

Chapter 5

Disease sampling procedures

Circumstantial evidence suggests that wild birds may play a role in the transmission and spread of the H5N1 HPAI virus. Yet, despite the fact that disease surveillance programmes in Europe, Asia, Africa and the Americas have collected samples (2004-2007) from several hundred thousand wild, apparently healthy birds, there is still no irrefutable evidence demonstrating that wild birds are acting as H5N1 HPAI viral reservoirs capable of travelling long distances and shedding the virus. Thus far, the H5N1 virus has been isolated primarily in sick, moribund or dead wild birds.

As the H5N1 HPAI virus continues to sporadically reappear in poultry farms, active disease surveillance programmes will become increasingly important for determining if wild birds are indeed acting as vectors in the transmission and geographic spread of the virus. Fortunately, H5N1 disease sampling in wild birds involves minimally invasive techniques that can be quickly learned following training in the basic procedures. These techniques are relatively straightforward and can be completed in just a few minutes with little or no detrimental effects to the bird. This means that active disease surveillance can be incorporated into most studies where wild birds are captured and handled. In addition, collection of fresh faeces of peridomestic and wild species can be a relatively simple and cheap process of collecting samples for the detection of avian influenza viruses, especially where catching of wild birds is not feasible.

Proper specimen collection is essential for providing samples that ensure reliable isolation and identification of any pathogens present. This chapter presents a brief description of the most practical disease sampling techniques used for the H5N1 AI virus in free-ranging wild birds. Please note that while these sampling techniques are for live, apparently healthy free-ranging birds, the use of personal protective equipment (PPE) appropriate to the level of risk level is recommended whenever wild birds are handled, because apparently healthy birds may be infected without exhibiting clinical signs of H5N1 infection. Clean PPE should be used at each sampling site to prevent the spread of disease among wild bird populations and between wild populations and domestic flocks. Good biosecurity practices should be followed, where the same PPE should not be used for sampling wild and domestic bird populations or between collection sites or between poultry holdings.

In countries where no outbreaks have been recorded, minimal PPE measures may include gloves, a mask and proper post-handling hygiene. However, working with sick and dead birds at suspected disease outbreak sites requires full PPE (including latex or vinyl gloves, a mask, goggles and coveralls or medical gown) and special handling and sampling procedures described in FAO (2006). If free-ranging birds captured during active surveillance programmes exhibit clinical signs (see below) of suspect infectious disease (i.e. H5N1 infection), immediately stop all bird handling activity and contact the appropriate governmental, veterinary or wildlife agencies in the country.

Possible clinical signs of H5N1 HPAI include (but are not limited to): diarrhoea; regurgitation; sneezing; emaciation; open sores; discharges from the mouth, nose, ear or vent; swelling or discolouration of head tissues including the conjunctiva; behavioural/neurological abnormalities (falling over, head tilt, head and neck twisting, seizures, circling, paralysis); and feather abnormalities in chickens. Some susceptible wild bird species would also show some of these signs but their presence or severity will vary greatly. These clinical signs are not specific for H5N1 infection, but suggest the presence of serious clinical disease that needs to be investigated and diagnosed in a timely manner.

The disease sampling techniques are presented with the following assumptions:

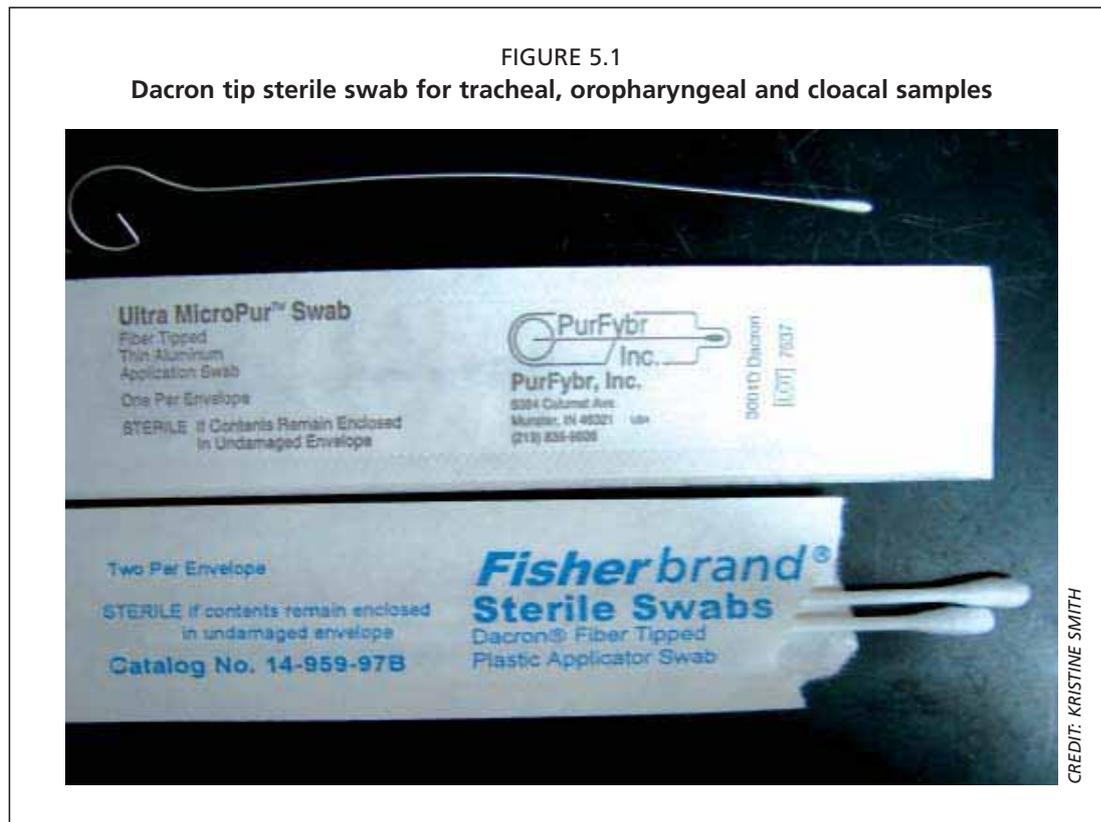
- all investigations will be performed by appropriately trained personnel;
- each bird sampled is correctly identified by an appropriately trained individual, and information relative to the bird (species, and when possible, sex and age) is properly recorded; if uncertain take a photo (See guidelines for taking good quality photographs in Annex A).
- proper human health and biosafety precautions will be adhered to (see FAO 2006);
- consent from the responsible local, state and federal veterinary and wildlife agencies has been obtained prior to any investigation;
- disease outbreak investigations should be conducted in collaboration with the responsible government agencies, and with FAO and OIE representatives when appropriate.

TRACHEAL AND CLOACAL SWABS

Swabs taken from the cloaca (vent) or trachea can be used for viral cultures or reverse-transcription polymerase chain reaction (RT-PCR) to test for the presence of many viral pathogens, including AI viruses. While non-pathogenic AI viruses replicate primarily in the avian intestinal tract, recent strains of H5N1 HPAI viruses have been detected both from cloacal and tracheal/oropharyngeal samples. Research has revealed that, unlike other AI viruses, the H5N1 HPAI subtype replicates to higher levels and for longer periods in the res-

EQUIPMENT LIST FOR TRACHEAL AND CLOACAL SWAB SAMPLES

1. Personal protective equipment (PPE)
2. Rayon or Dacron-tipped swabs
3. 2-2.5 ml screw-top cryovials
4. Viral transport medium (VTM)
5. Scissors/pliers
6. 70% alcohol solution
7. Cooler and ice and/or liquid nitrogen to store samples
8. Cryovial labels and pencils or indelible markers
9. Data forms
10. Headlamps or pen-lights



piratory tract compared to the gastrointestinal tract (Sturm-Ramirez *et al.* 2004, Hulse-Post *et al.* 2005). Furthermore, after experimental exposure, higher concentrations of the virus have been found in tracheal samples than in cloacal samples on any given day. Therefore, tracheal and cloacal swabs are currently the preferred samples for H5N1 surveillance in wild birds.

Swabbing techniques require Dacron or rayon-tipped swabs (Figure 5.1); avoid using swabs with cotton-tipped or wood stems as they may inhibit genetic detection or viral growth (due to inherent RNase activity of cotton or wood cellulose). Wire-stemmed swabs may also be used, especially for very small-sized birds. Cryovials containing a viral transport medium (VTM) will also be needed to store and transport the samples. Cryovials and cryolabels that are appropriate for the intended storage temperatures should be chosen, because some are certified only for use in dry ice and are unsuitable for use in liquid nitrogen.

VTM can be prepared locally at a laboratory (see instructions at WHO website⁷) or purchased as kits from commercial dealers (e.g. TBD Universal Viral Transport Media or Cellmatics Viral Transport Pack⁸). VTM should be stored at a low temperature (<4°C) in the field before use.

Rapid detection tests using tracheal swabs to detect the presence of a type A virus (in the case of AI, any of the possible 144 sub-type combinations) are available for use in the field, but these tests are relatively insensitive and require a substantial viral titre to return a positive result; thus the value of a negative test may be low (i.e. infection is present but

⁷ http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_ARO_2006_1/en/index.html

⁸ <http://www.bd.com/support/locations.asp>

not of sufficiently high level for the diagnostic strip to show up as a positive). However, a positive test in conjunction with a clinical scenario consistent with H5N1 AI infection warrants immediate notification of appropriate authorities, although an actual H5N1 diagnosis requires confirmation by laboratory tests.

Swabbing procedures

Other than the sampling site, the equipment and techniques for tracheal and cloacal swabs are similar. Tracheal swabs may not be possible for small birds (passerines) with narrow tracheal openings. In such cases, an oropharyngeal swab should be conducted. Be sure to use a swab size appropriate for the bird.

- Open the swab package from the stem end, being sure not touch the swab tip with anything prior to or after sampling
- **Tracheal swabs** are collected from the air passage (trachea) at the back of the bird's mouth. To gain access to the opening of the trachea, it is often useful to gently pull the tongue forward, exposing the trachea at the rear end of the tongue. Wait until the bird breathes and the cartilage protecting the trachea is open before inserting the swab and gently touching the sides and back of the trachea (Figure 5.2); moving the tongue forward can help expose the trachea.
- **Oropharyngeal swabs** are conducted by gently rolling the swab tip around the inside of the bird's mouth and behind the tongue (Figure 5.3).
- **Cloacal swabs** are collected by inserting the entire tip of the swab into the cloaca and swabbing with two to four circular motions while applying gentle pressure against the mucosal surfaces (Figure 5.4); gently shake any large faecal residues from the swab before placing it in the cryovial.

FIGURE 5.2
Proper location for a tracheal swab



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Arrow points to tracheal opening

FIGURE 5.3
Proper procedure for an oropharyngeal swab



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FIGURE 5.4
Proper procedure for a cloacal swab



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- Carefully remove the swab, open the cryovial and place the swab tip in the VTM about $\frac{3}{4}$ of the way toward the bottom of the vial; avoid overfilling the cryovial as the contents may expand and leak during the freezing process.
- Cut or break the swab stem so that the swab tip remains in the VTM and close the vial (Figure 5.5); if wire-stemmed swabs are used, these can be cut with wire cutters.
- If scissors or wire cutters are used to cut the swab stem, disinfect them after each use by cleaning the blades with a 70% alcohol solution.

FIGURE 5.5
Proper placement of swab sample in the viral transport medium



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FIGURE 5.6
Labelled cryovial with date, species, sample type and an ID number specific to each sampled individual that refers to a database containing all the information for that bird

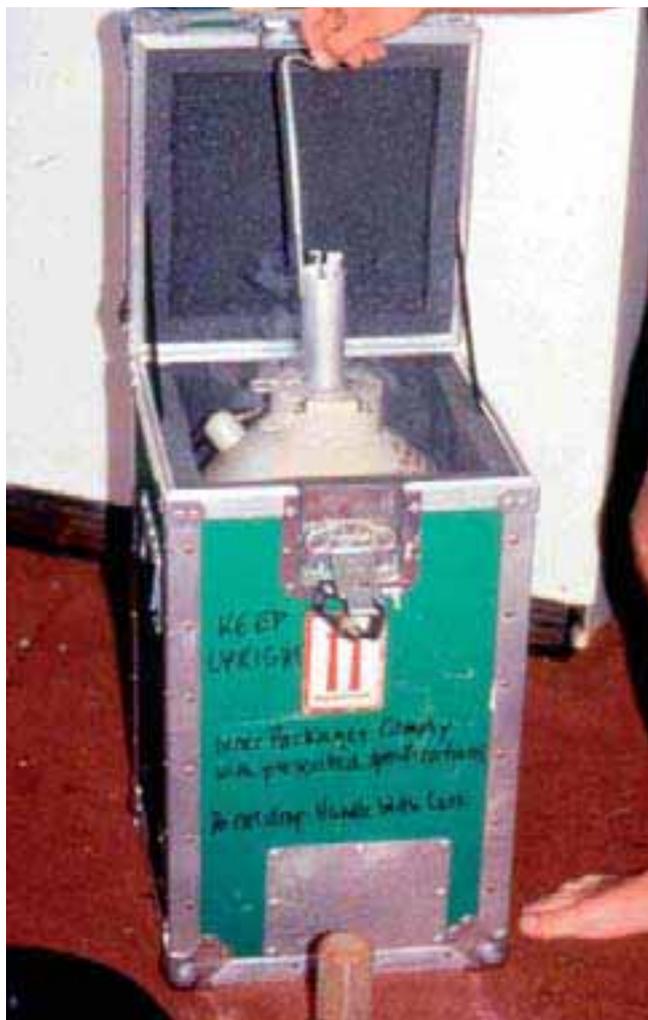


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- Label each cryovial sample with the date, species, sample type (tracheal or cloacal), and an ID number specific to each sampled individual that refers to a database containing all the information for that bird (Figure 5.6); labels should be written with a material that will not dissolve when wet, then placed in liquid nitrogen (Figure 5.7) or ethanol, or stored at temperatures below -70°C .

Check with the supplier of the VTM to determine the proper storage methods for that medium. If using a VTM that requires refrigeration or freezing, store samples in a sealable plastic bag on ice at or below 4°C , or in liquid nitrogen. It is important to maintain the "cold chain" during the entire storage and transport process as loss of the cold chain can result in samples being rendered non-diagnostic. Commercially available kits that inactivate the virus and are stable at room temperature may be a convenient back-up option for remote field sites where cold chain storage for transport media cannot be guaranteed. If samples cannot be transported to the laboratory within 24-48 hours, longer-term storage in liquid nitrogen or in a freezer at temperatures less than -70°C will be needed.

FIGURE 5.7
Liquid nitrogen container used to freeze and preserve samples when working at remote locations



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BLOOD SAMPLING

Serological testing of blood samples indicates prior exposure to the virus by detecting antibodies in the blood rather than viral antigens or specific genetic targets. Blood samples can be collected by different methods depending on the size of the bird. For small birds (e.g. passerines and small waders) blood should be collected from the jugular vein (right side of neck; Figure 5.8) using a 0.3-0.5 ml insulin syringe with a 0.33 mm hypodermic gauge (22-30G) needle depending on the size of the bird. For larger birds (e.g. ducks, coots, gulls and herons), blood can be collected from the jugular vein or medial metatarsal (leg) vein (Figure 5.9) using a 1-2 ml syringe and 23-27 gauge hypodermic needle. Sampling from the brachial (wing) vein is also an option for some larger birds.

In general, it is safe to collect 0.3-0.6 cc of blood for every 100 g of body mass from live birds (total volume collected should not exceed one percent of body mass), although it is a good practice to collect only enough blood needed for conducting the required tests.

The optimal venipuncture site (location where the hypodermic needle penetrates the vein) will vary depending upon the species being sampled. Intuitively, venipuncture techniques are easier on larger birds with larger veins, but the techniques become easier for all species as one gains experience. After the proper amount of blood is collected, a gauze square should be pressed against the venipuncture site as the needle is removed from the bird, and pressure applied to the venipuncture site for 30 seconds. This will prevent the bird from developing a painful haematoma (blood clot) under the skin which may affect movement of the wing or leg.

To reduce the risk of haemolysis, it is advisable to remove the needle from the syringe (for non-mounted syringes) when transferring blood into the tube by gently expelling the blood against the inside wall of the vial.

FIGURE 5.8
Blood sampling procedure from the jugular vein



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FIGURE 5.9
Blood sampling procedure from the medial metatarsal vein



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- When sampling from the jugular or brachial vein, expose the venipuncture site by using alcohol to wet the feathers, then separate the feathers with the fingers.
- Sample collection from the brachial or medial metatarsal vein is best accomplished by holding the vein off (applying pressure to the vein), proximal (towards the heart) to the desired venipuncture site to temporarily block blood flow and make the vein easier to locate.
- Sample collection from the jugular vein is most easily accomplished by holding off the vein on the right side of the neck, at the level of the clavicle.

EQUIPMENT LIST FOR BLOOD SAMPLING

1. Personal protective equipment (PPE)
2. Hypodermic or butterfly needles of various sizes (22-30 gauge)
3. Syringes of various sizes (1cc -12 cc)
4. Red top (serum) and green top (plasma) separator tubes
5. Portable centrifuge (if available)
6. 70% alcohol solution and cotton gauze
7. Cryovials
8. Sterile pipettes
9. Indelible marker and cryovial/separator tube labels
10. Cooler, ice and/or liquid nitrogen to store cryovials
11. Previously designed data sheets
12. Sharps Container

- Prior to inserting the needle into the bird, pull the plunger back to release the vacuum in the syringe, and then press it all the way forward so there is no air in the syringe.
- Carefully insert the hypodermic needle under the skin and into the vein with the bevel pointing upward so that the needle opening faces the inside and not the wall of the vein; for jugular vein sampling, the needle can be bent slightly to form a curve that eases insertion into the vein.
- Once assured that the hypodermic needle is in the vein, pull back very gently on the syringe plunger to draw blood.
- Any bird, regardless of size, may experience stress, cold or other factors that can cause vasoconstriction and impede the flow of blood; in conditions where blood does not flow smoothly, gentle digital massage above the venipuncture site may aid sample collection.
- After blood is collected, cover the venipuncture site with gauze and apply digital pressure until bleeding stops, usually 30-60 seconds.
- Dispose of used hypodermics and other veterinary-related wastes in appropriate and safe containers.
- Immediately transfer blood from the syringe to a **serum** (red top) or **plasma** (green top) separator tube to prepare samples for centrifugation.
- **Plasma tubes** should be immediately refrigerated or kept in a cool water bath before they are spun down in the centrifuge.
- **Serum samples** should be allowed to clot at room temperature (22-25 °C) before refrigeration; clotting can be facilitated by slightly inclining the tubes.
- Spin down blood samples in a centrifuge after collection to separate the fractions for later laboratory analyses; separation of **serum** samples is aided by refrigerating samples for several hours and carefully ringing the sample with a sterile, round "stick" to free the clot from the vial.
- After centrifugation, transfer **serum** and **plasma** samples to cryovials (preferably screw top cryovials with rubber o-rings) with a sterile transfer pipette; if pipettes are not available the samples can be carefully poured into the cryovials.
- Label each cryovial sample with the date, species, sample type (plasma or serum), and an individual ID number.

Choice of serum and/or plasma separator tubes will depend on the laboratory assays to be performed and should be confirmed with the laboratory before conducting field work. Cryovials with the separated serum or plasma fractions can be kept in a zip-lock bag for storage and transport. Samples can be stored on ice at 4° C if they can be shipped to the laboratory within 24-48 hours. Otherwise, store samples on dry ice, in liquid nitrogen or in a -70° C freezer.

If an electric centrifuge is not available during field work, consider use of a battery powered or hand-powered crank centrifuge, or send non-centrifuged blood samples to the laboratory if they can be shipped to the laboratory within 24-48 hours and maintained at 4° C. Transport samples on ice blocks by placing tubes in zip-lock bags and wrapping the bag in a cloth towel before placing in the cooler. Plasma and serum tubes with whole blood samples should not be frozen or come into direct contact with ice as this may damage the red blood cells causing haemolysis, which may interfere with diagnostic results.

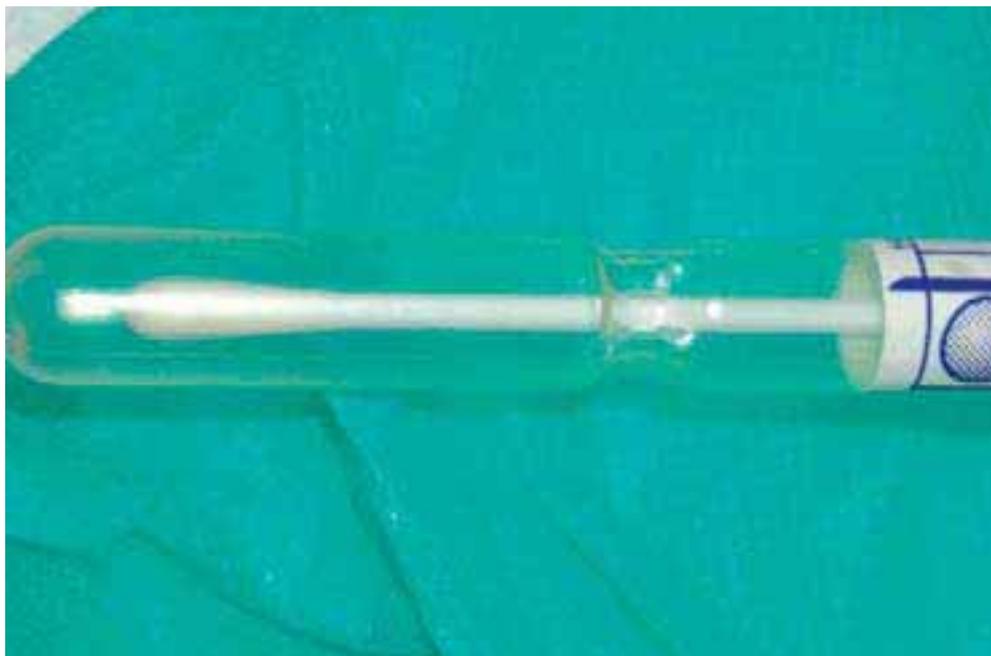
FAECAL SAMPLING

The collection of fresh faeces of peridomestic and wild species for the detection of avian influenza viruses can be a relatively simple process and an inexpensive way of collecting large number of samples, especially where catching of birds is not feasible. Faecal samples are also called “environmental samples” in some countries (such as in the United States of America).

The following guidelines should be followed for collection of faecal samples from a single individual or from a flock of birds:

- Observe the bird(s) from a distance and carefully note the area where it (they) are gathered. Birds may roost on the ground, within the poultry farms, in fields or around wetland areas, on wires, posts or on roofs or other structures on which they will defecate.
- Identify the species of birds that are to be sampled and to ensure that the bird(s) are roosting as either single species flocks or at least in mixed flocks where it is possible to be sure of the species from which faeces is subsequently collected. For example mixed goose flocks are problematic as the faeces may be difficult to separate. But a single goose species mixed with gulls should not create any problem as there would be no risk of misidentifying the faeces, based on size, colour and content.
- Walking rapidly towards a group of roosting birds normally causes them to move or fly away and in the process some individuals will defecate.
- Try to minimize the opportunity for resampling the same individual by limiting the number of faecal samples collected from each flock and ensuring that samples are collected evenly across the area where a single species flock had been observed.

FIGURE 5.10
Sterile swab to be used for faecal sampling



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EQUIPMENT LIST FOR FAECES SAMPLING

1. Personal Protective Equipment (PPE)
2. Sterile Rayon or Dacron-tipped swabs
3. Prelabelled cryovials with transport medium
4. Indelible marker and cryovial labels
5. Cooler, ice and/or liquid nitrogen to store cryovials
6. Previously designed data sheets

- Only collect fresh faecal samples, ideally those that are still moist. Dried and powdery faeces usually indicate old specimens and should not be collected as they are of poor diagnostic value. High temperatures may inactivate viruses within a few hours.
- Collect the faeces using a sterile swab (Figure 5.10) and place in a prelabelled vial with transport medium. If the swab is going to be placed in the viral transport medium, collect the faeces using a Rayon or Dacron-tipped swab.
- Resist the temptation to scoop faeces into the tube. It is better to roll the swab over the faeces and shake off excess matter.
- Where possible, try to sample the underside, or shaded side (since direct sunlight may reduce viral survival)
- Developing of a photo file of faeces of different bird species can assist in improving sample collection. A scale for identifying the size of the faeces is useful to include in the photos.

REFERENCES AND INFORMATION SOURCES

- European Commission, DG SANCO.** 2006. Guidelines on the implementation of survey programmes for avian influenza in poultry and wild birds to be carried out in the Member States in 2007. (also available on http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/surveillance4_en.pdf).
- FAO.** 2006. *Wild Bird HPAI Surveillance: Sample collection from healthy, sick and dead birds*, by K. Rose, S. Newman, M. Uhart & J. Lubroth. FAO Animal Production and Health Manual, No 4. Rome.
- Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., Scholtissek, C., Puthavathana, P., Buranathai, C., Nguyen, T.D., Long, H.T., Naipospos, T.S., Chen, H., Ellis, T.M., Guan, Y., Peiris, J.S. & Webster, R.G.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proceedings of the National Academy of Sciences of the United States*, 102: 10682-10687.
- Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., Poon, L., Guan, Y., Peiris, M. & Webster, R.G.** 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of Virology*, 78: 4892-4901.