described on pages 9-10) to offset the lack of processing, ineffective or insufficient processing methods, and the inherent high risk of the material.

The enhancements for BSL-2 and ABSL-2 are:

**Air handling:** There are no specific requirements for an air handling system. However, where there is no air handling system, APHIS mandates the restriction of avian species in close proximity to the facility. Where there are susceptible animals or avian species in the same facility, the air handling system must be independent or isolated from these laboratory areas.

**Decontamination of solid animal wastes:** Each laboratory must have a written protocol or procedure for the disposal of solid animal wastes. APHIS prefers waste disposal of carcasses, tissues, and bedding by incineration, but will accept validated methods of decontamination by heat treatment (e.g., use of a tissue autoclaving).

**Protective clothing:** Personnel must change clothing prior to entering the laboratory. Disposable clothing should not be reused and disposed of appropriately. Reusable clothing (e.g., scrubs) must be autoclaved out of the laboratory and laundered. Protective clothing should not leave the laboratory.

**Personnel quarantine policy:** APHIS requires entities to implement a written policy for visitors as well as their staff, which restricts contact with susceptible avian species for a minimum of 5 days after last working with the virus. The prohibition includes avian wildlife, pet birds, backyard poultry, fair birds, commercial poultry operations, and zoos. The policy must be read and signed by staff to ensure compliance.

Additionally, there should be no commercial poultry within 1/2 mile of the facility, or any other avian colonies (e.g., aviaries and zoos) within 100 meters of the facility if HEPA filtration of exhaust is not a component of containment.

**Administrative:** The permittee must notify the biosafety officer for the facility that the laboratory intends to receive the viral agent.

## Transfer and Permitting of Highly Pathogenic Avian Influenza Viruses and Low Pathogenic Avian Influenza Viruses

HPAIV may not be moved or transferred from one entity to another, or imported, unless the receiving entity is registered with a Federal Select Agent Program to possess, use, or transfer HPAIV. The appropriate document for such a transfer is the "Request to Transfer Select Agents and Toxins" (APHIS/Centers for Disease Control Form 2), and the transfer must be executed in accordance with 9 CFR §121.16 (Transfers). The Form 2 is available at http://www.selectagents.gov.

In addition, APHIS requires that the receiving entity of any domestic transfer or importation of LPAIV has a valid VS permit. The application for a VS permit, "VS 16-3: Application for Permit to Import Controlled Material or Transport Organisms or Vectors or Animal Products and By-Products", may be obtained at https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-and-animal-product-import-information/ct_organisms_and_vectors/. If an individual is already a current holder of a VS permit for agents other than LPAIV, it may be possible to amend the current permit.

LPAIV, reassortant influenza viruses that have been excluded from 9 CFR Part 121, nucleic acids of AIV, and intermediaries (e.g., cDNA and mRNA) also require a VS permit (Application form VS 16-3) for importation and interstate transport under most circumstances. These are not select agents; therefore, Form 2 is not required. For additional information concerning transfer of select agents, interstate transport of excluded select agents, and/or importation, please contact the DASAT Organisms and Vectors Unit, or Select Agent Unit (contact information on the last page).

## Summary

HPAIV is a pathogen of significant agricultural concern worldwide, mainly because of its potential economic impact. The zoonotic potential of some AIVs, particularly the H5N1 Asian lineage, has also contributed to this virus' high consequence. Our understanding of this virus has advanced in recent years, but there remains a great deal more to be learned. As advances are made, APHIS will modify its regulations and/or this document to remain transparent about the principles and practices essential to prevent unintentional releases of AIVs and exposure to domestic poultry. These guidelines are not a one-size-fits-all approach; APHIS will consider alternative approaches to a given scenario or work description. All inquiries by registered entities should first be made by contacting your CDC or APHIS file owner. Non-registered entities or individuals should contact DASAT for additional information or guidance.

# References

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