

manual

WILD BIRD HIGHLY PATHOGENIC AVIAN INFLUENZA SURVEILLANCE

sample collection from healthy, sick and dead birds



Zoological Parks Board
of New South Wales



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Introduction

The purpose of this document is to provide brief guidelines on the sampling methods to use when conducting wildlife surveillance, or a morbidity / mortality investigation. Topics covered include animal handling, proper methods for collecting and transporting diagnostic samples related to investigation of avian diseases such as avian influenza, West Nile virus, and Newcastle disease. Since highly pathogenic H5N1 avian influenza (H5N1 AI)¹ poses a potential serious human health risk, procedures to avoid exposure while working with live or dead wildlife are included.

If you are conducting wildlife surveillance in a country previously determined to have confirmed positive highly pathogenic H5N1 avian influenza virus (H5N1 AI) in wildlife, or you plan to conduct an investigation in a location where H5N1 AI could be the cause of wildlife sickness or mortality, follow the personal safety recommendations set out in this manual.

While not all species infected necessarily exhibit signs of disease, the current strains of H5N1 AI 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 (including disease testing from hunted birds, rehabilitation centres, zoos and beached bird monitoring programmes), and systematic investigation of morbidity and mortality in wild birds will provide a monitoring programme that has the highest probability of detecting the H5N1 AI virus. It is important to realize that proper sample collection from dead wildlife is vital since H5N1 avian influenza is only one of many diseases or problems that can result in the deaths of large numbers of wild birds.

This manual is based on the following assumptions:

- 1) all investigations will be performed by appropriately trained personnel;
- 2) proper human health and biosafety precautions will be adhered to;
- 3) consent from the responsible government veterinary agency will be obtained prior to any investigation; and
- 4) all disease outbreak investigation activities should be coordinated with FAO and OIE representatives.

¹ The term highly pathogenic avian influenza is usually reserved for the characteristic virulence exhibited of avian influenza virus in chickens, and is not appropriate to refer to high pathogenicity in other species (avian or mammalian). Throughout this manual, the H5N1 HPAI virus that has caused outbreaks in poultry and affected species in Asia and Europe (2003-2006) is referred to as H5N1 AI.

For **information on FAO** offices around the world, see:

http://www.fao.org/countryprofiles/physical_presence.asp?lang=en

For a list of **OIE member countries** and official delegates, see:

http://www.oie.int/eng/OIE/PM/en_PM.htm

For **OIE regional representations**, see:

http://www.oie.int/eng/OIE/organisation/en_RR.htm

Chapter 1

Clinical signs of infectious disease

Waterfowl and shorebirds are considered to be the natural reservoirs for all avian influenza virus sub-types and, in general, most sub-types 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 the highly pathogenic H5N1 AI strain that is now causing morbidity and mortality in many wildlife species. Although some surveillance has started, more research is necessary to determine which species of wildlife may serve as vectors, carrying and shedding the disease while not becoming sick or dying.

For many avian diseases, including H5N1 AI, clinical signs of illness can include:

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

If any of these signs are observed in free-ranging wildlife species, either in a few birds or many, contact the appropriate wildlife authorities, veterinary service or OIE/FAO representatives, and consider conducting a disease outbreak investigation.

Reports about sick wildlife from the general public are often the first indication that a larger incidence of mortality 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 preventing the spread of the disease to agricultural flocks and other wildlife, and ultimately, this will be more cost-effective than managing a large scale disease outbreak.

Zoos, wildlife sanctuaries, rehabilitation centres or similar institutions that house birds in outdoor settings 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 birds immediately, follow appropriate procedures for collecting information and samples (Chapter 4 and Annex 1), and pass on this information to the responsible government veterinary service (who's director is often the OIE delegate) or an FAO or OIE 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 exhibiting these clinical signs, the 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 members of the public who originally reported cases of sick wildlife if they observed other birds exhibiting the same clinical signs; this is necessary to be able to determine if there is a larger outbreak under way in the same location. In all cases, whether captive birds becoming sick or members of the general public bringing a sick bird into the facility, government veterinary services should be made aware of the species affected and the clinical signs in order to update their medical records.

These records should include contact details of the person bringing sick wildlife to the facility or of persons reporting sick animals outside the facility. This will facilitate further epidemiological investigation should the bird(s) test positive for H5N1 AI or other reportable diseases and allows such persons to receive public health information about possible exposure to the virus if necessary.

Chapter 2

Live bird handling

If a disease outbreak investigation site involves both healthy and moribund birds, 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 (see Chapter 12). 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 Chapter 12.

Before planning to capture wild birds, check with 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 using a number of methods including nets, live traps and spotlighting. Note that surveillance for AI viruses and other infectious diseases, particularly in the 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 minimise stress. If possible, use a thin cloth to cover the bird’s head while it is being handled to minimize visual stress.

BLOOD SAMPLING

Blood can be collected from the jugular vein (right side of the bird’s neck), brachial/ulnar vein (wing vein) (see figure 1) or medial metatarsal vein (leg vein) using a 22 g, 23 g, 25 g, or 27 g hypodermic needs or butterfly needle and a 12 mL, 10 mL, 6 mL, 3 mL or 1 mL syringe, depending on the size of the bird and the amount of blood to be collected (see figure 1). In general, it is safe to collect 0.3-0.6 cc of blood per 100 g of body mass from live birds, however, it is always best to collect as little blood as is necessary to conduct the testing required. If you plan to do hematology tests in addition to disease surveillance, it is recommended that you use a 22 g through 25 g needle as a 27 g needle or smaller damages cells as they pass through this narrow diameter needle. After blood is collected, cover the venipuncture site with gauze and apply digital pressure until bleeding stops (30-60 seconds).

Immediately transfer blood from the syringe to a serum (red top) gel separator tube or plasma (green top) gel separator tube (some labs prefer serum while others prefer plasma depending on the tests being performed – check before conducting field work). Plasma tubes should immediately be kept refrigerated or in a cool water bath until spun in a portable centrifuge. Serum samples should be allowed to clot at environmental temperature, and then be kept refrigerated or in a cool water bath until centrifugation. After being centrifuged, serum or plasma should be transferred to a cryovial with a sterile transfer pipette or, if unavailable, carefully poured into the cryovial and then frozen.

All cryovials should be labelled with the date, species, ID number that refers to a database where additional information can be found, and sample type (plasma or serum). Ensure that labels are marked with pencil or permanent ink, which will not dissolve when they get wet or are placed in liquid N, or temperatures of -70 °C or below.

Tracheal and cloacal swabs should also be collected from all live birds (See Chapter 7), and in many cases, additional morphometric information including mass, culmen, tarsus and wing cord should be recorded, and stainless steel leg bands placed for future monitoring (provided one has the appropriate banding permit).

In some cases, other samples may be requested to facilitate additional research; these include feather samples for heavy metal analyses, or additional blood or feather samples for genetic or isotope work. In rare cases, birds may also undergo minor or major surgery to implant telemetry units which will facilitate understanding of migration and habitat use.

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 or enteritis, wear a high filtration surgical face mask (i.e. N-95 or P2 mask²). 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.

EUTHANASIA

If clinical signs are consistent with AI or other reportable disease such as Newcastle disease (i.e. 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.

Collect blood samples before euthanizing birds. Detailed descriptions of methods to be used for euthanasia are provided below. Bear 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 operators must avoid direct, unprotected personal contact with the animal.

Acceptable methods of euthanasia for restrained birds include barbiturates, inhalant anaesthetics, and CO₂ and CO (in order of preference). If birds are to be euthanized using barbiturates, use recommended doses and titrate the dose to effect. Excessive quantities of barbiturates can severely damage tissues that may be required for histological examination.

If this method of euthanasia is not possible in the field, consider using physical methods such as cervical dislocation, decapitation, use of Burdizzo clamps³, 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*⁴.

² N-95 facemasks, 3M brand, part number 3M9320. For a local supplier, consult http://www.3m.com/FFP2_facemask (<http://www.greenham.com/c/ss/937190002/3M-FFP2-Disposable-Respirators>).

³ 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.

⁴ http://www.nwhc.usgs.gov/publications/field_manual/chapter_5.pdf and http://www.avma.org/issues/animal_welfare/euthanasia.pdf (page 686 and appendices 1, 2, 3 and 4).

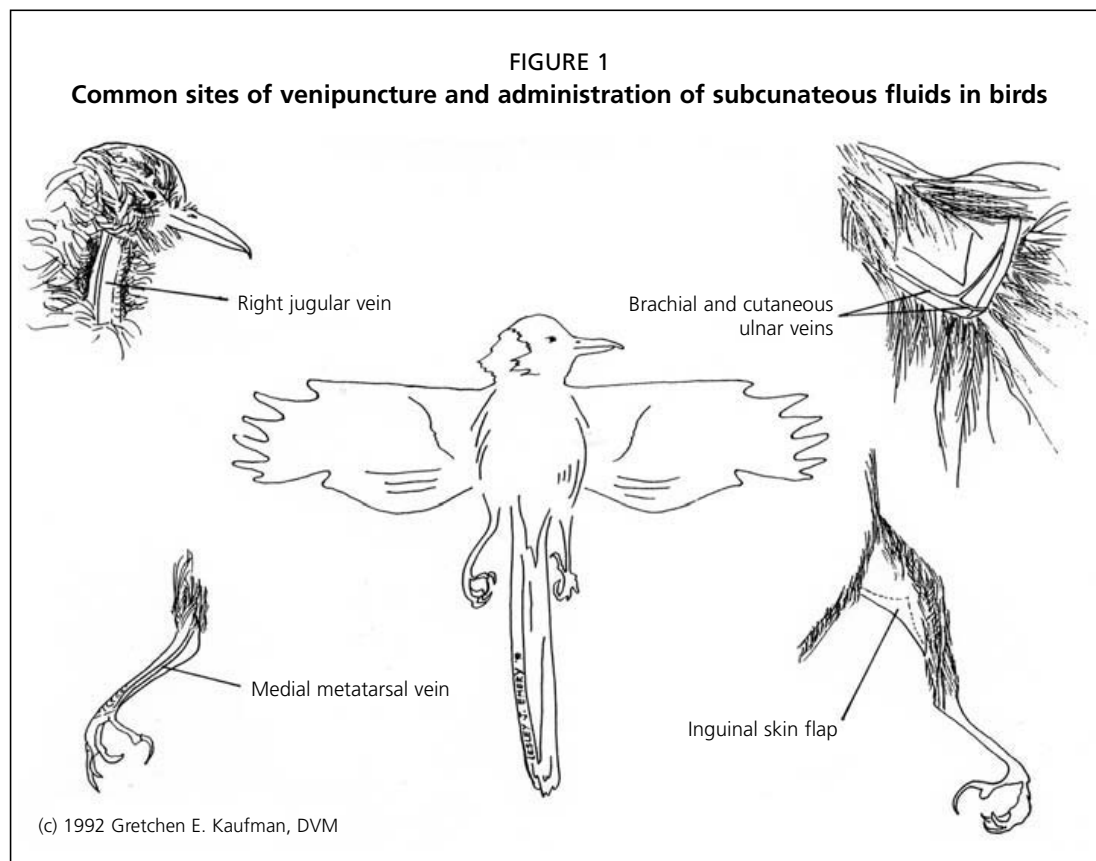
For collection of sick birds, that can not readily be captured, firearms (shotguns) are recommended. Birds should be killed outright using an ammunition load appropriate for the species to be collected. Wounded birds should be humanely killed quickly via cervical dislocation or other techniques described above.

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 or application of burdizzo clamps. Though less humane than decapitation, CO₂ narcosis can be used in the field to avoid contaminating oneself with blood splatter. Euthanasia by injection is another method that minimizes potential exposure to blood. If drugs are 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 is 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 (16 or 18 g) needle just below the keel but aim the tip of the needle cranially, towards the head at a 45-50° angle, and withdraw blood. Alternative needle sizes may be needed for different size birds.

Place blood into a serum separator tube (red top), 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 unavailable, carefully pour off serum into the cryovial. Collection of additional samples after euthanasia are described in the following sections.



Chapter 3

Dead bird collection

In the event of mass deaths and before going to the location in question, it is extremely important to:

- 1) contact the responsible government veterinary agency;
- 2) make sure all required permits are obtained prior to investigation activities; and
- 3) coordinate disease investigation activities with appropriate FAO and OIE representatives where necessary.

Before leaving for a site to carry out a disease investigation, make sure you have all the appropriate supplies and equipment (personal safety gear, bird sampling supplies, necropsy supplies, die-off investigation forms, necropsy forms, etc.). It may be useful to put together an *emergency response kit* that contains all the appropriate supplies and is restocked each time you return from a field investigation. Maintaining an inventory list of required supplies for the kit also facilitates restocking items (Chapter 4).

On arrival at the site, evaluate the extent of the mortality rate, including number of birds, species directly involved, other wildlife or domestic animals involved, and geographic range of mortality. This information should be recorded in a *Sick or Dead Bird Sample Collection Log* (Annex 1). 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 elements you think may have played some role in the deaths. If it is possible to get a GPS locations that characterize the extent of the die-off area, this is preferable to a general verbal description.

Make sure you wear 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 animals away from your face. Before handling a dead bird, you must at the very least wear vinyl or latex gloves. The best method for collecting 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. Seal the bag tightly (double bag if required for strength and cleanliness) and clearly and indelibly label the bag with an Animal Identification Number (which must match the number entered in the *Sick or Dead Bird Sample Collection Log*, Annex 1), species, date, time and location. If more than one species has been affected, collect several specimens of each for diagnosis. In general, carcasses of birds that have been dead for less than 24 hours (fresh carcasses) are sufficiently adequate (moribund or viraemic birds are best) for diagnostic purposes. In colder climates, carcasses may last in relatively good condition for longer periods of time; in warm climates, carcasses will decompose faster.

When possible, *fresh* carcasses should be refrigerated (NOT frozen); a *decomposing* carcass is dessicated, 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. In field settings and/or far from appropriate diagnostic facilities, collect samples on site and place them in an ice chest 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 most recently died (less than 24 hours) or more if practical, 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 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 virus is probably faecal contamination of water, shores or banks.

It is best to collect as many carcasses as possible and to place them in a central location for processing. Removal of dead birds from the site may also help prevent secondary contamination of scavengers or the environment. It is very important to complete the *Sick or Dead Bird Sample Collection Log* (Annex 1) as carcasses are being collected and processed.

If possible, try to collect and examine sick animals as well as newly dead birds, making sure that you have the appropriate permits to capture live samples. If there are too many dead to be able to individually bag and label, 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 space well separated from the occupants of the vehicle.

If you are working in a remote area, you may have to perform field necropsies on site.

Human exposure: special considerations for HPAI virus exposure

Anyone who handles birds suspected of being affected with avian influenza must use their best judgement and be aware of all possible routes of infection. 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. Thus, in short, infection occurs only as a result of direct exposure to live virus in aerosol droplets or contaminated fluids. Transdermal infection (infection across intact skin) has not been described and the virus is not vector-borne.

To date, with the exception of 1 case, all known human deaths resulting from H5N1 AI have been from exposure to poultry or areas where poultry are raised. Only 1 human case can be attributed to a person plucking the feathers of an infected swan. However, similar precautions should be taken when conducting a wild bird die-off investigation and depopulating a chicken barn.

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, and disinfect all equipment adequately (see Chapters 10, 11 and 12). 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 Chapter 11 on *Disinfection* for more details). Do not wash used field investigation clothing in household machines or in 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 disinfected 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 (-196°C) 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.

Chapter 4

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 humans 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 or FFP2 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. Biosafety 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 can be performed within a biosafety cabinet.
8. Carcasses should be maintained frozen (-70 °C) 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 pathogen 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/private-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 (i.e. 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 tracheal swabs prior to beginning the necropsy.

Ensure that you do/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
- Inspection of feathers around the vent; are they 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, larynx and syrinx. 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 and placing 0.5 cm samples of each organ into 10% buffered formalin. If you come across a lesion, place half of the lesion in formalin and half into a sterile vial for culture or freezing back pending histopathological examination.

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 or Bursa of Fabricius.

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 *ante-mortem***, 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 discolouration 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 caecae (**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, place the eye in formalin.

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

Sterilize instruments between each necropsy by immersing 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), sl (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. Select only one identification number per animal, even if you are collecting several samples from the animal.

Data recording

Complete a detailed necropsy report, or a sample collection log (Annex 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 Annex 2).

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 g sodium phosphate (monobasic) or 3.6 g sodium hydroxide]

Tissue descriptions (normal/abnormal)

Tissue	Normal	Abnormal
Lung	pink, "fluffy," collapsible	dark red, purple, heavy
Heart	consistently deep red	pale, mottled
Gut	light pink or brown with visible, but not prominent, red to purple vasculature	reddened, black, blue or with deep red to black prominent vasculature
Spleen	dark red, relatively consistent colouration	bright or purplish red, mottled with pale spots (consider effect if barbiturates were used to euthanize the bird)
Liver	deep red to brown, consistent colour	pale, yellow, green, black, mottled or in any way not consistently coloured
Cecal tonsils	barely discernable	swollen, deep red to black (necrotic)
Testicles	smooth, white surface	haemorrhagic
Ovarian follicles	progressive sized, yellow	haemorrhagic
Kidney	consistent dark reddish brown	pale, black, mottled
Pancreas	consistent off-white to pinkish brown	haemorrhagic, mottled
Trachea	free of exudate	haemorrhagic, containing exudate

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 only enough dry ice to leave some remaining when the samples 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 (-78 °C). Wear protective gloves and work in a well-ventilated area.

Avian Necropsy Equipment List

Personal Safety Equipment:

- Tarps and rope to create a tent to ward off rain or sun
- Insect repellent
- Sunscreen, hat, sunglasses
- Drinking water
- Change of clothes
- Coveralls
- PVC apron
- Latex gloves and/or dish-washing gloves
- Googles or face shield
- Surgical face masks
- Rubber boots and good walking shoes
- Wash bucket, nail brush, antiseptic soap, paper towels, spray disinfectant
- Torch – hand-held and head lamp
- First aid kit
- Mobile/satellite phone
- Emergency locator beacon if on water or very remote site
- Toilet paper

Carcass Collection Equipment:

- Heavy duty rubbish bags
- String
- Bag tags and pencil or indelible pen
- Sample Collection Log

General Equipment:

- Good pest-proof packs for carrying equipment
- Necropsy worksheet or sample collection log
- Pencils and sharpener
- Clipboard with a clear piece of plastic to keep rain off
- Sharps disposal unit
- Camera/batteries
- Masking tape and packing tape
- Ruler/spring scale
- GPS unit and maps

Necropsy Equipment:

- Knives and steel (knife sharpener)
- String and manila labels
- Scalpel handle (x 4) and disposable blades (x 24) or disposable scalpels
- Forceps - various
- Scissors - various
- Poultry shears or large bandage scissors

Clean-up Equipment:

- Tarp
- Water, scrub brush, detergent
- Heavy duty rubbish bags
- Disinfectants
- Pail (previously marked 1L, 2L, 5L)
- Pressure or hand sprayers
- Foot bath

Carcass disposal

- Lime
- Fuel oil or other fuel
- Shovels
- Lighter/matches

Sample Collection Equipment:

- Permanent marking pen
- Syringes – 1, 3, 6, 10, 12, 20 ml
- Needles – various gauges – 17 through 27
- Serum collection tubes
- Sterile plastic bottles - 90 ml
- Sterile cryovials - 2 and 5 ml
- Sterile plastic bags (Whirl-pak® bags)
- Zip-lock bags of various sizes
- One litre plastic containers filled with 10% neutral buffered formalin (x 3), distilled water, salt
- 100 ml of 70- 90% ethanol
- Bacterial culture swabs
- Viral transport medium and sterile polyester swabs
- Dry sterile polyester swabs
- Capillary tubes
- Glass microscope slides and slide storage box
- Microscope (may require mirror as light source if no access to power) –optional
- 12 volt portable centrifuge
- Saline
- Parasite preservative (or 5% formalin)
- Methanol to fix blood films. Acetone
- Faecal flotation vials and solution
- Cooler and ice packs
- Liquid-nitrogen dry shipper or dewar
- Dry ice*
- Burdizzo's
- Wire cutters
- Barbiturates
- Transfer pipettes
- Cryovials
- CO₂ (can be used to make CO₂s in the field)

Chapter 5

Necropsy sample collection

A necropsy is performed to determine the cause of death; it involves careful examination of the carcass both externally and internally. The ability of a laboratory to diagnose the cause of death depends on how well the necropsy is performed and how carefully samples are collected, labelled, stored and delivered to the laboratory. If a 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 Identification Number, 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 Identification Number per bird, even if you are collecting several samples from the animal. Ensure that labels are marked 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 Sample Collection Log*. On both the *Sick or Dead Bird Sample Collection Log* and the samples themselves, it is important to label samples legibly so that laboratory personnel can read the information you have provided. If an abbreviation system is used to identify collected tissues, provide the abbreviation code to the laboratory/epidemiology unit. (See page 17 for an example).

It is highly recommended that you contact the government veterinary service (whose director is often the OIE delegate) and the FAO representative prior to sample collection to obtain diagnostic kits, or to discuss sample collection or transport procedures. An Avian Necropsy Protocol is attached (see Chapter 4) 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:

http://www.nwhc.usgs.gov/publications/necropsy_manuals/index.jsp

Chapter 6

Sample collection

Samples to collect for H5N1 AI Investigation

All live birds

- 2 tracheal and 2 cloacal swabs with each swab being placed in a separate viral transport media tube (do not pool samples).
- Blood into red or green top tube, refrigerated, spun down; serum or plasma placed into a cryovial and frozen.

All dead necropsied birds. In addition to swabs and blood (as described for live bird sampling) also include:

- Piece (at least 2 cm x 2 cm but larger is acceptable) of spleen and lung, and any obviously abnormal tissue placed in sterile vials and frozen.

Note: Sterilize instruments between each necropsy by immersing instruments in alcohol and flaming them, or by leaving them in an acceptable disinfectant for the proscribed time prior to thorough rinsing in sterile water (see Chapter 11).

DETAILS REGARDING SAMPLES TO BE COLLECTED

- **Tracheal** swabs (see Chapter 7).
- **Cloacal** swabs (see Chapter 7).
- **Serum** or **plasma** - from centrifuged heart blood from a dead animal (see Chapter 3).
- **Fresh tissue** – placed in sterile vials and frozen.
 - liver, kidney, trachea, lung, air sacs, brain, spleen, pancreas, intestine, proventriculus, 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, thyroid/parathyroid, skin including feather follicles.

For AI investigations, 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.

Chapter 7

Swabbing techniques

Swab Collection Equipment List

- Latex or vinyl gloves +/- N95 or FFP2 mask, protective eyewear, etc. (see Chapter 12)
- 2 - 2.5 ml screw-top cryovials (able to be placed in liquid Nitrogen) containing transport media
- Rayon or dacron-tipped swabs (no cotton tipped swabs and no wooden sticks as they can inhibit viral growth or molecular diagnostic techniques)
- Scissors
- Cooler and ice blocks or liquid N container to store transport medium and swabs
- Lab marker/sample labels that can be placed in liquid N
- Data form on which to collect bird data
- Packing tape and courier forms

Swabs taken from the cloaca (vent) and trachea (located in-between the 2 cartilage structures located in the back of the mouth of the bird that open and close with breathing) and stored in a viral transport medium can be used for viral culture or RT-PCR to detect the presence of a variety of viral pathogens. Consider purchasing different sized swabs (normal size and paediatric or male urethra size) for large and small bird species respectively, to avoid injuries.

A variety of viral transport media exist and these can either be prepared locally at a laboratory (2.5% veal infusion broth, 0.5% BSA, 100 µg/mL gentamicin sulfate, 2 µg/ml amphotericin B in distilled water OR brain-heart infusion added with penicillin (10 000 IU/ml) streptomycin (200-10 000-µg/ml) gentamicine sulfate (10 000 µg/ml) and kanamycin sulfate (650 µg/ml⁵) or commercial kits may be purchased. Some commercial viral transport media are stable at room temperature such as the TBD Universal Viral Transport Media⁶ 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 media) must be stored refrigerated or frozen prior to use and frozen after sample collection, their appli-

⁵ American Association of Avian Pathologists 4th Ed pp. 150-155.

⁶ Universal Viral Transport vial, 3ml, catalogue number 220220 (50 x pckg). Cellmatics™ Viral Transport Pack, catalogue number 252171 (50 x pckg) Supplier: Becton Dickinson. Worldwide search: <http://www.bd.com/support/locations.asp>

cability for field use in remote areas is sometimes limited. Alternatives include commercial viral lysis buffer⁷, 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.

For instructions on how to prepare viral transport media and details on sample collection and storage for diagnosis of avian influenza virus, see:

http://www.who.int/csr/disease/avian_influenza/guidelines/animalspecimens/en or other peer reviewed journal articles describing these methodologies.

In a situation where dead birds are being necropsied, and viral transport medium is not available, nasal turbinates or trachea may be a suitable substitute for a tracheal swab and the cloaca with faeces 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.

PROCEDURE FOR SAMPLING

1. Wear appropriate PPE when handling birds (see Chapter 12) and opening sample vials.
2. Unwrap a Dacron swab from the stem-end of the packaging (choose appropriate size swab for bird) and be careful not to touch the swab tip.
3. Remove swab and insert the entire tip of the swab into the cloaca. Use gentle pressure and in a circular motion, swab the inside of the cloaca two to four times.
4. Shake off any large (>0.5 cm) pieces of feces.
5. Open the cryovial and place the swab tip in the transport media approximately $\frac{3}{4}$ of the way towards the bottom of the cryovial.
6. Cut or snap the stem of the swab so that the swab remains in the vial and the cap can be screwed on tightly. The entire swab end and a portion of the stem should be left in the cryovial.
7. Wipe scissors with 70% alcohol if they were used to cut the swab stem.
8. Label the tube with appropriate information (sample ID and type of sample (cloacal vs tracheal)) making sure that the ID on the tube can be cross referenced to the datasheet where additional information about the sample exists.
9. Record sample tube number on data sheet along with ID number, date, species, type of sample (cloacal vs tracheal), age, sex, location (GPS coordinates preferred), band number, comments, or other information.
10. For tracheal swabs, repeat steps 1, 2, however instead of steps 3 and 4, gently insert the swab tip into the trachea by waiting until the bird breathes and the cartilage protecting the trachea opens to allow the passage of air. **Gently** touch the swab tip to the back and sides of the trachea and remove it. Then follow steps 5-9.

Note that if birds are very small (passerines), it may not be possible to actually conduct a tracheal swab due to the narrow diameter of the opening into the trachea. In these

⁷ RNAlater lysis buffer. 50 ml catalogue number 76104, 250 ml catalogue number 76106. Supplier: Qiagen. Worldwide search: <http://www1.qiagen.com>

cases, an oropharyngeal swab should be collected by gently rolling the swab tip around the inside of the bird's mouth contacting the upper and lower portions of the mouth, and behind the tongue.

If scissors or wire cutters are used to cut the swabs, they should be disinfected between bird samples. Other commercial swabs are pre-cut so they can easily be broken by hand. Note that applicator sticks on many small sized swabs may be metal. In this case, if wire cutters are not available, insert the swab in the viral transport medium, mix 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 Sample Collection Log* or a live bird data sheet as you collect each sample (Figure 5).

FIGURE 2
Oropharyngeal swab



CREDIT: TARONGA ZOO/KARRIE ROSE

FIGURE 3
Cloacal swab



CREDIT: TARONGA ZOO/KARRIE ROSE

FIGURE 4
Breaking the swab



CREDIT: TARONGA ZOO/KARRIE ROSE

FIGURE 5
Sample label



CREDIT: TARONGA ZOO/KARRIE ROSE

Chapter 8

Sample handling and transport

SWABS AND VIRAL TRANSPORT MEDIA

The storage methods for viral transport media can vary depending on the type of media that is used. Please check with the diagnostic laboratory or company that has provided the transport media to determine proper storage techniques before and after sampling is conducted.

Some viral transport medium needs to be stored at 4 °C or in an ice-chest cooler containing ice blocks before and after use. If at a remote location, use either a viral transport medium that can be stored at room temperature or one that can be frozen 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 take place within 24-48 hours, transport the samples on ice blocks and store refrigerated. If samples cannot 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 into contact with the samples since vials contract during freezing. Never place dry ice (COB₂ (s)) in a hermetically sealed container as it may explode.

If using transport media that must be refrigerated or frozen, it is important to ensure that the samples are maintained cold (cold chain) throughout the entire storing and shipping process. Loss of cold chain can result in samples being rendered non-diagnostic.

SERUM, PLASMA AND FRESH TISSUES

Keep serum, plasma and fresh tissue samples at 4 °C if they can be shipped and arrive at the lab within 24-48 hours of sample collection. Transport the samples on ice blocks making sure that blood tubes (red or green top tubes) are placed in zip-lock bags and then wrapped in cloth towels before being placed in a cooler. Blood tubes (red or green top tubes) should never directly be in contact with ice as this may result in damage to cells and cellular morphology.

If red or green top tubes have already been spun in the centrifuge and serum or plasma has been placed into cryovials, they should be placed in a zip-lock bag, and these tubes

can be in direct contact with the ice. Alternatively freezing cryovials in a -70 °C freezer or liquid nitrogen and transport using dry ice is acceptable.

Freezers that can guarantee -70 °C temperatures are best. Inform the receiving laboratory of the method and temperature at which samples were stored⁸. 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.

Should such freezers (0 to -20 °C) be used, ensure that the receiving laboratory is aware of the sample preservation history.

FORMALIN FIXED TISSUES

Samples must be fixed in 10% neutral buffered formalin (see Chapter 4: Avian Necropsy Protocol). Samples should be no thicker than 0.5 cm so that the fixative penetrates the entire sample. The 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; this 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. Do not remove all of the formalin from the tissue samples, but remove enough to ensure that samples can be shipped as non-dangerous materials.

Fresh or frozen tissues that could contain infectious agents should be shipped within a three layer packaging system that meets IATA regulations⁹. Make sure you are aware of necessary permits and transport regulations particular to the country where the investigation has taken place.

Contact a FAO or OIE reference diagnostic laboratory (see Annex 2) to obtain instructions on how to proceed with sample shipment. Make sure you obtain the necessary government permits from the veterinary and wildlife authorities (note that the Convention on the International Trade of Endangered Species – CITES - export and/or import permits are required for listed species).

For more details, see Information for Shipping International Diagnostic Specimens http://www.fao.org/ag/againfo/subjects/en/health/diseases-cards/avian_fao.html

⁸ This information could be most relevant in result interpretation.

⁹ Dangerous Goods Regulations Manual, 47th edition; available in a range of languages, can be purchased from <http://www.iata.org/ps/publications/9065.htm>

Significant changes to the latest edition of the *IATA Dangerous Goods Regulations* effective January 2006: <http://www.iata.org/NR/rdonlyres/FBA32FAF-482A-4A04-8147-23C8E93508BC/0/SIGNIFICANTCHANGESANDAMENDMENTSTOTHE47THEDITION.pdf>

Infectious substances guidance: <http://www.iata.org/NR/rdonlyres/B8B91553-49BE-4DCC-901B-50DA4E57A98E/0/GuidanceDocument18Nov05.pdf>

Packing instructions: <http://www.iata.org/NR/rdonlyres/F9D6D81A-71FB-46C3-BD6A-DDE849FD8A56/0/PACKINGINSTRUCTION650.pdf>

Guidelines for the Submission of Diagnostic Samples to Reference Laboratories
http://www.fao.org/docs/eims/upload/208595/gui_labsample_en.pdf and
http://www.who.int/csr/disease/avian_influenza/guidelines/transport/en/index.html

Fresh or frozen tissues must be shipped as quickly as possible to a laboratory. Same day courier service is preferable, but overnight courier delivery is acceptable. Do not ship samples prior to weekends or extended holidays. 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, and give the airway bill number and expected time of arrival.

Chapter 9

Diagnostics

Although H5N1 AI has similarities to other avian influenza viruses, the current understanding is that this virus can be detected from the respiratory tract (trachea) more easily than from the cloaca or feces, making it different from other influenza viruses routinely found in healthy birds.

For this reason, analysis of tracheal swabs is viewed as the most likely method for detecting the virus, with cloacal swabs being the 2nd most likely sample from which H5N1 AI can be isolated.

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

LABORATORY DIAGNOSIS FOR AI VIRUSES

Identification of the agent

Suspensions in viral transport medium of tracheal and cloacal swabs (or faeces) 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 fowl 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 the immunodiffusion assay 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 sub-types

Avian influenza viruses are sub-typed on the basis of their hemagglutinin (H) and neuraminidase (N) antigens. There are 16 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
- 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 the agar gel immunodiffusion precipitin (AGDP) test or enzyme-linked immunosorbent assay (ELISA), or by testing for sub-type/specific antibody to the H or N antigens using a haemagglutination inhibition (HI) test or ELISA respectively.

Detailed methodological instructions and internationally accepted standard procedures can be found at: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, Chapter 2.7.12, Avian Influenza (version adopted May 2005): http://www.oie.int/eng/normes/mmanual/A_00037.htm

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

<http://www.offlu.net>

<http://www.fao.org/ag/aga/agah/VS/Default.htm> or

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 tracheal swabs collected from sick and/or dead animals. Numerous commercial rapid test kits for the detection of influenza A viruses are available¹⁰. For example: Flu Detect (Synbiotics™)¹¹, Directigen Flu A® (Becton Dickinson)¹² and Flu OIA® (Biostar Inc)¹³.

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

http://www.who.int/csr/disease/avian_influenza/guidelines/RapidTestInfluenza_web.pdf.

All diagnostic testing should be coordinated through the Chief Veterinarian Officer.

Ensure that you wear a full range of personal protective equipment to conduct the test, as outlined in the personal safety section (see Chapter 12). Note that even though there are several other commercial Influenza test kits available, results are often unreliable. Caution should be exercised because positives could be true positives, but negatives cannot be ruled out on the basis of these rapid tests.

¹⁰ 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 under way.

¹¹ Flu Detect™, Manufacturer Synbiotics. Product code 96-6800 (20 tests). Information at: <http://www.synbiotics.com/>

¹² Directigen™ Flu A+B Test Kit. Manufacturer TBD. Catalog number 256010 (20 tests). Information at: <http://www.bd.com/ds/productCenter/256010.asp>

¹³ Biostar® OIA® Flu. Manufacturer Biostar Inc. Order number FLU30 (30 tests). Information at: http://www.biostar.com/products/oia_flu.html

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. Therefore, any positive test result from the rapid antigen detection test should be further examined in a BSL 3 level environment, preferably at government veterinary laboratory or OIE/FAO reference laboratories to confirm these findings (see Annex 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 the sub-type 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 sub-typing of influenza A viruses should be confirmed at one of the OIE/FAO reference laboratories (see Annex 2).

Chapter 10

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 considering ground water circulation, drainage, and potential for erosion leading to carcass exposure. Place carcasses in a pit, cover with a thin layer of soil, then sprinkle lime over the top, and finally cover completely with at least one meter of soil to discourage scavengers.

For detailed instructions on carcass disposal procedures in field situations, visit the *Field Manual of Wildlife Diseases* (NWHC, USGS):

http://www.nwhc.usgs.gov/publications/field_manual/chapter_4.pdf

Additional information on disposal procedures can be found in the AUSVETPLAN Operational Procedures Manual: Disposal (Edition 2, Version 2.0, 1996).

Chapter 11

Disinfection

The purpose of disinfection is to prevent the mechanical spread of disease agents from one location to another by people, equipment or supplies. Before leaving a 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.

The avian influenza virus is easier to destroy than many viruses since it is very sensitive to detergents which destroy the fat-containing outer layer of the virus. This layer is needed to enter cells of animals and therefore destroys the infectivity. However, since the virus survives well in water and simple washing may help the virus enter areas where it can be picked up by other birds, any washing to remove contamination should always be with detergents (soapy water) or specific disinfectants.

Suitable decontamination procedures include wipe down with 10% bleach (0.5% hypochlorite), Lysol ® or similar quaternary ammonium compounds, Virkon ®, Virocid ® or 70% ethanol (see box below for detailed list of products and methods). Wash boots and the outsides of plastic bags containing collected specimens with a 5% solution of household chlorine bleach.

Give special attention to vehicles leaving an outbreak site. Disinfect the undersides of vehicles that have been at a site – pressure or hand sprayers can be used to dispense dis-

Details on disinfectant procedures good for use in HPAI virus contaminated environments

Item to be disinfected Item	Disinfectant/chemical/procedure (see key table)
Dead bird/carcass	Bury or burn
Animal housing/equipment/cages	1, 2a, 2b, 2c, 2d or 3
Humans	1
Electrical equipment	5
Water tanks	Drain to pasture if possible
Ponds used by poultry/ducks	Drain to pasture if possible
Feed	Bury
Effluent, manure	Bury or burn, 43, 3 4
Human housing	1, 2a, 2b, 2c or 2d
Machinery, vehicles	1, or 3
Clothing	1, 2a, 2b, 2c, 2d or 3
Aircraft	1, 2c or 2d

Key

Disinfectant Key	Form and final concentration	Contact time and notes
1. Soaps and detergents		
1. Soaps and detergents		Leave in contact for 10 minutes
2. Oxidising agents		
2a. Sodium hypochlorite	Liquid, dilute to final 2-3% available chlorine	Leave in contact for 10-30 minutes Not good for organic materials
2b. Calcium hypochlorite	Solid or powder, dilute 2-3% available chlorine (20 g/l powder, 30 g/l solid)	Leave in contact for 10-30 minutes
2c. Virkon®	2% (20 g/l)	Leave in contact for 10 minutes
2d. Virocid®	1:400 dilution	Leave in contact for 10 minutes Not tested on porous surfaces
3. Alkalis		
3a. Sodium hydroxide (caustic soda) (NaOH).	2% (20 g/l)	Leave in contact for 10 minutes Recommended for use in presence of organic materials Do not use in presence of aluminium and similar alloys
3b. Sodium carbonate -anhydrous (Na ₂ CO ₃) -washing soda (NaCO ₃ .10 H ₂ O)	4% (40 g/l) from powder 100 g/l from crystals	Leave in contact for 10 minutes (anhydrous) Leave in contact for 30 minutes (washing soda) Recommended for use in presence of organic materials 10 mins (anhydrous), 30 mins (washing soda)
4. Acids		
4a. Hydrochloric	2% (20 ml/l)	Corrosive, use only when better not available
4b. Citric	0.2% (2 g/l)	Leave in contact for 30 minutes Safe for clothes and body decontamination
5. Gas		
5a. Formaldehyde gas	Special generation required	Leave in contact for 15-24 hours in enclosed environment Toxic, only if other disinfectant procedures cannot be used

infectant. Wash vehicles thoroughly before moving to other areas. Detailed information on disposal procedures can be found in the *AUSVET Plan Decontamination Manual* (Edition 2, Version 2.1, 2000), or http://www.fao.org/ag/againfo/subjects/en/health/diseases-cards/avian_qa.html#7

The table above provides guidance for veterinary officers and others coming into direct with poultry on the selection and application of decontamination procedures – these procedures can be applied to wildlife outbreaks as well but remember that adaptation to specific country circumstances will always be necessary.

Chapter 12

Personal safety recommendations

Wildlife caretakers and health professionals, as well as people coming into contact with sick, injured or dead birds, should observe standard precautions to prevent exposure to pathogens, particularly when it is suspected that the birds in question are suffering from respiratory infections or when working in areas where H5N1 avian influenza is suspected or has been confirmed.

Special considerations for HPAI

Human infection from H5N1 AI occurs only as a result of direct exposure to live virus in aerosol droplets or contaminated fluids. Influenza may infect humans via contact with any mucous membrane (e.g. inhalation, ingestion, introduction into the eyes, and via open skin wounds).

Exposed or 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 may be considered and should be discussed with a physician.

The following recommendations for minimising droplet, contact and airborne transmission of disease have been taken from the World Health Organisation's *Standard Precautions and AUSVET Plan 2000*.

Do not eat, drink or smoke while working with sick/dead birds.

Wash your hands

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

- Wash your hands with hot water and soap before putting on gloves and after removing them.
- Always wash your hands before and after eating, smoking and using the toilet.
- Do not handle cigarettes, lighters, cell phones before thoroughly washing your hands.

When washing your hands, make sure the backs and palms of both hands are wet with warm water, apply soap, detergent or hospital grade antiseptics, lather and wash the backs, between the fingers and the palms of each hand. Rinse well and dry using a paper towel. Be careful when turning taps on and off if there is no 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.

Wear personal protective equipment

There are 4 key items of personal protective equipment (PPE) that will keep you safe from respiratory disease:

- Face mask (N-95 or FFP2 masks are recommended for the examination of animals with signs of respiratory illness or in locations where H5N1 AI has been found in either poultry or wildlife)
- Goggles face shield, or protective glasses
- Gloves (need not be sterile)
- Long-sleeve gown or coverall (plastic apron if splashing is foreseen)

When using PPE, **wash your hands** then put on your PPE in the following order:

- 1) Coverall
- 2) Hair cover
- 3) Plastic apron
- 4) Boot covers
- 5) Mask – fit the mask making sure it is secure around the face, especially around your nose
- 6) Goggles
- 7) Finally put on your gloves
- 8) Ideally wear two sets of gloves (making sure that the cuffs of the outer gloves go up and over the cuffs of your coverall)



Order for Putting on PPE

Please note that the order for putting on PPE becomes important when you go to remove it.

Once the task at hand is completed, it is important that you remove your PPE in such a way as not to expose yourself or others to potentially infectious matter. Have your disposal bag and container for reusable ready beforehand.

Order for Removing PPE

Remove your PPE in the following order:

- 1, 1a) Outer gloves, goggles (these are recyclable and should be put into your container for disinfection)
- 2) Apron (the thick PVC style aprons are recyclable and should be put into your container for disinfection),
- 3) Boot covers,
- 4, 4a, 4b, 4c, 4d, 4e) Coverall (taking care to roll the coverall downwards),

Order for removing PPE

Now wash your hands

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- 5) Mask (do not touch the front of your mask; remove it by taking hold of the straps at the back of your head, first bring the bottom strap up and over your head and finally the top strap, lifting the mask away from your face and into the disposal bag)
- 6) Finally, remove your hair cover
- 7) **Wash you hands**

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.

Treat waste, and used clothing and equipment with special care

All waste produced from handling and examining birds with signs of infectious disease must be treated as potentially contaminated. Disposable gloves, coveralls, shoe covers, masks and hair covers should be used once only. Disposable items and bird carcasses should be eliminated 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 table in Chapter 11 and list below). It is important to wash and rinse all materials thoroughly prior to disinfection.

Disinfectants active against avian influenza viruses include:

- 2% sodium hypochlorite (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

Annex 1

Sick or dead bird sample collection log

COVER PAGE (SAMPLE)

Submitter information	Incident information
Submitter's name: <u>Florence Smith</u> Dept/organisation: <u>Birds United</u> Address: <u>23 Wetlands Avenue</u> <u>Migration, Ukraine</u> Phone: <u>0724-1698-322</u> Fax: <u>0724-1698-320</u> Mobile #: <u>07399-149-2777</u> Email: <u>Fsmi@birdunit.org</u> Signature: <u>Florence Smith</u>	Date of observation: <u>10/10/06</u> Date of report: <u>14/10/06</u> Location (exact location – with GPS data if possible): <u>Edge of Wetland habitat</u> <u>32.39 longitude</u> <u>46.13 latitude</u> Landowner and land access: _____ <u>Wetland habitat-part of</u> <u>conservation park</u>
Animal details: Species affected (common name, genus and species): <u>Gargany</u> Total of Each Species: <u>62</u> Unaffected/Normal: <u>50</u> Sick: <u>10</u> Dead: <u>2</u> Approximate Ages of Affected Animals: <input type="checkbox"/> Chick <input type="checkbox"/> Juvenile <input checked="" type="checkbox"/> Adult Sex of Affected Animals: <input checked="" type="checkbox"/> Unknown <input type="checkbox"/> Male <input type="checkbox"/> Female Description of incident: <u>2 gargany found dead on the shoreline,</u> <u>10 gargany swimming in circles, head tilted, isolated from others</u> Environmental conditions: Weather, recent rainfall, sea conditions, recent local use of chemicals, changes in ground water levels, changes in domestic animal management: _____ Clinical signs of animals: <u>circling, head tilt, lethargic</u> Gross pathology findings: <u>pale liver, no food in GI tract, bird in good body condition, no fractures/trauma</u> Management actions taken: <u>called CVO + Ministry of Agriculture</u> Please add as many pages as necessary for thorough descriptions and additional observations	

COLLECTION LOG SHEET (SAMPLE)

Species	Animal Identification Number	Location	Live/Dead		Carcass kept fresh/frozen	Serum/Plasma	Swabs collected			Tissues collected		Photos		Sample collector	Comments
			Euthanised/Method	Method			Tracheal	Cloacal	Fresh	Fixed	Yes/No	Yes/No			
<i>Gargary</i>	06-001	Wetlands park	dead		fresh	serum	✓		✓	liver/brain fresh+fixed		Yes		Smith	
<i>Mallard</i>	06-002	Wetlands park	dead		fresh	serum	✓		✓	liver/brain fresh+fixed		Yes		Smith	
<i>Ruddy Shelduck</i>	06-003	Wetlands park	euthanized		fresh	plasma	✓		✓	full set according to protocol		Yes		Smith	
<i>Gargary</i>	06-004	Wetlands park	euthanized		fresh	plasma	✓		✓	full set according to protocol		Yes		Smith	
Specimens stored/sent where? <i>Specimens sent to Padova Reference Laboratory by DHL - Tracking # DL 461397</i>															
<i>Sent on 14 October 2006</i>															
Name of all people present during the sample collection															

Annex 2

OIE/FAO Network (OFFLU) and reference laboratories for avian influenza

OFFLU is a joint network of expertise on influenza, created and endorsed by the OIE and FAO in April 2005. Its objectives 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 influenza research needs, promote their development and ensure coordination.

For more information, visit: <http://www.offlu.net>

FAO OFFICES AROUND THE WORLD

For information on the location of FAO regional, sub-regional, 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 visit 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/FAO REFERENCE LABORATORIES AND OTHER EXPERTS FOR AVIAN INFLUENZA

** denotes an FAO reference laboratory for avian influenza*

(for updates please go to http://www.oie.int/eng/avian_influenza/vaccines.htm)

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For more information, visit the OFFLU website www.offlu.net

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Annex 3

Gross Pathology Illustrations

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.

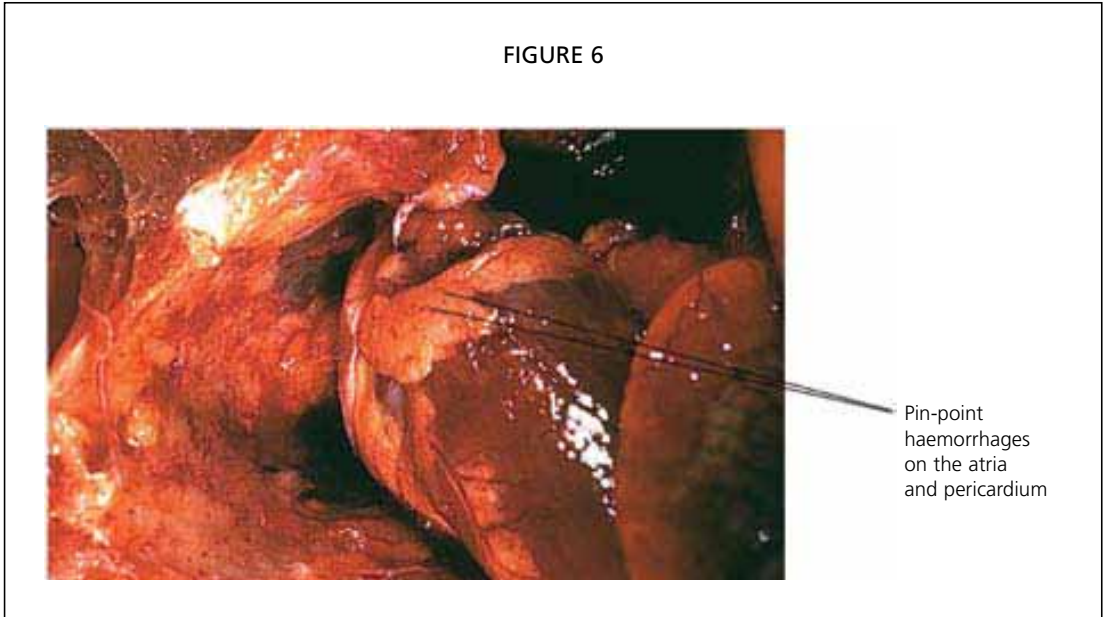
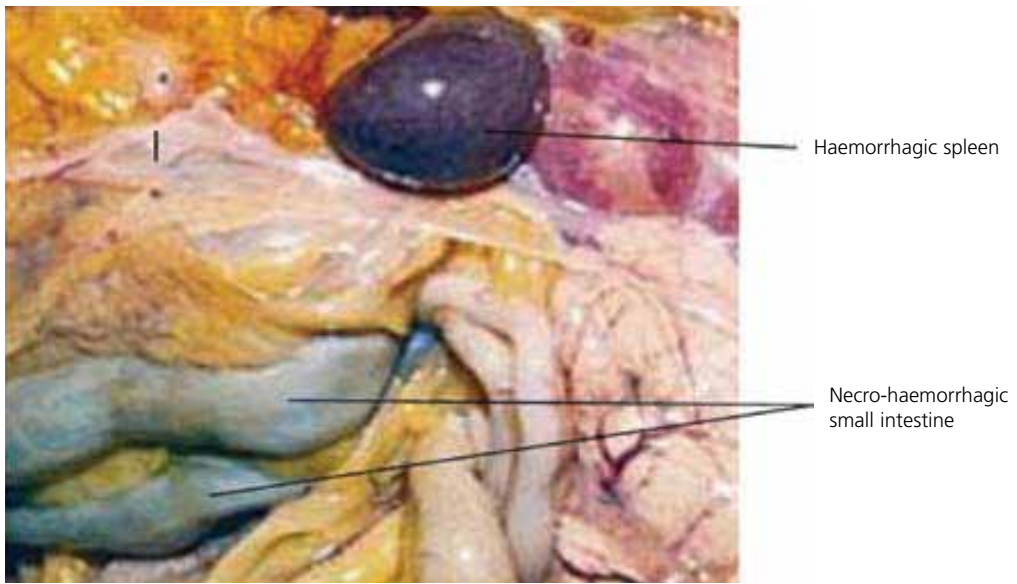


FIGURE 8



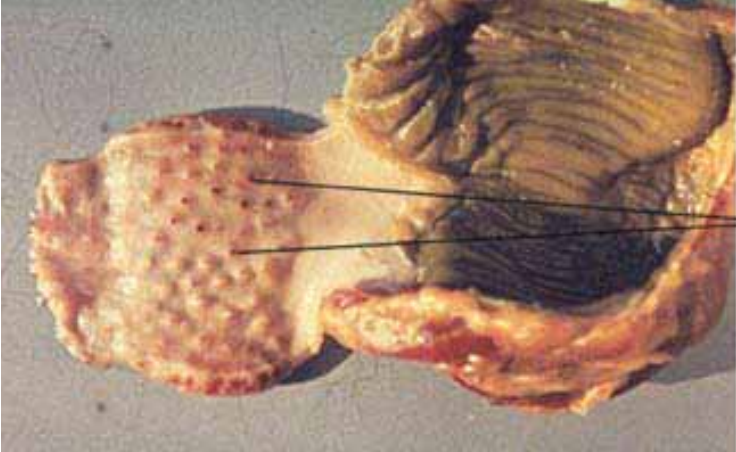
CREDIT: USDA

FIGURE 9



CREDIT: USDA

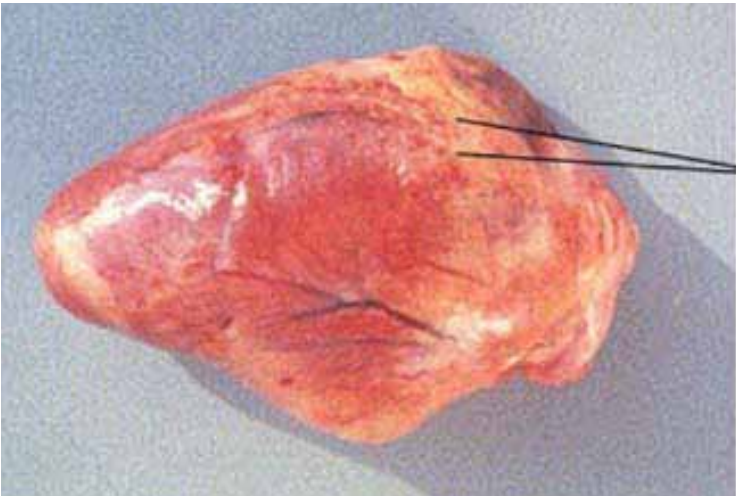
FIGURE 10



Thickened proventriculus with pin-point haemorrhages (normal gizzard)

CREDIT: USDA

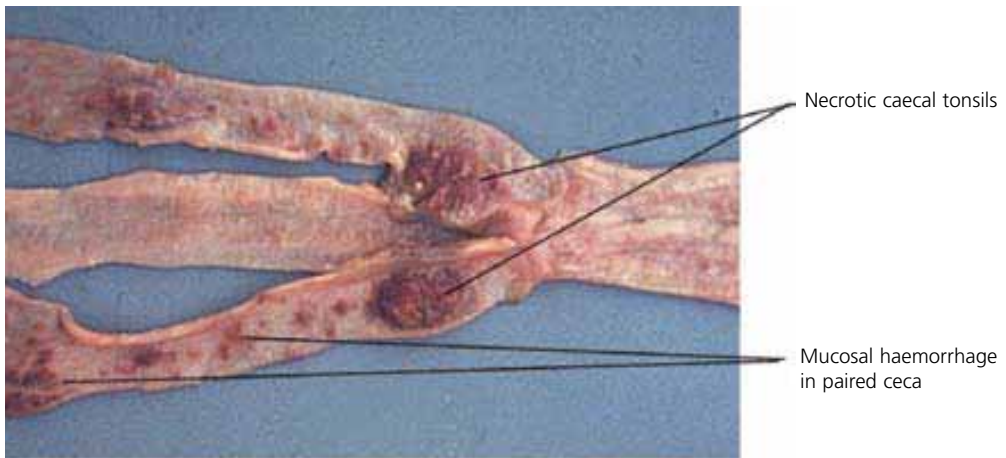
FIGURE 11



Cardiac necrosis and petechial haemorrhage

CREDIT: USDA

FIGURE 12



CREDIT: USDA

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1. Small-scale poultry production, 2004 (E, F)
2. Good practices for the meat industry, 2006 (E, F)
3. Preparing for highly pathogenic avian influenza, 2006 (E)
4. Wild Bird HPAI Surveillance – sample collection from healthy, sick and dead birds (E)

Availability: December 2006

Ar - Arabic	Multil - Multilingual
C - Chinese	* Out of print
E - English	** In preparation
F - French	
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13. Recognizing contagious bovine pleuropneumonia, 2001 (E, F)
14. Preparation of contagious bovine pleuropneumonia contingency plans, 2002 (E, F)
15. Preparation of Rift Valley fever contingency plans, 2002 (E, F)
16. Preparation of foot-and-mouth disease contingency plans, 2002 (E)
17. Recognizing Rift Valley fever, 2003 (E)

Submitter information**Incident information**

Submitter's name: _____

Date of observation: _____

Dept/organisation: _____

Date of report: _____

Address: _____

Location (exact location – with GPS data

if possible): _____

Phone: _____

Fax: _____

Mobile #: _____

Landowner and land access: _____

Email: _____

Signature: _____

Animal details:

Species affected (common name, genus and species): _____

Total of Each Species: _____ Unaffected/Normal: _____ Sick: _____ Dead: _____

Approximate Ages of Affected Animals: Chick Juvenile AdultSex of Affected Animals: Unknown Male Female**Description of incident:** _____

Environmental conditions: Weather, recent rainfall, sea conditions, recent local use of chemicals, changes in ground water levels, changes in domestic animal management: _____

Clinical signs of animals: _____

Gross pathology findings: _____

Management actions taken: _____

Please add as many pages as necessary for thorough descriptions and additional observations

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 H5N1 AI virus strain causing morbidity and mortality in many wildlife species. Although some surveillance has started, more research is necessary to determine the role that healthy wildlife plays in transporting and shedding virus.

This manual provides basic guidelines for wildlife surveillance and disease investigation whatever their cause. It contains chapters on clinical signs of infectious disease, bird handling and sample collection methods, sample handling and transportation, and diagnostic techniques. It also contains important recommendations on disinfection and personal safety.

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