USDA Animal and Plant Health Inspection Service
U.S. DEPARTMENT OF AGRICULTURE

Sample Collection for Avian Influenza and Newcastle Disease

December 26, 2024

This document contains sample collection recommendations from the National Veterinary Services Laboratories (NVSL) in Ames, IA, specifically for the detection of avian influenza (AI) viruses and avian paramyxoviruses, such as Newcastle disease (ND). This document supersedes all previous versions of *Sample Collection for Avian Influenza and Newcastle Disease* (NVSL-WI-0023).

> Testing for AI and ND for all commercial poultry must occur at NAHLN-approved or NPIP-authorized laboratories. All non-negative samples must be forwarded to NVSL-Ames for confirmatory testing. Refer to **Table 1** for information on expected testing and turnaround times.

- Please note that sample collection is conducted for different purposes and while this document serves as a quick reference on how to collect samples, please refer to other documents as needed for specific guidance on how many to collect such as the National Poultry Improvement Program, and the HPAI FAD PreP site.
- For guidance on the appropriate use of influenza A antigen capture immunoassay, refer to www.aphis.usda.gov/sites/default/files/acia_testpolicy.pdf.
- For guidance on the collection of environmental samples, please refer to the Post C&D Environmental Sampling Guidance.
- For foreign animal disease (FAD)[1] investigations (FADI), the fastest route for confirmation is by the collection of duplicate samples for submission to a National Animal Health Laboratory Network (NAHLN) lab and NVSL in parallel – refer to the *Foreign Animal Disease (FAD) Investigation Manual (FAD PReP Manual 4-0)* for further guidance.

IMPORTANT: Regardless of the purpose of testing, target sample collection from birds with the following priority. **NOTE: For FADIs**, a minimum of **two** pools is required for gallinaceous birds, **seven** 5-bird swab pools for non-gallinaceous species.

1) Recent mortalities
2) Sick birds
3) When birds specified in items #1 or #2 above are not available, target birds next to building inlets or in cages adjacent to sick/dead birds; pool these samples separately from the sick and mortality samples.

1.1. Swab collection routes[2]

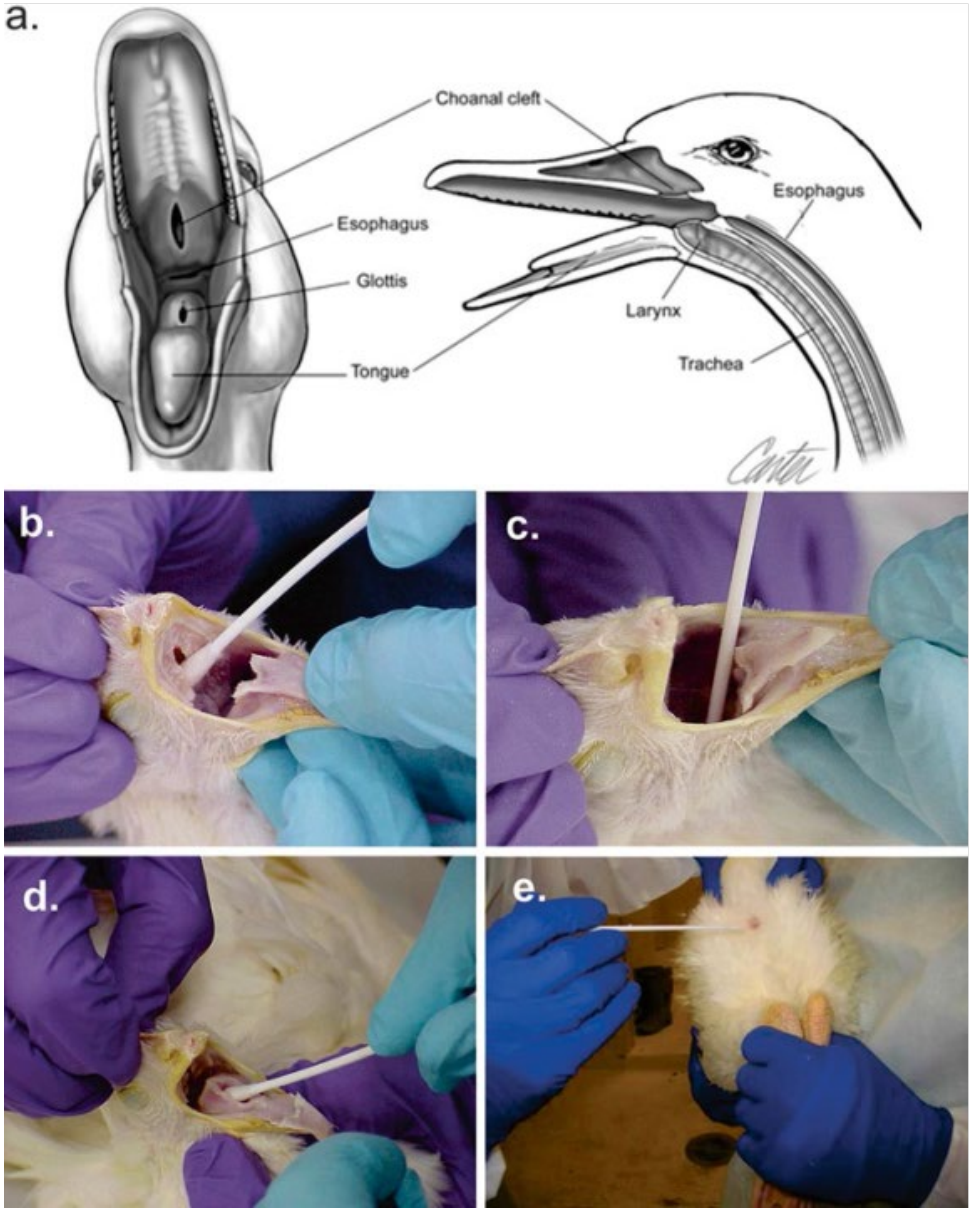
1.1.1. Oropharyngeal (OP) swabs are preferred for gallinaceous poultry (Figure 1b-1d). See NVSL Kit and Instrument Catalog.

1.1.2. Cloacal (CL) swabs are preferred for domestic waterfowl and other wild birds unless H5 goose/Guangdong lineage viruses are suspected in which case both OP and CL swabs are recommended (**Figure 1e**).

[1] www.aphis.usda.gov/animal-emergencies/fadprep

[2] See Figure 1

Figure 1. Swab collection: a) schematic of oral cavity; b-d) tracheal/oropharyngeal (TR/OP) swabs preferred for gallinaceous poultry; e) cloacal (CL) swabs preferred for domestic waterfowl and wild birds. From: Methods in molecular biology, vol. 436: Avian influenza virus. E. Spackman, ed. Humana Press, Totowa, NJ. pp. 95. 2008.



2.1 Swab Pooling procedures[3]

- Swab samples may be pooled in accordance with **Table 2** and by:
  - the same species,
  - the same premises/house/barn,
  - the same sampling route – do NOT pool TR/OP and CL together, and
  - For FADI/sick bird calls, collect at least two 11-swab pools for gallinaceous birds where possible; if the number of birds on the premises/house/barn is 11 or less, **divide samples among at least 2 pools**. Obtaining specimens from healthy birds is generally not recommended; however, when needed to complete sampling, these should be collected in a separate pool, target birds next to building inlets or in cages adjacent to sick/dead birds.

Important Notes

- The 5-swab pool in at least 3 mls of viral transport media (VTM) was validated for both TR/OP and CL swabs from gallinaceous poultry and domestic ducks tested for AI and ND.
- **The 11-bird swab pool is only valid for AI/ND testing of TR/OP swabs from gallinaceous poultry and must be in 5 mls BHI.**
- Submit the **entire swab suspension** for diagnostic testing, removing the swabs at the time of collection or during first line testing. After collecting the sample, swirl the swab vigorously in the VTM, squeeze the excess liquid from the swab inside the specimen tube and collect the swab in an appropriate container for proper disposal at the laboratory. Avoid leaving swabs or other collection devices in the tube; swabs left in the media may reduce the volume available for testing.
- Clearly label containers with appropriate ID using a waterproof marker or other label (barcode ID labels are available from NVSL using the same request form used for BHI).
- For routine NPIP surveillance only (e.g. not FADI), a single 11-bird swab lab pool may be generated from one 5-bird swab pool and one 6-bird swab pool at the testing laboratory[4] (testing labs should refer to *NVSL SOP-AV-0068* for further details).

NOTE: The NVSL supplies 5ml BHI which usually contains antibiotics and is not appropriate for testing of bacterial diseases. Refer to **Appendix 1** for additional BHI information.

Tissue Collection

Refer to **Table 2**. Pool tissues by system (respiratory, enteric, reproductive) typically from a single bird; it is not recommended to pool tissues from more than one bird especially for free-living waterfowl.

Environmental sample collection

Refer to https://www.aphis.usda.gov/sites/default/files/env_sampling_proced.pdf.

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[3] Pooling procedures are based upon work conducted by Spackman et al. in coordination with the NVSL (BMC Veterinary Research 2013, 9:35 http://www.biomedcentral.com/1746-6148/9/35).

[4] While pools of 6 swabs have not specifically been evaluated, it is considered an acceptable practice where seeking to conduct recommended 11-bird swab surveillance using 2 pooled samples instead of 3 (refer to 3.1.3); NOTE: Testing of a single 11-bird swab pool only applies to TR/OP swabs from gallinaceous poultry.

Table 1. For informational purposes only. APHIS approved and authorized laboratories are expected to initiate testing and provide test results according to the timeframes below.

NAHLN (PCR) and NPIP (Serology) Testing	
Complete testing	• FADI Priority 1, or A typically same day (includes control area surveillance) • FADI/sick bird Priority 2 within two business days or as indicated by SAHO/AVIC • Routine NPIP surveillance per laboratory accession order
Forward non-negative samples to NVSL	• FADI Priority 1, or A typically same day (includes control area surveillance) • Others within 24 hours (one business day) of non-negative
NVSL Testing	
Estimated turn-around time	• Priority 1, or A FADI: PCR within 4 hours of sample receipt including pathotype assay where available (H5 clade 2.3.4.4-specific) • Cleavage site attempt within 9 hours • Testing for lower priorities initiated within 24 hours, subject to testing burden
Virus characterization (method as appropriate)	• Whole genome sequencing attempted directly from sample 2-4 days for high priority, ~10-14 days standard timeline • Virus isolation ~7-14 days when needed

3.1 Specimen transfer and storage

- Maintain cold chain for all samples. Specimens should be held on ice pack immediately following collection until transferred to the testing laboratory or other refrigerated storage.
- Tubes should be stored and transferred in an upright position to reduce chances of leakage.
- AI and ND have been shown to be stable in BHI when stored at refrigeration (4ºC) for up to **96 hours**, with consideration given to the length of time needed at the laboratory for sample processing. If samples have been frozen (-70ºC), they should remain frozen until delivered to the testing laboratory.
- Specimens should **never** be stored in the freezer portion (-20ºC) of a standard refrigerator/freezer unit with an automatic defrost cycle (specimens will go through freeze/thaw, which is detrimental to the survival of virus and viral nucleic acid).

4.1 Forwarding samples to the NVSL in Ames, IA

4.1.1 **Email ahead of shipment, especially for Priority 1, A, or 2** to: nvsl.ai.nd@usda.gov - Please include: 1) number of samples, 2) completed 10-4 form[5] or NCAH portal packing list including animal location and premises identification number, 3) tracking number, 4) pertinent case and contact information on the day the package is shipped.

a. Format for Avian/Wild species Email Subject line:

MN / wild bird / UPS XXXXX
WA / FAD 22WAXXXX / FedEx XXXXX
WI / wild mammal / UPS XXXXX
MN / post C&D / location ID / FedEx XXXXX
PA / LBM / UPS XXXXX

Please use 8 AM delivery option (FedEx First) when shipping high priority especially for Friday delivery.

Contact the Diagnostic Virology Laboratory (refer to: USDA APHIS | NVSL Diagnostic Virology Laboratory) for instructions when sending driver/courier after hours/holidays/weekends.

[5] VS Form 10-4 Specimen Submission

Table 2. Preferred specimens for Influenza A and Newcastle disease diagnostics.

Sampling source	Preferred Specimen	Sample Collection	Comment
Gallinaceous poultry (e.g., chickens, turkeys, pheasants, quail)	**Tracheal or oropharyngeal (TR/OP) preferred** Cloacal swab (CL) may be used	• FOR FADs – typically 5 swabs/ pool in at least 3 mls of VTM • Up to 11 swabs/pool in at least 5mls of VTM pooled is valid only for TR/OP swabs from gallinaceous species [a] Up to 5 swabs/pool at least 3 mls of VTM pooled by sample route and species	Virus usually shed via respiratory route; may be strain dependent For egg production drops in turkeys cloacal and oviduct swabs are preferred, semen can also be tested
Domestic waterfowl (production)	**CL preferred**, TR/OP swab may be used	Up to 5 swabs/pool from a single flock and species in at least 3 mls of VTM	Virus usually shed via the enteric route; may be strain dependent – e.g., for H5 goose/Guangdong lineage, both OP and CL are recommended
Wild/captive waterfowl species[c]	**TR/OP and CL** swabs may be used	• Collect USDA Wildlife Services Surveillance samples by pooling 1 CL and 1 OP swab from a **single bird** in one 3ml VTM tube[b]; this approach may also be used for captive waterfowl that are openly housed • Captive flocks in closed, common housing may be pooled 5 swabs/pool in at least 3mls VTM by sample route and **species**	Wild migratory waterfowl are the natural reservoir for influenza A viruses (typically enteric shed)
Other wild/free living/captive /pet species	Typically, **CL swabs**; fresh fecal samples may be used – call the NVSL for guidance	• Captive flocks in closed, common housing may be pooled 5 swabs/pool in at least 3mls VTM by sample route and **species group** (e.g., passerines)	Shedding of influenza from non-host species can be variable and dependent on other factors such as immune status and virus strain
Any avian species	Tissue samples	Pool by system from a **single** bird (e.g., respiratory, enteric, reproductive) [b] - mince tissue and place in 3mls VTM	vND viruses may replicate to higher titres in tissues; brain tissue is preferred if neurological signs are noted

[a] The 5-bird swab pool in 3 mls of VTM was validated for AI and ND testing of both TR/OP and CL swabs from gallinaceous poultry and domestic ducks; pooling of up to 6 swabs from the same species, location, and sampling route in 3 mls allows for collection of 11 samples per Secure Supply plans and NPIP surveillance using two tubes rather than three. Either 3ml (any for domestic species) or ~5 ml with antibiotics (for TR/OP swabs from gallinaceous poultry only) may be used for zone surveillance.

[b] Antibody from one bird may neutralize virus from another (e.g., mixed backyard poultry); avoid potential of mixing viruses from different birds in a single sample when sampling migratory waterfowl.

[c] The H5 goose/Guangdong HPAI viruses may be shed higher via the OP route in waterfowl; sampling of both routes is recommended.

Serum collection

For routine monitoring, serum samples should be collected from apparently healthy birds. Do not use cull birds that are sick or appear distressed. In caged housing systems, it is important to select birds from various locations throughout the house. A minimum of 10 samples[6] per flock should be collected to ensure adequate detection of antibody.

For disease investigations, blood samples can be collected from the same birds that are being swabbed, e.g., targeting those exhibiting clinical signs or lesions.

A good quality serum sample will appear clear to pale yellow in color. Cloudy or hemolyzed samples should be avoided. To increase the volume of sera obtained immediately after collecting blood, lay the tube on its side to increase surface area exposure to air at room temperature. Do not shake, roughly handle, or freeze the blood while the clot is forming, or hemolysis will occur. Overall sample quality is achieved by separating the sera from the clot prior to shipping.

Supplies:

- Sterile 3-5 cc syringes
- 20-22 gauge needles (wing vein collection)
- Sterile 3 ml plastic or glass blood tubes with leak-proof caps

Collect 2 to 3 mL of blood from each bird not to exceed 1% of the body weight of bird. This volume of blood will generally yield 1 to 1.5 mL of serum. NOTE: The minimum volume of serum required for testing is 0.5ml –1.0ml.

- Do not leave samples in a hot car or in direct sunlight
- If birds are dehydrated (hot weather, stress), they produce poor serum samples that are gelled. Additionally, serum from birds after a recent meal appear cloudy due to excess fat in the serum. Lipemic (fatty) samples are not ideal for laboratory testing as the fat will interfere with any optical based test or antibody fixing test such as ELISA and AGID.
- Keep the serum samples cool and send immediately to the laboratory on gel ice packs.
- Do not send serum samples to the lab that:
  - Contain less than 0.25 ml of serum
  - Are excessively hemolyzed
  - Are excessively lipemic (fatty)
  - That contain blood clot(s)
  - Are gelled, slimy, or contain white particles

[6] See NPIP Program Standards (December 2019). *A suggested number of samples would be 5 samples from pens or houses with less than 500 birds; 10 samples from pens or houses with 500 to 2,500 birds; and 15 samples from pens or houses with more than 2,500 birds.*

Appendix 1: Viral Transport Media (VTM) and Sample Collection Devices

- Brain heart infusion broth (BHI; BD Bacto #237400) is the recommended VTM for these specimens as it contains a protein component which protects the virus from degradation during storage and shipping and is available from the NVSL for national avian influenza testing. NOTE: BHI when stored appropriately and visually inspected is fit-for-purpose for several years. States should coordinate media purchase for NPIP testing, the order form can be accessed here.
    - 3 ml BHI Broth (40 tubes/box; no antibiotics): blue cap plastic tube for **up to** 5-bird swab pools from avian species.
    - ~5 ml BHI with antibiotics (40 tubes/box): black cap glass tube for up to 5-bird swab pools for any avian species, and **up to** 11-bird swab pools from gallinaceous poultry only. Preferred for environmental samples; however BHI alone is acceptable due to supply chain issues with the antibiotic components.
- Other acceptable VTM include any salt-balanced, buffered media with a protein component such as tris-buffered tryptose broth (TBTB)[7], nutrient broth (NB), and peptone broth (PB); or commercially available media, e.g., BD™ Universal Viral Transport 3 mL Collection Kit, and Primestore MTM (currently for Wildlife Services wild bird surveillance; and where approved by APHIS – contact NVSL for appropriate sample volume).
- If not an FADI, In the absence of appropriate VTM, phosphate buffered saline (PBS) or saline solution (contact lens solution – *not* disinfectant) may be used as a last resort to keep the swab moist during transport – dry swabs are **not** acceptable. **NOTE:** PBS or saline should only be used when none of the preferable media are available as **negative results are not acceptable** for confirmation of disease status; appropriate samples should be obtained.
- Dry swab specimens should be avoided – heat and desiccation can inactivate AI and ND in ≤24 hours; therefore, **negative results are not acceptable** for confirmation of disease status; appropriate samples should be obtained.

Sample collection devices (swabs)

- Use synthetic or semi-synthetic swabs (e.g., polyester, rayon, nylon) with a plastic handle (flocked or spun head).
- **Avoid** cotton or calcium alginate swabs or swabs with wooden handles which have been shown to inactivate virus and inhibit PCR invalidating the laboratory test results.

BHI Stability

Stability studies were conducted on 3 separate lots of BHI media containing antibiotics (NVSL media # 50067) and 3 separate lots containing no antibiotics (media # 10009). Each lot of media was spiked with live virus then frozen and thawed two days later to mimic shipping conditions from field to lab. Virus isolation was conducted and harvested material was evaluated for bacterial contamination and confirmation of virus recovery. Testing was conducted three separate times for each of the 3 lots of media over the course of approximately one year.

[7] TBTB Formulation (NVSL media #10088): 1.21 g Trizma Base 77-86-1, 26 g Tryptose Broth, 1000 ml QH2O

BHI broth was determined to be stable a **minimum of 6 years from manufacture date**. Expiration dates are provided on the outer box containing BHI tubes prepared at the National Veterinary Services Laboratories for quality management. The expiration of unopened BHI tubes may be extended in 6 month increments up to 6 years past expiration using the following criteria.

1) The expiration on the box applies to the tubes provided in the original shipment. Tubes can be individually labeled with the expiration date after receipt.
2) Individual laboratory policies for expired reagents should be used if applicable. If the policy is to NOT use expired reagents, then a deviation including a plan must be put into place to proceed. A laboratory may have a policy to requalify media or obtain documentation supporting an extended expiration. If there is no policy regarding expired media, follow the recommendations below.
    - BHI expiration can be extended as long as the unopened media has been stored at 4°C or colder (-20°C recommended), the tubes have not been compromised, no obvious color change is observed, and the media does not appear to be cloudy.
    - The following should be documented: storage conditions of the expired media since the time of receipt, who confirmed the criteria in bullet 3 and when the media was evaluated.