Laboratory Diagnosis of Avian Influenza and Newcastle Disease

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Objectives: Laboratory Diagnosis of AI/ND

- **Serologic Diagnosis**
  - AI – AGID, ELISA, HI, NI
  - ND – HI, ELISA

- **Virus isolation**

- **Antigen capture** – pen-side diagnostic tests (AI)

- **Molecular diagnostics** (rRT-PCR) (AI/ND)

- Types of samples needed for above tests

- Advantages and disadvantages of above tests
Diagnosis of AI/ND

- **Presumptive diagnosis**
  - Serologic diagnosis
  - Clinical signs/lesions (HPAI, VVND only)
  - Antigen capture tests (AI)

- **Definitive diagnosis**
  - Isolation and characterization of the virus
  - Molecular detection with subtyping/pathotyping
Diagnosis of AI/ND

Source of Samples

- **Passive surveillance**
  - Investigations of clinical cases

- **Active surveillance (random, organized and targeted)**
  - Live bird markets
  - Processing plants—slaughter, eggs
  - Export testing
  - Pre slaughter/movement
  - Backyard poultry
Diagnosis of AI/ND

Source of Samples

- Newcastle Disease vaccinated commercial flocks
  - Monitor feed and water consumption, daily mortalities, egg production
  - Collect swabs from daily mortality (dead birds) for virus isolation/detection
Diagnosis of AIV

Serologic Tests:

- **Type-Specific Tests (type A, B, C):**
  - Agar gel immunodiffusion (AGID) test
    - IgM, (some IgG)
  - Enzyme-linked immunosorbent assay (ELISA)
    - IgG
  - Detects all subtypes (H1-H16)

- **Subtype-Specific Tests (H or N subtype):**
  - Hemagglutination-inhibition test
  - Neuraminidase-inhibition test
  - Detects only homologous subtype
Diagnosis of NDV

Serologic Tests:

- Limited value because of routine use of vaccine
- Hemagglutination-inhibition test (HI)
- Enzyme-linked immunosorbent assay (ELISA)
**AIV (Antibody Detection)**

**Samples Versus Tests:**

<table>
<thead>
<tr>
<th>Sample</th>
<th>AGID</th>
<th>ELISA</th>
<th>HI/NI</th>
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<tbody>
<tr>
<td>Serum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Plasma</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Egg Yolk</td>
<td>Yes</td>
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![Serum/Plasma](image1.png) ![Egg Yolk](image2.png)
Type-Specific Tests for AIV: AGID Test

**Advantages:**
- Gold Standard (screening)
- Detects Antibody to all influenza A virus
- Easy, inexpensive, requires few reagents/equipment

**Disadvantages:**
- Semi quantitative
- Moderate sensitivity
- Subjective interpretation
- Requires 24 hours
- Further testing of positives
- Antibodies not detectable for several days
AGID Test (AI)

1. Pour Agar
2. Cut Agar
3. Remove Agar Plugs
4. Fill Wells
CAUTION!

- The AGID and ELISA tests should be used to determine the immune status of a flock, not an individual bird
Enzyme-Linked Immunosorbent Assay (ELISA)
Type-Specific Tests for AIV: \textit{ELISA}

- **Advantages**
  - Commercial kits available
  - Rapid (same day)
  - Can be semi-automated

- **Disadvantages**
  - Requires expensive equipment
  - False positive reactions
  - Positives require confirmation
AIV ELISA

Source of Diagnostic Kits:

- IDEXX Laboratories, Inc., Westbrook, Maine
  - FlockChek®

- Synbiotics International, San Diego, CA
  - ProFLOK®
Subtype-Specific Tests for AIV

HI/NI (antibodies)

- **Advantages**
  - Gold standard
  - Quantitative (titer)
  - Rapid (same day)

- **Disadvantages**
  - Requires many reagents (antigens/antiserums)
  - Non-specific (steric) inhibition
  - Requires pre-treatment of serum to remove normal serum agglutinins (false negatives)
HI Test – AIV

**Interpretation of Results:**

- Serum HI titers of $\geq 1:8$ are suggestive of previous exposure to AIV/NDV, provided the antigen used in the HI test was devoid of homologous neuraminidase.
  - For example: a serum with H9N2 antibodies could give a positive HI titer against the H5N2 antigen because of steric inhibition with the N2.
AIV Neuraminidase-Inhibition Test

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Neuraminidase-Inhibition Test

Virus + Antibody

Fetuin

Homologus Ab + Virus

Heterologus Ab + Virus

Bound NANA

Unbound NANA

Periodate Reagent

Formation of a chromophore (Pink color)

Heat (56°C)

Thiobarbituric Acid

Sodium Arsenite

β-formal Pyruvic Acid
Diagnosis of AIV:  
*Virus Detection Methods*

Virus Isolation
- Required for virus characterization

Rapid Diagnostics – Antigen Capture (AI)
- Make quick decisions in the field

Real Time Reverse Transcription-Polymerase Chain Reaction (rRT-PCR)
Virus Isolation

- **Samples** – any (tissue, swabs)
- **Advantages**
  - Gold standard
  - Sensitive – all subtypes
- **Disadvantages**
  - Expensive and labor intensive
  - False negatives (sample mishandling)
  - Special facilities needed
Flow Chart for AI/ND Testing

1. **Specimens Received** → **Prepare Worksheet** → **Process Specimen** → **Inoculate Embryos** → **Candle Eggs Daily**

2. **Review Case** → **Run HI** If HA+ → **Run HA Test** → **Check for Bacteria** → **Harvest AAF**
   - Yes → **Dead Embryos?** → **Day 4**
   - No → **No**

3. **Dead Embryos?** → **Yes** → **HA Positive** → **Repassed Before?** → **No**
   - Yes → **Inoculate Additional Embryos**
   - No → **Inoculate Chickens**

4. **Dead Chickens?** → **No** → **Final Report**
   - Yes → **Necropsy Chickens**

5. **Report Negative** → **Sequence if H5 or H7** → **Notify Field** → **Yes** → **Inoculate Chickens**
   - No → **No**

6. **HA+?** → **Yes** → **Notify Field** → **Yes** → **Inoculate Chickens** → **Dead Chickens?** → **No** → **Final Report**
   - No → **No**
Isolation of AIV: Sample Collection

Tissues (do not pool tissues from different birds)
- Lung
- Spleen

Swabs (pool up to 5/tube in BHI)
- Tracheal or oropharyngeal
- Cloacal

Note: Keep tissue and swabs cold (on ice)
Isolation of AIV: *Embryonating Eggs*

- Specific-pathogen-free (SPF) flocks
- Commercial flocks negative for AIV
- Inoculate between 9-11 days of incubation
  - Chorioallantoic sac (CAS) route
  - Yolk-sac (YS) route
Characterization of H5 and H7 Subtypes of AIV

- Usually performed by reference laboratories
- Determine H and N subtype
- Intravenous inoculation of chickens:
  - 8 chickens (4-8-weeks of age)
  - Observe for 10 days
  - Isolates killing 6 of 8 chickens (75%) = HPAI
- Sequence cleavage site of HA gene
  - Presence of multiple basic amino acids = HPAI
Characterization of NDV

- Usually performed by reference laboratories
- Intracerebral Pathogenicity Index (ICPI) in day-old chicks
  - 10 day-old chicks
  - Observe for 8 days
  - Isolates with ICPI ≥ 0.7 = virulent
- Sequence cleavage site of F gene
  - Presence of multiple basic amino acids = virulent NDV
Alternate Methods to Detect AIV

Antigen Capture

- Directigen™ Flu A Test (Becton Dickinson)
- Flu DetectTM (Synbiotics)
- 15-20 minute tests, 70%-80% sensitivity
- Most useful for sick and dead birds (acute)
Antigen Capture:
*Directigen/Flu Detect*

Samples – swabs only

**Advantages**
- Rapid (15-20 minutes)
- Highly specific
- No special facilities required

**Disadvantages**
- Cost ($8-$25/test)
- Moderate sensitivity (70-80% compared to VI)
- False positives (bacterial contamination)
- Interference by blood (alkaline phosphatase)
Molecular Diagnostics:
AIV rRT-PCR

Samples: tracheal/oropharyngeal swabs preferred, cloacal swabs, tissue (lung, spleen)

Advantages

- Rapid (2.5 hours)
- Highly sensitive/specific
- Differentiates type A, H5, and H7

Disadvantages

- Expensive equipment
- Moderate per test cost ($8)
- Special facilities required
- False negatives – genetic variation
Molecular Diagnostics: NDV RRT-PCR

Samples: tracheal/oropharyngeal swabs preferred, cloacal swabs, tissue (lung, spleen)

Advantages
- Rapid (2.5 hours)
- Highly sensitive/specific
- Can differentiate between virulent and avirulent strains

Disadvantages
- Expensive equipment
- Moderate per test cost
- Special facilities required
Avian Influenza Diagnostic Tests (LPAI): Range of Detection in a Flock (Unvaccinated)

- **Virus Level**
- **Days Post-Infection**: 0, 7, 14, 21, 28

**Antigen Capture**
- AGID (IgM, may start to decrease after 30 days)
- ELISA (IgG)
- HI (IgG)

**rRT-PCR**

**Virus Isolation**

**Range of Detection**
- HI (IgG) detection can occur as early as 7 days post-infection.
- AGID (IgM) may start to decrease after 30 days.
Remarks:

- Virus isolation is the gold standard test
- Sequence is important to define or predict a change in pathogenicity
- Real-time PCR for the detection of AI and the differentiation of H5/H7
- Pen-side antigen detection tests provide a quick screen of respiratory cases in 15 minutes with 70-80% sensitivity
If ELISA tests are used for screening, positive results should be confirmed with AGID, followed by HI for H5 or H7.

For vaccinated populations, sentinel birds must be used and diagnostic tests must be able to differentiate between infected and vaccinated animals (DIVA).

Serologic tests used for AI surveillance in absence of or following outbreaks – AGID, ELISA, HI

Positive AI AGID and ELISA serums should be submitted to reference laboratory for subtyping

Virus isolation is needed to determine the pathogenicity of new field isolates of AIV/NDV

Antigen detection kits are useful pen-side tests to quickly confirm AI infections

Molecular diagnostics (rRT-PCR) are rapidly replacing conventional isolation procedures for AI/ND
Surveillance Tools for Influenza: Agent Detection

- Virus isolation (embryonating chicken eggs or cell culture)
  - Gold standard

- Molecular detection assays
  - Conventional RT-PCR assays
  - Real-time RT-PCR
  - Nucleic acid sequence based amplification (NASBA)

- Antigen capture immunoassays
  - On-farm testing – quick diagnosis
Antigen Capture Immunoassays - AI

- **Samples** – best suited for testing sick or dead birds (need 3-5 logs of virus)
- **Advantages**
  - Rapid (15-20 minutes)
  - Highly specific
  - No special facilities required
  - Cost varies ($8-25/test)
- **Disadvantages**
  - Moderate sensitivity (70-80% compared to VI)
  - False positives (poor sample quality)
  - Low sensitivity in vaccinated populations
Molecular Detection Assays - AI

- **Advantages (PCR, NASBA)**
  - Rapid (2-6 hours)
  - Sensitivity similar to VI (85-95%), high specificity
  - Type or subtype specificity (H5 and H7)
  - Can determine pathogenicity of H5 and H7 virus from clinical specimens (sequence the HA gene)
  - Cost varies ($8-50/test)
  - Potential for high throughput (96, 384)
  - Live virus not required
Molecular Detection Assays - AI (cont’d)

- **Disadvantages**
  - High cost of equipment ($25,000-90,000)
  - False positives (lab contamination)
  - Does not differentiate live from inactivated virus (not good for environmental testing to show freedom from virus)
  - False negatives (PCR inhibitors, extraction inefficiency, genetic diversity of isolates)
Avian Influenza Diagnostic Tests (HPAI): Range of Detection in a Flock (Vaccinated)

- Antibody levels (AGID, ELISA, HI) will remain high, but of little value unless DIVA testing is used.

Antigen Capture (not likely to detect infection)