United States Department of Agriculture

Guidelines for Avian Influenza Viruses

Prepared by

U.S. Department of Agriculture

Animal and Plant Health Inspection Services

Division of Agricultural Select Agents

and Toxins

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H5 Avian Influenza Virus Exemption:

On June 6, 2024, the Administrator of the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) has utilized his exemption authority under 9 C.F.R. § 121.5(f) in the select agent and toxin regulations to temporarily exempt H5 avian influenza viruses from the requirements of the regulations listed in 9 C.F.R. Part 121 for a period of three years.

Such an exemption is consistent with protecting animal health and animal products while allowing more laboratories to conduct research and develop solutions to address the disease. This exemption replaces all previously issued exemptions related to H5 avian influenza viruses.

For the duration of the exemption, APHIS, Veterinary Services (VS), Organisms and Vectors (OV) Permitting Unit will issue permits for importation and interstate transportation of all H5 avian influenza viruses pursuant to 9 C.F.R. Part 122. The APHIS, VS, OV Permitting Unit can be contacted by email at apie@usda.gov.

Additionally, an import permit from the Centers for Disease Control and Prevention's Import Permit Program will be required for all H5 Avian Influenza variants that are known or suspected to cause human disease. If any imported agent is determined to not cause disease in humans (e.g., attenuated strains that are no longer infectious), then an importer certification statement should be included to avoid potential shipping delays. The Import Permit Program can be contacted by email at: importpermit@cdc.gov.

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Purpose

The Division of Agricultural Select Agents and Toxins (DASAT) prepared this guidance document to assist individuals and entities develop policies and implement procedures for working safely with avian influenza viruses (AIVs) 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), Part 121 (Possession, Use, and Transfer of Select Agents and Toxins).

Introduction

AIV is one the most significant viruses of concern to the poultry industry in the United States and around the world. The chief 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 a broader definition, domestic poultry may also include but not limited to ducks, quail, and pheasants. (See 9 CFR §53.1)

Avian influenza (AI) is an infectious disease of birds caused by type A strains of the influenza virus. Type B and C strains of 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 (4). 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 (Section 1 (usda.gov)). AIV is also transmitted by airborne dissemination. However, AIV is among the easiest viruses to inactivate using disinfectants or heat treatment (2).

The highly pathogenic form of the disease affects multiple organs, and symptoms may include a lack of energy or appetite, 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 (29). Outbreaks of this highly pathogenic form of the virus may result in cumulative morbidity and mortality rates of 90%-100% within 1–2 weeks in susceptible poultry. The low pathogenic form of the disease usually causes mild illness unless concurrent bacterial or viral infections, and environmental stressors complicate it. (Section 1 (usda.gov)).

The size of an outbreak, and the measures and time taken to respond, control and eradicate the virus determine the degree to which trading partners may implement regional or country-wide restrictions. The resulting action may cause significant economic losses in the poultry industry and lead to increased costs for consumers. The magnitude and duration of an outbreak ultimately determines the overall impact to the economy. Outbreaks in the United States occurred in 1924, 1983, 2004, 2015, and 2022. The 2015 United States outbreak cost approximately $1.6 billion in lost birds and $3.3 billion in economy-wide losses. In addition, USDA committed $500 million to address emergency efforts to block the disease and paid out $190 million for indemnity payments (6). The most recent outbreak began in early 2022 and is ongoing. (See USDA APHIS | 2022-2024 Confirmations of Highly Pathogenic Avian Influenza in Commercial and Backyard Flocks.)

Categorizing Influenza A and Nomenclature

Influenza A viruses are divided into subtypes and are identified based on two surface glycoproteins or antigens: hemagglutinin (HA or H) and neuraminidase (NA or N). Subtyping is based on serologic reactions of the HA and NA surface glycoproteins or sequence analysis of HA and NA gene segments. 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, in order, the influenza type, the host origin, the place of isolation, the strain number, and the influenza 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 based on specific molecular determinants of the HA protein and the biological behavior of the virus using *in-vitro* and *in-vivo* tests (29). 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, influenza A viruses of the subtypes H5 or H7 have caused all outbreaks of the highly pathogenic form of the disease. However, it is important to emphasize that the majority of H5 and H7 subtypes isolated from birds are LPAIVs. Of note, nephrogenic strains of H10 AIVs that did not possess the multi-basic motif at the cleavage site were lethal in chickens when administered intravenously. (12,20,23).

All known influenza A viruses circulate in their natural bird hosts (i.e., a reservoir host): wild aquatic birds (e.g., ducks, geese, and swans), and shorebirds (e.g., gulls and terns). However, investigators have only found subtypes H17N10 and H18N11 in bats. Traditionally, investigators have not observed reservoir hosts as carriers of HPAIVs. However, there is evidence to suggest that HPAIV has adapted to endemic circulation in some wild bird species and can now carry the virus in its highly pathogenic form in many parts of the world (8,16,17,18).

The virus adapts to poultry hosts and may mutate to a HPAIV through multiple replication cycles and/or bird transmissions (4,9). Low pathogenic H5, H7, and H9 subtypes have adapted to and circulate in domestic poultry (21). HPAIV field isolates are often utilized in laboratories and are subject to the select agents and toxins regulations found in 9 CFR Part 121. Simply knowing the subtype of an AIV does not allow determining its pathotype. For example, highly pathogenic H5N1 strains and low pathogenic H5N1 strains exist.

The ability of LPAIV H5 and H7 subtypes to mutate into highly pathogenic forms or overspills resulting in outbreaks of HPAI often leads to immediate restrictions on trade in poultry and poultry products. Therefore, the World Organisation for Animal Health (WOAH [OIE]) classifies all H5 and H7 subtypes of AIV as notifiable AIVs and mandates reporting them (29). However, only HPAIVs are regulated as select agents in the United States and subject to the select agents and toxins regulations in 9 CFR Part 121; importation and interstate movement of all LPAIVs is regulated by APHIS, Veterinary Services (VS), Organisms and Vectors (OV) Permitting Unit and subject to the permitting regulations in 9 CFR Part 122.

Defining Highly Pathogenic Avian Influenza Virus

Highly Pathogenic Avian Influenza (HPAI) or virulent AI describes a highly contagious viral infection and/or disease affecting many avian species including poultry, wild and exotic birds, ratites, shorebirds, and migratory waterfowl, caused by influenza A strains of the virus. The defined and internationally recognized criteria used to classify an AIV as low or highly pathogenic are 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 (10,29).

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. Researchers have identified other gene segments and factors that contribute to virulence in poultry, making virulence in poultry a multigenic trait (5,21,24). However, the HA is the protein that is understood most and is a prerequisite for the virus' high pathogenicity classification in poultry. For replication to occur, the HA must cleave into two subunits (HA1 and HA2) to infect host cells. Without cleavage, there will be no infection, no replication, and or disease.

The cleavage site of the HA protein of most AIVs is comprised of only one to two basic amino acids at specific positions. Trypsin-like proteases expressed at the surface of cells lining the respiratory and gastrointestinal tracts recognize this motif in the critical position. Therefore, virus replication is restricted to these tissue types and causes only mild disease or no clinical signs. These viruses are classified as LPAIV. By contrast, AIVs expressing multiple basic amino acids (i.e., arginine (R) and lysine (K)) in the critical position at the cleavage site are recognized by a wide range of proteases distributed ubiquitously, resulting in virus replication in many tissues and causing systemic disease. These viruses are classified as HPAIV (29). 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 (1,10).

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 (14). The eight gene segments of influenza A virus that encode the ten proteins listed are bold and italicized 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 (11). Advancements in biotechnology have led to methods for generating reassortants in the laboratory (i.e., created by using reverse genetics systems) and allows for the strategic creation of many influenza A virus reassortants for study in the laboratory (7). Theoretically, two different parent influenza viruses could contribute 16 RNA segments to create 254 viral progenies (excluding the parental genomic constructs). Progeny viruses inheriting 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 laboratory-generated reassortant influenza virus is often unpredictable, particularly with reassortants composed of avian–avian gene segments or avian–mammalian gene segments. Rare exceptions have occurred where field isolates have not conformed to the multi-basic cleavage site correlating with virulence and vice versa (12,13,22,23). 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 laboratory-generated reassortant influenza viruses 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 influenza type A viruses that have not been subtyped or subtyped as H5 or H7 and not classified as a low pathogenic strain. The regulatory requirement under 9 CFR § 121.9(c)(1) states that a registered entity is to immediately report such identifications on APHIS/CDC Form 4, unless excluded from the provision under 9 CFR § 121.3(d)(9). CFR § 121.5 provides an exemption for diagnostic laboratories and other entities but reporting on APHIS/CDC Form 4 is still required. In summary, uncharacterized influenza A viruses or uncharacterized H5 or H7 subtypes are considered highly pathogenic, unless determined to be consistent with a strain of low pathogenicity. The importation and interstate movement of low pathogenic influenza viruses may be regulated under 9 CFR Part 122.

Laboratory-Generated 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 (28). 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 (14,15,24). While the highly pathogenic H5N1 viruses do not spread efficiently among humans, infection has resulted in high human mortality and morbidity (14,15). The highly pathogenic H5N1 variants and strains continue to circulate in avian species and occasionally transmit and infect humans (15). As a result, research has increased for intraspecies and interspecies transmission, and pandemic preparedness. On March 29, 2023, USDA first published data on cases of HPAI spillover in mammals. The data is regularly updated and is located at: 2022–2024 Detections of Highly Pathogenic Avian Influenza (usda.gov).

APHIS regulates AIVs and laboratory-generated reassortant influenza viruses which meet the internationally recognized definition of HPAIV – as defined in the current WOAH Terrestrial Manual (29) – as select agents, under 9 CFR §121.3(c). The use of reverse genetic approaches to create laboratory-generated 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 laboratory-generated 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 cell lines, e.g., Madin–Darby canine kidney cell line (1,10,19), are acceptable.

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[a]	As described in the current WOAH Terrestrial Manual	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

[a]Next-generation sequencing of the entire HA gene may be used as an alternative to pathogenicity testing in chickens.
Quer, J., et. al. (2022). Next-generation sequencing for confronting virus pandemics. Viruses, 14, 600. https://doi.org/10.3390/v14030600.

Because the virulence of HPAIV is multigenic and the predominant virulence determinant of HPAIV in poultry is the multi-basic cleavage site of HA, APHIS provides reasonable deductions regarding the regulation of laboratory-generated reassortant influenza viruses to assist in reducing time and financial burden on laboratories. The outline below may be used to determine whether 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 (excluding H5 and H7) 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 subject to regulation by APHIS, Veterinary Services (VS), Organisms and Vectors (OV) Permitting Unit as an "organism," pursuant to 9 CFR Part 122.

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 is subject to regulation by APHIS, Veterinary Services (VS), Organisms and Vectors (OV) Permitting Unit as an "organism," pursuant to 9 CFR Part 122.

For reassortant constructs with a PR8 background, DASAT will accept exclusion requests absent of live bird lethality testing data for avian influenza constructs in which each of the following are demonstrated:

- 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;
- For H5 and H7 constructs, the H5/H7 cleavage site has been altered from highly pathogenic avian influenza (HPAI) to low pathogenic avian influenza (LPAI); or, viruses used for gene inserts have been well characterized as LPAI prior to use in reverse genetically derived processes;
- All reverse genetically derived H5/ H7 viruses with PR8 or similar human vaccine strain backbone must 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 by cell culture system with/without exogenous trypsin.

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 select agent and toxin 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 the select agent and toxin regulations, they may still be 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 laboratory-generated reassortants. However, most laboratory-generated reassortants bear some reference to the parent viruses. Thus, the nomenclature for laboratory-generated reassortants 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 may regulate it pursuant to 9 CFR Part 122. However, to ensure that the fully intact genome is not regulated as a select agent, the method by which the 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, to ensure that the individual RNA segments are not regulated as select agents, the method by which the nucleic acid is extracted from a system must ensure that no viable virus will cross-contaminate preparations. APHIS may regulate the 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 – pursuant to 9 CFR Part 122.

Introduction to Biocontainment Practices and Procedures

Biocontainment may be described as the practices, procedures, and equipment implemented in a laboratory facility to safely manage and contain infectious materials or agents. Proper mitigation reduces the risk of agent exposure to personnel and unintentional release into the environment. From an agricultural perspective, the primary concern while working with AIV in the laboratory is maintaining proper biocontainment. The 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 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 guidance document is not intended to focus on how to design a facility for proper biocontainment or to address the generally accepted requirements of basic laboratory practices. Those standards can be found elsewhere (26,27). Instead, this guidance document aims to align the scope of work conducted in a laboratory with appropriate 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)

Conducting laboratory work with HPAIV in BSL-4 and ABSL-4 laboratories does not necessitate additional provisions. Meeting the BSL-4 and ABSL-4 criteria is adequate for ensuring proper biocontainment. While a personal quarantine policy is not mandatory, the entity incident response plan should cover the scenario of a breach in containment that could result in potential exposure to the agent.

Biosafety Level 3 Agriculture (BSL-3Ag)

In cases where animals infected with HPAIV are housed loosely on the floor or in open caging systems, they are required to be contained in a BSL-3Ag facility. However, in most situations, the BSL-3Ag facility is reserved for large animal species, such as adult swine, for which the use of primary biocontainment housing would not be practical. The animal room functions as an airtight barrier serving as primary containment for the animal. Additionally, as part of risk mitigation, a personal quarantine policy is implemented based on DASAT policy (3).

Biosafety Level 3 (BSL-3)

Laboratories engaging in in-vitro work with HPAIV can have diverse activities and may include clinical, diagnostic, teaching, and research facilities. They may aerosolize, amplify, or propagate HPAIV in eggs, cell culture, or tissue culture. HPAIV can also be used in procedures involving known concentrated virus preparations. These activities should be conducted in BSL-3 laboratories with the following provisions:

Air handling: recommendations include using high efficiency particulate air (HEPA) filters on the laboratory exhaust air system. The exhaust system should feature a sealed ductwork system from the containment barrier to the exhaust filter, and an interlocking supply and exhaust air handling system. Ideally, independent air supply and exhaust systems should be in place. While APHIS does not mandate independent air handling systems, isolating them from other areas in advisable.

Showers: it is recommended to enforce a gown-in, shower-out procedure to change out of street clothing and 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: laboratories should collect liquid effluents locally and either chemically disinfect, or heat treat them, or collect and process them in a central effluent decontamination system before being released into the local sanitary system. While APHIS does not mandate the decontamination of shower and toilet effluents, appropriate practices and procedures for primary containment are recommended.

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.

Personal quarantine policy: it is DASAT policy that as part of risk mitigation a personal quarantine policy is implemented (3).

Animal Biosafety Level 3 (ABSL-3)

When studying HPAIV in the laboratory, researchers may use animal species that vary in size, necessitating special attention to concerns such as containing higher viral loads, aerosols, primary containment caging, and animal husbandry practices. This work can be carried out in Animal Biosafety Level 3 (ABSL-3) laboratories if adequate biosafety measures are in place, as described in more detail below.

AIVs inherently possess the ability to reassort. An example of high-risk research is when conducting two or more experiments at the same time with HPAIV and the 1918 influenza virus (a U.S. Department of Health and Human Services select agent), which could potentially lead to the natural generation of a reassortant influenza virus that is both lethal and highly transmissible among humans. It is recommended to separate these experiments temporally, meaning they should not be carried out simultaneously in the same laboratory room. If such experiments are conducted simultaneously in the same facility, it is advised to conduct them in separate laboratory rooms with independent air systems to prevent the sharing of airspace.

APHIS recommends implementing the following additional biosafety procedures in ABSL-3 laboratories to ensure the adequate containment of HPAIV:

Air handling: APHIS recommends using high efficiency particulate air (HEPA) filters on the laboratory exhaust air system. The exhaust system should feature a sealed ductwork system from the containment barrier to the filter. It is advisable to have interlocking supply and exhaust air handling systems. While it is not mandatory for air handling systems to be independent, they should be isolated from other areas.

Special caging: APHIS recommends placing animals infected with HPAIV in appropriate biocontainment units for animal housing. Achieve containment at the cage level in various ways depending on preference and animal size. Examples include using containment cages or rack systems, flexible film isolators, or glove boxes for primary biocontainment housing. Ensure that caging is ventilated, and the exhaust air is HEPA filtered in all instances. Note that static micro-isolators are ineffective in preventing air leakage into the laboratory space.

Showers: a gown-in, shower-out procedure is recommended to enforce a change of street clothing and 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: laboratories should locally collect and either chemically disinfect or heat treat them, or collect and process them in a central effluent decontamination system before releasing them into the local sanitary sewer system. APHIS does not mandate the decontamination of shower and toilet effluents; nevertheless, APHIS recommends having appropriate practices and procedures in place for primary containment.

Decontamination of solid animal wastes: before leaving the containment barrier, personnel should decontaminate all animal tissues, carcasses, and bedding using an effective and validated method, such as utilizing a tissue autoclave.

Protective clothing: it is important to change clothing before entering the laboratory. 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.

Personal quarantine policy: it is DASAT policy that as part of risk mitigation a personal quarantine policy is implemented (3).

NB: when conducting laboratory work in a facility where the exhaust air is HEPA filtered, AHPIS does not impose a facility proximity restriction on avian species located outside of the laboratory facility.

Transfer of Highly Pathogenic Avian Influenza Viruses and Permitting of 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 the Federal Select Agent Program to possess, use, or transfer AIV. The appropriate document for such a transfer is the "Request to Transfer Select Agents and Toxins" Form (APHIS/CDC 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 or in eFSAP.

APHIS, Veterinary Services (VS), Organisms and Vectors (OV) Permitting Unit regulates the importation into the United States, and interstate transportation, of organisms and vectors of pathogenic diseases of livestock and poultry.

Under most circumstances, LPAIV, laboratory-generated reassortant influenza viruses that have been excluded from 9 CFR Part 121, nucleic acids of AIV, intermediaries (e.g., RNA or DNA extracts), and materials derived from animals or exposed to animal-source materials require a VS permit for importation and interstate transport. These materials are not select agents; hence, a Form 2 is not required.

For additional information concerning importation and interstate transport of non-select agents, contact APHIS, VS, OV staff at (301) 851-3300 or email APIE@USDA.GOV.

Summary

HPAIV is a pathogen of significant agricultural concern worldwide, primarily due to its potential economic impact. The zoonotic potential of some AIVs, particularly the H5N1 Eurasian lineage, has also contributed to this virus' high consequence. Our understanding of this virus has advanced in recent years, but there is still much more to learn. As advances are made, APHIS will modify its regulations and/or this guidance document to maintain transparency regarding the essential principles and practices to prevent unintentional releases of AIVs and exposure to domestic poultry. This guidance document does not follow a one-size-fits-all approach; APHIS will consider alternative approaches to a given scenario or work description. All inquiries from registered entities should first

contact their Centers for Disease Control (CDC) or APHIS point of contact. Entities or individuals not registered with the Federal Select Agent Program should reach out to DASAT for additional information or guidance.

Animal and Plant Health Inspection Service
Division of Agricultural Select Agents and Toxins
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E-mail: DASAT@usda.gov

References

1. Council of the European Communities (1992). Council Directive 92/40/EEC, May 19, 1992, introducing Community measures for the control of avian influenza. *Official Journal of the European Communities*, L167, 1-15.

2. De Benedictis P, Beato MS, Capua I. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. (2007). *Zoonoses and Public Health*. 54(2):51-68. doi: 10.1111/j.1863- 2378.2007.01029.x.

3. Division of Agricultural Select Agents and Toxins (DASAT) Policy Statement: Personal Quarantine Policy for Specified Veterinary Services (VS) Select Agents. (December 3, 2021).

4. Fichtner G.J. (1986). The Pennsylvania/Virginia Experience in Eradication of Avian Influenza (H5N2). In: Proceedings of the Second International Symposium on Avian Influenza, 33-38.

5. Gohrbandt S., Veits J., Hundt J., Bogs J., Breithaupt A., Teifke J.P., Weber S., Mettenleiter T.C., and Stech J. (2011). Amino acids adjacent to the haemagglutinin cleavage site are relevant for virulence of avian influenza viruses of subtype H5. *Journal of General Virology*, 92, 51-59. doi:10.1099/vir.0.023887-0.

6. Greene, J.L. (2015). Update on the highly pathogenic avian influenza outbreak of 2014-2015. Congressional Research Service Report R44114. Online reference https://fas.org/sgp/crs/misc/R44114.pdf.

7. Hoffman E., Krauss S., Perez, D., Webby R., & Webster R.G. (2002). Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine*, 20, 3165-3170.

8. Kaplan, B.S and Webby, R.J. (2013). The avian and mammalian host range of highly pathogenic avian H5N1 influenza. *Virus Res*. 178(1): 3–11. do: 10.1016/j.virusres.201 3.09.004.

9. Kawaoka, Y., and R.G. Webster. (1985). Evolution of the A/Chicken/Pennsylvania/83 (H5N2) influenza virus. *Virology*, 146: 130-137. https://doi.org/10.1016/0042-6822(85)90059-5.

10. Lee, C.W., Lee, Y.J., Swayne, D., Senne, D., Linares, J., and Suarez, D. (2007). Assessing Potential Pathogenicity of Avian Influenza Virus: Current and Experimental System. *Avian Disease*, 51, 250-263. Doi: 10.1637/7572-033106R.1

11. Lee, D.H., Criado, M.F. and Swayne, D.E. (2021). Pathobiological Origins and Evolutionary History of Highly Pathogenic Viruses. *Cold Spring Harb Perspect Med*. Feb 11(2) doi: 10.1101/cshperspect.a038679.

12. Lee, D. H., Torchetti, M. K., Killian, M. L., Brown, I., & Swayne, D. E. (2024). Genome sequences of haemagglutinin cleavage site predict the pathogenicity phenotype of avian influenza virus: statistically validated data for facilitating rapid declarations and reducing reliance on *in vivo* testing. *Avian Pathology*, 1–5. https://www.tandfonline.com/doi/full/10.1080/03079457.2024.2317430.

13. Londt, B.Z., Banks J., and Alexander D.J. (2007). Highly pathogenic avian influenza viruses with low virulence for chickens in *in vivo* tests. *Avian Pathology*, 36 (5), 347-350. https://doi.org/10.1080/03079450701589134.

14. Malik Peiris J.S., de Jong, Menno D., & Guan Yi. (2007). Avian Influenza Virus (H5N1): A Threat to Human Health. *Clinical Microbiology Reviews*, 20 (2), 243-267. doi: 10.1128/CMR.00037-06.

15. Neumann, G., Chen, H., Gao, G.F., Shu, Y., and Kawaoka, Y. (2010). H5N1 influenza viruses: outbreaks and biological properties. Cell Research, 20, 51-61. 10.1038/cr.2009.124.

16. Normile D. (2006). Wild Birds Only Partly to Blame in Spreading H5N1. Science, 312, 1451. doi: 10.1126/science.312.5779.1451.

17. Normile D. (2005). Are Wild Birds to Blame? *Science*, 310, 426-428. 10.1126/science.310.5747.426.

18. Pohlmann, A., et. al. (2022) Has Epizootic Become Enzootic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. *American Society for Microbiology* 13(4) doi: 10.1128/mbio.00609-22.

19. Shankar B.P., Sreenivas Gowda R.N., Pattnaik B., Manjunatha Prabhu B.H., Kamal R.P., Sreenivas B.K., Madhusudhan, H.S., Ranjith D., and Pradhan H.K. (2009). Assessment of Pathogenic Potential of Avian Influenza Viruses by MDCK Cell Culture. *International Journal of Poultry Science*, 8 (5), 462-464. doi: 10.3923/ijps.2009.462.464.

20. Si, Y.J., et. al. (2022). Pathogenesis and genetic characteristics of low pathogenic avian influenza H10 viruses isolated from migratory birds in South Korea during 2010-2019. *Transboundary and Emerging Diseases*, 69, 2588–2599. doi: 10.1111/tbed.14409.

21. Song, J., Feng, H., Xu, J., Zhao, D., Shi, J., Li, Y., Deng, G., Jiang, Y., Li, X., Zhu, P., Guan, Y., Bu, Z., Kawaoka, Y., and Chen, H. (2011). The PA Protein Directly Contributes to the Virulence of H5N1 Avian Influenza Viruses in Domestic Ducks. *Journal of Virology*, 85(5), 2180-2188. doi: 10.1128/JVI.01975-10.

22. Suarez D.L., Senne D.A., Banks J., Brown I.H., Essen S.C., Lee C., Manvell R.J., Mathieu-Benson C., Moreno V., Pedersen J.C., Panigrahy B., Rojas H., Spackman E., and Alexander D.J. (2004). Recombination Resulting in Virulence Shift in Avian Influenza Outbreak, Chile. *Emerging Infectious Diseases*, 10 (4), 693-699. doi: 10.3201/eid1004.030396.

23. Swayne, D.E. & Alexander, D.J. (1994). Confirmation of nephrotropism and nephropathogenicity of three low pathogenic chicken-origin influenza viruses for chickens. Avian Pathology, 23, 345–352. doi: 10.1080/03079459408419002.

24. Tada T., Suzuki K., Sakurai Y., Kubo M., Okada H., Itoh T., and Tsukamoto K. (2011). NP Body Domain and PB2 Contribute to Increased Virulence of H5N1 Highly Pathogenic Avian Influenza Viruses in Chickens. *Journal of Virology*, 85 (4), 1834-1846. doi: 10.1128/JVI.01648-10.

25. Taha F.A. (2007). How Highly Pathogenic Avian Influenza (H5N1) Has Affected World Poultry-Meat Trade. In: A Report from the Economic Research Service. LDP-M-159-02, October 2007.

26. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, & National Institutes of Health. (2020). Biosafety in Microbiological and Biomedical Laboratories (6th ed.)

27. U.S. Department of Agriculture, Research, Education, and Economics. (2012). ARS Facilities Design Standards Manual, 242.1-ARS.

28. World Health Organization (WHO). (JAN 2023). Media Center Fact Sheet Influenza (Seasonal). https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal).

29. World Organization for Animal Health (OIE). (2025). Terrestrial Manual. Chapter 3.3.4., Avian Influenza (Including Infection with High Pathogenicity Avian Influenza Viruses). Avian influenza (woah.org); https://www.woah.org/en/disease/avian-influenza/.