

# **Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection**

## **Guide for field operations**

October 2006



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This document is a work in progress and is based on the best information available at the time of production. The document will be updated regularly as more information becomes available.

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## Abbreviations

AI	avian influenza A(H5N1)
ARO	Alert and Response Operations
BSA	bovine serum albumin
CDS	Communicable Disease Surveillance and Response
EDTA	ethylenediaminetetraacetic acid (anticoagulant used for blood samples)
EPR	Epidemic and Pandemic Alert and Response
HPAI	highly pathogenic avian influenza
H5N1	avian influenza subtype
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
NAMRU 3	Naval Medical Research Unit 3 (US Navy) based in Cairo, Egypt
PCR	polymerase chain reaction
PPE	personal protective equipment
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
UN	United Nations
VTM	viral transport medium
WHO	World Health Organization

## 1. Introduction

Highly pathogenic avian influenza (HPAI) caused by the A(H5N1) influenza subtype in animal populations, particularly wild waterfowl and domestic poultry such as chickens and ducks, poses a continuing global human public health risk. The virus has expanded its geographical range increasing the size of the population at risk. Each new human case gives the virus an opportunity to evolve towards a transmissible pandemic strain.

Collection of appropriate specimens from human and animal cases for rapid viral RNA detection by any qualified laboratory, together with rapid and precise characterization of virus isolates of the specimens at specialized reference laboratories, is essential for early detection of cases, proper management of patients and understanding the epidemiology of the disease. In addition, the development of resistance to antivirals can be determined, effective vaccines produced in a timely manner and quality control improved.

The rapid confirmation of the precise nature of isolates of the virus permits effective surveillance and in particular the proper documentation of the spread of the infection in human and animal populations and detection of changes in the virus that could indicate improved transmissibility to humans.

This protocol is designed to simplify the previously published guidelines on specimen collection, packing and shipment (see Annex 1) to allow for ease of use at field level and to provide the data needed to confirm the diagnosis of A(H5N1) infection as specified/described in the case definitions recently produced by WHO (see Annex 1 for link). It is a work in progress and may change as circumstances demand. It is intended to:

- describe the minimum number and types of specimens collected;
- enhance the chances of obtaining a positive result if the patient is infected with A(H5N1);
- allow the potential identification of respiratory pathogens other than A(H5N1) (including other strains of influenza virus);
- contribute to work designed to increase understanding of the pathogenesis of A(H5N1) disease including the potential duration of infectiousness.

As a WHO document, the protocol is designed primarily for sampling from humans by medical staff. Specimens from birds and mammals should ideally be taken by veterinarians, their assistants, or trained wildlife professionals and detailed guidelines are available on the FAO/OIE web site (see Annex 1 for links). However there may be instances where professionally trained persons are not available for sampling from animals and details of the specimens to take and how to take them are given in Annex 12. The same procedures for subsequent handling, storage and shipping of the specimens apply as for those taken from humans and the relevant parts of this protocol may be useful as an advisory document for the veterinary services.

This protocol covers the following aspects of sampling:

- the specimens required from humans;
- collection of the specimens;
- preservation before and for shipping;
- correct packing of the specimens;

- shipment to laboratories;
- the information that needs to accompany the shipment.

While this can act as a stand-alone document it is also intended to accompany a new WHO kit (Annex 4) which has been designed to facilitate all these aspects of sampling.

## 2. Safety

The majority of the human infections with influenza A(H5N1) virus to date have been caused by close (usually direct) contact with infected birds. Epidemiologic observations suggest human-to-human transmission of the virus by very close contact (e.g., face-to-face) with infected individuals may have occurred on some occasions. A(H5N1) infection is acquired by inhalation of infectious droplets or droplet nuclei or by direct (and possibly indirect) contact and self-inoculation of infectious virus into the nose, eye, or possibly mouth. Infection via the eyes could occur either by infection of the conjunctiva or (possibly) if the virus is washed through the lachrymal ducts and into the nasopharynx.

- There is some evidence that infection via the pharynx and the gastrointestinal tract may occur following ingestion of the virus.
- Infection across intact skin has not been described.
- The virus is transmitted on respiratory droplets larger than 5 microns and possibly by fomites. The relative importance of short-range fine particle (airborne) transmission in A(H5N1) infection is unknown.
- The virus cannot survive long in a dry environment but can survive for weeks in a moist environment protected from direct sunlight.
- Viral RNA and less often infectious A(H5N1) virus are detectable early in illness (see Fig 1). Infection from percutaneous inoculation of the virus during care of patients or specimen collection (needle sticks, cuts from contaminated surgical instruments) or during postmortem examination has not been recognized to date (although such injury obviously carries a real risk of transmission of known blood borne pathogens).

The use of personal protective equipment (PPE) is therefore mandatory if direct close contact with a patient is anticipated, when entering a room where aerosol-producing procedures in AI-infected patients are being performed, when sampling from wild birds or mammals and when taking specimens from poultry either in intensive rearing or laying facilities or from backyard flocks.

The level of PPE to be employed will be determined by the exposure risk. For example work in intensive poultry units, where virus contaminated material can accumulate and there is a great deal of potentially contaminated dust in the air, requires greater personal protective equipment levels than sampling from wild birds. Yuen & Wong (2005) recommend that: “In view of the high fatality of the disease, a combination of contact, droplet, and airborne precautions are recommended as long as resources allow despite the fact that the relative importance of these three modes in nosocomial transmission of avian influenza is still unknown.”

In general PPE should include:

- a suitable form of respiratory protection (Annex 6)
- non-sterile latex gloves (or equivalent if allergic)
- goggles or a face shield
- gown
- head covering.

It may also be necessary to include:

- impermeable apron
- suitable rubber boots.

High risk activities such as post mortem examination of a confirmed or strongly suspected human case, capture of birds in poultry sheds or on farms, euthanasia of infected or potentially infected birds or other animals, and procedures such as decontamination in an intensive agriculture system should only be conducted in a full body cover-all, with easily cleaned waterproof boots, heavy rubber gloves and eye protection. In poultry sheds or other situations where the virus load is likely to be high, the best form of respiratory protection is powered air pressure respirators (PAPR). If a PAPR is not available then a NIOSH-certified N95, EU FFP2, or equivalent respirator should be used.

PPE is essential to prevent infection during sampling but is not the whole answer. Those taking specimens should comply with all recommended infection control precautions including specific personal hygiene measures, and the correct use of disinfectants.

Further details of safety procedures are given in Annex 5 (Hand hygiene technique), Annex 6 (Respiratory protection) and Annex 7 (Disinfection).

## Monitoring medical or veterinary personnel

1. If an incident that could lead to infection occurs during a sampling procedure (such as a breakdown of protective procedures) the staff member(s) involved should be monitored for signs of illness for a week (including daily temperature measurement). Post-exposure chemoprophylaxis with a neuraminidase inhibitor for 7 to 10 days is a consideration under such circumstances (see: *WHO rapid advice guidelines on pharmacological management of humans infected with avian influenza A(H5N1) virus*. Link provided in Annex 1).
2. All staff working with human or animal cases of AI should monitor their own health and any evidence of influenza-like illness within seven days of exposure to a confirmed or suspected human case or to a potential avian source should be viewed as suspected avian influenza and treated appropriately by a medical doctor.

### 3. Taking specimens

- A checklist for the various stages of taking, handling and shipping specimens is given in Annex 2. This should be filled in for each batch of specimens and kept for reference. Note that the checklist covers a wide range of activities from taking the specimen to activities such as taking aliquots from specimens (sub-sampling) which may occur before the specimen is sent to the reference laboratory.
- For each type of specimen two specimens should be taken in separate specimen tubes on each occasion that sampling is undertaken. One can be used for immediate analysis and the other retained for reference purposes, retesting, etc.
- Each patient or animal sampled or each environmental sample taken should be given a unique identifier number and accompanied by a field data sheet (Annex 9)
  - All specimens taken from that source should be marked with that unique identifier as well as any other numbers needed to identify the particular specimen.
  - This identifier should be used on all documentation concerning the specimen from that source.
  - Specimen tubes should also be marked with information about the type of specimen in the tube and the date when the specimen was taken.
  - Specimens of animal origin should be accompanied by a detailed epidemiological investigation form (see Annex 9).

#### a) What specimens to collect from suspect cases

##### 1. Preferred samples

- **Upper respiratory tract** ( take both types of specimen to allow detection of A(H5N1) and other influenza viruses):
  - Posterior-pharyngeal (throat) swabs are currently the highest yield upper respiratory tract specimen for detecting A(H5N1) (unlike human influenza). Naso-pharyngeal swabs may be collected if necessary (see below).
  - Nasal swabs with nasal secretions (from the anterior turbinate area) or nasopharyngeal aspirates or swabs are appropriate specimens for detecting human influenza A and B and therefore useful if the influenza is not due to A(H5N1).
- **Lower respiratory tract:**
  - If the patient is intubated, take a tracheal aspirate or collect a sample during bronchoalveolar lavage.
- **Blood:**
  - Serum (acute and convalescent if possible).

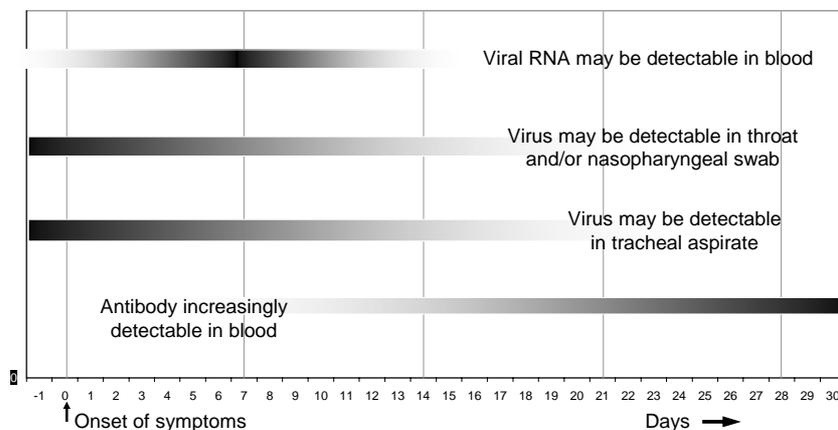
2. Secondary specimens ( **these are not essential but can be useful if materials are available**)

- Plasma in EDTA (for detection of viral RNA)
- Rectal swab —especially if the patient has diarrhoea
- Spinal fluid if meningitis is suspected and a spinal tap is to be performed for diagnostic/therapeutic purposes.

b) When to collect the specimens from suspect cases

The figure below (Fig 1) is a summary of the data available at the time of the publication (October 2006) and will be changed as necessary as more data become available. It must be emphasized that the bars indicate **approximate** periods of time after onset of symptoms when taking specimens is **likely** to yield results and **not** periods when sampling will always be effective.

**Fig 1: Virus excretion, viral RNA in blood and antibody response in H5N1 infection in humans**



- A throat swab should be taken (if possible) within three days of onset of symptoms. Note that the virus is generally detectable in throat swabs from most patients from the point of onset of symptoms (or even just before) until towards the end of the second week, and infrequently beginning of the third week, after onset of symptoms. Cases whose initial specimens are negative for A/H5 but who continue to show symptoms suggestive of this type of infection and/or who have a history of exposure that would also support the diagnosis should therefore be sampled at least once again as soon as possible.
- Virus may be detectable in tracheal aspirates from onset of lower respiratory complaints (dyspnoea, difficulty breathing, marked cough) or pneumonia until the second or third week of illness.

- An acute phase serum sample should be taken seven days or less after symptom onset (this will usually be done when the patient presents and begins treatment) and a convalescent sample after 3 to 4 weeks. Note that the limited data available on antibody kinetics indicate development of positivity (initially ELISA and not necessarily neutralizing antibody) from day 10 onwards.
- Single serum samples. To be collected at day 14 or later after symptom onset since the likelihood of detecting neutralizing antibodies increases over time, certainly during the first 3 to 4 weeks after onset of symptoms.
- Blood serum or plasma for the detection of viral RNA should be taken during the first 7 to 9 days after the development of symptoms because the patient is most likely to be RNAemic (have detectable RNA in the bloodstream) at that time (Fig 1).
- Initial specimens (respiratory and blood) should ideally be collected from suspected patients before antiviral therapy is begun but treatment must not be delayed in order to take specimens. (Note that standard treatment may render throat swabs negative for virus after three or more days of treatment but probably has no effect on the development of neutralizing antibody).
- Specimens should be collected from deceased patients as soon as possible after death.

### c) Sampling human contacts

Taking single respiratory tract or blood specimens from contacts of human cases who remain healthy in the days immediately after potential contact with HPAI is unlikely to yield useful results. Individuals who are contacts of suspect or known human patients or have had exposure to sick animals should be observed for seven days after the last contact (take the temperature daily). If they become ill with an influenza-like illness they should be sampled as outlined above.

Blood specimens for serological studies can usefully be taken from contacts for several reasons:

- as a tool for searching for asymptomatic/subclinical cases;
- for studies of the prevalence of A(H5N1) infection;
- to assess possible susceptibility to A(H5N1) infection.

#### d) Specimens from the respiratory tract

Sampling from the respiratory tract is hazardous as the operator is very close to the patient (Fig 2) and the procedure can generate aerosols and droplets. Full PPE is therefore essential.



Fig 2. Taking a throat swab - operator is close to the patient

Choose a sitting position for adults and a supine position for infants and younger children. Children often find sampling from the respiratory tract very distressing and need to be reassured. They may also need to be restrained during the sampling process.

If the child's parents or guardians are present they must be fully informed of what is to take place, and must be made aware that the child may become distressed. The parent(s) should not usually be in the room during the sampling procedure. The sampling procedure can generate aerosols which could present a risk to others in the immediate vicinity, and also the parent(s) may react to the child's distress by attempting to interfere with the sampling procedure and risk injury to the child. It is also not usually appropriate for a parent to help restrain the child, mainly because the assistant needs to be in PPE and the parent would not have been trained to don, wear and remove PPE safely. In addition, the parent could be upset by their child's distress and, if restraining the child, could release the child at a critical moment, which could risk injury to the child. (These are general considerations and should be adjusted accordingly to the local cultural sensitivity and social circumstances).

Very young children can be restrained by being held by an assistant against the assistant's chest with the child's arms restrained by the assistant's enfolding arms and the child's legs held between the assistant's legs (Fig 3).



Fig 3. Restraining a small child

Even older children will attempt to defend themselves with their hands or by leaning their head backwards. Lay the subject supine and with extended ('hold-up') positioning of the subject's arms above the head. The assistant holds the child's arms with elbows and arms pressed against the subject's forearms and the assistant also uses his/her palms to hold the subject's head in place (Fig 4 and Fig 5). An additional assistant may be needed to restrain the child's legs.



Fig 4 and Fig 5 restraining an older child

When taking throat (or nasal) swabs, the swabs must be held correctly. They should be held between the thumb and the first and second fingers with the shaft protruding beyond the web of the thumb (like a pencil) (Fig 6) and not between the thumb and forefinger with the base in the palm of the hand (Fig 7). The main reason is that if the patient makes a movement as a reaction to the swabbing the swab will slide out of harms way if held the first way (Fig 8 - with the patient represented by the open gloved hand of the operator) but not if held the second (Fig 9). In this case discomfort would be caused and the patient could be injured. In addition control over the swab is much greater if it is held correctly.

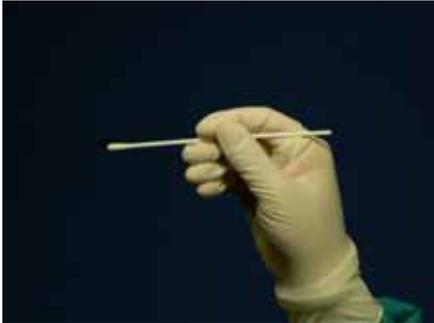


Fig 6. Swab held correctly

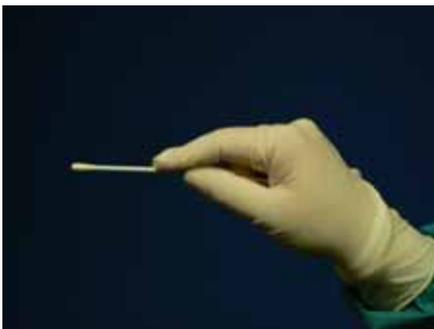


Fig 7. Swab held incorrectly



Fig 8. Correctly held swab can slide out of the way



Fig 9. Swab can injure patient

- Use only sterile dacron or rayon swabs with plastic shafts. Calcium alginate or cotton swabs, or swabs with wooden sticks may contain substances that inactivate some viruses and inhibit PCR testing and should only be used if dacron or rayon swabs are not available.
- Prepare two vials containing at least 2–3 ml of a suitable transport/preservative medium (Viral Transport Medium - VTM - Annex 8) for each specimen. These should be marked with:
  - the unique identifier
  - the specimen date
  - the type of specimen in the tube (e.g. blood serum, throat swab etc.).

**Note:** Always mark the tube itself with identifying details, never the cap as this can get switched during handling). Use an indelible and alcohol resistant marker pen. Be aware that stick-on labels can easily come off, especially when the specimen is chilled to very low temperatures. Relevant field data sheets (see Annex 9) should be filled in.

- Take two specimens and put one into each vial.
- If VTM is not available or alternatively specimens cannot be stored at appropriate temperatures (e.g. no freezers are available - see Table 1 below), swabs can be stored and shipped in absolute (100%) ethanol. (If pure ethanol cannot be used, 99% Industrial Methylated Spirit - without additives other than methanol - may be substituted). Put 1–2ml ethanol into a vial and put the swab tip into the tube (see below). Note that such specimens are suitable only for PCR.
- After a specimen is taken, the tip of the swab should be put into the vial and the shaft broken or cut off sufficiently short for the lid to be closed. Plastic swab handles usually have a weak point in them to allow them to be broken off for insertion into a specimen tube. Others have a handle made of a brittle plastic that will snap easily.

If the shaft cannot easily be broken off short enough to be put into a small tube such as a cryovial it will have to be cut. To do this:

- cut the shaft with scissors taking care not to touch the tip;
- allow the tip to slide into the VTM and then cap the tube. Do not let cut portions of the bag or wrap fall into the tube.

Sterilize the cutting edge of the scissors by the use of flame (e.g. by the use of a spirit burner, a Bunsen burner or another suitable heat source). Allow scissors to cool before reuse.

If this procedure cannot be followed, agitate the swab tip in the medium for 30 seconds and squeeze it against the side of the tube before removing it from the medium and disposing of it in a safe manner (not suitable for ethanol storage).

#### Posterior pharyngeal and nasopharyngeal swabs

Posterior pharyngeal swabs are the best upper respiratory tract to take because the evidence so far available suggests that they are more likely to be positive than anterior nasal swabs in sporadic A(H5N1) illness. However if difficulty is experienced in obtaining the former (e.g. from babies and young children) nasopharyngeal swabs should be obtained instead.

### Posterior pharyngeal swab (throat swab)

- Hold the tongue out of the way with a tongue depressor;
- Use a sweeping motion to swab the posterior pharyngeal wall and tonsillar pillars. Have the subject say "aahh" to elevate the uvula. Avoid swabbing the soft palate and do not touch the tongue with the swab tip. (N.B. This procedure can induce the gag reflex);
- Put the swab into VTM.

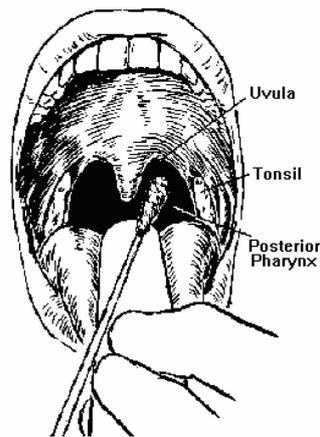


Fig 10. Taking a throat swab

### Nasopharyngeal swab

- Insert a flexible, **fine-shafted** polyester swab into the nostril and back to the nasopharynx. The swab should be slid straight into the nostril with the patient's head held slightly back (Fig 11). The swab is inserted following the base of the nostril towards the auditory pit and will need to be inserted at least 5–6 cm in adults to ensure that it reaches the posterior pharynx. (Do NOT use rigid shafted swabs for this sampling method—a flexible shafted swab is essential).



Fig 11. Taking a nasopharyngeal swab

- Leave the swab in place for a few seconds
- Withdraw slowly with a rotating motion

- Put the swab into VTM
- A second swab should be used for the other nostril and put into a second tube. This can serve as the second sample from the patient.

**Note:** Nasopharyngeal sampling is an invasive process that can cause considerable distress to the patient.

#### Nasopharyngeal aspirate

- Easier and safer than swabbing in infants and young children.
- Use an aspiration trap. Insert silicon catheter in the nostril towards the auditory pit and aspirate secretion gently by suction (Fig 12).

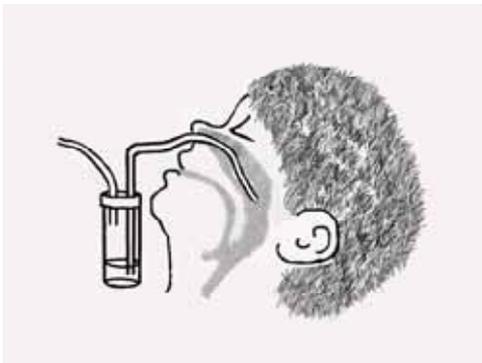


Fig 12. Nasopharyngeal aspiration

#### Anterior nasal swab

Use the same type of rigid swab as for sampling from the throat. Advance the swab tip past the vestibule (anterior nares) to the nasal mucosa (approximately 2–3 cm from the nostrils in adults) and gently rotate to collect nasal secretions from the anterior portions of the turbinate and septal mucosa (Fig 13).

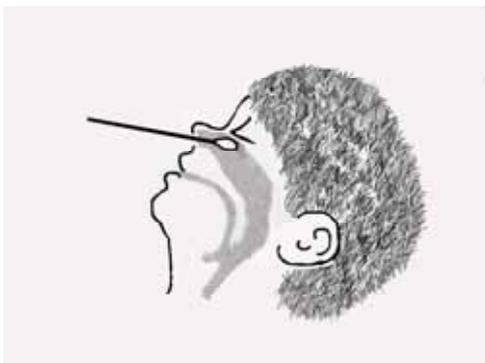


Fig 13. Anterior nasal swab

#### e) Blood specimens

- Standard precautions should always be observed when taking and handling blood specimens because the patient may be infected with a blood born pathogen (for example HIV or Hepatitis B).
- Use PPE — at least gloves (plus face-shields, masks and gowns if splashes are anticipated).

- Remove and discard PPE items **immediately** after completion of task.
- Perform hand hygiene every time gloves are removed.

The best "all round" specimen to collect is serum. Acute and convalescent sera are useful for detection of changes in antibody titre and serum can be used for detection of viral RNA. An acute-phase serum specimen should be taken soon after onset of clinical symptoms and not later than seven days after onset.

EDTA-anticoagulated plasma is also valuable for detection of viral RNA in blood and may be better than serum for this particular purpose since EDTA inactivates RNAses present in the specimen. Heparin is not suitable as an anticoagulant for this type of specimen because of potential inhibition of PCR reactions. Note that specimens for the detection of viral RNA in the blood should be collected during the first week after the development of symptoms (Fig 1).

At least 1ml of whole blood is needed to obtain a sufficient amount of serum or plasma for tests. This is the maximum that should be taken from infants. However larger specimens of 3–5 ml should be taken from older children and adults as this will allow a greater range of tests or repeat tests to be done.

A convalescent-phase serum specimen should ideally be collected 3–4 weeks after the onset of symptoms. When a patient is critically ill, a second ante-mortem specimen should be collected.

Blood should be collected either by use of a vacuum venepuncture system or syringes and needles. The specimens should be collected either in a serum separator tube (SST) or a clotting tube (for serum) and in an EDTA tube (for plasma).

#### Taking a blood specimen

1. Label the tubes, including the unique patient identification number, using an indelible marker pen. Always check to ensure that the correct tubes are used for each patient.
2. Place a tourniquet above the venepuncture site, palpate and locate the vein (Fig 14).



Fig 14. Tourniquet on, palpating the vein

3. Disinfect the venepuncture site meticulously with 70% isopropyl alcohol (an alcohol swab) or 10% polyvidone iodine by swabbing the skin concentrically from the centre of the venepuncture site outwards (Fig 15). Let the disinfectant evaporate. Do not re-palpate the vein.



Fig 15. Disinfecting the venepuncture site

4. Perform venepuncture. (Fig 16)



Fig 16. Venepuncture with a vacuum venepuncture system

5. If withdrawing blood with conventional disposable syringes, withdraw 3–5 ml of whole blood from adults and older children and 1ml from infants. Under asepsis, transfer the specimen to appropriate transport tubes. Secure caps tightly.
6. If withdrawing blood with a vacuum system (e.g. Vacutainer®), withdraw the desired amount of blood directly into each transport tube (Fig 17).



Fig 17. Drawing blood into a Vacutainer® serum-separator tube

7. Remove the tourniquet. Use a cotton swab to apply pressure to the venepuncture site until bleeding stops (Fig 18) and apply a band-aid.



Fig 18. Applying pressure to the venepuncture site

8. Never recap used sharps. Discard directly into a suitable container (a proper sharps disposal container if available or a container such as a coffee or other metal can which should be appropriately labelled before use).
9. Recheck that the tubes used for sampling have been correctly labelled.
10. After taking all the samples, complete the appropriate field data sheets or case investigation forms and the required laboratory request forms using the same identification numbers used on the tubes.

#### Separation of serum and plasma

Blood samples need to be centrifuged for at least five minutes at 1500g (3000 rpm). This requires an electric centrifuge (ideally with a swing out head rather than an angle head rotor). Hand centrifuges are not adequate for the separation of serum or plasma from red cells.

#### **Serum separator tubes (SST)**

The instructions for use of these tubes must be followed carefully if the tubes are to work properly. The tubes contain a gel (with an intermediate density between blood cells and blood plasma) and (usually) a coagulation (clot) activator (Fig 19).

- Put the blood sample into the tube and then follow the instructions for mixing the contents.
- Allow the clot to form ( follow the instructions with the tube — do not cut the clotting process short).
- Centrifuge the tube according to the relevant instructions.

When a filled SST has been properly centrifuged, the sample will separate into a top layer of serum separated by a gel barrier from the cell/clot layer and the clot activator.



Fig 19. Serum separator tube

### **Clotting tubes**

If a basic sampling tube without any additives is used the clot can be allowed to form overnight and the serum pipetted off the next day. Serum should not be left in contact with the clot for more than 12 hours, as lysis of the red cells can occur.

Whichever type of tube is used, once the serum has been separated it should be pipetted off without disturbing the gel barrier or the clot. Put the serum into a vial such as a cryovial (without VTM). Ideally vials for transport of serum should have external caps and internal O-ring seals. If there is no internal O-ring seal, ensure the cap is closed tightly and then sealed with an inert sealing film such as Parafilm®.

### **EDTA tubes**

Centrifuge the tubes at high speed (ca 10 000 g) to compact the cellular fraction and then pipette off the plasma taking care not to draw blood cells off at the same time.

### **Filter paper**

Blood or serum specimens can also be shipped in air dried form on filter paper discs or special filter paper strips (Nobuto strips). Volumes of 0.1 ml of whole blood or serum are put onto the strip which is then air dried. Strips of this sort can be stored for months at room temperature.

### **Transport**

Blood specimen bottles and tubes should be transported upright and secured in a screw cap container or in a rack in a transport box. They should have enough absorbent paper around them to soak up all the liquid in case of spillage. See Table 1 below and the notes following the table for details of storage and shipment conditions.

Samples air dried on filter paper are exempt from shipping regulations and do not have to be sent to a laboratory via a specialist courier. They can be sent by airmail. (Note that to avoid damage, they should not be sent in hermetically sealed triple packages ).

#### f) Specimens from patients who have died

- If the corpse has an endotracheal tube in place, collect a deep endotracheal aspirate. If the circumstance allows, perform tissue sampling by incision or by needle from the affected lung(s). The operator may use chest radiograph results to guide the sampling and aim for areas at the margins of interstitial infiltrates which is most likely the site of active virus replication for the best diagnostic yield. The lung tissue sample will provide excellent material for various laboratory tests including RT-PCR, virus isolation, histopathology, bacterial cultures, direct antigen detection or immunohistochemistry, and cytokine-chemokine analyses. The needle aspiration or the core needle sampling may give sufficient sample for microbiologic studies. Clean a small area on the lateral chest wall between two ribs and make a small incision between the ribs overlying the lungs with a sterile scalpel. Cut wedge sample(s) from the lung (1–2 cm<sup>3</sup> minimum) or insert a large-bore needle (e.g. 18G) into the lung tissue and aspirate or cut available material into the needle/syringe. Put the specimen into VTM. The needle sampling should be performed as soon as possible after death.
- Throat swabs, nasopharyngeal aspirates or stool samples may be collected if time, sampling materials and safety considerations permit but this should not supersede or delay the collection and sending of the deep endotracheal or lung material.

## 4. Storing specimens

Table 1 below gives the different storage and shipment conditions that can be used and which methods are recommended (based on the likelihood of obtaining a positive A(H5N1) result on laboratory analysis).

Table 1. Suitability of various storage/shipment conditions for different specimen types

Storage/shipment conditions	Swabs or other specimens in VTM for isolation of virus	Swabs or other specimens in VTM for PCR	Swabs in ethanol for PCR**	Blood serum for virus isolation	Blood serum for PCR	Blood serum for antibodies
<b>-70 °C or dry ice or Liquid N<sub>2</sub></b>	SR	SR	N/A	SR	SR	SR
<b>-20°C</b>	NR	A	N/A	NR	A	SR
<b>+4°C</b>	A*	A	A	A***	A	A
<b>Room temperature</b>	NR	A	A	NR	A*	A*
<b>Dried blood spot on filter paper</b>	N/A	N/A	N/A	N/A	A	A

SR= Strongly recommended method    A = Adequate method.    NR = Not recommended    N/A = Not applicable  
 \* For up to 7 days storage    \*\* Where refrigeration is not available    \*\*\*For up to 4 days storage

- Aliquots of specimens should be taken before the specimens are frozen.
- Repeated freezing and thawing of specimens must be avoided to prevent loss of infectivity. Note that certain types of freezer are designated "frost free" and these should not be used for specimen storage as the temperature cycling involved in keeping them free of ice accumulation can damage specimens.
- If specimens in VTM (or blood sera/plasma) for viral isolation can be taken to the laboratory within four days, they may be kept at +4 °C and frozen at -70 °C on arrival if they are to be stored. Otherwise they should be frozen at or below -70 °C until they can be transported to the laboratory. Freezing at -20 °C is not recommended because the virus does not survive well at this temperature, particularly in frost-free freezers.
- In the absence of freezers (or of VTM), ethanol-preserved swabs are a possible alternative. Storage of such specimens at +4 °C (in a standard refrigerator) is better than at room temperature.
- Blood serum samples should be frozen at -70 °C for PCR and at -20 °C or lower for antibody determination but they can be stored at +4 °C for approximately one week.

- Specimens for influenza virus isolation should not be stored or shipped in dry ice (solid carbon dioxide) unless they are sealed in glass or sealed, taped and double plastic-bagged. Carbon dioxide can rapidly inactivate influenza viruses if it gains access to the specimens. (Note: Take care not to place dry ice in an hermetically sealed containers as it could cause an explosion).

## 5. Taking aliquots of specimens

It is better to take more than one specimen when sampling from a patient than to subdivide specimens later. However this may not be possible and if specimens have to be sub-divided, the smallest volume of VTM or serum that should be stored is 0.5ml (thus a 3ml sample can be divided into six separate sub-samples). Fresh sterile or disposable pipettes should be used for each sample and these should be discarded safely (into 1/100 chlorine solution - see Annex 7).

Taking aliquots of samples should only be undertaken under appropriate levels of laboratory safety (e.g. preferably in a certified<sup>1</sup> a Class II biosafety cabinet).

Great care must be taken not to contaminate or cross contaminate specimens. This is especially so when they are intended for analysis by PCR because PCR procedures are especially vulnerable to cross contamination after amplification and uncapping of the tube.

Taking aliquots should be done before the specimen is frozen as repeated freezing and thawing of specimens can reduce the content of virus and should therefore be avoided.

## 6. Packing specimens and shipping them by air

The packing of specimens and their shipment to external laboratories by air is complex and is governed by international and national regulations and operator variations. These are summarized below. Links to other relevant documents are given in Annex 1. Readers are also referred to the WHO document *Guidance on regulations for the Transport of Infectious Substances* (Annex 10). The FAO/OIE reference laboratory requirements for shipment of animal specimens can be found on the FAO/OIE web site (see Annex 1 for the link).

International air transport of human specimens known or suspected to contain the avian influenza agent, or of specimens from avian influenza infected animals must follow the current edition of the International Air Transport Association (IATA) *Dangerous Goods Regulations (Infectious Substances Shipping Guidelines 2006)*. However be aware that specimens shipped by air need to be transported to and from the airport(s) (e.g. by road or by rail) and that different regulations may govern transport by such means — information should be obtained from the ministry of health or ministry of agriculture/chief veterinary office (for animals specimens) of the country(s) involved.

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<sup>1</sup> Biological safety cabinets should be recertified annually by an independent qualified technician.

## Shipment of specimens from humans and animals

There are two categories covering shipment of specimens by air:

- Category A covers infectious substances (included in an indicative list of specified pathogens) that are capable of causing permanent disability, life threatening or fatal disease in otherwise healthy humans or animals. Highly pathogenic avian influenza is part of the indicative list with the mention "cultures only" (more information can be found in Annexes 1 and 10). Thus only cultures of HPAI ( i.e. virus isolates) must be transported as Category A.
- Category B covers all other infectious substances that are not included in Category A.

As far as shipment of human or animal samples suspected or confirmed to contain A(H5N1) viruses is concerned, **human blood and other human samples or animal blood and other animal samples** known or suspected to contain the A(H5N1) subtype, can be transported as "diagnostic specimens" (UN 3373) and are included in **Category B**. Note that individual airlines may adopt their own policies and these may be stricter than those issued by IATA.

Shipment of specimens in Category A requires shippers who have undergone special training. For the transport of Category B infectious substances there is a requirement for clear instructions on the use of the packaging to be supplied to the user; and this is regarded as sufficient "training" for the shipping of these substances. However, if such specimens are consigned with other dangerous goods (including **liquid nitrogen** or **dry ice**), then shippers trained in the proper procedures for the transport of those substances must be used.

Detailed instructions for packing specimens for shipment in the two categories can be found in the WHO document *Guidance on regulations for the Transport of Infectious Substances* (Annex 10).

## Shipment of frozen specimens

Specimens that are to be shipped by air can be preserved either in dry ice or in liquid nitrogen. IATA regulations require that both types of shipments must be consigned by trained shippers. Details of the labelling required are given in the WHO document *Guidance on regulations for the Transport of Infectious Substances* (Annex 10).

### Note

Dry ice and liquid nitrogen both give off gases that can cause asphyxiation and should only be handled in well ventilated areas. In addition any containers in which they are shipped must be able to vent evaporated gases to the air to avoid the risk of explosions.

Specimens stored and shipped at very low temperatures should be handled with appropriate protective gauntlets and eye protection should be worn, especially when handling liquid nitrogen.

### Dry ice

Dry ice can be sent into a country in an appropriate container by the shipping company. Alternatively dry ice may sometimes be obtainable in-country from a dry ice manufacturer, a brewery or sometimes from an importer of frozen products such as ice cream. A third possibility is to make dry ice as required by use of a dry ice maker. This is only possible if cylinders of liquid CO<sub>2</sub> can be obtained in the country concerned (it can sometimes be obtained from hospitals or biological research institutes).

Specimens to be shipped in dry ice need to be shipped in specially insulated boxes capable of releasing gaseous CO<sub>2</sub>. These can be obtained from shippers or provided by WHO or FAO's Animal Health Service. Note that dry ice is very cold and can injure unprotected skin.

## Liquid nitrogen

Liquid nitrogen is a dangerous compound (it is very cold) and can cause injury to persons and severe damage to materials such as metals if spilt. For this reason shipment at liquid nitrogen temperatures is done in a device called a **dry shipper**. These are large Dewar flasks (vacuum flasks) that contain an absorbent material that will hold liquid nitrogen. The dry shipper is filled with liquid nitrogen and charged for the period specified by the manufacturer. Just before the specimens are put into the shipper, the excess nitrogen is poured out (see Annex 11 for the proper use of dry shippers).

Liquid nitrogen can sometimes be obtained at international airports — contact the engineering departments of international airlines operating from the relevant airports to see if this is possible. Liquid nitrogen can also be obtained from services that deal with artificial insemination (i.e. beef/dairy industry), and these are often strategically located in many rural areas for animal production and husbandry improvement programmes.

Dry shippers should be well marked with ownership details. Shipment procedures should always include arrangements to return the shipper to the originating laboratory.

## Shipping specimens in alcohol

For specimens of Category A, follow Packing Instruction P602, ( for details see *WHO document Guidance on regulations for the Transport of Infectious Substances*) respecting the volume limitations and adding the package orientation label if necessary (primary containers containing more than 50 ml).

For specimens in Category B, a quantity of 30 ml or less of dangerous goods included in Classes 3, 8 or 9 (i.e. alcohol) may be packed in each primary receptacle containing infectious substances. When these small quantities of dangerous goods are packed with infectious substances in accordance with Packing Instruction 650, no other requirements need to be met ( for details see *WHO document Guidance on regulations for the Transport of Infectious Substances*).

## Information about shipments of specimens

The recipients should be sent full details of the shipment in advance. Similar information should accompany the shipment. A form that can be used for this purpose is given in Annex 3<sup>2</sup>. This form includes details of the materials included and details of the laboratory and of the person sending the specimens. The WHO Avian Influenza Response team in Geneva requests that a copy should be sent to them (AIRresponse@who.int) so that they can assist with problems should they occur and so that a complete listing of specimens shipped and results obtained can be maintained for detailed surveillance purposes.

## Other relevant matters

- Passengers and crew members are specifically prohibited from transporting infectious substances or diagnostic specimens on passenger aircrafts either as or in carry-on baggage or checked baggage, or on their person.

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<sup>2</sup> This form is not part of the international transport requirements but is designed to inform relevant individuals or organizations

- Infectious substances cannot be shipped in diplomatic bags.
- Transport of specimens within national borders should comply with the procedures detailed within each country's regulations.
- Authorization for shipment of specimens out of a country may be required. Contact the appropriate authorities (usually the ministry of health or ministry of agriculture/chief veterinary office) for further information. Wildlife samples may require clearance from the ministry of the environment or of natural resources.
- Appropriate import permission **must** be obtained from the recipient country before the specimens are shipped. Contact the recipient laboratory for help (usually reference laboratories have the appropriate import licences).

## 7. References

(See Annex 1 for links to key documents)

- IATA. *IATA Dangerous Goods Regulations*. 48th Edition, 2006. International Air Transport Association.
- ICAO. *Technical Instructions for the Safe Transport of Dangerous Goods by Air*. 2005–2006 Edition. International Civil Aviation Organization.
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