Virus Isolation and Specimen Processing

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Biosafety Cabinets II

- Turn the cabinet on 3-5 min. before use
- Adjust the viewing screen
- Don’t block the air flow (grills)
- Separate clean from dirty items
- Arrange items for logical workflow
- Disinfect material in cabinet before removal
- Control traffic directly behind the hood
Collection of Tracheal/oralpharyngeal and Cloacal Swabs

- Swab the TR/OP or cloaca area with a sterile Dacron swab
- Disperse swab contents in media
- Remove the swab from media
- Can pool 5 tracheal or 5 cloacal swabs in a single tube – do not pool TR/OP and CL swabs if processing specimens for RRT-PCR
Tissue Pooling

- **Respiratory**
  - Lung, trachea, air sacs (posterior)

- **Digestive**
  - Liver, pancreas, small intestine (duodenum, jejunum, ileum), cecum, proventriculus, ventriculus, large intestine

- **Urinary – kidney**

- **Lymphoreticular system – spleen & Bursa of Fabricius**

- **Nervous – brain**

- **Cardiovascular – heart**

- **Reproductive – oviduct and ovaries**

- **Swabs – Tracheal/oral pharyngeal or cloacal (5 swabs per tube)**
Do’s of Tissue Processing

- Do’s
  - Do pool tissues from an organ system
  - Digestive and nervous systems are processed separately
  - Heart can be pooled with spleen and bursa
  - Lung and spleen can be pooled
  - Liver and kidney can be pooled

- Keep tissues cold (on ice)
Don'ts of Tissue Processing

- Don’t pool tissues from more than one bird – Each bird should be separate
  - Antibody from one tissue can neutralize the virus from another tissue
- Don’t pool the brain with any other tissue
- Don’t pool digestive organ tissues with other organ system
Processing Tissues for Isolation of AlV and APMV

- Prepare 10% suspension in antibiotic diluent
- Centrifuge at 1,500 x g - 20 minutes
- Remove supernatant with pipette and place in sterile vial
- Incubate 1 hour at room temperature
Processing Swab Pool Specimens
Isolation of AI V and APMV
Sample Processing:

- **Antibiotics (final concentration)**
  - Penicillin (10,000 IU/ml)
  - Streptomycin (2,000 - 10,000 μg/ml)
  - Gentamicin sulfate (1,000 μg/ml)
  - Kanamycin sulfate (650 μg/ml)
  - Amphotericin B (5-20 μg/ml)
Isolation of AI V and APMV
Sample Processing:

- **Swabs (tracheal and cloacal)**
  - Vortex (mix) to resuspend material and rinse all swab material from the sides of the tubes
  - Centrifuge 1,500 x g – 20 minutes
  - Remove supernatant with pipette
  - Add supernatant to antibiotic diluent (3-4 ml total)
  - Incubate 1 hour at room temperature
Embryos for Virus Isolation

- **Specific-pathogen-free (SPF) flocks**
- Commercial flocks negative for AI V and APMV
- Embryos from SPF and commercial flocks must be negative for bacteria
- Ab + flocks – limited use
  - After day 14 the antibody will move from the yolk to the rest of the egg, neutralizing the isolated virus
  - Will reduce the ability of the virus to grow and the success of virus isolation
  - No YS inoculation
Isolation of AIV and APMV Embryonating Eggs

- **Allantoic sac (AR) route**
  - 9-11 day old embryos
  - Incubate 5 days (APMV) and 4 (AIV)

- **Chorio-allantoic sac (CAS) route**
  - 10-11 day old embryos
  - Incubate 7 days APMV and AIV

- **Yolk-sac (YS) route**
  - 6-7 day old embryos
  - Incubate 7 days APMV and AIV
Embryo Inoculation

Routes of Egg Inoculation

Method B -
23 gauge 1” needle

22 gauge 1” needle

22 gauge
1½” needle

25 gauge 5/8” needle

22 gauge
1½” needle
Insert a small hole through the shell (not the eggshell membrane) on the lateral side.
Dropped CAM with air cell on side of embryo
Candle Embryos Prior to Inoculation

- Check embryo for:
  - Proper fertility
  - Proper growth of embryo
  - Placement of air sac
  - Development of chorio-allantoic membrane
Egg incubator
37-39 C
relative humidity 60-70%
Candle eggs daily
Checking for embryo mortality
Live 12 day old chicken embryo prominent vessels in CAM

Embryos

Dead chicken embryo – no CAM

Refrigerate embryo – embryos is dead and blood vessels are constricted
Equipment Needed for Egg Candling and Inoculation

- 4 eggs/student
- Egg flat
- Drill
- 70% Ethanol spray bottle
- Marker
- Glue
- 23 guage 1” needle
- Egg labels
- Sharps container
Harvesting AAF from Dead Embryos

1. Disinfect egg shell surface
2. Only open eggs from a single specimen at one time in the BSC
3. Open egg from air cell end with forceps
4. Break allantoic sac with sterile forceps
5. Hold membranes and embryo away from pipette tip with forceps
6. Harvest AAF
7. Streak BA plate
8. Centrifuge 1,500 x g for 15 min.
Equipment Needed for Harvesting AAF from Dead Eggs

- 2 embryos/student
- 2 Forceps
- Iodine box for forceps
- 5 ml pipettes and pipette aid
- Gloves
- Blood Agar plate
- Plastic loops

- Snap cap tube with labels
- Ethanol spray bottle
- Iodine bucket for pipettes
- Discard bucket with bags for embryos
- Plastic bags to discard flats
Harvesting AAF from Dead Embryos

1. Disinfect egg shell surface
2. Only open eggs from a single specimen at one time in the BSC
3. Open egg from air cell end with forceps
4. Break allantoic sac with sterile forceps
5. Hold membranes and embryo away from pipette tip with forceps
6. Harvest AAF
7. Streak BA plate
8. Centrifuge 1,500 x g for 15 min.
Harvesting AAF for Live Embryos

1. Refrigerate embryo to kill embryo and constrict chorioallantoic vessels
2. Disinfect shell
3. Drill small hole above air cell line
4. Aspirate AAF with 3cc 22 gauge 1½” syringe
5. Discard AAF with red blood cells – RBC will bind with virus
Candling Live Embryos

Live 12 day old chicken embryo prominent vessels in CAM

Refrigerated chicken embryo – embryo is dead and the blood vessels are constricted
Harvesting AAF for Live Embryos

1. Refrigerate embryo to kill embryo and constrict chorioallantoic vessels
2. Disinfect shell
3. Drill small hole above airsac
4. Aspirate AAF with 3cc 22 gauge 1½” syringe
5. Discard AAF with red blood cells – RBC will bind with virus
AIV and PMV-1 Virus Isolation & Identification

- AAF from all dead and live embryos are tested by the hemagglutination test (HA) for detection of a hemagglutinating virus.

- All hemagglutinating viruses are identified by the hemagglutination-inhibition (HI) test using monospecific antisera.

- AAF from HA negative dead embryos are processed for 2nd passage
Flow Chart for Avian Influenza Testing

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

Review Case → Run HI If HA+ → Run HA Test → Check for Bacteria → Harvest AAF

Dead Embryos? Yes → HA Positive → Repassed Before? No → Yes → Inoculate Additional Embryos

Yes → Notify Field

Harvest AAF

HA+? Yes → Notify Field

Dead Embryos? No → Yes → Final Report

Harvest AAF

Inoculate Chickens

Dead Chickens? Yes

Necropsy Chickens

Sequence if H5 or H7

Report Negative
Equipment for Harvesting AAF from Live Eggs

- Drill
- 4 eggs/student
- 3 cc syringe with 22 gauge 1½” needle
- Snap cap tube with label/student
- Plastic bags to discard flats
- Ethanol spray bottle
- Sharps container
Thank you for your participation in the virus isolation portion of the training
Dropping CAMS for Chorio-allantoic sac (CAS) Inoculation
Candle 10-11 day-old embryos and check for embryo vitality – mark the air cell line
Check for embryo location and mark the side opposite the embryo midway along the long axis where the vein structure is well developed.
Disinfect egg shell surface on both the air cell end and side.
Drill a small hole through the shell and eggshell membrane in the air cell end of the embryo.
Apply gentle pressure to the eggshell membrane which detaches the membrane from the shell.
Loosen the egg membrane to allow the air cell to move from the end of the egg to the side.
Eggs should lie in a horizontal position with the inoculum and air cell holes glued shut.
Embryo Lesions
Avian Respiratory Disease

Caseous exudate, mucous and hemorrhage in the trachea may be induced by a variety of avian viruses.
Newcastle Disease Virus

The type and extent of lesions in the embryo is dependent upon the strain and pathogenicity of NDV.
Infectious Bronchitis Virus

CAM inoculation results in very stunted and tightly curled embryos.

Multiple passages are required to produce typical lesions. Urates in the kidney are one of the visible signs that may occur in early passages.
Infectious Laryngotracheitis Virus

Inoculation by dropped CAM reveals formation of pox lesions
Infectious Bursal Disease

Classic and variant IBDV can be distinguished Based on embryo mortality and lesions.

Standard embryo lesions – hemorrhagic embryos, “parboiled” livers and pale spleens

Variant embryo lesions – cream colored embryos, liver necrosis and splenomegaly
Infectious Bursal Disease

Classic and variant IBDV can be distinguished based on embryo mortality and lesions as well as bursal lesion in susceptible birds 4 DPI.
Marek’s Disease Virus

“Red Leg” a prominent gross lesion associated with the early phase of virulent MDV in young chickens

Chicken Anemia Virus – thymic atrophy

Avian Encephalomyelitis Virus – Muscle atrophy