Sick & Dead Wild Bird Disease Surveillance *

SAMPLE COLLECTION PROTOCOL

*with emphasis on Highly Pathogenic Avian Influenza

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Some information in this manual has been adapted from An Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds U.S. Interagency Strategic Plan http://www.nwhc.usgs.gov/publications/other/index.jsp

Adapted by the Food and Agriculture Organization of the United Nations in collaboration with The Wildlife Conservation Society

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* with particular reference to Highly Pathogenic Avian Influenza

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Introduction

The purpose of this document is to provide brief guidelines on methods necessary to conduct a wildlife mortality event investigation and to properly collect samples for the investigation of avian disease of concern to FAO, OIE or WHO such as Avian Influenza, West Nile Virus, and Exotic Newcastle Disease. Since highly pathogenic H5N1 Avian Influenza (H5N1 AI) poses a potential human health risk, procedures to avoid exposure while working with live or dead wildlife is included.

While not all species infected necessarily exhibit signs of disease, the current strain(s) of H5N1 circulating in Asia, Europe, and Africa have been shown to cause morbidity and mortality in a wide variety of species. Combining targeted active surveillance (capture and sampling of free ranging “apparently healthy birds”), passive surveillance (disease testing from hunted birds, rehabilitation centers, zoos, beached bird monitoring programs), and systematic investigation of morbidity and mortality events in wild birds will provide a monitoring program that has the highest probability of detecting the H5N1 AI virus. It is important to realize that proper sample collection from wildlife involved in mortality events is vital, as H5N1 avian influenza is only one of many diseases or problems that can result in the deaths of large numbers of wild birds.

The following document is based on the following assumptions:

1) the disease or die-off investigation will be performed by appropriately trained personnel,

2) proper human health and biosafety precautions will be adhered to,

3) proper consent from the responsible government veterinary agency will be obtained prior to investigation activities, and

4) all disease investigation activities will be coordinated with the knowledge of FAO and OIE representatives.

For information on FAO offices around the world, visit: http://www.fao.org/countryprofiles/physical_presence.asp?lang=en;

For a list of OIE member countries and official delegates go to: http://www.oie.int/eng/OIE/PM/en_PM.htm;

For OIE regional representations go to: http://www.oie.int/eng/OIE/organisation/en_RR.htm
If you are planning an investigation and suspect highly pathogenic avian influenza virus could be the cause of wildlife mortality, be sure to follow strict occupational health and safety measures as described in the personal safety recommendations included in this document.

This mortality event investigation manual contains the following sections:

1. Clinical signs of infectious disease: triggers for disease investigation
2. Live bird handling
3. Dead bird collection
4. Necropsy Sample collection
5. Sample collection list
6. Swabbing technique
7. Sample handling and transport
8. Diagnostics
9. Carcass disposal
10. Disinfection
11. Personal safety protocols for handling of wild birds with signs of infectious disease
12. Appendix 1a - Sick or Dead Bird Mortality Event - Sample Collection Log:
   Submitter’s Cover Page
   Appendix 1b - Sick or Dead Bird Mortality Event - Sample Collection Log
13. Appendix 2 - OIE/FAO Network (OFFLU) and Reference Laboratories for Avian Influenza
14. Appendix 3 - Avian Necropsy Protocol
1. General Clinical Signs of Avian Infectious Diseases

Waterfowl and shorebirds are considered to be the natural reservoirs for all Avian Influenza virus subtypes and in general, most subtypes cause little or no disease in wildlife. However, type A influenza has undergone a combination of genetic drifts and shifts that have resulted in the production of a highly pathogenic H5N1 strain that now causes morbidity and mortality in many wildlife species. Although some surveillance is starting to be conducted, more research is necessary to determine which species of wildlife can carry this disease and not become sick or die. For many avian diseases including H5N1 AI, clinical signs of illness can include:

- Sudden death
- Diarrhea
- Regurgitation
- Sneezing
- Unexplained emaciation
- Open sores
- Discharge (clear or cloudy) from the mouth, nose, ears, or vent
- Behavioural abnormalities - falling over, head tilt, head and neck twisting, circling, paralysis, seizures
- Locomotion abnormalities - unable to stand or flap wings properly with no traumatic injuries
- Extensive swelling and/or purple discoloration of the tissues of the head (including the conjunctiva)
- Abnormal feathers: annular constrictions of the shaft, shaft haemorrhages, or, retained waxy sheaths
- Mass mortality or clusters of wild bird mortality (mortality unexpected considering the natural history of the species)

If any of these clinical signs are observed in free ranging wildlife species, either in a few birds or many individuals, one should consider conducting a disease outbreak investigation.

Reports about sick wildlife from the general public are often the first indication that a larger mortality event is about to occur, and given the economic and political implications of the emergence of H5N1 AI in a new location, it is best to know at an early stage of disease emergence, that it is present. This will allow management steps to be taken, potentially prevent the spread of the disease to agricultural flocks and other wildlife, and ultimately, be more cost-effective than managing a large scale disease outbreak.

Zoos, wildlife sanctuaries, rehabilitation centres or similar institutions that house birds in outdoor setting should also be briefed on clinical signs to watch for in their captive wild birds. If they observe any of these clinical signs, they should follow proper isolation procedures for the sick birds, have their staff veterinarian examine the bird immediately, follow appropriate procedures for collecting information and samples (Appendix 1a, 1b, and 3), and pass on this information to the responsible government veterinary service or OIE/FAO representative. Photographs and/or videos of animals (alive with clinical signs or dead) are also very useful in investigating wildlife disease.

If these facilities also routinely receive sick or injured wildlife that are exhibiting these clinical signs, these birds should be immediately isolated to prevent the spread of disease to the in-house collection or other birds receiving care. It is important to ask the member of the general public if they observed other birds exhibiting the same clinical signs to determine if there is a larger event ongoing where the one bird was found. Whether in-house captive birds become sick, or the general public brings a sick bird into the facility, government veterinary services should be made aware of the species affected and the clinical signs.

Medical record keeping is becoming more important with the emergence of more zoonotic diseases and included in the medical information should be the contact information of the person submitting the bird or reporting additional sick animals that are still out in the environment. This will facilitate further epidemiological investigation should the bird(s) test positive for H5N1 AI or other reportable diseases and if public health information needs to be conveyed to the person potentially exposed to this disease.
2. Live Bird Handling- in the field or a facility

Always handle “apparently” healthy live birds before working with sick live birds or dead birds. Wear proper protective clothing, latex gloves, face mask and protective eye cover when examining affected birds. Do not smoke, eat, drink or answer mobile telephones while handling birds (live or dead). Be sure to wash your hands and disinfect /dispose of your instruments and clothes prior to leaving the field site. Additional personal safety information is available in Section 11 this manual.

Before planning to capture wild birds, check with the local government, wildlife park, or protected area managers to determine whether it is necessary to obtain permits before wild birds are captured and sampled. Additional permits may be required to handle endangered species. Free-ranging birds may be captured by a number of methods including nets, live traps, and spotlighting. Note that surveillance for AI viruses and other infectious diseases, particularly in absence of a disease outbreak or dead birds in the area, can be performed by sampling healthy live birds. Once wild birds are captured, it is important to keep them in a well ventilated, cool, quiet environment to prevent them from overheating and to minimize stress. Blood can be collected from the jugular vein (right side of the bird), basilic vein (wing vein), or medial metatarsal vein (leg vein) and immediately transferred to a serum or plasma separator tube. Standard samples that should be collected include blood for serology, oral and cloacal swabs which should be refrigerated if possible for AI surveillance, and if possible, additional morphometric information including mass, culmen, tarsus, and wing cord. Blood samples should be kept refrigerated or in a cool water bath until spun in a portable centrifuge. The serum or plasma should be transferred to a cryovial with a transfer pipette, or if either or both are unavailable, carefully poured into the cryovial and then frozen. If necessary, swabs can be immediately frozen after collection.

In certain instances, other samples may be requested to facilitate additional research such as feather samples for heavy metal analyses, or additional blood or feather samples for genetics or isotope work. In rare cases, birds may also undergo minor or major surgeries to implant telemetry units which will facilitate understanding migration and habitat use. Finally, when possible, it is best to place a stainless steel band on the bird's leg for individual identification purposes at a later time.

If you are in an area where highly pathogenic avian influenza (H5N1 AI ) has been reported or if sick or dead animals exhibit signs of a respiratory infection, wear a high filtration surgical face mask (preferably an N-95 mask). Please read details on using this type of mask at http://www.fda.gov/cdrh/ppe/masksrespirators.html#1 or obtain training in applying and wearing these face masks from a medical professional.

If clinical signs are consistent with AI or other reportable disease such as Newcastle disease (ie. animals suffering respiratory, neurologic or gastro-intestinal disease) or if animals are moribund (non-moribund, sick birds should be feverish, while moribund birds may be hypothermic), consider euthanizing the bird.

If birds are to be euthanized, collect blood prior to euthanizing the bird. Detailed descriptions of methods to be used for euthanasia are provided below. Keep in mind that the method of euthanasia should not compromise the diagnostic value of the specimen. Euthanasia of birds suspected of being infected with H5N1 AI must be performed with great caution and avoiding direct personal contact with the animal.

Acceptable methods of euthanasia for restrained birds include barbiturates, inhalant anaesthetics, CO₂ and CO (in order of preference). If birds are to be euthanized using barbiturates, then this should be undertaken using recommended doses and titrating the dose to effect. Excessive quantities of barbiturates can severely damage tissues that may be required for histological examination. Keep in mind that the method of euthanasia should not compromise the diagnostic value of the specimen. Euthanasia of birds suspected of being infected with H5N1 AI must be performed with great caution and avoiding direct personal contact with the animal.

1 N-95 facemasks, 3M Brand, part number 3M9320. For a local 3M Supplier, search http://www.3m.com/ or FFP2 facemask (http://www.greenham.com/c/ss/937190002/3M-FFP2-Disposable-Respirators).
If this method of euthanasia is not possible in the field, consider the following using physical methods such as cervical dislocation, decapitation, use of Burdizzo clamps\(^2\), stunning and exsanguination (removal of blood) and gunshot. Detailed description of some of these methods can be found in The Field Manual of Wildlife Diseases [http://www.nwhc.usgs.gov/publications/field_manual/chapter_5.pdf](http://www.nwhc.usgs.gov/publications/field_manual/chapter_5.pdf) and [http://www.avma.org/issues/animal_welfare/euthanasia.pdf](http://www.avma.org/issues/animal_welfare/euthanasia.pdf) (page 686 and appendices 1, 2, 3 and 4).

For collection of sick birds, firearms (shotguns) are recommended. Birds should be killed outright by use of ammunition loads appropriate for the species to be collected. Wounded birds should be killed quickly by appropriate techniques.

**Special considerations for euthanasia of birds suspected of H5N1 AI infection**: In general, it is best to euthanize birds suspected of suffering from avian influenza by cervical dislocation (neck wringing) only. Though less humane than decapitation, CO\(_2\) narcosis or injectable euthanasia, it is necessary in the field to avoid contaminating oneself with blood splatter. If drugs are to be used, a veterinarian and animal handler are required. Euthanize birds using an IV barbiturate overdose. Beware that restraining animals for IV injection may put animal handlers at inappropriate risk of exposure. If it was not possible to collect a blood sample prior to euthanasia (greatly preferred) do so immediately after euthanasia via cardiac puncture. For duck-sized birds, insert a 4 cm needle just below the keel but aim the tip of the needle cranially, towards the head at a 45-50\(^\circ\) angle, and withdraw blood. Alternative needle sizes may be needed for different size birds. Place blood into a serum separator tube (red top clot tube), and allow to clot at room temperature. Spin tubes in a portable centrifuge and transfer serum to a cryovial with a transfer pipette, or if either or both are unavailable, carefully pour off serum into the cryovial.

\(^2\) Use in [veterinary] medicine as an emasculator of mammalian species, crushes the vessels quickly, and can be used in long/toughed necked avian species – applied to the upper part of the neck behind the mandible and held tightly for 15-30 seconds.
3. Dead Bird Collection

In the event of mass mortalities, before going to the field, it is extremely important to: 1) contact the responsible government veterinary agency, 2) make sure no permits are necessary prior to investigation activities, and 3) coordinate disease investigation activities with appropriate FAO and OIE representatives, where needed.

Before leaving for a field site for a disease investigation, make sure you have all of the appropriate supplies and equipment (personal safety gear, bird sampling supplies, necropsy supplies, die-off investigation forms, necropsy forms, etc.). It may be beneficial to create an “emergency response kit” that contains all of the appropriate supplies and is restocked each time you return from a field investigation. Maintaining an inventory list of supplies that should be in the emergency response kit also facilitates restocking supplies.

Upon arrival at the site, evaluate the extent of the die off including how many birds, what species, are other wildlife or domestic animals involved, and over what geographic range has this event occurred. This information should all be recorded on the Sick or Dead Bird Mortality Event Sample Collection Log: Submitter’s Cover Page (appendix 1a). In addition to preparing for animal sample collection, you may want to also consider collection of other environmental samples including water, soil, vegetation, or other samples that you think may have played some role in the mortalities.

When you are ready to work on birds, please make sure you are wearing the appropriate level of personal protective equipment, based on the situation you are investigating. Try to minimise direct contact with dead birds and always keep the animal away from your face. Before handling a dead bird you should at a minimum, be wearing vinyl or latex gloves. The best method to collect a dead bird is to invert a plastic bag around your gloved hand and then surround the animal with the bag so that you do not directly touch the animal and have a double protective barrier. Seal the bag tightly (double bag if required for strength and cleanliness) and clearly and indelibly label the bag with the Animal Identification Number (that matches the Sick or Dead Bird Mortality Event Sample Collection Log- appendix 1b), species, date, time and location where the animal was found. If more than one species is affected, at a minimum, collect several specimens of each species for diagnostics. In general, carcasses that have been dead for less than 24 hours are best for diagnostic purposes. In colder climates, carcasses may persist in relatively good condition for longer periods of time, and in warmed temperatures, carcasses will decompose faster.

When possible, carcasses that are “fresh” should be refrigerated (NOT FROZEN). A decomposing carcass is one that is bloated, green, foul smelling, and has feathers that pull out easily. To increase diagnostic value, fresh carcasses must be transferred to the appropriate veterinary or pathology facility and examined as soon as possible. If in field settings and/or far from appropriate diagnostic facilities, collect samples at the site and place them in an ice crest or cooler. Keep carcasses away from refrigerators used for animal or human food.

Sampling strategy for H5N1 AI: For each affected species, select up to 3 birds that have recently died (less than 24 hours), up to 3 sick birds (suffering respiratory, neurologic or gastro-intestinal disease or moribund) and up to 3 apparently healthy birds in direct contact with the currently sick birds. If possible, also conduct a survey of other live birds that share the same habitat (cloacal swabs and/or tracheal swabs only). Priority should be given to birds that share wetlands with affected birds since the main mode of transmission of AI is probably fecal contamination of water, shores, or banks.

It is best to collect as many carcasses as possible and to place them at a central location for processing. Removal of dead birds from the environment may also help prevent secondary contamination to scavengers or the environment. Complete the Sick or Dead Bird Mortality Event Sample Collection Log : Submitter’s Cover Page and the Sick or Dead Bird Mortality Event Sample Collection Log (appendix 1a & 1b), as carcasses are being collected and processed.

If possible, try to collect and examine sick animals as well as freshly dead ones being sure that you have the appropriate permits to capture live individuals. If there are so many dead animals present that individual bagging and labelling are not possible, try to bag or examine well preserved animals that will be most useful for diagnostic purposes, and keep these separate from decomposing carcasses. If possible, transport carcasses (in sealed bags) in a separate air space from the vehicle occupants.
If you are working in a remote area, you may have to perform field necropsies on site. In this case, follow strict personal safety measures, particularly if in an area where H5N1 AI has been reported or is highly suspected. It is also important to make sure you dispose of examined carcasses and used equipment properly as well as disinfecting all equipment adequately (see Sections 9, 10 & 11 of this manual for details). If clothing or other elements must be taken back to urban/rural areas with you, place them in double bags after dipping them in disinfectant for at least 30 minutes (see Section 10 on Disinfection for more details). Do not wash used field investigation clothing in household machines or at laundry shops.

If H5N1 AI is strongly suspected, do not move birds prior to sampling; euthanize, sample and dispose of properly on site in order to minimize the risk of contaminating previously uninfected areas. Also be sure that clothing, vehicles, and other fomites are properly sterilized before leaving the suspected disease positive area.

For AI virological analysis, if samples can be transported to the lab for assay or archiving within 4 hours, storage on ice is appropriate. Since in most field investigations this may not be possible, a system should be established to place samples directly into liquid nitrogen in the field, with subsequent preservation at --70°C or below (liquid nitrogen is -196°C) in order to preserve the virus and its RNA pending laboratory investigation. Without proper preservation samples may be non-diagnostic.
4. Necropsy Sample Collection

A necropsy is performed to determine the cause of death and it involves careful examination of the carcass both externally and internally. The ability of the lab to diagnose the cause of death is dependent on how well the necropsy is preformed, how carefully samples are collected, labelled, stored and delivered to the lab. If the field necropsy is done well, it will increase the likelihood of diagnosing the cause of death.

Each sample collected must be properly labelled with the Animal ID, species, location, date, and organ or sample type. Always label the jar or vial rather than the lid to ensure that the identification is not lost when lids are removed during sample handling. Use only one Animal ID per bird, even if you are collecting several samples from the animal. Ensure that labels are made with pencil or permanent ink, which will not dissolve in the fixative that you are using (alcohol-based fixatives will dissolve the ink of many “permanent/indelible” markers).

The label on the sample should always be linked to the information on the Sick or Dead Bird Mortality Event Sample Collection Log. On both the Sick or Dead Bird Mortality Event Sample Collection Log and the samples themselves, it is important to label things legibly so that the lab personnel can read the information you have provided. Do not abbreviate the names of the tissues, but instead, write out the name of the organ sampled (tracheal swab, cloacal swab, spleen, feces, serum, nasal turbinates, lung, etc.

It is highly recommended that you contact the government veterinary service and the FAO representative prior to sample collection to obtain diagnostic kits, or to discuss any sample collection or transport procedures. An avian necropsy protocol is attached (appendix 3 or section 14 of this manual) to assist with the sample collection process and identification of lesions consistent with H5N1 AI (in poultry). An additional resource on avian necropsies can be found at:

5. Sample Collection List

**PRIORITY RANKED:**

- Oropharyngeal swab
- Cloacal swab
- Serum or Plasma - from a live animal or centrifuged heart blood from a dead animal
- Fresh tissue samples collected into sterile vials and frozen:
  
  Liver, kidney, trachea and lung, air sacs, brain, spleen, pancreas, intestine, proventriculus, ceca and heart

  **Plus:**
  
  Half of any lesion
  
  Cecae and intestine if the animals exhibit diarrhoea.

- Formalin fixed tissues (minimum collection list):
  
  Brain, trachea, lung, heart, liver, kidney, spleen, pancreas, bursa of Fabricius if present, proventriculus/ventriculus, duodenum, cecae, skin including feather follicles, half of any lesion.

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For AI investigation, always take duplicate samples (one for real-time polymerase chain reaction (RT-PCR), one for possible virus isolation). Place samples into polypropylene screw-top, gasketed cryovials with liquid nitrogen safe labels only.

**Samples to collect for H5N1 AI (always in duplicate)**

**All birds**

Cloacal swabs ± tracheal swab in viral transport media

**All necropsied birds**

Piece (at least 2cm x 2cm but larger is acceptable) of spleen and lung, and any obviously abnormal tissue

**Sick and healthy in-contact birds**

Blood into red or green top tubes, refrigerated, spun down, serum or plasma placed into a cryovial and frozen

**Note:** If possible, all bird sampling should include both swabs and blood sampling.

Sterilize instruments between each necropsy by immersing instruments in alcohol and flaming them.
6. Swabbing Techniques

Swabs taken from the cloaca (vent) and oropharynx (back of the mouth/throat) then stored in viral transport medium can be used for viral culture or RT-PCR to detect the presence of a variety of viral pathogens.

Viral transport media can either be prepared locally at a lab (2.5% veal infusion broth, 0.5% BSA, 100 µg/ml gentamicin sulfate, 2 µg/ml amphotericin B in distilled water) or commercial kits may be purchased. Some commercial viral transport media are stable at room temperature such as the BD Universal Viral Transport Media^3^, which can also be found as a kit (Cellmatics™ Viral Transport Pack) containing a sterile rayon-tipped swab and a vial of medium.

However, because many viral transport media (particularly locally prepared ones) must be stored refrigerated or frozen prior to and after sample collection, their applicability for field use in remote areas is sometimes limited. Alternatives include commercial viral lysis buffer^4^ which can be kept at room temperature prior to sample collection. Note that samples collected in lysis buffer can only be used for RT-PCR agent detection.


When viral transport medium is not available, nasal turbinates or trachea may be a suitable substitute for a tracheal swab and feces may be a suitable substitute for a cloacal swab. To take a tracheal sample, incise the skin of the neck and dissect until the trachea is identified. To take a nasal turbinate sample, cut off the upper beak or bill near the head and take a sample of the tissue from above the roof of the mouth.

### Swab Collection Equipment List:

- Latex or vinyl gloves
- Swabs and transport medium
- Cooler and ice blocks to transport medium and swabs
- Pencil, pen and sick/dead bird event form to label your samples and record your findings
- Packing tape and courier forms
- Scissors and a large vial of ethanol

Wear disposable latex or vinyl gloves during the swabbing procedure to decrease the risk of contaminating the swabs with bacteria from your skin.

Use either a sterile plain polyester swab on a plastic applicator and then cut or break off the part of the applicator that you touched while depositing the swab into the vial of viral transport medium, or use commercially available sterile swabs that come with a tube containing transport medium, as described previously. When sampling live birds, consider purchasing different size swabs (normal size and paediatric or male urethra size) for large and small bird species respectively, to avoid injuries.

Handle the swab from the very end of the plastic handle only. Do not touch the portion of the applicator stick that will enter the tube containing the viral transport medium. Insert the swab well inside the cloaca, or oropharynx (different swabs for each site please) and twirl it around before pulling it out (Figures 1 & 2).

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^4^ RNALater lysis buffer. 50 ml catalogue number 76104, 250 ml catalogue number 76106. Supplier: Qiagen. Worldwide search: [http://www1.qiagen.com](http://www1.qiagen.com)
If using a sterile swab, break off or cut the swab so that the polyester bud falls into the viral transport medium (Figure 3). If scissors are used to cut the swabs, the scissors should be disinfected in a large vial of ethanol between birds (some commercial swabs are pre-cut so this can be done with your hands). Note that applicator sticks on many small sized swabs are metal. In this case, insert the swab in the viral transport medium, shake well and discard the used swab in a disinfectant filled container.

Label each sample so it can be cross-referenced with relevant information on the Sick or Dead Bird Mortality Event Sample Collection Log as you collect each sample (Figure 4).
7. Sample Handling and Transport

**Swabs**
Store the viral transport medium at 4°C or in an ice-chest cooler containing ice blocks before and after use. If at a remote location, use either viral transport media that can be stored at room temperature as mentioned previously, or freeze the transport media in liquid nitrogen prior to and after use. If using lysis buffer, store at room temperature prior to use and refrigerate after sample collection.

If transport to the laboratory will occur within 24/48 hours, transport the samples on ice blocks and store refrigerated. If samples can not be shipped to an appropriate laboratory within 2 days of collection, they should be stored in a -70°C freezer or liquid nitrogen. When shipping samples on dry ice, make sure samples are inside an airtight container, wrapped in adhesive tape and double bagged. CO₂ may inactivate AI virus if it comes in contact with the samples as vials contract during freezing.

**Serum, plasma, and fresh tissues**
Keep serum, plasma, and fresh tissue samples at 4°C until they can be shipped (if in less than 24 hours). Transport the samples on ice blocks. Alternatively freeze the samples in a -70°C freezer or liquid nitrogen and transport these on dry ice.

If possible avoid freezing any swabs or tissue samples between 0°C and -20°C (such as in many domestic freezers), although this is preferable to not freezing samples at all. Freezers that can guarantee -70°C temperatures are best. Inform the receiving laboratory the method (and temperature samples were stored)⁵.

**Formalin fixed tissues**
Samples must be fixed in 10% neutral buffered formalin (see APPENDIX 3: Avian Necropsy Protocol). Samples should be no thicker than 0.5cm so that the fixative penetrates the entire sample. Formalin to tissue ratio in containers should be 10:1. Fixed samples can be stored at room temperature, and never frozen.

**Sample shipment**
Formalin in quantities greater than 50 ml is considered a dangerous good by courier companies, which increases the costs and complexity of shipping. The tissues can be more readily shipped by courier or post if the fixative is decanted after the samples have been fixed for a period of at least 48 hours.

Fresh or frozen tissues that could contain infectious agents should be shipped within a three layer packaging system that meets IATA regulations⁶. Make sure you are aware of necessary permits and transport regulations particular to your country.

Contact a FAO or OIE reference diagnostic laboratory (see appendix 2) to obtain instructions on how to proceed with sample shipment. Ensure to obtain the necessary government permits from the veterinary and wildlife authorities (note that CITES export and/or import permits are required for listed species).

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⁵ This information could be most relevant in result interpretation.


For more details, see Information for shipping international diagnostic specimens

Fresh or frozen tissues must be shipped as quickly as possible to the laboratory. Same day courier service is preferable, but overnight courier delivery is acceptable. Do not ship samples on Fridays. Samples that go missing in the courier system on a weekend are often of little value when finally found. Always advise the receiving laboratory in advance that specimens are being dispatched, the airway bill number and the expected time of arrival, as international transport is full of obstacles.
8. Diagnostics

Because pathological lesions are not definitive for many diseases (including H5N1 AI), diagnosis must be confirmed by the isolation and characterisation of the causative agent. If possible, bacteriology should be performed to exclude bacterial septicemias from the differential diagnosis.

**Laboratory diagnosis for H5N1 AI**

**Identification of the agent**
Suspensions in viral transport medium of tracheal and cloacal swabs (or feces) taken from live birds, or of feces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated fowls eggs. The eggs are incubated at 35-37°C for 4-7 days. The allantoic fluid of any eggs containing dead or dying embryos as they arise and all eggs at the end of the incubation period are tested for the presence of haemagglutinin activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses.

**Tests for virus subtypes**
Avian influenza viruses are subtyped on the basis of their hemagglutinin (H) and neuraminidase (N) antigens. There are sixteen different H subtypes and nine N subtypes, with all combinations possible. So far, all HPAI viruses have been of the H5 or H7 subtypes.

**Tests for pathogenicity**
Pathogenicity can be determined by one or more of the following tests: a) chicken pathogenicity tests; b) cell culture tests and c) molecular pathotyping. The quickest method is molecular pathotyping. Once an outbreak virus has been characterized, immunohistochemistry, immunofluorescence, virus detection and virus isolation can be used to confirm virulent infections.

**Tests for previous infection**
Evidence of previous AI infection can be obtained by testing for influenza A group specific antibody using agar gel immunodiffusion precipitin (AGDP) test or enzyme-linked immunosorbent assay (ELISA), or by testing for subtype/specifc antibody to the H or N antigens using a haemaglutination inhibition (HI) test or ELISA, respectively.

Detailed methodological instructions and internationally accepted standard procedures can be found at:


See appendix 2 for the list of OIE and FAO avian influenza reference laboratories, or visit the following:

[http://www.offlu.net](http://www.offlu.net)
[http://www.oie.int/eng/avian_influenza/List_lab_ref_2006.pdf](http://www.oie.int/eng/avian_influenza/List_lab_ref_2006.pdf)

**Field tests (Point-of-care)**
In some cases, and if available, it may be wise to conduct a rapid influenza antigen detection tests on cloacal swabs or oropharyngeal (back of the mouth) swabs collected from sick and/or dead animals. Numerous commercial rapid test kits for the detection of influenza A viruses are available\(^7\). For example: Flu Detect\(^8\), Directigen Flu A® (Becton Dickinson) \(^9\), and Flu OIA® (Biostar Inc) \(^10\).

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\(^7\) Neither the authors nor their agencies verify the reliability, reproducibility, accuracy, sensitivity, or specificity of the assays listed. The information is provided for source information only. These tests are typically highly specific for all A viruses, but have lower sensitivity. As such a negative result may not mean that Influenza A virus is not present. The authors recognise that there are other manufacturers and that current research in developing improved field tests (Point-of-care) is underway.


Additional information is available regarding human point-of-care assays via the WHO website:

Note that even though there are several other commercial Influenza test kits available, results are often unreliable. Caution should be exercised as positives could be true positives, but negatives can not be ruled out based on these rapid tests. Ensure that you wear a full range of personal protective equipment to conduct the test, as outlined in the personal safety section (Section 11). The results of these tests must only be viewed as indicative, since their sensitivity is not as high as for other diagnostic tests available, and are not specific for H or N antigen. Any animal that tests positive using the rapid antigen detection test should be further examined in a BSL 3 level laboratory, preferably at a state government veterinary laboratory and diagnosis must be confirmed at one of the OIE/FAO reference laboratories (see appendix 2).

**RT-PCR**

The presence of influenza virus can be diagnosed by the use of reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific or matrix-specific conserved primers. Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers. Note that negative results do not preclude influenza virus infection and should not be used as the sole basis for decisions. Diagnosis and definitive antigenic subtyping of influenza A viruses should be confirmed at one of the OIE/FAO reference laboratories (see appendix 2).

10 Biostar® OIA® Flu. Manufacturer Biostar Inc. Order number FLU30 (30 tests). Information at:
http://www.biostar.com/products/oia_flu.html
9. Carcass disposal

The goal of carcass disposal is to prevent spread of the disease agent to other animals or humans through environmental contamination. This activity requires proper training and supervision, as well as observation of strict personal safety precautions.

**In the field**

Incineration is generally the preferred method for disposal of carcasses and contaminated materials associated with wildlife disease outbreak investigations. Carcasses may be burned above or below ground. The fire must be kept contained and with sufficient air movement under the carcasses to maintain a hot fire and completely burn the carcasses. Wood, coal, fuel oil and other fuels have been successfully used.

When burning is not feasible or needed, burial is often a suitable alternative. Select burial sites carefully (consider ground water circulation and drainage and potential for later carcass exposure). Place carcasses in a pit, cover with a thin layer of soil, then sprinkle lime over it and finally cover completely with at least one meter of soil, to discourage scavengers.


10. Disinfection

The purpose of disinfection is to prevent the mechanical spread of disease agents from one location to another by people, equipment and supplies. Before leaving the site, adequately dispose of non-reusable materials and disinfect clothes and boots and all equipment to the extent possible. Care should be taken to decontaminate all objects that have come in contact with potentially infectious materials, e.g., necropsy instruments, clothing, cages, restraint or capture equipment, vehicles, boots, etc. Suitable decontamination procedures include wipe down with 10% bleach (0.5% hypochlorite), Lysol® or similar quaternary ammonium compounds, Virkon®, or 70% ethanol. (see detailed list of product and methods in personal safety section 11). Wash boots and outside of plastic bags containing collected specimens with a 5% solution of household chlorine bleach.

Give special attention to vehicles leaving the field/outbreak site. Disinfect underside of vehicles at the site -pressure or hand sprayers can be used to dispense disinfectant. Wash vehicle thoroughly before moving to other areas. Detailed information on disposal procedures can be found in the AUSVET Plan Decontamination Manual Decontamination (edition 2, version 2.1, 2000), available at http://www.animalhealthaustralia.com.au/shadomx/apps/fms/fmsdownload.cfm?file_uuid=2B50B4BD-E62D-ECF1-C6AB-FA21B96AED7&siteName=aahc;


Details on disinfectants good for use for HPAI virus contaminated environments

<table>
<thead>
<tr>
<th>Item</th>
<th>Disinfectant / chemical/ procedure (see key below)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead bird / carcass</td>
<td>Bury or burn</td>
</tr>
<tr>
<td>Animal housing / equipment / cages</td>
<td>1, 2a, 2b, 2c, or 3</td>
</tr>
<tr>
<td>Humans</td>
<td>1</td>
</tr>
<tr>
<td>Electrical equipment</td>
<td>5c</td>
</tr>
<tr>
<td>Water tanks</td>
<td>Drain to pasture if possible</td>
</tr>
<tr>
<td>Ponds used by poultry / ducks</td>
<td>Drain to pasture if possible</td>
</tr>
<tr>
<td>Feed</td>
<td>Bury</td>
</tr>
<tr>
<td>Effluent, manure</td>
<td>Bury or burn, 4, 3</td>
</tr>
<tr>
<td>Human housing</td>
<td>1, 2a, 2b, or 2c</td>
</tr>
<tr>
<td>Machinery, vehicles</td>
<td>1, or 3</td>
</tr>
<tr>
<td>Clothing</td>
<td>1, 2a, 2b, 2c, or 3</td>
</tr>
<tr>
<td>Aircraft</td>
<td>1, or 2c</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key</th>
<th>Form and final concentration</th>
<th>Contact time and notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Soaps and detergents</td>
<td>Leave in contact 10 minutes</td>
<td></td>
</tr>
<tr>
<td>2. Oxidising agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a. Sodium hypochlorite</td>
<td>Liquid, dilute to final 2-3%</td>
<td>Not good for organic materials.</td>
</tr>
<tr>
<td></td>
<td>available chlorine</td>
<td>10-30 minutes contact</td>
</tr>
<tr>
<td>2b. Calcium hypochlorite</td>
<td>Solid or powder, dilute 2-3%</td>
<td>Not good for organic materials.</td>
</tr>
<tr>
<td></td>
<td>available chlorine (20g/litre</td>
<td>10-30 minutes contact</td>
</tr>
<tr>
<td></td>
<td>powder, 30g/l solid)</td>
<td></td>
</tr>
<tr>
<td>2c. Virkon®¹¹</td>
<td>2% (20 g/litre)</td>
<td>10 minutes. Excellent disinfectant.</td>
</tr>
<tr>
<td>3. Alkalis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a. Sodium hydroxide (caustic soda) (NaOH). Do not use with aluminium and similar alloys</td>
<td>2% (20g/litre)</td>
<td>10 mins. Do not use in presence of aluminium</td>
</tr>
</tbody>
</table>

| 3b. Sodium carbonate  
- anhydrous (Na₂CO₃)  
- washing soda (NaCO₃·10 H₂O) | 4% (40g/litre) from powder  
100 g/l from crystals | Recommended for use in presence of organic materials as above. 10 mins (anhydrous), 30 mins (washing soda). |
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>4. Acids</td>
<td></td>
<td></td>
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</tbody>
</table>
| 4a. Hydrochloric            | 2% (20 ml/litre)            | Corrosive, use only when better not available  
30 mins, safe for clothes and body decontamination  
15-24hs. Toxic, only if others cannot be used. |
| 4b. Citric                  | 0.2% (2 g/l)                |                                                                 |
| 4c. Formaldehyde gas        | Special generation required |                                                                 |
11. Personal Safety Protocols - Wildlife with Signs of Infectious Disease

Wild birds are the hosts for a number of pathogens that can infect people. It is recommended that wildlife care takers, wildlife health professionals and people contacting sick, injured or dead birds undertake standard precautions to prevent exposed to diseases. Increased precautions should be taken when animals are suspected to be suffering from respiratory infections, and in areas where H5N1 AI is present or suspected.

The following recommendations are extracted from the World Health Organization’s Standard Precautions and AUSVET Plan 2000 to minimise droplet, contact, and airborne transmission of disease.

Hand washing

The first line of defence against transmitting or contracting an infection is hand washing.

- Hands must be washed appropriately using hot water and soap before putting gloves on and after removing them
- Always wash hands before and after eating, smoking and going to the toilet.

When washing hands make sure the backs and palms of both hands are wet with warm water, apply soap, detergent or hospital grade antiseptics (suggestions below) lather and wash backs of hands, between fingers of each hand, and palms. Rinse well and dry using paper towel. Be careful when turning taps on and off if there is not an automatic sensor or foot operated system in place. Ensure tap handles are clean.

If clean running water is not available use an alcohol based hand rub and wash hands as described above. Do not eat, drink or smoke while working with dead/sick birds.

Personal Protective Equipment

There are 4 key items of personal protective equipment (PPE) that will protect against respiratory disease:

- Face mask (N-95 masks are recommended for the examination of animals that have signs of respiratory illness)
- Face shield, glasses or goggles
- Gloves (need not be sterile)
- Long sleeved gown (plastic apron if splashing is anticipated)

When using this PPE fit your mask first, making sure it is a secure fit around your face, then your goggles and gown and finally your gloves (having washed your hands prior to fitting your mask) When protection is no longer required remove your PPE in the reverse order you put it on, making sure that you wash your hands immediately after removing your gloves and that all PPE is disposed of in a hazardous waste bag or appropriately disinfected if applicable.

Waste

All waste produced from handling and examination of birds with signs of infectious disease must be treated as potentially contaminated. Gloves, gowns and masks should not be used again. Disposable items and carcasses should be disposed of through a biohazard incineration service whenever possible.

In field situations, gowns, clothing and other reusable equipment should be washed with detergent and hot soapy water and disinfected. Most avian viruses are sensitive to a broad range of detergents and hospital grade disinfectants (see list and tables below). It is important that materials are washed thoroughly and rinsed prior to disinfection.

Disinfectants active against avian influenza viruses include: 2% sodium hypochlorite for 10 - 30 minutes, 4% quaternary ammonium salts, 2% synthetic phenols, sodium carbonate (washing soda) 10% weight/volume for 30 minutes, citric acid 0.2% weight/volume for 30 minutes (good for clothing and body).
**Special considerations for HPAI**

Possible routes of infection and use of judgment are incumbent upon all who handle birds suspected of avian influenza. Influenza may infect humans via contact with any mucous membrane (e.g., the entire respiratory and gastro-intestinal tracts and the eyes). Infection could occur by accidental stab with a needle or necropsy instrument contaminated with fresh, moist tissue or fluids from infected animals and conceivably through contamination of a break in the skin. Transdermal infection (infection across intact skin) has not been described and the virus is not vector-borne. Thus, in short, infection occurs only as a result of direct exposure to live virus in aerosol droplets or contaminated fluids. Also, infection with H5N1 virus in particular appears to be dose-dependent. To date, no human cases of H5N1 AI are known to have resulted from exposure to wildlife. Generally speaking, chickens shed much more virus than wild birds and therefore depopulation of a chicken barn, for example, requires greater personal protective equipment safeguards than sampling from a wild bird die-off investigation. Contaminated skin should be washed with soap and water. Influenza-like illness within 4 days of working with birds should be viewed as suspected avian influenza and treated appropriately by a medical doctor. Post-exposure treatment with an antiviral should be discussed with a physician.

**Supplies necessary for cleaning and disinfecting clothing and equipment**

Plastic buckets, brushes, towels (disposable, paper towels), plastic refuse bags, footbath pans, antiseptic soap, detergent and disinfectants.
12. Sick or Dead Bird Mortality Event Sample Collection Log: Submitter’s Cover Page: see APPENDIX 1a.
13. Sick or Dead Bird Mortality Event Sample Collection Log: see APPENDIX 1b.
14. OFFLU Influenza Expertise Network. See APPENDIX 2
15. Avian Necropsy Protocol: See APPENDIX 3
### Submitter Information

<table>
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<tr>
<th>Submitter's Name:</th>
<th>Dept/Organisation:</th>
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<tr>
<th>Mobile #:</th>
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<th>Signature:</th>
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### Incident Information

<table>
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<tr>
<th>Date of Observation:</th>
<th>Date of Report:</th>
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<table>
<thead>
<tr>
<th>Location (Exact Location - with GPS data if possible):</th>
<th>Landowner and land access:</th>
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### Animal Details:

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<th>Species Affected (Common name, genus and species):</th>
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<table>
<thead>
<tr>
<th>Total of Each Species:</th>
<th>Sick:</th>
<th>Dead:</th>
</tr>
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<tbody>
<tr>
<td>Unaffected/Normal:</td>
<td></td>
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<table>
<thead>
<tr>
<th>Approximate Ages of Affected Animals:</th>
<th>Chick:</th>
<th>Juvenile:</th>
<th>Adult:</th>
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<table>
<thead>
<tr>
<th>Sex of Affected Animals:</th>
<th>Unknown:</th>
<th>Male:</th>
<th>Female:</th>
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<tbody>
<tr>
<td>Unaffected/Normal:</td>
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<td></td>
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### Description of Incident:

<table>
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<tr>
<th>Description of Incident:</th>
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### Environmental Conditions:

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<tr>
<td>Weather, recent rainfall,</td>
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<tr>
<td>local use of chemicals,</td>
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<tr>
<td>changes in domestic animal</td>
</tr>
<tr>
<td>management</td>
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<td></td>
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### Clinical Signs of Animals:

<table>
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<tr>
<th>Clinical Signs of Animals:</th>
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### Gross Pathology Findings:

<table>
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<th>Gross Pathology Findings:</th>
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<tbody>
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### Management Actions Taken:

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<th>Management Actions Taken:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Please add as many pages as necessary for thorough descriptions and additional observations
### APPENDIX 1b: Sick or Dead Bird Mortality Event Sample Collection Log Sheet (*Sample*)

<table>
<thead>
<tr>
<th>Species</th>
<th>Animal ID</th>
<th>Location</th>
<th>Live/Died/ Euthanized Method</th>
<th>Carcass kept fresh / frozen</th>
<th>Serum or plasma?</th>
<th>Swabs Collected?</th>
<th>Tissues Collected?</th>
<th>Sample Collector</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Specimens Stored/Sent Where**

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APPENDIX 2:
OIE/FAO NETWORK (OFFLU)

OFFLU is a joint network of expertise on avian influenza, created and endorsed by the OIE and FAO, in April 2005. The objectives of OFFLU are: 1) to exchange scientific data and biological materials (including virus strains) within the network, and to share such information with the wider scientific community; 2) to offer technical advice and veterinary expertise to member countries to assist in the diagnosis, surveillance and control of avian influenza; 3) to collaborate with the WHO influenza network on issues relating to the animal-human interface; and 4) to highlight avian influenza research needs, promote their development and ensure co-ordination. For more information, visit: http://www.offlu.net

FAO offices around the world
For information on location of FAO regional, subregional, liaison offices and country representations, visit http://www.fao.org/countryprofiles/physical_presence.asp?lang=en
Information on country representations can be obtained by clicking on the country dot on the map, which is linked to the country profile.

Additional information can be found at http://www.fao.org/countryprofiles/selectiso.asp?lang=en by clicking on each of the member country names on the list below the map.

OIE members and regional representations
For a list of OIE member countries and official delegates go to: http://www.oie.int/eng/OIE/PM/en_PM.htm Contact information can be found by clicking on the country name.

The OIE maintains representations in the following regions: Africa, the Americas, Asia-Pacific, Eastern Europe, and the Middle East. For details on OIE regional representations go to: http://www.oie.int/eng/OIE/organisation/en_RR.htm

List of the OIE reference laboratories and other experts for Highly Pathogenic Avian Influenza (*notes an FAO reference laboratory for Avian influenza)
(for updates please go to http://www.oie.int/eng/avian_influenza/vaccines.htm)

VLA Weybridge *
New Haw, Addlestone, Surrey KT15 3NB , UNITED KINGDOM
Tel: (44.1932) 34.11.11 Fax: (44.1932) 34.70.46
Contact person: Dr Ian Brown
Email: i.h.brown@vla.defra.gsi.gov.uk

CSIRO, Australian Animal Health Laboratory (AAHL)*
5 Portarlington Road, Private Bag 24, Geelong 3220, Victoria, AUSTRALIA
Tel: (61.3) 52.27.50.00 Fax: (61.3) 52.27.55.55
Contact person: Dr Paul W. Selleck
Email: paul.selleck@csiro.au

National Veterinary Services Laboratories*
P.O. Box 844, Ames, IA 50010, USA
Tel: (1.515) 663.75.51 Fax: (1.515) 663.73.48
Contact person: Dr B. Panigrahy  
Email: brundaban.panigrahy@aphis.usda.gov

Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio Virologia*  
Via Romea 14/A, 35020 Legnaro, Padova, ITALY  
Tel: (39.049) 808.43.69 Fax: (39.049) 808.43.60  
Contact person: Dr Ilaria Capua  
Email: icapua@izsvenezie.it

Graduate School of Veterinary Medicine, Hokkaido University, Department of Disease Control  
Kita-18, Nishi-9, Kita-ku, Sapporo 060-0818, JAPAN  
Tel: (81.11) 706.52.07 Fax: (81.11) 706.52.73  
Contact person: Dr H. Kida  
Email: kida@vetmed.hokudai.ac.jp

National Reference Laboratory for Highly pathogenic avian influenza and Newcastle disease,  
Institute of Diagnostic Virology, Federal Research Centre for Virus Diseases of Animals (BFAV)  
Insel Riems, Boddenblick 5a, 17493 Greifswald - Insel Riems, GERMANY  
Tel: (41) 383.517.152 Fax: (41) 383.517.151  
Contact person: Dr Ortrud Werner  
Email: ortrud.werner@rie.bfav.de

OTHER OIE and FAO EXPERTS

Dr David Swayne  
Southeast Poultry Research Laboratory  
USDA/ARS  
934 College Station Road, Athens, Georgia, USA  
Tel: 001-706-546-3433  
Fax: 001-706-546-3161  
Email: dswayne@seprl.usda.gov

Dr Véronique Jestin  
Unité de pathologie aviaire Zoopôle Beaucemaine-Les Croix  
BP 53, 22440 Ploufragan, FRANCE  
Tel: (33 (0)2) 96.01.62.81  
Fax: (33 (0)2) 96 01 62 73  
Email: v.jestin@ploufragan.afssa.fr

Dr William Karesh  
Department Head, Field Veterinary Program  
Wildlife Conservation Society  
2300 Southern Blvd.  
Bronx, New York 10460, USA  
Tel: (1 (718)) 220-5892 phone  
Fax: (1(718)) 220-7126 fax  
Email: lstarr@wcs.org (Lisa Starr)
APPENDIX 3: Avian Necropsy Protocol

Necropsy Occupational Health and Safety

1. The necropsy room should be a sole purpose isolation-type room. Necropsy equipment, instruments and cutting boards should not be used for other purposes. Necropsy equipment and surfaces must be thoroughly cleaned and then disinfected after each use. Ideally, a footbath should be set up at the doorway(s) of the necropsy room.

2. The necropsy area as well as refrigerators and freezers used to store pathology samples should not be used to store food for human or other animals.

3. Support staff should be thoroughly briefed on the hazards of zoonotic disease, potential methods of disease transmission, and be informed of biohazard and chemical spill management.

4. Individuals conducting or observing gross post-mortem examinations and those cleaning the post-mortem room should wear appropriate protective clothing. Protective clothing should include a face mask (N-95 masks are recommended for the examination of animals that have signs of respiratory illness), disposable (non-sterile) gloves, waterproof splash-aprons, long sleeved gown with tight fitting cuffs, safety glasses, and rubber boots. A hand washing station should be accessible within the necropsy room.

5. Animal feathers should be wet down with a very dilute detergent solution and water prior to commencing the examination to reduce the risk of aerosolizing infectious agents.

6. A biohazard safety cabinet (Class II) should be used to examine birds that exhibited signs suggestive of infectious disease.

7. Referral laboratories should be notified when tissues bearing potential zoonotic agents are submitted (avian tissues where chlamydiosis or avian influenza is suspected). Conducting in-house impression smears or other diagnostic testing in these cases is not recommended unless they may be performed within a biohazard safety cabinet.

8. Carcasses should be maintained frozen until a diagnosis has been established, and then they should be disposed of in a means approved by local regulations, preferably through a biohazard incineration service.

9. Animal tissues and remains should be retained frozen until the presence of zoonotic disease is ruled out, prior to being disseminated to museums or other researchers.

The overview of safety measures described above applies to diagnostic procedures undertaken at appropriately equipped facilities. When necropsies must be performed in isolated or remote areas, particular attention must be paid to personal safety precautions as well as to avoiding dispersal of the disease by contaminated personnel, equipment and vehicles. When in remote locations, the same necropsy protocol (detailed below) should be followed and samples collected as indicated. However, in addition to these procedures, special precautions on carcass and waste disposal, as well as disinfection of reusable equipment must be taken, as described in previous sections.

Remember that under field conditions, you must collect all possible samples at once, as this will be your only chance. Examined carcasses must be destroyed and disposed of appropriately after examination.
Avian Necropsy Protocol:

An experienced person can perform the following necropsy in 15-20 minutes.

History
A history should include:

- Species, origin (wild/zoo/rehabilitation/privately owned), date and location of collection

In captivity:
- Diet, food and water sources
- Environmental conditions or housing conditions - ventilation, substrate, cage type, etc
- Exposure to other birds
- Exposure to toxic substances - lead, plants, fumes
- Any recent changes in the environment
- Clinical signs of disease, the onset and progression of these signs
- Treatment offered, including whether the animal was euthanized or died

In the wild
- who informed of the mortality / disease outbreak
- how many birds affected / dead
- what species / age category
- other wildlife affected (ie. scavengers, predators)
- has the mortality been going on for days? weeks? months?
- proximity to poultry operations
- domestic animals affected
- proximity to urban centres / backyard poultry

An examination of the bird’s environment can provide invaluable information

Photographs/ video of site and dead / affected birds can provide invaluable information

External examination
An external general physical examination of the bird should be conducted following the same systematic method that would be used for a live bird

Collect cloacal and combined oropharyngeal/tracheal swabs prior to beginning the necropsy.
Ensure that you examine the following:

- Verify the carcass species, age, and look for identifying bands
- Plumage and skin for evidence of parasites, moulting, bruising, laceration, punctures, abrasion, swelling, anaemia, dermatitis
- Nostrils, eyes, ears, cloaca, and oral cavity for exudates, parasites, foreign bodies
- Quantity of muscle mass and presence of subcutaneous fat
- Long bones and joints for evidence of fracture, luxation, swelling
- State of feathers around the vent. Are these feathers pasted with faeces or urates?
- Cloacal mucosa
- Feet for evidence of trauma or bumblefoot (thickened or ulcerated plantar surfaces)

**Internal Examination**

Several protocols for avian necropsy are available. Your protocol should be one that you feel comfortable with and one that is thorough and systematic.

Spray or dip the carcass in a dilute solution of detergent to wet the feathers and reduce the risk of aerosolising infectious particles.

Cut across the upper beak at the level of the oral commissure to examine the nares and sinuses. Cut through the mandible and make an incision in the skin extending from the mandible to the thoracic inlet. Cut the oesophagus from the oral cavity, through the crop and down to the level of the thoracic inlet.

Examine the soft palate and larynx. Longitudinally incise the trachea beginning at the larynx and proceeding to the level of the thoracic inlet. Explore the trachea for parasites, fungal plaques, exudates, foreign bodies, congestion, or blood clots.

Incise the skin from the thoracic inlet to the vent. Disarticulate the coxofemoral joints. Reflect the skin off of the abdomen and breast. Tightly adherent skin and dark tissues may be an indicator of dehydration.

Make serial incisions into the pectoral musculature to rule out the presence of lesions. Palpate the coracoid and furcula for any subtle fractures. Remove the sternum by cutting through the abdominal muscles, ribs and coracoid bones and furcula.

As soon as the internal body cavity is exposed, use clean instruments to collect fresh tissue samples. Do this prior to touching the organs with your gloved hands. Then take the opportunity to examine the position and general appearance of the organs. Pay particular attention to evidence of free coelomic fluid, parasites, abscesses or masses. Carefully lift the ventriculus and intestines to investigate the abdominal air sacs and reproductive organs.

Coelomic surfaces coated with fibrin are consistent with infection caused by bacteria, including *Chlamydophila* species. White chalky material upon the surfaces of the heart, liver and other organs are most often uric acid crystals and are secondary to hyperuricemia from nephritis or urate nephrosis secondary to water deprivation. Excessive quantities of barbiturates used during euthanasia can produce white crystals along surfaces of the heart and greater vessels. Barbiturates often also partially liquefy these tissues, making them soft.
and brown.

Large blood clots in the abdomen or a haematoma within the liver are often a result of trauma. Blood clots may also be a result of haemorrhage from a large tumour, rupture of the aorta, or fungal vasculitis. Ascites may result from heart disease, liver disease, ingestion of toxins or neoplasia. White-yellow lesions on the air sacs, within the tracheal lumen or lungs are most often due to fungal infection (aspergillosis), but can also be due to bacterial infection, or tumours.

In chicks, check the navel and yolk sac for evidence of infection.

Begin to examine the tissues of the body while collecting 0.5 cm samples of each organ into 10% buffered formalin.

Examine the circulatory system and immune system. Examine and sample the thyroid glands as they disappear quickly upon dissection of other organs. The thyroid glands are found just at the base of the internal carotid artery. Sampling the whole gland and portions of the blood vessel around it will often provide a sample of the parathyroid gland, ultimobranchial body, artery, vein, air sac, and in a young bird, the thymus.

Remove the heart by severing the major vessels at the base of the heart. Make a transverse cut along the apex of the heart to expose the ventricular chambers and valves. If blood was not collected antemortem, an excellent method is to collect it from the heart chambers using a syringe and then ejecting the fluid gently into a serum collection tube, allow time for clotting or blood to settle if no centrifuge is available, and decant the clear serum into a clean tube.

Birds that are anaemic have pale tissues and watery blood. Birds that are hypovolemic often have a conical and contracted appearance of the cardiac ventricles.

Cut the oesophagus at the level of the bifurcation of the trachea. Grasp the caudal oesophagus with forceps and gently lift it as you cut the peritoneal membranes that attach the liver and intestinal tract to the dorsal body wall. Reflect the liver and intestinal tract onto the table beyond the cloaca. Stretch out the intestinal tract and examine the serosal surface carefully. Examine the pancreas and spleen. The pancreas is the tan tissue located between the descending and ascending loop of the duodenum. The spleen is usually nestled between the liver and the serosa of the stomach, at the junction of the proventriculus and ventriculus.

Test the patency of the bile duct by expressing the gall bladder or bile duct prior to removing the liver from the intestinal mass. Create serial sections through the liver to observe the integrity of the hepatic parenchyma and biliary system.

Yellow discoloration of the liver may be a physiological change in a laying hen or a very young chick when lipid metabolism is occurring at a high rate.

Peel out the lungs. Examine the pulmonary parenchyma and incise several major bronchi.

Examine the adrenal glands and gonads. Open the oviduct if one is present. Confirm the sex of the bird by the shape of the gonads. Most female birds have only a left ovary and oviduct, except for brown kiwi and some birds of prey that have two ovaries.
Examine the kidneys and ureters. Attempt to find the bursa of Fabricius, which is only present in young birds. The bursa is pale white or tan and can be found in the caudal coelomic cavity, just dorsal to the cloaca.

Starting at the proventriculus, cut through the wall of the entire intestinal tract, including the caeca (ensure that samples for bacterial and viral culture have been collected prior to opening the intestinal tract). Examine the digestive tract for evidence of normal or abnormal ingesta, haemorrhage, necrosis, ulceration, parasites or vascular accident.

Examine the skin, integument, muscles, bones, and joints. Reduced muscle mass, lack of fat deposits, a small liver, contracted ventricles, a full gall bladder and serous atrophy of fat are indicators of prolonged anorexia. Check bone strength by breaking one of the long bones. Place half of the tibiotarsus in formalin to allow examination of bone marrow. Incise the soft tissue surrounding several joints to look for evidence of degenerative change, infection or articular gout.

Disarticulate and remove the head from the cervical spine. Using scissors or bone rongeurs gently snip away the dorsal portions of the cranium beginning at the foramen magnum. Grossly examine the cranial vault and brain. Either place the entire head in formalin, or remove the brain from the cranial vault and place half of the brain in formalin, and freeze the other half.

If the bird was blind, or has an eye lesion, collect an eye into formalin.

If the bird had a drooping wing or lameness, collect samples of femoral nerve and brachial plexus into formalin.

**Sterilize instruments between each necropsy by immersing instruments in alcohol and flaming them.**

**Labelling**

Mark all samples with the date and a distinctive acronym or abbreviation representing the sampling site, e.g., MB = My Backyard. Then D, S or N for dead, sick or normal. Then T (Tracheal swab), C (Cloacal swab), S (Spleen), F (Faeces), Se (serum), Nt (Nasal turbinates), Tr (Trachea), L (Lung), Li (Liver), P (Pancreas), H (Heart), Cr (Crop), Pr (Proventriculus), G (Gizzard), St (small intestine), Du (Duodenum), I (Intestine - colon), Ce (Cecum), Ct (Cecal tonsil), B (brain), Te (testicle), O (Ovary), K (Kidney). Then the sequence number of the bird sampled. Only select one identification number per animal, even if you are collecting several samples from the animal.

**Data recording**

Complete a detailed necropsy report, or a sick/dead bird event form (Appendix 1) to document your observations and list the samples that you have collected. Forward a copy of the report to the responsible government veterinary service and OIE/FAO reference laboratory (see Appendix 2, page 17) even if you did not collect any samples.

**Tissue fixative for pathological diagnosis:**

For one litre solution:

100 ml formalin (38-40% formaldehyde)
900 ml distilled water
4 g sodium chloride (one tablespoon of salt) [or 4.5 gm sodium phosphate (monobasic) or 3.6 gm sodium hydroxide]
**Tissue descriptions (normal/abnormal):**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>pink, &quot;fluffy,&quot; collapsible</td>
<td>dark red, purple, heavy</td>
</tr>
<tr>
<td>Heart</td>
<td>consistently deep red</td>
<td>pale, mottled</td>
</tr>
<tr>
<td>Gut</td>
<td>light pink or brown with visible, but not prominent, red to purple vasculature</td>
<td>reddened, black, blue or with deep red to black prominent vasculature</td>
</tr>
<tr>
<td>Spleen</td>
<td>dark red, relatively consistent coloration</td>
<td>bright or purplish red, mottled with pale spots</td>
</tr>
<tr>
<td>Liver</td>
<td>deep red to brown, consistent colored</td>
<td>pale, yellow, green, black, mottled or in any way not consistently colored</td>
</tr>
<tr>
<td>Cecal tonsils</td>
<td>barely discernable</td>
<td>swollen, deep red to black (necrotic)</td>
</tr>
<tr>
<td>Testicles</td>
<td>smooth, white surface</td>
<td>hemorrhagic</td>
</tr>
<tr>
<td>Ovarian follicles</td>
<td>progressive sized, yellow</td>
<td>hemorrhagic</td>
</tr>
<tr>
<td>Kidney</td>
<td>consistent dark reddish brown</td>
<td>pale, black, mottled</td>
</tr>
<tr>
<td>Pancreas</td>
<td>consistent off white to pinkish brown</td>
<td>hemorrhagic, mottled</td>
</tr>
<tr>
<td>Trachea</td>
<td>free of exudate</td>
<td>hemorrhagic, containing exudate</td>
</tr>
</tbody>
</table>
*Where to get dry ice?* Prior to the investigation check with local hospitals, semen banks or ice-cream factories. If using dry ice for sample shipment, you must use enough dry ice so that there is still some remaining when the simples arrive at the laboratory. This requires a minimum 1 kg of dry ice for every kg of samples. For shipments that require more than 2 days travel, you might need 2 or more kg of dry ice per kg of samples. Be careful when handling dry ice (-70°C). Wear protective gloves and work in a well-ventilated area.

**Avian Necropsy Equipment List**

<table>
<thead>
<tr>
<th>Personal Safety Equipment:</th>
<th>General Equipment:</th>
<th>Sample Collection Equipment:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Tarps and rope to create a tent to ward off rain or sun</td>
<td>• Good pest proof packs for carrying equipment</td>
<td>• Permanent marking pen</td>
</tr>
<tr>
<td>• Insect repellent</td>
<td>• Necropsy worksheet or sick/dead bird event form</td>
<td>• Syringes - 5, 10, 20 ml</td>
</tr>
<tr>
<td>• Sunscreen, hat, sunglasses</td>
<td>• Pencils and sharpener</td>
<td>• Needles - various gauges</td>
</tr>
<tr>
<td>• Drinking water</td>
<td>• Clipboard with a clear piece of plastic to keep rain off</td>
<td>• Serum collection tubes</td>
</tr>
<tr>
<td>• Change of clothes</td>
<td>• Sharps disposal unit</td>
<td>• Sterile plastic bottles - 90 ml</td>
</tr>
<tr>
<td>• Coveralls</td>
<td>• Camera / batteries</td>
<td>• Sterile cryovials - 2 and 5 ml</td>
</tr>
<tr>
<td>• PVC apron</td>
<td>• Misting tape and packing tape</td>
<td>• Sterile plastic bags (Whirl-pak® bags)</td>
</tr>
<tr>
<td>• Latex gloves and/or dishwashing gloves</td>
<td>• Ruler / spring scale</td>
<td>• various size zip-lock bags</td>
</tr>
<tr>
<td>• Surgical face masks</td>
<td>• GPS unit and maps</td>
<td>• One litre plastic containers filled with 10% neutral buffered formalin (x 3)</td>
</tr>
<tr>
<td>• Rubber boots and good walking shoes</td>
<td>• Torch - hand held and head lamp</td>
<td>• 100 ml of 70- 90% ethanol</td>
</tr>
<tr>
<td>• Wash bucket, nail brush, antiseptic soap, paper towels</td>
<td>• First Aid Kit</td>
<td>• Bacterial culture swabs</td>
</tr>
<tr>
<td>• Torch - hand held and head lamp</td>
<td>• Mobile/ satellite phone</td>
<td>• Viral transport medium and sterile polyester swabs</td>
</tr>
<tr>
<td>• Emergency locator beacon if on water or very remote</td>
<td>• Emergency locator beacon</td>
<td>• Dry sterile polyester swabs</td>
</tr>
</tbody>
</table>

**Carcass Collection Equipment:**

| • Heavy duty rubbish bags | • Knives and steel (knife sharpener) | • Capillary tubes |
| • String | • String and manila labels | • Glass microscope slides and slide storage box |
| • Bag tags and pencil or indelible pen | • Scalpel handle (# 4) and disposable blades (#24) or disposable scalpels | • Microscope (may require mirror as light source if no access to power) -optional- |
| • Sick/Dead Bird Event Form | • Forceps - various | • 12 volt portable centrifuge |

**Clean-up Equipment:**

| • Tarp | • Scissors - various | • Saline |
| • Water, scrub brush, detergent | • Poultry shears or large bandage scissors | • Formalin, distilled water, salt |
| • Heavy duty rubbish bags | | • Parasite preservative (or 5% formalin) |
| • Disinfectants | | • Methanol to fix blood films |

**Carcass disposal**

| • Lime | • Faecal flotation vials and solution |
| • Fuel oil or other fuel | • Cooler and ice packs |
| • Shovels | • Liquid-nitrogen dry shipper or dewar |
| • Lighter / matches | • Dry ice* |
The following are illustrations of gross pathology frequently observed in chickens suffering from highly pathogenic avian influenza. These gross pathology signs may or may not be applicable to wild birds exposed to highly pathogenic avian influenza viruses.

- Pin point hemorrhages on the atria and pericardium
- Ovarian follicle hemorrhages
- Hemorrhagic spleen
- Necro-hemorrhagic small intestine
- Necrotic colon
hemorrhagic pancreas
necro-hemorrhagic small intestine

Thickened proventriculus with pinpoint hemorrhages (normal gizzard)

Cardiac necrosis and petechial hemorrhage

necrotic cecal tonsils
mucosal hemorrhage in paired ceca
May 2006