SRI LANKA EXOTIC DISEASE EMERGENCY PLAN

SEDEP

2009

HIGHLY PATHOGENIC AVIAN INFLUENZA

CONTROL PROGRAMME

Part II

1 Epidemiological surveillance

1.1 Migratory Water-Birds

The role of migratory birds in spreading AI has now become an important, but poorly understood issue. Currently available epidemiological data suggest that wild migratory waterfowl are most likely to play a role in the AI cycle and could be the initial source of the AI virus into a country. Being at the southernmost tip of the South Asia, Sri Lanka is the final stop over for many migratory species. These birds cannot proceed further and therefore spend their entire winter here. They arrive in Sri Lanka during mid-August to late November, and return to their countries during March to May of the succeeding year.

Disease surveillance in migratory birds will be confined to the birds of following Orders only.
Anseriformes **:** Water birds such as ducks, swans, geese etc.
Charadriformes **:** Sea birds such as gulls, turns etc.

Locations	Thirty locations have been identified based on the information collected from the Department of Wildlife and the Ceylon Bird Club
Type of sample	Wet droppings/cloacal swabs/tracheal swabs
Collection By	Wildlife staff, VIOs, VSs, VRI staff
Period of Collection	September - November and February – April
Sample Size	Five sites identified in each location and fifteen samples collected at each site. 7-8 samples collected at each site to be pooled. Accordingly, 10 pooled samples (2 each from 5 sites) to be sent to the laboratory from each location.
Laboratory Testing	Three hundred pooled samples to be received by the VRI during each collection period for virus isolation followed by HA/HI.

1.2 Backyard poultry in the vicinity of migratory bird locations

Backyard poultry reared in the vicinity of migratory bird locations can easily come in contact with exotic birds. Contaminated water reservoirs could play a vital role in transmitting the infection to local birds.

Locations	Areas in the vicinity of Migratory Bird locations, within a radius of 3 km. Type of BirdsAll poultry such as chicken, duck, turkey, quail, guinea fowl, geese etc. reared under extensive management
Type of sample	Cloacal swabs/wet droppings
Collection By	Field VSs (co-ordinated by VICC)
Period of Collection	September – October and March – April
Sample Size	Five sites identified in each location and 15 samples collected at each site. 7-8 samples collected at each site to be pooled (site = 1-5 adjoining farms in contact)
Laboratory Testing	Three hundred pooled samples (2 each from 5 sites at 30 locations) to

	be received by the VRI during each collection period for virus isolation followed by HA/HI.

1.3 Sero-surveillance in ducks

Ducks play an important role in maintaining AI infection and transmitting it to other poultry species. Hence, the LPAI virus has an opportunity to mutate into a HPAI virus. Though duck farming is not popular in Sri Lanka, considerable numbers of ducks are found in the North and East, North-Western, Western and also in North-Central Provinces. The presence of this population together with other poultry species further increases the risk of possible emergence of HPAI viruses.

Locations	Western Province, North-Western Province, North & East Province and North-Central province
Type of sample	Cloacal swabs/wet droppings, Serum/blood
Collection By	VRI
Duration	One Year
Frequency	Once / year
Sample Size	750 Samples (15 samples at 50 sites/farms)
Laboratory Testing	ELISA

1.4 Live–bird markets

Live bird markets are considered to play an important role in spreading the infection within the country. The initial study will be confined to the Western Province where this operation is common.

Locations	Western Province. Outlets belonging to six municipal councils and other Local government institutions such as urban councils (e.g. Ja-ela) and Pradeshiya sabha's.
Type of Sample	Cage swabs (swabs of fresh fecal material from cages used to hold birds in markets) .Dirty cages or cages housing mixed species of poultry to be selected
Collection By	Municipal Council VSs and selected field VSs
Frequency	Six Months
Sample Size	15 Samples per site to be collected and subsequently, 2 pooled samples to 50, be obtained at each site. The total number of selected sites to be varying from 1-5 in each location depending on the number of birds handled at each location.
Lab Testing	One hundred pooled samples to be received every six months by the VRI for virus isolation followed by HA/HI.

1.5 Surveillance at poultry processing establishments

Surveillance is to be undertaken in all the large-scale and selected medium-scale processing operations to be included.

Locations	Fifteen processing establishments
Type of birds	Broilers
Collection By	Private VSs /plant supervisors
Frequency	Monthly collection
Sample	Cloacal swab/wet droppings
Sample Size	30 Samples/Processor/Month; 10 samples to be pooled
Lab Testing	Forty five (45) pooled samples to be tested at VRI monthly by HA/HI

1.6 Poultry breeder farms

There are only 35 poultry breeder farms in operation in the country and these farms renew their registration with the DAPH annually. These farms import their parent stock from approved exporting farms and only ten such farms currently hold approval for the same. Though disease monitoring and surveillance are carried out effectively in the breeder farms, the following activities may be introduced in relation to the AI surveillance program.

Testing of day old parent/grand-parent chicks on arrival

Sample	Wet droppings
Collected By	Animal quarantine staff
Consignment	2 consignments/importing hatchery/year
Sample Size	30 samples each from 20 consignments (10 samples pooled)
Lab Testing	Sixty pooled samples per year for HA/AI at VRI

- ***Testing of specified consignments during on-farm quarantine***

Day old birds imported from non-infected countries where vaccination against AI is practiced will be serologically screened before they reach the age of four weeks.

Consignments	2 per month
Sample	Serum/blood
Collected By	Animal quarantine staff
Sample Size	30 samples per consignment in 12 months
Lab Testing	ELISA at Animal quarantine laboratory

1.7 Pet-bird breeding establishment

There are very few Breeding establishments that are involved in breeding pet-birds for commercial purpose in Sri Lanka. However, it is important to include these establishments in the surveillance program.

Locations	Ten identified locations
Type of Birds	All types of pet birds
Sample	Cloacal swabs/wet droppings
Sample Size	Two pooled samples from each location
Collection By	VRI, VIC, field VS

Frequency	Once a year
Lab Testing	Twenty pooled samples (2 pooled sample at 10 locations) to be received at the VRI annually for virus isolation and HA / HI

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2 Clinical Examination of Affected Poultry and Completion of a Standard Form to document the Findings

(Details to be entered in Form 1 - Preliminary Inspection Report)

Preparatory measures

When undertaking a clinical examination of poultry suspected to be affected with Highly Pathogenic Avian Influenza (HPAI), it is essential that the veterinarian takes all precautions to ensure that,

- the veterinarian and all helpers get protected themselves of getting infected, by wearing appropriate PPEs and
- strict bio-containment measures are adopted to prevent the dispersal of pathogens

As the preliminary examination may have to be followed by a necropsy examination, the items of equipment and supplies to be taken for a preliminary examination are the same as those for a necropsy examination (see checklist of items to be carried for a necropsy examination).

Step 1

Before proceeding to undertake the clinical examination, Part A of Form 1 is to be completed in consultation with the owner or the Manager of the poultry operation. Particular attention is to be paid to symptoms observed by owner/Manager, mortality pattern, possible sources of infection, and vaccinations to ascertain whether appropriate procedures were adopted including the maintenance of relevant transport and storage requirements.

Step 2

Thereafter, the shed(s) in which the affected birds were housed are examined to see whether any environmental factors e.g. heat stress or water deprivation due to a faulty supply system could have contributed to the reported events. If there are live birds at the time, numbers of sick and live birds and the symptoms shown by the sick birds are recorded (Form 1 Item No: 27). If the sick birds have already been separated, observations are to be made on them.

It is likely that sick birds may not all show the same symptoms. In that case, two or three birds showing different symptoms are selected. A thorough clinical examination is then performed, starting from the head and ending in the vent region, observing the nature of all abnormalities such as abnormal discharges, color changes, edematous conditions, soiling of feathers etc. and the observations noted in Form 1 under Item No: 28.

An animal identification number is determined so that cross referencing between any samples submitted with the VS's (or VIO's) Records would be possible.

2.1 Form 1 – Preliminary Inspection Report

Part A (location, production system, history, possible sources of infection etc.)

1. Veterinary Range:
2. Name of V.S.:
3. District:
4. Province:
5. Informant a. Name:
   b. Contact details:
6. Affected farm a. Owner/manager at site:
   b. Address:
   c. Telephone:
7. Production type: Layer/Broiler/Breeder
8. Management: Intensive/Semi intensive/Free range
9. Date symptoms first observed:
10. No of birds on that day:

	Sick	Healthy	Dead
Affected Shed			
Whole Farm			

11. Mortality a. Average prior to showing symptoms (per week):
    b. Average after showing symptoms (per day):
    c. Numbers for last three days: Yesterday
    Day before Previous day
12. Average water intake: No change/Reduced by %
13. Average feed intake: No change/Reduced by %
14. Average egg production: Not applicable/No change/Reduced by %
15. Abnormal eggs: Not applicable/Absent/Present (about %)
16. Other symptoms observed:

17. Vaccination history:

Vaccine						
Date(s)						
Remarks*						

* Explain negative remarks:
18. Possible sources of infection:

19. Origin of birds/day old chicks:
20. Introduction of any birds within 21 days prior to onset of clinical sign: Yes/No
    If yes, details:

21. Exit of birds/eggs within 21 days prior to onset of clinical sign: Yes/No
    If yes, details:

22. Possibility of contact with wild birds/feral birds: Yes/No
    If yes, species:
23. Any other birds present on site (captive or free): Yes/No
    If yes, species:
    If affected: date(s) of onset mortality pattern symptoms

24. Existence of ponds, lakes or water reservoirs within/bordering premises: Yes/No
25. Location of other Farms/Poultry establishments owned by the same owner:

Part B (Veterinarian's observations and clinical examination findings)

26. Environment
    a. water supply system:
    b. ventilation/shed temperature:
    c. any other remarks:

27. Birds in normal environment (shed/open area/other)
    a. No: of birds: Healthy Sick Dead

(If all sick birds have been removed to another shed/area, make observations on them)

b. Sick birds (write Y or N in the box to indicate 'yes' or 'no' respectively on the clinical signs observed)

Remain isolated from others		Depressed		Coughing	
Cyanosed comb/wattles/shanks		Gasping		Sneezing	
Dropping of wings		Ruffling of feathers		Diarrhea	
Swelling of comb/head					

28. Other clinical findings (write Y or N in the box to indicate 'yes' or 'no' respectively)

Ocular discharge		If 'yes', give details
Nasal discharge		If 'yes', give details
Soilingof vent area		If 'yes', give details

Other significant observations:

29. Samples submitted (indicate single or pooled –minimum of 5 nos.)

Moribund Organs (single or pooled)

Trachea	Brain	Lungs	Liver	Heart	Spleen	Kidney	Contents of large intestine

30. Time and date of sample collection:

31. Farm/Animal Identification No:

Signature of Veterinary Surgeon & date

3 Standard Operating Procedures for Necropsy Examination of Poultry Suspected to be affected with Avian Influenza

3.1 Preparatory Measures

3.1.1 Safety measures and equipment

When undertaking a necropsy examination of poultry suspected to be affected with Highly Pathogenic Avian Influenza (AI), it is essential that the veterinarian takes all precautions to ensure that,

- the veterinarian and all helpers protect themselves of getting infected, by wearing appropriate PPEs and
- strict bio-containment measures are adopted to prevent the dispersal of pathogens

Ensure the list of the equipment and supplies to be carried when proceeding for a necropsy examination is in order.

3.1.2 Preliminary information

Before proceeding to undertake the necropsy examination, all the necessary information will be collected from the owner or the Manager of the poultry operation, a clinical examination of sick birds carried out, if available, and all relevant forms completed. In fact, necropsy examination and completing the Necropsy Examination Report will be the last activity to be carried out by the range veterinarian or the District Investigation Team before attending to the decontamination procedures and leaving the premises.

As the objective of a necropsy examination when HPAI is suspected is to, rule out or confirm HPAI, if HPAI, to identify the virus, and if not HPAI, to identify other cause of death, a systematic approach has to be adopted, and appropriate samples must be collected, packed and dispatched to the VRI in the prescribed manner. Collection, Packaging and Transport of Specimens for Submission to VRI is explained in a separately.

3.1.3 Selection of a place for Conducting Necropsy Examination

In consultation with the manager of the farm or the owner of the farm select a suitable location within the premises for conducting the necropsy examination, paying attention to the subsequent decontamination and movement control measures that may become necessary. For the examination the carcass is best placed in a post-mortem tray which can be conveniently decontaminated. If the carcass is too big it may be placed on a surface which can be conveniently decontaminated or disposed of together with the carcass.

3.1.4 Suitability of Carcass

It would be best to perform a necropsy examination on a poultry carcass kept in the shade within about three hours after death. Older carcasses may be used if they had been kept under refrigeration. Freezing of carcasses can lead to tissue damage from the formation and thawing of ice crystals, and make them unsuitable for histological examination. If suitable carcasses are not available, and if sick birds are available, it would be best to use a freshly slaughtered sick bird(s) for necropsy examination.

3.2 Examination process

3.2.1 External Examination of the Carcass

A thorough external examination of the carcass will then be performed, starting from the head and ending in the vent region, observing the nature of all abnormalities such as abnormal discharges, color changes, edematous conditions, soiling of feathers etc. and the observations noted in Necropsy Examination Report.

After the external examination tracheal and cloaca swabs are collected and placed in transport medium, to be dispatched to the laboratory together with other samples.

3.2.2 Necropsy Examination

As it is inconvenient to take down notes while performing an autopsy examination, the observations made by the examiner are noted down by another person to be subsequently transferred to the Necropsy Examination Report by the examiner.

Several protocols are available for avian necropsy. Any of them may be used, provided a thorough and systematic procedure is adopted, as described below.

In preparation for the necropsy examination, the carcass may be dampened with soapy water to reduce the chances of small feathers and dust particles carried on the feathers getting airborne. It is then laid in dorsal recumbency and the coxofemoral joint is disarticulated by pulling on the femur in a dorsal direction, or if necessary, by incising the skin, abductor muscles of the medial thigh and the coxofemoral joint capsule. The knees are then pressed craniolaterally to spread out each leg and place them in a horizontal position.

The upper beak is transected at the level of the oral commissure to examine the nares and the sinuses. An incision is then made on the mandible at its anterior extremity and continued on the skin to the thoracic inlet. The esophagus is opened from the oral cavity through the crop and down to level of the thoracic inlet and observations made. Thereafter, the trachea is longitudinally incised from the larynx to the thoracic inlet and observations made

A ventral midline incision is then made in the skin, starting at the thoracic inlet and encircling the vent caudally. This would enable the separation of the skin from the body by blunt dissection

revealing keel and the pectoral and abdominal muscles. An incision is then made in the abdominal muscles along the caudal border of the sternum, and is continued cranially on both sides of the sternum through the pectoral muscles taking precautions to prevent injury to the underlying viscera. When the sternum is lifted from the caudal end, the abdominal and thoracic air sacs will be visible. Any abnormalities in these air sacs are noted and appropriate samples taken before proceeding further.

The lateral incisions are then extended cranially through the thoracic musculature and using scissors or poultry shears to transect the ribs, coracoid bones and clavicles to remove the sternal plate. A midline incision is continued caudally towards the vent area through the abdominal musculature and relevant parts of the overlying abdominal muscles removed to expose the underlying viscera, without incising the cloaca and taking precautions to prevent injury to the viscera.

At this stage, before disturbing the viscera, gross observations are made of the thoracoabdominal cavity and the visible viscera. The observations relate mainly to the presence of fluids or exudates in the cavity and the appearance, size and color of the heart (pericardium), lungs, liver, proventriculus and ventriculus.

3.2.3 Examination of organs and collection of samples

Thereafter, organs or groups of organs are removed from the carcass in the following order and placed in a dissecting tray, and a detailed examination carried out on each and samples collected in appropriate transport media (see below). No material is discarded until the Necropsy Examination Report is completed to ensure that the components are available for re-examination to clear any doubts that may arise while completing the report.

1. Heart, transected at the main aorta
2. Ligametous attachments, air sacs, ureters, oviduct and attached blood vessels together with the vent area with a margin of skin around it
3. The entire gastro-intestinal tract along with the liver and spleen

Gross observations in situ are again made on the remaining organs and they are removed and subjected to detailed examination in the following order, collecting samples and transferring them to appropriate transport media.

1. Lungs together with bronchia and remaining part of trachea
2. Kidneys, gonads and adrenals
3. Brain, after carefully nipping away the dorsal cranium.

3.2.4 Reporting of observations

The observations and findings of the necropsy examination are then entered in the Necropsy Examination Report given at the bottom of this document and send to the relevant authorities either separately or along with the samples for further investigations if any.

4 Standard Operating Procedures – Collection, Packaging and Transportation of appropriate Specimens for submission to the VRI for Confirmatory Diagnosis

4.1 General Information

The successful diagnosis largely depends on the quality of the specimen and condition for transport and storage of the specimen before it is processed in the laboratory. Specimens for isolation of respiratory virus in cell cultures or embryonated chicken eggs and for the direct detection of antigen or nucleic acid should generally be taken during the first three days after onset of clinical symptoms of influenza.

Before handling any poultry suspected to be affected with Highly Pathogenic Avian Influenza (HPAI), it is essential that the veterinarian takes all precautions to ensure that,

- the veterinarian and all helpers protect themselves of getting infected, by wearing appropriate PPEs and
- strict bio-containment measures are adopted to prevent the dispersal of pathogens.

4.2 Equipment and materials needed for collection and dispatch of samples (includes PPE)

I. 1 thermic container (ice box)
II. viral transport medium
III. instruments for postmortem examination
IV. pairs of forceps
V. 2 pairs of surgical scissors
VI. 1 knife
VII. sealing tape
VIII. labels and pens
IX. 100 syringes of 2.5 ml with needles
X. Polyester or cotton fiber tipped sterile swabs
XI. 50, 1-3 ml plastic screw capped sterile tubes with viral transport media
XII. 10 leak proof containers
XIII. 2 disposable suits
XIV. pairs of disposable shoe covers
XV. 5 pairs of latex gloves
XVI. Disposable caps and face marks including goggles
XVII. 10 black waste bags
XVIII. 50 rubber bands
XIX. disinfectant solution
XX. cardboard container

4.3 Transport medium

(A) Medium 199 for transport

1. Tissue culture medium 199 containing 0.5% BSA
2. Penicillin G ($2X10^6$ units/) Streptomycin 200mg/liter, Nystatin $0.5X10^6$U/liter
3. Sterilize by filtration an distribute 1-2 ml volumes in screw capped tubes

OR

(B) Glycerol medium

1.	PBS	NaCl	8 gms
		KCl	0.2 gm
		NaH2PO4	1.15 gm
		KH2PO4	0.2 gm
		D /Water	QS to 1 liter

2. Autoclave the PBS an mix 1:1 with sterile glycerol to make 1 Liter to 1 litr PBS/Glycerol add: Penicillin G Penicillin G ($2X10^6$ units/) Streptomycin 200mg/liter, Nystatin $0.5X10^6$U/L

4.4 Samples to be collected

Samples to be collected for a Highly Pathogenic Avian Influenza outbreak investigation are as follows: The samples should be placed in isotonic phosphate buffer solution (PBS), pH 7.0-7.4 containing antibiotics.

From Live Birds:
Two tracheal and two cloacal swabs
3 – 5 ml of whole blood

From Necropsied birds:
Two tracheal and two cloacal swabs
Fresh tissue: 2 cm x 2 cm pieces of air sacs, heart, lung, trachea, liver, spleen, pancreas, proventriculus, caecum, intestine, kidney, brain, and half of any obvious lesion
Formalin fixed tissue: Same as for fresh tissue, but sample size not exceeding 0.5 cm. and in addition, duodenum, thyroid/parathyroid and skin include feather follicles.

4.5 Collection and transport of Samples

4.5.1 Preparing the sample vials and labeling of specimens

To sterile vials (plastic or glass screw capped) dispense 1-2 ml of transport media. It is preferable to store these vials at -70C until use but can be stored at 4 C (or room temperature for short period 1-2 days only).

Whenever possible the following data should be recorded.

- Type of animal sampled
- Species
- Type of sample
- Date time and location where the sample was collected

Tissue culture medium 'A' is widely used for collection and transport of clinical samples from all species of birds. The glycerol based medium 'B' provides longer term stability of samples when cooling is not immediately available and is suitable for egg inoculation but is not suited for tissue culture inoculation.

Each Sample is to be labeled (written on the wall of the container/vial and not on the lid) in such a manner that cross referencing to the VS's or VIO's records would be possible. An example would be:
VS : 1 VIO : 2
Station : Two or three letter code, e.g. Kur for Kurunegala
Suspected outbreak No: for each station : 1, 2, 3 etc. (in the order they were reported)
Farm registration or location No: for each outbreak : 1, 2, 3 etc. (in the order they were visited)
Normal, sick or dead bird : N, S or D respectively
Animal No: 1, 2, 3 etc for each N or S of each suspected outbreak (each sample will have the same animal number even when more than one sample is submitted per tissue)
Organ/tissue ID: Two letters to denote the organ or tissue e.g. Tracheal swab – TS;
Trachea – TR; Lung – LU; Liver - LI etc.
E.g. 2/Pan/1/2/D/1/IN would refer to a intestinal sample from the 1st dead animal necropsied in the 2nd location/farm visited by the VIO/Pannala in outbreak No: 1.

4.5.2 Collection of samples

Clinical specimens should be collected as describe below and added to transport medium

Tracheal swabs

Tracheal swabs are obtained from live birds by inserting the swab into the trachea (when the two cartilage structures at the back of the mouth open to allow the passage of air) and gently touching the back and sides of the trachea with its tip. In dead birds pressure may be applied on the tracheal gate with the tip of the swab to enter the trachea or they may be swabbed after the lungs and trachea have been removed from the animal. The trachea is held in a gloved hand and the swab inserted to its maximum length with vigorous swabbing of the wall. The swabs are placed in transport medium immediately after collection.

Cloacal swabs

Whenever possible, cloacal swabs should be collected from live or freshly killed birds. The cloaca of live bird is swabbed by inserting the entire tip of the swab into the cloaca and using gentle pressure on the cloacal wall in a circular motion two to four times. Any large fecal particles (>0.5 cm) are then shaken off and the swab placed in transport medium as described above.

Fecal samples

Fecal samples from the cages of poultry in live bird markets or from wild birds in the field are collected by sampling freshly deposited wet feces so that the swab is heavily coated with feces which is then placed in transport medium.

Tissue samples

Tissue samples are collected during necropsies.

Fresh tissue samples are best collected in sterile vials without transport media and later grounded in transport medium prior to inoculation of eggs or for use in tissue culture. Small samples of tissues however are suspended in transport medium.

Formalin fixed tissue samples are collected in vials containing 10% neutral buffered formalin. The samples should be no more than 0.5 cm thick and the ratio of formalin to tissue should be 10:1. These samples may be stored at room temperature and should not be frozen.

Blood Samples

In live birds, blood may be collected from the jugular vein, brachial/ulnar vein (wing vein) or medial metatarsal vein (leg vein) using a 22-25 G needle and a 5-10 ml syringe. From necropsy examinations, blood samples may be collected from the heart.
Optimally an acute phase serum specimen (3-5 ml of whole blood) should be taken soon after onset of clinical symptoms but not later than 7 days, and a convalescent phase serum should be collected 2- 4 weeks later. In serological surveillance studies, samples from slaughter houses or from free living wild birds that are released after bleeding only a single serum sample is available.

Where facilities are available, the blood may be allowed to clot and centrifuged at 2500 rpm for 15 minutes to separate the serum. The serum is pipetted off and the rest discarded. Single serum specimens cannot be used for individual diagnosis. Serum samples may be stored at 4^0C for about a week, but for longer term use that should be stored at -20^0C.

4.6 Transport and storage of specimens

Specimens should be collected and transported in a suitable transport medium with antibiotics and in ice or in liquid nitrogen. Hanks balanced salt solution cell culture medium, Phosphate buffered saline Veal infusion broth and sucrose phosphate buffer are commonly used transport media. They should be supplemented with protein such as bovine serum albumin or gelatin to a concentration of 0.5% to 1% to stabilize he viruses. The addition of antibiotics and antimycotics help prevent microbial growth.

Clinical samples for viral isolation should be placed in ice packs and transported to the laboratory promptly. If clinical specimens are transported to the laboratory within 2 days the specimens may be kept at 0^0 to 4^0 C, otherwise they should be frozen at -70^0 C until transported to the laboratory. In order to prevent loss of infectivity repeated freezing and thawing must be avoided. Sera may be stored at 4^0 C for approximately one week, but afterwards should be frozen at -20^0 C.

4.7 Packaging the Samples

All samples have to be in leak proof containers/vials, which are then wrapped in at least two plastic bags. An identification number should be written with a permanent marker on the wall of each container/vial (see 'Labeling of Samples' above). The containers with samples for viral isolation and blood samples should be placed on ice packs in a polystyrene box. The walls of the tubes containing blood samples should not come in contact with the ice packs. Formalin fixed tissue samples, also wrapped in plastic bags, may be placed in another polystyrene box without ice packs, as they can be kept at room temperature.

A data sheet with the sample numbers and other relevant data should accompany the samples. It should be placed in a plastic bag and kept just under the lid of the polystyrene box (to be seen as the box is opened).

The outside of the polystyrene boxes is thoroughly decontaminated before taking it out of the premises.

4.8 Prior notice to the laboratory

Arrangements should be made in advance with the VRI to be prepared to receive the samples on arrival. Samples should be transported to the laboratory promptly, preferably by a special messenger or vehicle to ensure their arrival as early as possible and in any event, not later than 24-36 hours after collection.

5 Standard Operating Procedure – Rapid Antigen Test for Avian Influenza

5.1 General Information

The Rapid Antigen Test is a convenient immuno-assay test designed to aid in the qualitative detection of Influenza type A virus in tracheal and cloacal samples from avian species including poultry. Rapid test kits are available commercially and it is important that a product that is sensitive to all 16 sub-types of Influenza type A virus and especially sensitive to poultry serum is used in the testing process. Also a positive result in the rapid testing is only an indication of a probable infection by Type A Influenza virus and the samples found positive should be submitted to a reference laboratory for confirmation and subtype determination. Negative result indicates that no detectable influenza Type A virus is present.

The test is therefore not conclusive, but could be used to monitor the exposure of poultry and birds to influenza virus and as such could be used in surveillance work for AI.

There are many Rapid Antigen Test kits (Antigen Capture Test Strips) and it is essential that the investigator follows the protocol specified by the manufacturer. The following is the principle and the process to be followed when using FluDETECT by Synbiotcs Corporation.

A comparison of two Rapid Antigen Test kits available commercially is given as an annex to this SOP.

5.2 Test Principle

The Rapid antigen tests are based on Rapid Immuno-Migration technology. The test strips in the kit uses two antibodies that are specific to the p56 nucleoprotein on Influenza type A virus. Anti-Influenza A antibody bound to Influenza A antigen present in the sample forms a complex which migrate along a strip and is captured on a sensitized reaction line by the second antibody. The accumulation of the complex causes the formation of a clearly visible pink/purple band. The presence of a control band, located above the reaction line ensures that the test was performed correctly.

5.3 Preparation of Materials for Testing

The commercial products available for Rapid Antigen Detection usually come with the following.

- test strips
- sufficient amount of extraction buffer
- sufficient number of swabs to go with the test strips
- test tubes to match the number of swabs
- test tube rack
- instruction on the use of the test strips

The swabs and the test tubes together with the extraction buffer is used for the sample collection and extraction of viral suspension from the sample.

5.3.1 Sample collection

The test kits are provided with sterile swabs. These swabs are used to collect samples from trachea and cloaca from the suspected birds. Also, since virus excretion is at its peak at the early stages of the infection, it is better that samples are collected earlier in the course of an infection since they will contain the highest amount of viruses.

The tracheal or oropharyngeal samples should be taken from behind the tongue and into the tracheal or oropharyngeal area and not just from the mouth.

Similarly, care should be taken to take the cloacal samples from within the cloacal area avoiding excess solid fecal matter or visible blood.

5.3.2 Sample Extraction

Sample extraction may be performed either by using the extraction buffer provided or using viral transport media.

- place about 200 µl of Extraction Buffer provided (about 8 drops) in the test tube provided
- place the swab containing the specimen in the tube and rotate the swab 5-10 times in the buffer
- press the swab against the wall of the test tube repeatedly until no more fluid comes from th swab
- discard the swab in an appropriate biohazard container

Note: i. if viral transport medium is used to extract the sample, then it is advisable to use about 500 µl of buffer in the test tube

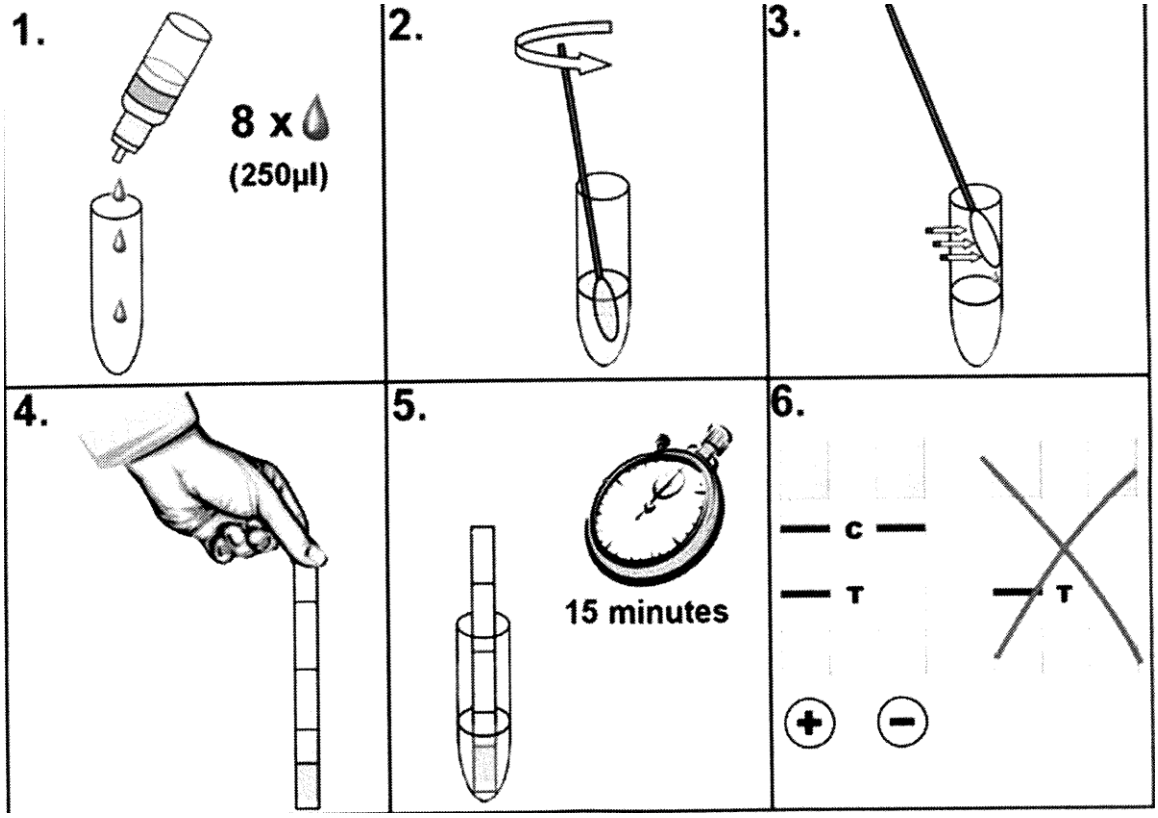
Note: ii. If samples are not tested immediately they should be stored at 4^0 C but not exceeding 48 hours. If any prolonged storage is needed then samples should be stored frozen at – 70^0 C and multiple freeze- thaw cycles should be avoided.

5.4 Test procedure

- remove the test strip from the desiccant vial for each sample to be tested. Care should be taken to handle the strip on the labeled portion. The indicator bands on the strip will appear yellow before adding the sample.
- place the test strip directly into the test tube containing the sample, so that the pink pad is submerged in the sample
- incubate the test strip in the sample for 15 minutes.

- Remove test strip from the test tube to read the results.

Figure I Sample Extraction and Test Procedure



5.5 Reading the Results

After removing the strips, observe for pink/purple bands on the strip between the two absorption pads found on the strip.

The control band appears at the upper end of the test strip, while the sample test results are read in the lower part of the test strip.

5.6 Interpretation of results

The test is valid if the control line (pink/purple band) develops in the upper part of the test strip. The absence of a Control Line indicates that the is invalid and the test must be repeated (steps 1-6)

If two pink/purple lines (Control Line and Test Line) are clearly visible on the test strip, it indicates that detectable levels of Avian Influenza virus are present in the sample. The sample must then be referred to a reference laboratory for confirmation and sub type determination.

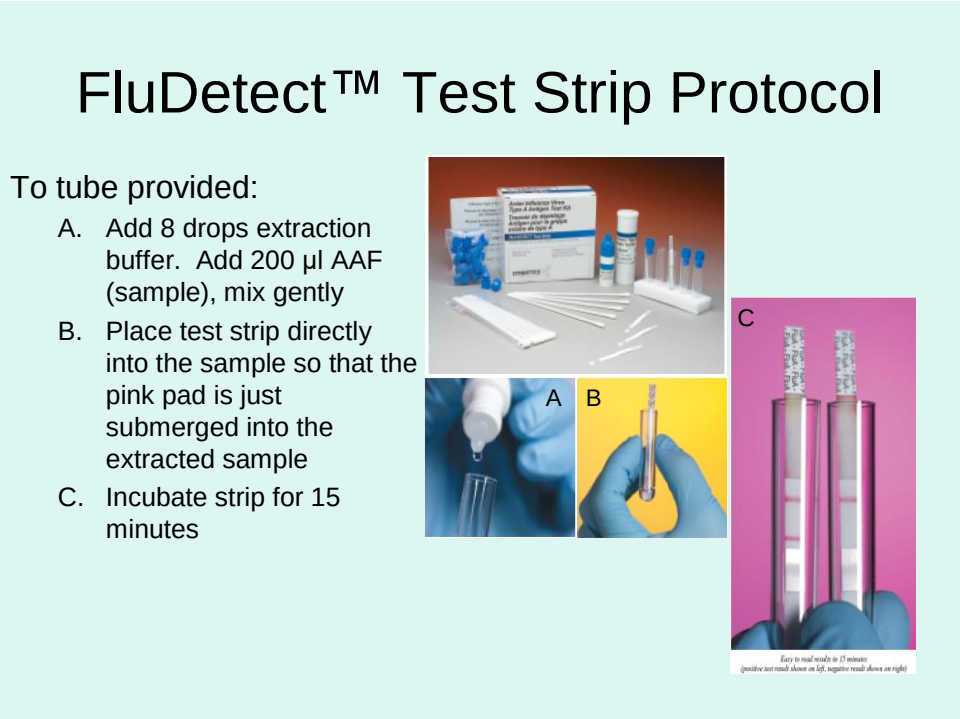
If only a single pink/purple line is visible in the upper part of the test strip, it indicates that there are no detectable Avian Influenza viruses in the sample.

Note: Very faint lines may be due to non-specific binding and should be further investigated.

5.7 General Precautions

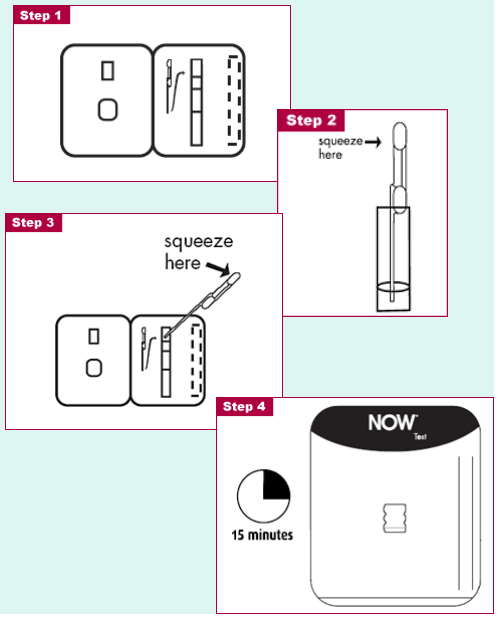
- Do not use test strips after the expiry date.
- If samples from storage are used, allow samples to come to room temperature before testing.
- The test strips are packed in a desiccant and the vial should be closed properly after use.
- The test strip should be used within 10 minutes after removing from the desiccant.
- Hold the test strips from the labeled part at the upper end and avoid touching the surface.
- The test strip should be placed vertically in the sample.
- Do not centrifuge samples before use.
- Samples containing visible blood may obscure a weak positive band.
- Cloacal swabs containing excess fecal material may interfere with the test. Allow the fecal matter to settle at the bottom of the tube before extracting the sample for testing.
- Use virus transport medium to extract samples from cloacal swabs.
- Do not through the used test strips haphazardly.

Illustrations:



Binax NOW® Influenza A&B Test Protocol

1. Remove the device from the pouch just prior to testing—lay flat
2. Draw 100 µl sample into the pipet—make sure there are no air bubbles
3. Add the entire contents (drop by drop) to the **white** pad at the top of the strip
4. Peel off the adhesive liner, seal the device and incubate for 15 minutes



Avian Influenza Antigen Detection Tests

- Advantages
  - Rapid (15-20 minutes)
  - Highly specific (nucleoprotein target)
  - No special facilities required
- Disadvantages
  - Expensive ($15-30/test)
  - Moderate sensitivity (70-80% compared to VI)
  - False positives (bacterial contamination)
  - Interference by blood (alkaline phosphatase)

Avian Influenza Virus Type A Antigen Test Kit

- **Recommended for testing sick/dead birds (high virus load)**
- FluDetect™–Conditional license for veterinary use
- Test strips contain antibody to influenza type A nucleoprotein
- FluDetect™: Tracheal, oropharyngeal, and cloacal swabs
- Binax NOW®: Tracheal and oropharyngeal swabs
- Confirmation of AI in hemagglutinating samples

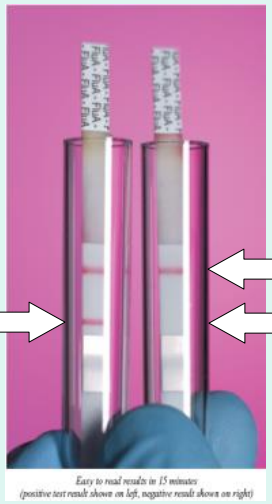
Viral Transport Media (VTM)

- Preferred Media for use with FluDetect™
  - Brain-Heart Infusion (BHI) Broth, porcine origin
  - Tris-Buffered Tryptone Broth (TBTB)
  - Nutrient Broth (NB)
  - Peptone Broth (PB)
  - Viral Transport Media
- Preferred Media for use with Binax NOW®
  - Amies Media
  - BHI broth
  - Dulbecco's Medium
  - Hank's Balanced Salt Solution (BSS)

Sucrose-Phosphate Buffer is NOT suitable for use with this test

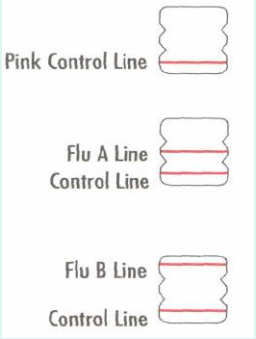
Interpretation of Results—FluDetect™

a **POSITIVE** sample is indicated by two pink/purple bands (control line and test line) that are clearly visible on the strip. The test line may be any intensity



A negative result indicates that no ***detectable*** influenza type A virus is present in the sample

Interpretation of Results—Binax NOW®

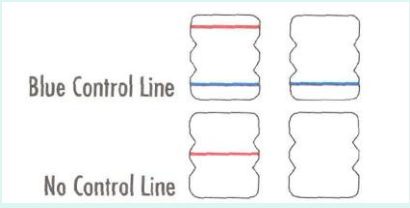


A NEGATIVE sample has a single pink/purple control line in the bottom third of the window

A sample POSITVE for INFLUENZA A has a pink/purple control line and a second pink/purple line in the MIDDLE third of the window

A sample POSITIVE for INFLUENZA B has a pink/purple control line and a second pink/purple line in the TOP third of the window

The test is INVALID if the control line remains BLUE or is not present at all, whether a sample line exists or not



6 Standard Operating Procedures – ELISA Assay

6.1 General information

Enzyme-linked immunosorbent assay, also called ELISA, enzyme immunoassay or EIA, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries.

Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, many variants of ELISA are performed, ie indirect ELISA, Sandwich ELISA etc. In HPAI we use the indirect ELISA and is a useful tool for determining serum antibody concentrations (such as with the HPAI test).

The antigen fixed plates for testing for HAPAI are commercially available as ELISA diagnostic kits including the reagents required for the washings and the pipettes for drawing and adding the samples and reagents. Different suppliers of kits have slightly differing procedures all within a standard framework of different steps used in the process. Therefore it is necessary that the guidelines and instructions as well as the results interpretation is carefully followed in performing the ELISA assay for HPAI.

6.2 The principles of the ELISA assay for HPAI.

An unknown amount of AIV antigen is affixed to the micro-titer plate, and the assay is designed to measure AIV antibody bound to AIV antigen coated plate. Serum obtained from chicken or turkeys exposed to AIV antigen contain specific anti-AIV antibodies. Serum diluted in Dilution Buffer is added to an AIV antigen coated plate. Specific AIV antibody in the serum forms an antibody-antigen complex with the AIV antigen bound to the plate. The plate is washed with a mild detergent solution (or four times with PBS) to remove any proteins or antibodies that are not specifically bound. After washing the plate and the unbound material has been removed, the next reagent (usually a peroxidase labeled conjugate - horse radish peroxidase (HRP), alkaline phosphatase (ALP) are the two widely used enzymes employed in ELISA assay) is added to each well, which comprised of a specific antibody and an enzyme covalently linked . The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step (washing the plate four times with PBS). Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, which interacts with the enzymatic portion of the conjugate to produce a colored compound, which can be detected by absorbance, fluorescence or luminescence depending on the substrate used. Regardless of the analyte, the same process steps are used in ELISA and catalysis by the enzyme leads to a change in color or fluorescence.

Thus when light of the appropriate wavelength (405-410nm in the case of ProFlock of Synbiotics) is shone upon the sample, any antigen/antibody complexes will fluoresce so that the

amount of antigen in the sample can be inferred through the magnitude of the fluorescence. The result is viewed/quantified using a spectrophotometer, spectrofluorometer, or other optical/electrochemical device. The Wash Buffers are either PBS, 0.02% Thimerosal, 0.05% Tween-20 and the secondary antibodies are Goat anti-rabbit IgG: 1:20000, anti-chicken IgY: 1:5000 or anti-goat IgG: 1:10000

6.3 General Protocol

The following is a general protocol for an indirect ELISA. The precise conditions should be optimized for a particular assay.

Solution Preparation

Coating Solution: Antigen or antibody are diluted in coating solution to immobilize them to the microplate. Commonly used coating solutions are: 50 mM sodium carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; or 10 mM PBS, pH 7.2. A protein concentration of 1-10 µg/ml is usually sufficient.
Blocking Solution: Commonly used blocking agents are: BSA, nonfat dry milk, casein, gelatin, etc. Different assay systems may require different blocking agents.
Primary/Secondary Antibody Solution: Primary/secondary antibody should be diluted in 1X blocking solution to help prevent non-specific binding. A concentration of 0.1-1.0 µg/ml is usually sufficient.
Wash Solution: Typically 0.1 M Phosphate-buffered saline or Tris-buffered saline (pH 7.4) with a detergent such as Tween 20 (0.02%-0.05% v/v).

Protocol

a) Apply Antigen
1. Add 100 µl antigen diluted in coating solution to appropriate wells.
2. Incubate 1 hour at room temperature.
3. Empty plate and tap out residual liquid.

b) Block Plate
1. Add 300 µl blocking solution to each well.
2. Incubate 5 minutes, empty plate and tap out residual liquid.

c) React Primary Antibody
1. Add 100 µl diluted primary antibody to each well.
2. Incubate 1 hour at room temperature.
3. Empty plate, tap out residual liquid.

d) **Wash Procedure**
1. Fill each well with wash solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat 3 to 5 times.

e) **Add Secondary Antibody Solution**
1. Add 100 µl diluted secondary antibody to each well.
2. Incubate 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash as in step 4.
4. Give final 5 minute soak with wash solution; tap residual liquid from plate.

f) **React Substrate**

1. Dispense 100 µl substrate into each well
2. If desired, after sufficient color development add 100 µl of the appropriate stop solution to each well.
3. Read plate with plate reader.

6.4 Commercial ELISA kits

ELISA is a test that can be used to detect either antibody (Ab) or antigen such as viral proteins and there are several test kits available commercially.

Bird Flu ELISA test kits, also known as Bird Flu EIA kits, can be divided into two subcategories according to the chemicals tested: Bird Flu antigen ELISA test kits and Bird Flu antibody ELISA test kits. Avian Influenza Antigen ELISA kits are two-step incubation assay and Avian Influenza antibody ELISA kits are one-step incubation test. Avian influenza Antigen EIA kits can be performed with both human samples and bird body fluid, while avian influenza Antibody ELISA kits are for human clinical testing only.

6.5 Sample Collection for ELISA testing

For routine monitoring, it is suggested that at least 30 or more sera per flock be randomly collected at standard time intervals (i.e. every four weeks). Proper sample collection procedures, serum harvest, and serum sample storage are needed to provide reliable results.

To achieve better specificity and to minimize possible false positive reactions, serum samples that are contaminated with bacteria or are very fatty materials should be excluded from testing.

6.6 Preparations of samples

The first step in the process is the preparation of the samples for testing. Usually the test sample is diluted 500-fold (1:500) with the sample diluent provided (1 of sample with 500 µl of sample diluent).

It is important to change pipette tips with each sample used.

Mix the sample thoroughly and dispense into a well of the coated plate.

Once the reagents are taken out from cold storage, it is important to allow the reagents to come to room temperature before they are used.

6.7 Protocols for Two Commercial Products

As stated above, there are several commercial ELISA test kits available in the market to detect Avian influenza virus antibody in chicken serum. For example the USDA has recommended two commercial ELISAs from two companies, ie IDEXX Inc., and Synbiotics.

The protocols and the reagents are different for the two products and it is **very important** that the manufacturers protocol is strictly adhered to .

The advantages and limitations and the general process and results interpretation are given below.

6.8 Advantages and limitations of ELISA tests for AIV antibody

Advantages

- Fast—90 samples can be tested in 2-3 hours
- Good sensitivity
- Many samples can be processed at once—about 90 samples per plate and several plates may be processed at once.
- Very little sample volume is needed, <10 μl in most cases
- Quantitative—can measure the amount of the Ab present, good for vaccine response monitoring, but not highly precise
- Easy to learn, simple procedures

Limitations

- May need specialized equipment: plate reader and washer
- Specificity varies by target; over all very good, but not great specificity AGID, HI, NI, and VI are needed to confirm AIV negative and AIV-infected chicken flocks
- Measures exposure and vaccine response, but not acute infection. For example, once a bird is ELISA Ab positive, the infection is probably over.
- Regulatory considerations

6.9 Materials needed

- ELISA plate and kit reagents IDEXX or Synbiotics
    - ELISA plate
    - Positive control
    - Negative control
    - Dilution buffer
    - Conjugate (secondary antibody)
    - Substrate
    - Stop Solution
    - Wash Solution (Synbiotics only)
    - Pipette and pipette tips

- Record Sheet
- Multi-channel pipette
- dilution tubes
- Wash bottle or mechanical washer
- Reader and computer
- Unknown test samples

6.10 A brief protocol for each kit

a) IDEXX brief protocol

I. Fill out the record sheet to record where each sample will be. Run samples in duplicate
II. Label dilution tubes
III. Add 1 ml of diluent to dilution tubes
IV. Add 2 µl of test serum to a dilution tube
V. Do NOT dilute controls
VI. Add negative control to plate: 0.1 ml to wells A1 and A2
VII. Add positive control to plate: 0.1 ml to wells A3 and A4
VIII. Add 100 µl of diluted test serum to the plate according to your record sheet
IX. Incubate for 30 minutes
X. Wash with distilled water
XI. Add 100 µl of conjugate to all wells of your test plate
XII. Incubate for 30 minutes
XIII. Wash with distilled water
XIV. Add 100 µl of TMB substrate to each well
XV. Incubate for 15 minutes
XVI. Add 100 µl of stop solution to each well
XVII. Read results

b) SYNBIOTICS brief protocol

I. Fill out the record sheet to record where each sample will be. Run samples in duplicate
II. Prepare wash solution
III. Label dilution tubes
IV. Add 0.3 ml diluent to each dilution tube
V. Add 6 µl of test serum to a dilution tube
VI. Dilute controls the same way as the test samples
VII. Add 50 µl of dilution buffer to each well of the ELISA plate
VIII. Add positive control: 50 µl to wells A1, A3 and H11
IX. Add negative control: 50 µl to wells A2, H10, and H12
X. Add 50 µl of diluted test serum to the plate according to your record sheet
XI. Incubate for 30 minutes
XII. Wash with wash solution
XIII. Dilute conjugate: Add 100 µl of conjugate to 10 ml of dilution buffer and mix
XIV. Add 100 µl of diluted conjugate to all wells of your test plate

XV. Incubate for 30 minutes
XVI. Wash with wash solution
XVII. Add 100 µl substrate to each well
XVIII. Incubate for 15 minutes
XIX. Dilute stop solution: Add 2.5 ml stop solution to 10 ml of distilled water
XX. Add 100 µl of diluted stop solution to each well
XXI. Read results

6.11 Reading and Interpreting the Results

- Results should be recorded by reading the optical densities of the plates in a plate reader at the correct absorbance: eg. IDEXX: 650nm, Synbiotics: 405-410nm
- Each manufacturer supplies computer software specific for their test which calculates which samples are negative and the titers of positive samples.
- The status of a sample is evaluated by the sample to positive ratio (S/P ratio):
- S/P ratio = Sample mean (mean of optical absorbance) – negative control mean positive control mean – negative control mean
- -IDEXX kit S/P ratios of greater than 0.5 are considered positive
- Synbiotics kit S/P ratios of greater than 0.299 are considered positive

Example:

Sample mean = 0.820
Negative control mean = 0.053
Positive control mean = 0.563
0.820 – 0.053 = 0.767 = 1.5 = Positive
0.563 – 0.053 = 0.510

Values are relatively quantitative: a higher value indicates more antibodies.

6.12 Valid ranges for the positive and negative controls for each kit

IDEXX

- Negative control: 0.150 or less
- The difference between the positive and negative control means must be greater than 0.075
- Example: if negative control = 0.100, the positive control must be 0.176 or greater

SYNBIOTICS

- Negative control: Less than 0.200
- Positive control: 0.250 – 0.900

IDEXX ELISA Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC-	NC-	PC+	PC+								
B												
C												
D												
E												
F												
G												
H												

Synbiotics ELISA Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC+	NC-	PC+									
B												
C												
D												
E												
F												
G												
H										NC-	PC+	NC-

7 Standard Operating Procedures- Performing Egg Inoculation Technique Allontoic Sac route

7.1 General information

Embryonic membranes of the chicken embryo provide varied substrates that allow the growth of many viruses. Because of the ability to alter their tropism and to adapt to a new host species, many viruses become capable of growing in chicken embryo tissues and may even attain a higher concentration than in the tissues of the natural host.

The avian influenza (AI) virus is usually isolated and propagated by inoculating either swab or tissue samples from infected birds into the chorioallantoic sac of embryonating chicken eggs. Chorioallantoic fluid is harvested from eggs with dead or dying embryos and is tested for the presence of hemagglutinating antigen. If hemagglutination-positive, this indicates that the isolate may be the AI virus or the New Castle Disease virus. To confirm the isolate is AI virus, the haemagglutination inhibition test is carried out using reference serum with AI antibodies.

Egg inoculation therefore is used to cultivate the suspected virus, then harvest the allantoic fluid from the chicken embryos and use the allantoic fluid verify whether the virus in the suspected material used for inoculation of embrocated eggs was AI or not.

7.2 Materials required

- 11 day old Embryonated chicken eggs (from SPF flocks or commercial flocks free for antibodies against AI and NCD)
- Egg Candler
- Sufficient quantity of 70 % alcohol (around 500 ml)
- Needle 22 gauge 1 1/2 inch
- Syringe 1ml (Tuberculin syringe)
- Egg whole punch
- Sealing wax
- 15ml tubes and rack
- 10ml pipettes
- Forceps, scissors (autoclaved)
- Sterile distilled water
- Sterile normal saline

7.3 Procedure

7.3.1 Preparation of samples

The samples are first thawed. Tissue samples are cut into pieces. Then using sterile sand, antibiotic solution and with sterile motar and pestle the tissues are ground. Normal saline is then added making a suspension. It is filtered and allowed to sediment.

7.3.2 Candling of eggs

Egg should be candled for fertility, embryonic growth, air sac placement, chorioallontoic membrane development and placement.

- Examine eggs with an egg Candler in a dark room and place eggs on egg trays with blunt end up
- Discard any eggs that are infertile, have cracks, appear to have porous shells or under-developed embryos or those with dead embryos.

7.3.3 Inoculation of eggs

- Mark the location of the air cell marked on the egg opposite the location of embryo
- Place eggs with blunt end up in egg trays and number them (3 eggs per specimen)
- Wipe the top of the eggs with 70% ethanol and punch a small hole in the shell over the air sac using a vibrating engraving device. Three eggs are usually inoculated per specimen.
- Aspirate 1 ml of processed clinical specimens into a tuberculin syringe with a 25 gauge, $^{5/8}$ inch needle
- Holding the egg up to the Candler, locate the embryo.
- Using a short stabbing motion insert the full length of the needle through the hole of the shell slightly away from the center of the egg., piercing the amniotic membrane and inoculate 100 µl of the processed sample into the allantoic cavity. Remove the syringe
- Inoculate the other two eggs in the same manner with the same syringe and needle (a total of three eggs inoculated per processed sample)
- Discard syringe into a proper safety container
- Seal the holes punched in the eggs with a drop of sealing wax
- Repeat the process for the other specimens
- Incubate these eggs at 36-37^{0}C for 5 days with control embryonated chicken egg inoculated with 0.1 ml distilled water or normal saline

7.3.4 Candling of inoculated eggs

The inoculated eggs should be candled after 24 hours of inoculation and continued thereafter every 12 hours. Any deaths of embryos at 24 hours could be considered as non-specific and due to shock or contamination. From 24 hours onwards, any dead embryos could be used for harvesting of allantoic fluid. The candling is continued up to 5-7 days and dead embryos are separated and allantoic fluid harvested for testing.

7.3.5 Harvesting of virus from inoculated chicken eggs

The egg inoculation using the processed samples from suspected material was carried out for virus culture. Therefore the important end product of the egg inoculation process is the amniotic fluid and this is harvested as follows.

- Chill the eggs at +4^{0}C overnight or for 4 hours before harvesting.

- Label one plastic tube (15 ml) for each egg with the specimen number

- Clean the top of each egg with 70% alcohol

- Break the shell over the air sac with sterile forceps and push aside the allantoic membrane. Using a 10 ml pipette aspirate the allantoic fluid and place in labeled plastic tube. Then using a syringe and needle pierce the amniotic sac and remove as much allantoic fluid as possible.

- Place the harvested material in a separate tube but because of the small amount of amniotic fluid obtained from each egg it is usually necessary to combine the amniotic fluid from all the three eggs inoculated per specimen.

8 Standard Operating Procedure – Haemagglutination Test

8.1 General information

Many viruses attach to molecules present on the surface of red blood cells. A consequence of this is that - at certain concentrations - a viral suspension may bind together (agglutinate) the red blood cells thus preventing them from settling out of suspension. The haemagglutination test can therefore be used check for the presence of HAPI viruses.

By serially diluting a virus suspension into an assay tray (a series of wells of uniform volume) and adding a standard amount of blood cells, an estimation of the number of virus particles present in the samples can be made. The haemagglutination assay is cheaper and quicker method (taking only just 30 minutes) for confirming the presence of HPAI virus in a suspension of suspected sample.

Haemagglutination (HA) and Haemagglutination inhibition (HA) tests are used to confirm, Avian Influenza virus in a suspected sample of (eg. Allentoic fluid of infected embryonated eggs) or antibodies for Avian Influenza in serum samples. (for serosurveillace work)

8.2 Principle

As stated above, many viruses including the AI virus will attach to the molecules present on the surface of red blood cells. Therefore if Haemagglutination is detected the sample could be suspected to contain AI virus. To confirm that the sample contain AI virus, assay is modified by adding known antiserum containing antibodies against AI where by the AI virus in the suspected serum is neutralized by this antiserum and therefore prevent the haemagglutination process. By using a standard amount of virus, a standard amount of blood cells and serially diluting the antiserum, one can identify the minimum inhibitory concentration of the antiserum (the greatest dilution which inhibits hemagglutination).

8.3 Preparaions for HA and HI testing

8.3.1 Equipment and materials required

- Single channel micropipettes - calibrated for 25 and 50 µl volumes
- Multi-channel micropipettes - calibrated for 25 and 50 µl volumes
- Any suitable, round-bottom, 96 well microliter plate.(“V” well microliter plates may also be used.)
- Hematocrit centrifuge
- Plate shaker

- +4°C storage
- Plate covers
- HI Plate Reading Platform
- Bench top centrifuge

8.3.2 Required reagents and chemicals

Alsever's Solution

- Citric acid 0.55 g
- Glucose 20.50 g
- NaCl 4.20 g
- Sodium Citrate 8.00 g
- Make up to 1 litre with distilled water and autoclave for 15 minutes at 110°C.

Phosphate Buffered Saline (PBS)

- NaC1 8.0 g
- KC1 0.2 g
- Na2HPO4 1.15 g
- KH2PO4 0.2 g
- Make up to 1 liter with distilled water and adjust pH to 7.3

8.4 Performing Hemagglutination Inhibition Assay for detection of antibodies against Avian Influenza (serosurveillance work)

8.4.1 The biologicals needed for the process

Chicken Red Blood Cells

Whole chicken blood is collected from an SPF chicken and added to sterile Alsever's solution. The cells may be stored at 4°C for up to one month but should be discarded if the Alsever's solution becomes too red, indicating that the cells have haemolysed.

Avian Influenza Virus Antigens

Avian Influenza monospecific antigens should be purchased from reference laboratories and should be stored at -80oC for longer term storage and at 4oC for short term storage. Antigens should be titrated before each use.

Positive Serum

Monospecific reference antisera against each H type should be purchased from reference Laboratories.

Negative Serum

Normal chicken serum should be collected from known antibody free chickens. The clean egg flock should be tested for presence of antibodies against Avian Influenza, Ranikhet by ELISA prior to commencing HA,HI.

8.5 Preparation of samples

Test samples are usually received as avian sera, however whole clotted blood may also be received. Clotted blood is centrifuged at 1000g for 15 minutes and serum decanted into clean containers. Samples in poor condition e.g. contaminated or haemolysed samples may be tested in this assay, however best results are obtained with fresh samples.

8.6 6. Antigen titration

- Add 25 l of PBS to all wells in two rows of a microtitre plate.
- Put 25 l of virus into the first well of each row.
- Make serial, two-fold dilutions of the virus from column 1 to column 12 and discard 25 l from the last well.
- Add a further 25 l of PBS to all wells.
- Add 50 l of 0.5% chicken red blood cells to all wells, cover the plate, shake for 10 to 15 seconds and then incubate at 4°C for 45 to 60 minutes.
- Examine the plate for haemagglutination. The endpoint is the highest virus dilution at which there is complete agglutination. At this dilution the virus is said to contain 1 haemagglutinating unit (HAU) per 25 l.
- Sera are tested against 4 HAU of virus. The working dilution of the virus is calculated by dividing the endpoint dilution by 4. If the endpoint of the titration is at a dilution of 1:256 the working dilution of this antigen preparation is 1:64 (ie. 256/4 = 64)
- 7. Haemagglutination inhibition assay procedure
- Sera may be screened at 1:4 dilutions or titrated. Sera may be tested singly or in duplicate.

- When screening sera at a single dilution add 25 l of a 1:4 dilution of each serum to two well of a microtitre plate. When titrating add 25 l of PBS to all wells of the microtitre plate, 25 l of serum to the first well and make 2-fold dilution of each serum from 1:2 to the desired final dilution. It is important to include a serum control for each serum to check for the presence of non-specific agglutinins in the serum.

- A titration of the positive and negative control sera, a back titration of the working dilution of virus and a cell control are included in each test. The negative control serum titration is begun at the same dilution as the test sera while the positive serum titration is begun at a 1:10 dilution. The back titration of virus is prepared by adding 25 l of the working dilution to the first two wells of four rows of a microliter plate. 25 l of PBS is then added to all wells in these four rows.

- Add 25 l of the working dilution of virus to each well containing serum, except for the serum control well. Cover the plates, shake for 10 to 15 seconds and incubate at room temperature for 1 hour.

- At the end of this incubation make a back titration of the working dilution of antigen to confirm that it contains 4 HAU of antigen. If large numbers of sera are being tested it is advisable to do the back titration of the working dilution of antigen before adding the antigen to the test sera. If the working dilution does not contain 4 HAU of antigen adjust the concentration accordingly.

- Calculate the volume of 0.5% chicken red blood cells needed, allowing 5.5 ml per plate.

- Add 50 µl of 0.5% chicken red blood cells to all wells, cover the plates, shake for 10 to 15 seconds and then incubate at 4°C for 45 to 60 minutes.

- Examine the plates for the presence or absence of haemagglutination.

8.7 Findings/results

- Examine the cell control wells to check for problems with the cells used in the test. Check the back titration to determine if the amount of virus used in the test was within acceptable limits.

- Check if the positive serum has an acceptable titre and that the negative control serum shows no significant inhibition. If all controls are acceptable the test sera are checked.

- The serum controls of each test serum are checked for non-specific agglutination of the cells. The wells to which virus were added were then checked for haemagglutination inhibition. If a sample is positive in the screening test, it may be titrated and re-tested to confirm the result or treated with RDE and re-tested.

8.8 Interpretation of results

- If the serum control wells show no non-specific agglutination and the test wells show no inhibition the serum is considered negative for antibodies. Sera showing inhibition at dilutions of 1:16 or greater against 4 HAU of antigen are considered positive for antibody. Chicken sera rarely give non-specific positive reactions in this test while sera from other species may cause agglutination of chicken red blood cells.

- Sera with serum control wells showing non-specific agglutination are adsorbed with chicken red blood cells and re-tested by HI.

- Sera showing haemagglutination inhibition may be re-tested by HI or treated with receptor destroying enzyme (RDE) if necessary before re-testing.

8.9 Quality control

The antigen titer is acceptable if the back titration shows that between 2 and 8 haemagglutinating units of antigen were used in the test. The titer of the positive control serum should be within one 2-fold dilution of the expected titer. The titer of the negative control serum should be 8 or less.

Titres of test sera duplicates should not differ by more than 4 fold. If paired test results differ by more than this amount the result may be accepted if there is no ambiguity in the interpretation.

9 Standard Operating Procedure –Haemagglutination (HA) and Haemagglutination Inhibition (HAI) for confirmation of the Avian Influenza virus

9.1 General

This test is mainly used in the diagnosis of Avian Influenza with virus isolation using egg inoculation method. (refer egg inoculation technique). Allentoic fluids of dead embryos are subject to haemagglutination and haemagglutination inhibition for confirmation of the AI virus.

9.2 Principle

- A loopful of allentoic fluid is mixed with a similar volume of chicken RBC suspension on a porcelain tile for few seconds and observed for the presence of any agglutination. If positive reaction is detected indicated by agglutination of RBCs, then allentoic fluid is suspected to contain an agent which has the characteristic of haemagglutination such as Avian influenza or the Ranikhet virus.

- To differentiate AI from Raniket, HA is performed using V bottom micro titer plates following the procedure mentioned above by which 4 HA titer could be calculated. Then haemagglutination inhibition is performed using 4HA antigen (ie virus) concentration with positive antiserum for avian influenza A H5.

- If haemagglutination inhibition is observed indicated by button formation of RBCS, the virus can be confirmed as corresponding to the antigen for the H5 known antiserum and hence the virus can be confirmed as avian influenza A, H5. Accordingly any of the AI types can be confirmed if the known positive sera are available.

- Additional advantage of this test is that the titer of the virus (virus concentration) suspension could also be estimated.

9.2.1 Antigen titration

- Add 25l of PBS to all wells in two rows of a microliter plate.

- Put 25 l of virus (allentoic fluid) into the first well of each row.

- Make serial, two-fold dilutions of the virus from column 1 to column 12 and discard 25 l from the last well.

- Add a further 25 l of PBS to all wells.

- Add 50 l of 0.5% chicken red blood cells to all wells, cover the plate, shake for 10 to 15 seconds and then incubate at 4°C for 45 to 60 minutes.

- Examine the plate for haemagglutination. The endpoint is the highest virus dilution at which there is complete agglutination. At this dilution the virus is said to contain 1 haemagglutinating unit (HAU) per 25 l.

- Sera are tested against 4 HAU of virus. The working dilution of the virus is calculated by dividing the endpoint dilution by 4. If the endpoint of the titration is at a dilution of 1:256 the working dilution of this antigen preparation is 1:64 (ie. 256/4 = 64)

9.2.2 Haemagglutination inhibition assay procedure with Avian Influenza A H5 positive serum

- Dispense 25µl of PBS into each well of "V" bottom microliter plate.
- Place 25µl of 4HA allentoic fluid into the first wells of two rows of the plate.
- Place 25µl of 4HA Avian Influenza A H5 inactivated virus (positive control) into first wells of another two rows of the microliter plate.
- Make two fold dilutions of 25µl volumes of the allentoic fluid and the AI-A H5 inactivated virus. Discard the 25µl of the last well of both rows.
- Add 25µl of Avian Influenza A H5 1:10 diluted antiserum into two wells (Allentoic fluid and Virus) and leave for 30 mts at room temperature or 60 mts at 4 C .
- Into another row with allentioic fluid dilution add 25µl water in place of serum and leave for 30 mts. at room temperature or 60 mts at 4C.
- Into the other row with AI –A H5 dilution add 25µl of water in place of serum leave for 30 mts. at room temperature or 60 mts at 4C.
- Add 25µl of 0.5% V/V chicken RBC to each well and after gentle mixing allow RBCs to settle for 40min. at room temperature or 60 min. at 4 C.
- In positive reactions the RBCS will settle to bottom of the wells. The HA titer is the highest dilution of antigen causing complete inhibition. Agglutination is assessed by tilting the microliter plate.

9.2.3 Interpretation of results

In positive reactions the RBCS will settle to bottom of the wells. The HA titer is the highest dilution of antigen causing complete inhibition. Agglutination is assessed by tilting the microtitre plate.

Avian influenza A H5 inactivated antigens (concentration 4HA) should give complete haemaggluination inhibition with AI - A H5 positive antiserum.

The wells using water as he negative control for serum should result in complete haemagglutination.

10 Standard Operating Procedures - Performing Real Time PCR Test and Interpreting the Results

10.1 General information

The real time PCR uses the principles of reverse transcriptase polymerase chain reaction (RT-PCR) and its key feature is that the amplified DNA is quantified as it accumulates in the PCR process in real time after each amplification cycle. In normal PCR methods the amount of the targeted DNA material produced in the PCR is measured as separately by electrophorasing the PCR product using a fluorescent dye.

In the real time PCR a fluorescent dye, such as Sybr Green, or fluorophore-containing DNA probes, such as TaqMan are also added to the reaction system where DNA multiplication is carried out so that the measurement of the amount of amplified product could be measured in real time. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.

Although the real time PCR uses the same principles of the RT-PCR , the reagents and probes used in the real time PCR are still covered by patent licenses. In performing the real time PCR it is therefore important to follow the protocol of the supplier of the PCR kits for detection of avian influenza virus. (the kits contain (a) Assay Beads for avian influenza virus and sub types together with the enzymes, the primers and probes (b) reconstitution buffers for 96 reactions, (c) positive and negative controls) In normal RT- PCR the reagents and dyes are prepared in the laboratory, which is a much cheaper process, for performing the PCR.

Since the real time PCR is developed on the principles of the RT-PCR, the procedures of the latter process is described first so that the logic of performing the steps in the real time PCR is appreciated.

10.2 Principles of RT-PCR

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA thus generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.

Since the genome of Avian influenza virus is a single stranded RNA, the PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA. Reverse Transcription PCR is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium Thermus aquaticus. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, using single-stranded DNA as template and DNA oligonucleotides (also called DNA primers) required for initiation of DNA synthesis. The process of PCR use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the strands (at high temperatures) in a DNA double helix (DNA melting) used as template during DNA synthesis (at lower temperatures) by the DNA polymerase to selectively amplify the target DNA.

The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. Primer sequence therefore is the most critical parameter in the process. Primer pairs used in PCR reaction are designed based on known sequences. In the method used in testing for AI virus, the primer pairs used in the reaction are the primer pair specific to haemagglutinins (HA) gene of currently circulating influenza viruses. Primers HA 1140 and HA reverse are influenza A specific primers which are designed to amplify the HA genes of H5 H6 an H9 subtypes. The subtype of the virus can be determined by sequencing the PCR product and comparing the sequences with sequences maintained in data bases.

The PCR products are then measured based on the wave lengths emitted by adding a fluorescent dye to get attached to the product.

10.3 The RT-PCR process

The different stages of the TR-PCR process include the following:

1. RNA extraction from the suspected material
2. Converting RNA to cDNA – reverse transcription.
3. A polymerase chain reaction (PCR) to amplify the viral target
4. Electrophoresis of the PCR products
5. Interpretation of results

10.4 Performing the RT-PCR

Step 1. Preparing RNA samples for RNA extraction

The first step in the process is the RNA extraction from viral isolates. It is important to use high quality viral RNA that is free of materials that can inhibit amplification in the performance of the assays.

The Materials required for RNA isolation are;

- Qiagen RNA assay min kit catalogue No. 74104
- NA easy mini spin column
- Collection tube 1.5 ml
- Collection tube 2ml
- Buffer RLT
- Buffer RW1
- Buffer RPE
- RNAse free water
- Beta Mercaptoethanol
- 70% Alcohol
- Sterie 1.5 micro-centrifuge tubes
- 10, 20 .100 µl adjustable pipettes and tips
- Micro-centrifuge adjustable to 13K
- Vortex

Procedure for RNA extraction

This step should be carried out in a class II biosafety cabinet preferably in a separate room. Personnel protective equipment (PPE) should also be used when the extraction process is on.

Using the QIAGEN RNA extraction kit, mix together the following materials

350 µl lysis buffer RLT
3.5 µl of Beta Mercaptoethanol
200 µl of viral suspension
550 µl 70%alcohol

In addition to suspected viral suspension positive antigens such as H5 and H7 should be used for RNA extraction simultaneously.

Then do the following;

I. Pipette mixture onto the column (Pink) and centrifuge for 15 seconds at 10000 rpm.
II. Remove the column and empty the liquid from the bottom collection tube replace the column in the collection tube

III. Add 700 µl wash buffer RW 1 to the column and centrifuge for 15 seconds at 10,000 rpm.
IV. Transfer the column to collection tube and add 500 µl wash buffer RPE to the column.
V. Centrifuge for 5 seconds at 10000 rpm.
VI. Remove the column an empty the liquid from the collection tube.
VII. Add 500 µl wash buffer RPE to the column and centrifuge for 2 mts. At 12,000 rpm.
VIII. Transfer column to 1.5 ml micro-centrifuge tube and pipette 20 µl RNAse free water directly on to the filter of the column .Wait for one minute.
IX. Centrifuge at 10,000 rpm one minute.
X. Centrifuge for 1 minute at 10,000 rpm. Sample is now ready for cDNA Synthesis. RNA can be stored at -200 C

Step 2. Synthesis of cDNA

The next step is to synthesize a copy DNA from the RNA extracted in step 1.
(From the RNA of suspected sample an positives)

The materials required are;

- Sterile 0.5 ml micro-centrifuge tubes
- µl, 20 µl, 100 µl adjustable pipettes and tips
- Micro-centrifuge to 13K
- Vortex
- Water bath or thermocycler for 90C and 420 C incubation
- Viral RNA (already extracted)
- 10mM dNTP mix
- Ultra-pure water
- Primer Uni-12AGCAAAAGCAGG (1 µg per µl)
- AMV Reverse Transcriptase (25units per µl)
- 5 reverse transcriptase buffer
- RNAse inhibitor

Procedure

I. All reactions should be carried on ice with a negative control also performed together using just water only.
II. Label 1 0.5ml micro-centrifuge tube for each RNA used
III. Place a negative control using just water
IV. To 4 µl of RNA and negative control (water) add 0.5 µl of Primer uni-12.
V. Incubate for 5 minutes at 72^0 C.
VI. Make the cocktail of the following

1.5 µl of water
2.00 µl of reverse transcriptase buffer
0.5 µl of 10mM dNTP mix
0.5 µl of RNAse inhibitor
1 µl of Reverse transcriptase

VII. d 5.5 µl of the cocktail to each tube
VIII. Incubate RNA primer mix with the cocktail, total volume 10 µl at 420 C for hour.
IX. Stop the reverse transcription reaction by incubating for 5 minutes at 950C.

Step 3 PCR Reaction – amplification of target DNA

With the cDNA that was produced in step 2 the PCR reaction is performed to amplify the viral target.

Materials required

A. Amplification of different sub types with one primer pair
Forward and reverse primer at 1 µg/ µl
HA 1144 and HA reverse

B. Amplification of different sub types with subtype specific primer pairs
Forward and reverse 1 µg/ µl
H5- HA – 1144 H5 – 1735 R
H6- HA 1144, H6 1480R
H9 – H9-1 an H9- 808 R

C. Positive control
To ensure that the RT PCR is working a reaction for the amplification of the M gene can be in parallel for the PCR reaction
Forward primer M-WSN -8
Reverse primer M- 1023 –R

D. Sterile 0,5 ml micro-centrifuge tubes
10, 20 , 100 µl adjustable pipettes and tips
Micro-centrifuge adjustable to 13 K
Thermal cycler for Polymerase Chain Reaction
Taq DNA Polymerase(5Units/ µl)
10X PCR buffer 500mM KCl, 100mM tris HCl, 1.0Triton, pH 9.0)
25 mM MgCl2
10 mM dNTP mix
Water
Sterile mineral oil
Ice

Procedure

1. From the cDNA synthesized take only 1.5 µl for each PCR reaction. This is added to 48.5 µl of the master mix.

   Suppose there are two suspected samples (S1 and S2), H5 and H7 from which the cDNA has been prepared. The following procedure for adding master mix and the primers should be followed very carefully.

   Sample 1(cDNA) +Primers HA 1144 and HA reverse +master mix

Sample 1(cDNA) +Primers H5 forward and reverse + master mix
Sample 1 (cDNA) + Primers H7 and reverse + master mix
Sample 1 (cDNA) +Primers M-WSN 8 an reverse +master mix
Sample 2 also should be prepared as above using the same primers
H5 (cDNA) + Primers H5 and reverse +master mix
H5 (cDNA) +Primers M-WSN-8 an reverse +Master mix
H5 (cDNA) +Primers HA 1144 and reverse +master mix
H7 (cDNA) + Primers for H7 and reverse +master mix
H7 (cDNA) + Primers M-WSN- 8 and reverse +master mix
H7 (cDNA) + Primers HA 1144 and reverse + master mix

2. Make a PCR reaction Master mix as follows

   5 µl PCR buffer
   38.65 µl water
   1 µl mMdNTP mix
   3 µl 25 mMMgCi2
   0.25 µl Taq DNA polymerase
   0.3 µl forward primer (0.1microgram/micrilitr)
   0.3 µl reverse primer (0.1 microgram/µl)
   Spin briefly to collect
   Add a drop of mineral oil to the top of the tube

3. Place tube in thermocycler (PCR machine). Programme for amplification
   94^0 C for 2 minute
   94^0 C for 1minute (denature)
   50^0 C for 1 minute (anneal)
   72^0 C for 3 minutes (extend)e Repeat from step 2, 30 times
   72^0 C for 8 minutes
   $4C^0$ C until usage

Step 4. Agarose gel electrophoresis of the PCR products

Procedure

1. All reactions should be carried on ice
2. Label 1 0.5ml micro-centrifuge tube for each RNA used
3. The negative control is water
4. To 4 µl of RNA and negative control (just water, blank) add 0.5 µl of Primer uni-12.
5. Incubate for 5 minutes at 72 c.
6. Make the cocktail of the following
   a. 1.5 µl of water
   b. 2.00 µl of reverse transcriptase buffer
   c. 0.5 µl of 10mM dNTP mix
   d. 0.5 µl of RNAse inhibitor
   e. 1 µl of Reverse transcriptase
   f. 5.5 µl of the cocktail to each tube

7. Incubate RNA primer mix with the cocktail, total volume 10 µl at 420 C for hour.
8. Stop the reverse transcription reaction by incubating for 5 minutes at 95 0 C.

Step 5, Agarose gel electrophoresis of the PCR products

Materials required

- Agarose Gel casting tray and electrophoresis chamber
- Power supply and electrode leads
- Hand held UV light (302nm) or UV light box
- Camera and film
- µl piptte and tips
- 1% Agarose Gel in 1X TBE
- 1X TBE buffer
- Ethidium Bromide (10 microgram per µl)
- Gel loading buffer
- Molecular weight marker (Low DNA mass ladder)
- Microtubes containing the PCR product from the overnight reactions

Procedure

1. Remove tape from Gel frame and place the gel into the electrophoresis chamber, cover with gel with 1X TBE

2. Label the 0.5 µl tubes separately

3. Remove 4µl of the PCR product from each tube to a corresponding 0.5 ml micro-centrifuge tube. (remove PCR product from underneath oil) mix with 3 µl gel loading buffer.

4. Load 4 µl molecular weight marker to the first well of the 1% agarose gel.

5. Pipette 7 µl of PCR reaction mix positive control negative control to wells of the gels separately.

6. Close lid of the chamber and attach the electrodes. Run the gel at 120 V for 30 t0 40 minutes.

7. Visualize presence of marker and PCR product bands with a hand held UV light. It is desirable to have an ultraviolet light source emitting light at 302 NM wave length.

8. Document gel with a photograph

9. Compare the size of the PCR- fragments with the marker.

10.4.1 Interpretation of results

In experiment A the expected size of the PCR product for H5, H6,H9 are 600bp. To determine the sub type the PCR fragments have to be sequenced and compared to sequences deposited in data bases. In experiment B specific amplification of each subtype is expected. For example A 591 bp fragment with the primer HA 1144 and H5 1735 R and no amplification with the H6 and H9 specific primers allows the conclusion that the analyzed virus isolate is an influenza virus with an H5 sub type. This result can be confirmed by sequencing the PCR product. The expected sizes of H6 gene amplification is 336 bp and for H9 genes 808 bp . In the positive control (C) for the M segment should yield 1015 bp PCR product. In all of the negative controls no PCR-fragment is expected.

10.5 Performing the real time RT-PCR

The real time RT-PCR uses the same principles of the RT-PCR described above. In the case of the procedure described above, the primers, some reagents and buffers and can be prepared in the laboratory by following the method described. However, for real time PCR commercially prepared readymade kits are available but are very expensive. Once the RNA is extracted from the viral isolates, the material is then mixed with the reagents supplied with the kits and placed in the PCR machine together with the primers and probes for PCR. Real time quantitative PCR uses fluorescent reporter probes and are very accurate, reliable but very expensive.

It uses a sequence-specific RNA or DNA-based probe to quantify only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and allows quantification even in the presence of some non-specific DNA amplification. This potentially allows for multiplexing - assaying for several genes in the same reaction by using specific probes with different-colored labels, provided that all genes are amplified with similar efficiency.

It is commonly carried out with an RNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

Reaction components

- DNA polymerase
- Buffers for DNA polymerase
- dNTPs
- Reverse Transcriptase
- Buffer for reverse transcriptase
- RNase inhibitors
- RNAse free water
- Primer for M gene

- Primer for H5 sub type
- Probes for PCR
- RNA isolated from epidemiological samples
- RNA positive controls (Extracted from H5)
- Negative control (ultra-pure water)

Using the above components a protocol for real time PCR can be developed in the laboratory. The most essential thing is optimization of the reagents. Series of tests with varying concentrations of each component is essential in this exercise.

Summary of the real time PCR procedure

1. RNA is reverse transcribe to cDNA
2. PCR cycles are performed.
    a. Primer anneal, or bind to the cDNA target sequence
    b. The DNA polymerase creates a new cDNA strand by
    c. Extending the primers with nucleotides
    d. c. If the NA target of interest is present in the amplification product
    e. the probe hybridizes to the sequence.
    f. The 5' to 3' nucleolytic activity of the rTh DNA polymerase cleaves the hybridized probe between the reporter dye and the quencher dye . The reporter dye fragments are displaced from the target, resulting in an increase in fluorescence.
    g. This process occurs in every cycle an oes not interfere with the exponential accumulation of product.
    h. The polymeraization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR.
    i. Accumulation of PCR product is detected directly by monitoring the increase in fluorescence of the reporter dye.

Note; the increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Nonspecific amplification is not detected in the absence of a pro-binding site.

Probes- The probe contains a fluorescence reporter dye at the 5' end of the probe and quencher ye at the 3' end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye suppresses the reporter fluorescence. Probe cleavage during the PCR reaction spatially separates the reporter ye from the quencher moiety and allows detection of the reporter dye fluorescence.

Example

Real time PCR protocol(Applied Biosystems Taqman Influenza A H5 detection kit version 1.0)

1. 1.Thaw RNA andreagents on ice
2. Prepare master mix. (for one sample)
    a. 5X EZ buffer 5 µl
    b. Mn(OAc) 2 µl
    c. dATP 10mM 0.75 µl

d. dCTP 10mM 0.75 µl
e. dGTP (10mM) 0.75 µl
f. dUTP (10mM) 0.75µl
g. rTth polymerase(25u/ 1 µl) 1µl
h. UNG(1u/ µl) 0.25 µl
i. 10X assay mix 2.5 µl
j. dd H2O 9.25 µl
k. Total 25 µl

3. Vortex, short Centrifuge
4. Aliquot master mix(transfer to 96 well plate)
5. Add RNA
6. Centrifuge 5 sec.
7. Run real time Detection
8. Reaction 72.C 1 minute
    a. 50 C 2 min.

    b. 60 C 30 min.
    c. 95 C 5 min.
    d. 94 C 20 seconds and 58 C 1 min. (40 cycles)-amplification

Fluorescence is detected and measured in the real-time PCR thermocycler (PCR machine), and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (CT) in each reaction.

Preventing contamination

PCR assays require special laboratory practices to avoid false positive amplifications.

- Wear a clean lab coat. Wear separate coat and gloves for sample preparation for PCR amplification, handling of amplified PCR products.

- Maintain separate areas; dedicate equipment and supplies for
    - sample preparation
    - PCR setup
    - PCR amplification
    - Post PCR analysis

- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully. Do not splay or spray PCR samples.
- Keep reactions and components sealed as much as possible.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10 % chlorine bleach solution.
- To avoid false positives due to amplified material in the work area, do not open tubes after amplification.

10.6 Preventing biohazards

Samples from suspected HPAI cases have the potential to transmit the infection to humans. It is therefore very important to wear appropriate protective clothing including protective eye wear, face shield, lab coat and gloves.

11 Standard Operating Procedures - Quarantining an infected place

11.1 Suspected Farm

The following restrictions should be enforced at the farm pending the receipt of test reports. For this purpose the District Veterinary Surgeon together with the other members of the District Emergency Team should visit the suspected farm and make the farm workers and the owner aware of the seriousness of the situation and the actions they are taking for a verification of the suspicion. However, care should be taken to prevent unnecessary fears and panic and the DVS should inform the strengths of the emergency preparedness of the DAPH.

- They must inform the Divisional Secretary about the current situation and obtain his assistance to mobilize the Grama Niladhari and the KRUPANISA to enforce the quarantine measures.

- The DVS will assign a LDI as the site supervisor at the suspected premises and he will be given required instructions to ensure the restrictions imposed on the farm.

- Together with the GN and the KRUPANISA the range veterinary surgeon and the site supervisor will enforce the following;

- Cordon off the entire suspected farm

- No vehicles should be allowed to move in or out of the farm premises. Any authorized person visiting the farm should leave the vehicles outside the farm. Also, the owner or any supplier of feed or any other essentials should leave their vehicles outside the farm

- No movement of poultry, eggs, dead carcasses, manure, used litter, farm machinery or any such material should be allowed both within the alert zone and from and to outside the zone.

- The farm personnel should wear protective clothing all the time inside the farm, including face masks, gloves, gum boots etc. When leaving the farm premises, farm personnel should leave the protective clothing etc at the farm and clean themselves thoroughly with suitable disinfectants.

- Movement of people to and from the suspected farm should be restricted to the bareset minimum.

- No other animals should be allowed in the farm.

- Inter-sectional movements of farm personnel should be strictly forbidden. Also, farm personnel should not visit any other poultry farm, bird sanctuary, zoo, poultry market etc.

- Disinfection procedures should be strictly applied at the entrance of the premises.

- Al records of the birds present at the farm are to be maintained properly and the range veterinary surgeon to give instructions accordingly.

- Once HPAI is confirmed then the stamping out procedures followed by decontamination of the farm will be carried out. However, the farm will be off-limits for visitors for a prescribed number of days and no birds will be allowed before it is declared safe for farming.

11.2 Village/s (Restricted zone)

General

- The HPAI being a highly infectious disease, the area within a 1km radius from the farm which is suspected to be affected with HPAI is identified first as an alert zone and once the disease is confirmed will be designated as the restricted zone.

- For enforcing the restrictions the Provincial Director will define the area to be defined as the alert/restricted zone and will inform the DG/AP&H and also the Divisional secretary and the District Secretary concerned.

- All the veterinary surgeons within the alert zone will be made responsible for ensuring the restrictions on movement of poultry.

- The DIG of the area will be briefed by the PD on the importance of the quarantine measures and will be requested for his assistance for enforcing the road blocks for movement restriction of poultry.

- The measures enforced around the alert zone pending the receipt of test reports and will continue to be enforced as a restricted zone if the test results are positive for HPAI.

- All villages, habitations and poultry farms within this area should be identified.

- The PD with the assistance of the Divisional Secretary, the GN/s and the KRUPANISAs should then mobilize village vigilant committees to guard all road and other accesses to the alert area to prevent any movement into and out of the alert area.

- All poultry farmers are informed of what they should do and what they should not do both for preventing the spread of the disease and also from minimizing the risk of human infection.

- Poultry farmers within the zone will be required to inform immediately any unusual death, drop in egg production or feed consumption to the veterinary authorities.
- The media should be properly briefed so that unnecessary fears and confusion is avoided and adverse effects on poultry consumption and poultry product prices are prevented. The

public should also be informed of the strengths of the emergency preparedness of the DAPH and the DHS.

- Notices in both official languages about the movement restrictions should be properly displayed at all entry/exit points to the restricted area.

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11.3 Slaughter-house

General

- The PD will inform the Provincial Commissioner of Local Government about the emergency situation as a result of HPAI.

- The PD will provide details of the boundaries of the restricted area and the actions taken to restrict the movement of poultry to and from the area.

- The Provincial Commissioner of Local Government will be requested to close all poultry markets, slaughter-houses and poultry processing plants within the alert zone until the area is declared safe.

- He will also be required to collect from the relevant people about the details of the movement of poultry and their transport vehicles into the restricted zone during the past 3 weeks.

- Details of the movement of poultry and poultry products from these markets, slaughterhouses and the processing plants during the past 3 weeks will also be collected through the offices of the Provincial Commissioner of Local Government.

12 SOP Depopulation and Disposal

The aim of depopulating the infected premises and pre-emptive slaughter of all poultry in the infected zone and disposing the carcasses in an appropriate way is to prevent the spread of the avian influenza agent from the infected area to the non-infected areas.

An outbreak of avian influenza is an emergency situation, and it will be required to destroy a large number of birds quickly, and to cover a wide geographical area. It is essential that the birds required to be slaughtered are speedily and humanely slaughtered and that they are indeed dead before the disposal of the carcasses actually begins. Demonstrating a high standard of slaughter procedure and respect for animals is an essential consideration during an emergency response. Apart from animal welfare considerations, this aspect is very important because some of the poultry that are required to be killed will be found living in the same environment with the local community. It is therefore essential that professional judgment and ethical considerations exercised throughout the depopulation and disposal exercise. It is also necessary to carry out the killing of the birds away from the public view. Furthermore, every effort must be made by the depopulating teams to avoid damage to any property in the infected premises while they carry out the destruction of birds.

13 Preparatory stage

Planning is essential so that the destruction and disposal of infected poultry and those at risk are carried out efficiently and the work is not impeded by lack of resources. It is important that D/AH and the PDs attend to the following in preparedness for any emergency due to an outbreak of AI.

I. Establishing two teams per district to carry out the tasks related to asset valuation, depopulation and disposal.
   Each team should comprise the following officers and helpers;
   Three government veterinary surgeons from the district, the senior of the two as the team leader.
   Two officers from the following category of village level officers, namely GramaNiladhari/Krupanisa/Samurdhi Niyamake
   Two LDIs
   8-10 helpers.

II. Training of team members; all members of the team should be very conversant with the tasks to be performed in connection with the DD and they should be well trained including the use of PPE, PR aspects, teamwork, personnel safety aspects etc. In selecting helpers attention should be paid to their attitudes about killing of birds and working in poultry farms. The helpers should be trained on bird catching without causing undue pain and distress to birds.

III. Identification of burial sites and burning sites in case there are no sites for disposing the birds in the farms itself and machinery for digging and covering of burial pits.

IV. Suitable trucks for transporting carcasses to be identified.
V. Identification of suppliers of transport containers of poultry carcasses for disposal and preliminary negotiations regarding the terms and conditions on use of their services. These matters need regular updating by the D/AH.
VI. Maintaining adequate inventories of materials and supplies required for DD and their strategic positioning in the different provinces.
VII. Arranging a system for cash advances and accounting with the PDs for local expenditures connected with an emergency response.

13.1 Pre- operational activities

As the initial alert is raised by the field veterinarian or the vet of a private establishment of a suspected outbreak of AI and informs the DG AP&H, the Director Animal Health of the AP&H, being a member of the National Emergency Response Team for animal diseases control, will come to know of the alert and will immediately initiate the emergency response to control the AI outbreak.

I. The D/AH with the help of GIS tools, digital location maps and databases of poultry farms will estimate the number of premises to be depopulated, the number of birds required to be destroyed, the staff requirements for depopulation and disposal, PPE requirements, carbon dioxide gas requirements, and other logistical needs.
II. He will then communicate the relevant information to PD/s and instruct the PD to be prepared for mobilizing the depopulation teams if the disease is confirmed.
III. The Director Animal Health will also prepare a list of the destruction sites based on the information available with his databases and also a plan regarding the order of destruction and a timeframe for the operation, broken down on a daily basis.
IV. He will also ensure that required supplies are available in adequate quantities so as to avoid any impediments to the operations.
V. The PD with the help of the relevant VSs will update the list of the destruction sites sent to him by Director Animal Health. He will also in consultation with the Director Animal Health, arrange the resources needed and transport requirements for the depopulation exercise.
VI. The PD will check the availability and preparedness of the DDTs, including the availability of sufficient number of trained and experienced helpers to carry out the depopulation and disposal work. He will contact the helpers and ensure that they can be mobilized at short notice.
VII. The PD and the VSs will also arrange additional burial sites in the infected zone, if no such facilities are available at the infected premises and inform D/AH about the need for transport vehicles for dead birds.
VIII. The PD will ensure that the members of the Depopulation and Disposal Teams are vaccinated with the latest available human vaccine against AI.
IX. The D/AH also will inform the Chief Accountant at the DAPH to be ready for the financial requirements.
X. The Director Animal Health will also arrange a cash advance for the PD to meet the emergency cash expenditure regarding the depopulation and disposal operation.

13.2 Depopulation activities

These activities will commence once the disease is confirmed by the VRI.

I. The Director Animal Health will inform the PDs and the respective VSs on the proclamations made by the DG/AP&H and instruct the PD to liaise with the Divisional Secretary's concerned and take action to control movement of animals.
II. He will also instruct the DDTs to carryout slaughter of the poultry in the infected premises, the poultry in the premises which have had contact/link with the infected premise and also all the poultry at risk in the infected zone.
III. The Provincial Director will then immediately inform the relevant Divisional Secretary/s to mobilize the valuation assessment team/s. He will also mobilize the depopulation team/s from the district concerned to the infected zones with the required supplies for the depopulation and disposal exercise.
IV. The depopulation and disposal teams should be provided with meals and refreshments in the IPs itself to prevent contamination of non-infected areas and the PD will make the necessary arrangements for same.
V. The PD will also keep the relevant District Secretary/s, the DPDHS, MOH, ASP informed of the operation. The PD will inform the DPDHS of the personnel involved in the depopulation exercise so as to be ready with antiviral drugs etc.

13.3 Euthanasia of birds

Euthanasia is the causing of death with minimal pain and distress and is a process of rapid loss of consciousness and a loss of brain function. Considering the importance of animal welfare and the need to destroy the affected birds rapidly, euthanasia by carbon dioxide gassing is the most efficient method to depopulate the infected premises of poultry as well as to destroy the poultry at risk in the infected zone.

However, when the number of birds involved is small, the preferred methods may be dislocation of the neck using a burdizzo, forceps or bear hands all done by an experienced person.
It is also important to remember that carbon dioxide gassing has a number of potential occupational health and safety risks. It is therefore essential that all operators are well trained in handling carbon dioxide gas and experienced in each step of the operation. The team leader of the disposal team will in addition to his other duties, play the role of the first aider and the safety officer to handle any accident during the operation.

I. Prior to the commencement of the operation, the members of the valuation assessment team and the depopulation and disposal team will wear the protective clothing and get a briefing on the sequencing of the tasks and the procedures to be followed by the respective team leader.
II. From the DDT, a sub-group of officers comprising a veterinary surgeon, GN/Krupanisa/SN and an LDI will operate as the valuation team.
III. The value assessment team will then enter the IPs and will make a list of the details of the birds and any structures that will be destroyed in the operation. Based on this list they will work out the asset valuation after they return to their base stations and submit same to the PD. The team leader of the valuation team must ensure that his team wears the PPE at all times while they are in the IPs.

IV. Immediately after the asset assessment is completed, the Depopulation and Disposal Team will enter the IP. The team leader for this exercise must ensure that his teams wear the PPE at all times when they are near the infected birds or in the infected premises.

V. The birds are then caught by the helpers, in the depopulation and disposal team (the helpers must be well trained in bird catching and handling) and are transferred to plastic bags for carbon dioxide gassing. The plastic bag should be of suitable thickness and it is placed inside another bag for gassing with carbon dioxide.

VI. Where the poultry houses are large, the birds are driven, using a movable partition to a catching area, where they are caught and transferred to plastic bags. Caged birds however are more difficult and the progress will be slower.

VII. The plastic bags are then filled with carbon dioxide gas. Co2 is a heavier gas and it will remain inside the bag. A lighter can be used to check the adequacy of gas in the bag. The bags are then securely sealed (tied) and left for about 20 minutes for the birds to get killed. Using transparent thick plastic bags is preferred because it is easy to confirm the birds are dead before they are disposed of.

13.4 Departure from IP

Once the depopulation and disposal is over, the DDT will assemble at the personnel decontamination site. They will then remove the PPE and put them in a plastic bag for disposal. They will the follow personal decontamination procedures before leaving premises.

14 Standard Operating Procedures - Environmentally Friendly Methods for the Disposal of Suspected/Infected Poultry, Poultry Products and Feed

14.1 General

Burial in pits or landfills, mounding, incineration, burning on pyres or in pits, composting and rendering are the main methods for the disposal of suspected/infected poultry, poultry products and feed. Even though incineration is an ideal method, its use is limited because of the capacity and the location of the few incinerators that are available. Out of all the available methods those that can be applied in Sri Lanka are burial in pits, burning on pyres and composting. Each method has its own drawbacks as stated below. Therefore, the decision as to the most suitable method will have to be made taking into consideration the conditions prevalent in the particular location, preferably in consultation with the environmental authorities.

14.2 Burial in Pits

This is the commonest method used in Sri Lanka for the disposal of carcasses of all species of animals and discarded animal products and feed. However, the precautions that has to be taken against the spread of the AI virus makes its use quite cumbersome, if the material is infected (or suspected to be infected). As the carcasses have to be buried at a depth of 2m or more, the pit will have to be significantly deep, especially when large quantities of material are to be buried, which would involve the use of heavy machinery. Whether the soil is strong enough to support their use without the sides collapsing as the depth is increased, is an important factor that has to be taken into account.

Many poultry farms may not have suitable land free of flooding and water logging and adequate space to dig large burial pits, when such aspects as soil type, probability of leachate contaminating water sources etc. are considered. If several farms in a locality have to be depopulated, however, a common burial pit(s) within the locality may be used for all or some of them, provided the carcasses and other material can be transported to the burial site without leaving room for the spread of the virus.

Excavators are the best machinery to be used for preparing large pits. In their absence, backhoes, which are more suitable for smaller pits may have to be used. Use of manual labor will be feasible only for the smallest of burial pits.

Depending on the type of soil, the depth of the pit may vary (but it has to be more than 2m) and the walls (sides) may be vertical or sloping. The maximum recommend depth and the width at the bottom are 5m and 3m respectively. This allows for 9 m2 of space per metre in the length of the pit to bury material, leaving 2m for stacking soil on top of the buried material. The length can be varied depending on the availability of land and the quantity of material to be buried.

The same machinery used for digging the pit may also be used to deposit the material to be buried and level it, as well as to cover it with soil. All the soil brought up when digging the pit must be laid back on the pit to form a mound and a drain dug up around the pit to prevent rain water from flowing into it.

14.3 Burning on Pyres

This is a suitable method to be used, especially when small quantities of material (including carcasses) are to be disposed of and when sufficient firewood or other combustible material and a site free of fire-hazards are available. This method is also to be considered when suitable lands for burial or suitable conditions for composting are not available.

Burning finds particular application when small numbers of poultry are housed in sheds constructed with material that cannot be conveniently decontaminated, such as wood and cadjans. These materials can be heaped in the form of a pyre and the carcasses and feed etc. burned on it.

Possible disadvantages of this method are the smoke pollution and the foul smell from partially burnt carcasses. The latter can minimized by spraying the carcasses with a combustible fluid such as kerosene oil before lighting the pyre

14.4 Composting

Composting has several advantages over other methods because of its low cost and convenience as it may be done either in the shed itself or outside (without digging pits) and the possibility to use the compost as fertilizer. AI viruses have been reported to survive for about 30 days in fecal matter at low temperatures of 00C. As much higher temperatures are achieved in compost heaps, the virus would be inactivated within a shorter period of time and it would be safe to use the compost one month after making the heap. The heap has to be protected from rodents and other scavengers to prevent the spread of the virus and this can be conveniently achieved if the composting is done within the poultry shed itself. As about five to six layers of carcasses can be placed in compost heap, less than 20% of the floor area of a poultry shed will be required for this purpose.

The main disadvantages of composting are the possibility of scavengers disturbing the heap and a foul smell emanating from it. While the former can be controlled by composting in a secure area such as a closed shed or poultry house, the latter can be minimized by covering the heap with a polythene sheet. A main requirement is to select a site that does not get flooded.

For making compost in the poultry shed itself, the poultry litter is first raked into an area of about 25% of the shed area required to house the poultry that had died and/or were slaughtered, and leveled. Any poultry feed to be discarded is then spread on the litter. A single layer of carcasses is then laid over the leveled poultry litter or the feed. The carcasses are then covered with a layer of dry organic matter (paddy husk, straw or wood shavings or preferably, a mixture of these) about 10 cm thick. Sufficient water is sprayed on the dry organic matter to make it just moist to the touch. This is followed by another single layer of carcasses covered by a layer of dry matter. The process is continued until the heap ends up in a layer of dry matter at a height of about 1.5

m. To minimize any foul smell the heap is then covered with a polythene sheet. Bacterial activity can be stimulated and compost formation expedited by turning the heap over in about two weeks' time. But, in the case of infected or suspected material, it may best be left undisturbed for about five weeks.

If composting is done in an open area, all precautions have to be taken to prevent scavenging and to keep off the rain water.

15 SOP - Decontamination Procedure

The aim of decontamination is to safely remove any contamination from the body or clothing of persons, from any buildings in infected premises (IP), equipment or vehicles used in the IP or used by visitors coming to the infected premises.

The decontamination should be done in a systematic way and by properly trained people. For this purpose each district will have three Decontamination Teams identified by the Provincial Director AP&H. These teams will work under the direct supervision of the PD and their actions are coordinated by the District VS. Each Decontamination Team will be headed by a Veterinary Surgeon assisted by a LDI and a dispensary laborer and 3-5 casual laborers hired for the task. All except the casual laborers should receive adequate training on the principles and procedures of decontamination of infected premises or suspected premises and related items.

Members of the decontamination teams should be vaccinated (with the latest available human vaccine against AI), and treated with antivirals if appropriate. They must be provided with adequate protective measures from infection by means of Personal Protective Equipment (PPE) which include cap, goggles, face mask, gloves, overall, boots and shoe-cover meeting the required standard in accordance with the occupational health and safety guidelines for Avian Influenza. These items should be worn at all times when they are near the infected birds or in the infected premises.

15.1 Planning of the Decontamination Procedure

Efficient and effective premises decontamination will only result from proper planning. Thus from the time an alert is raised about AI in a farm or any premises, District Decontamination Teams will have to start their planning for a possible mobilization of the teams.

The Veterinary Surgeon and the LDI of the appropriate Decontamination Team from the district will arrive at the site and a planning exercise started, including site selection for personnel decontamination, decontamination of vehicles and equipment and the assessment of materials and supplies for the decontamination process, including water requirement, high pressure equipment for washing, disinfectants, PPE etc.

The above two officers will;

- make an assessment and recording of contaminated areas, animals and articles;
- the selection of the most suitable decontamination techniques for each item and area.
- the acquisition of necessary equipment and materials and recruitment of personnel to undertake the tasks
- select a personnel decontamination site and
- decide on the adoption of an appropriate strategy.

They will assess also the amount of poultry litter to be removed for disposal and the amount of feed available in the farm for destroying. Every consideration should be given to utilising farm owners and staff on IPs. Their knowledge of operations on the premises is crucial.

In carrying out premises decontamination, realistic goals should be set. The following regime is recommended.

1. Inspect the IP and the suspected premises and prepare a map. Also, identify a personal decontamination site and a disposal site for the PPE used.
2. Start a logbook to record all events and recordings.
3. Indicate areas not requiring decontamination action.
4. Indicate areas or sites requiring specific decontamination action
5. List the actions needed in each area, in chronological order.
6. Estimate a timeframe for the decontamination program.
7. Seek approval from the PD for the proposed program.
8. Implement the agreed decontamination plan, maintaining liaison with the PD and submitting a daily progress report to the D/AH.

Do not take the vehicle used for FVS, VIO or any other person visiting suspected premises inside the site. Also, no vehicles from the IP should be allowed to leave the premises.

It is important before entering the IP to arrange a personal decontamination site (PDS) near the exit point from the IP. The site may be moved further into the IP as necessary during decontamination. This is best done in consultation with the farmer/owner of the IP. It must be possible to leave the IP directly from the PDS without becoming re-contaminated. Consideration must be given to privacy for changing clothes. Also, in selecting the PDS consideration should be given to the slope of the ground so that no run-off water from the PDS flows into clean areas. In addition the PDS should also have adequate bio-security arrangements. It must be possible to leave the IP directly from the PDS without becoming re-contaminated.

The officers should then wear the PPE before entering the IP.

15.2 Premises assessment

Carryout a premises assessment including identification of electrical connections, equipment, fuses boxes, meters, gas supplies etc. by the planning team.

I. Locate and mark all drains and run-off and prevent any effluent running out of the premises.

II. Identify unloading area where supplies, material and equipment can be unloaded without entering the IP.

III. If there are residences within the IPs arrange disposal operations and cleaning of contacted material.

IV. Post a notice as INFECTED PREMISES at the entrance to the IP.

V. Turn off all extractor fans.

15.3 Decontamination Process

15.3.1 Personal decontamination

The decontamination team should leave the vehicle outside the farm and should go to the changing area arranged at the personal decontamination site at the IP.

Each person in the team should wear the PPE properly before entering the IP.

After decontaminating the premises, it's buildings, vehicles, equipment etc. all members will then remove the PPE in the correct sequence as demonstrated at the training sessions and will wash themselves thoroughly with soap and water and decontaminate using 0.2% Citric acid for 30 minutes.

They will leave the PPE in the changing room itself for proper disposal, either by burying or by burning. If there is no facility for destroying at the site put all PPE in plastic bags for safe removal to an alternate site for destroying.

On returning home they will wash their clothes without allowing them to get mixed with other clothes.

15.3.2 Removal of person from an IP in an accident

In case of an accident or injury on an IP, the person affected may be allowed to be carried before personal decontamination can take place to a hospital. However, the hospital authorities must be informed of the risk and appropriate personal decontamination of the patient carried out as circumstances permit. All clothes appropriately destroyed and all contacts at the hospital must be thoroughly cleaned with proper disinfectants. Also, the hospital authorities must be told to wash and disinfect the vehicle/ambulance wheels, underside and interior at an appropriate place.

All people helping the dispatch of the injured to the hospital should also follow personal decontamination.

15.4 Decontamination of Premises

15.4.1 Cleanup

The aim is to remove all manure, dirt and debris and contaminated articles that cannot be disinfected. The surfaces of all buildings, pens, fittings and equipment must be exposed, ready for the first disinfection. This is the most important phase in the decontamination procedure, because the presence of organic material reduces the effectiveness of disinfectant. Encrusted dung, dirt and grease shield the underlying permanent surfaces from the disinfectant.

All litter and or droppings must be removed. Also, remove materials hung on the sheds that cannot be decontaminated.

All fixtures and fittings should be removed and stacked for cleaning and disinfection. Delicate electronic equipment must be protected for later specialist treatment. Earthen floors in buildings may need to be broken for better soaking with disinfectants.

15.4.2 Disinfection process

The aim is to rapidly reduce the amount and distribution of the AI virus from the infected premises. The process begins soon after the depopulation and disposal of poultry products from the IP.

I. All units which are physically or functionally connected to the establishment eg hatchery, egg storage rooms, packing rooms, egg trolleys, processing plants etc must be properly disinfected. All poultry sheds and buildings known to be contaminated are sprayed with appropriate disinfectants.
II. The decontamination should start with the roof, then the walls and fixtures, equipment etc and finally the floor. The shed, cages and other equipment need to be cleaned thoroughly and disinfected using 4% Sodium carbonate anhydrous for 30 minutes.
III. All equipment such as drinkers and feeders must be washed and treated with a disinfectant
IV. Litter – The surface of the litter has to be disinfected using 0.2% Citric acid and thereafter composted inside the shed or in the infected premises.
V. Washing and disinfection of walls, floors and ceilings of the infected establishment must be performed aiming at the removal of all organic material using high pressure equipment

VI. Water tanks and water sources - chlorination by hypo chlorite or oxidation by chlorine dioxide has to be carried out to bring the pH to 2.5.

VII. Clothing, footwear, crates, feed sacks etc. have to be destroyed or if not decontaminated appropriately.
VIII. Feed tanks and silos must be emptied, washed with high pressure water and subsequently fumigated.

Enclosed spaces that can be made air tight eg feed silos etc and spaces containing electrical machinery or electronic equipment and delicate equipment that can be enclosed in a plastic tent, heavy vehicle cabins, poultry incubator rooms and egg rooms should be fumigated with formaldehyde gas to prevent them from getting damaged by washing. Persons doing fumigation should follow the safety procedures for fumigation. There should be two people involved in the operation and both equipped with full face respirators effective against formaldehyde gas.

15.4.3 Vehicles and machinery

Contaminated cars, vehicles used for transport of poultry and poultry products pose a threat to spread of AI. Therefore no vehicle that is contaminated should be allowed to leave the IP without decontamination. Vehicles and other machinery have to be cleaned using soap or detergent allowing them to be in contact for 10 minutes and thereafter disinfected using 4% Sodium carbonate anhydrous for 30 minutes. Also, trace all vehicles that have been in contact with the disease agent, to take them off the road and decontaminate them thoroughly. The FVS/VIO should make inquiries about the origin and occupation of the cars' occupants and any contact they may have had with livestock.

A carwash is ideal for decontamination of vehicles if one can be sourced easily.

15.5 Disinfectants and use

The recommended disinfectants and their application is shown in table 1. The disinfection of specific items using the disinfectants in table 1 is given in table 2.

Table 1. Recommended chemicals for disinfection purposes

Key	**Form and final concentration**	**Contact time and notes**
1. Soaps and detergents		Leave in contact 10 minutes
2. Oxidizing agents		
a. Sodium hypochlorite	Liquid, dilute to final 2-3% available chlorine	Not good for organic materials. 10-30 minutes contact.
b. Calcium hypochlorite	Solid or powder , dilute 2-3% available chlorine (20 g/liter powder, 30g/l solid)	Not good for organic materials. 10-30 minutes contact.
c. Virkon®	2% (20 g/liter)	10 minutes. Excellent disinfectant
3. Alkalis		
a. Sodium hydroxide (caustic soda) (NaOH). Do not use with aluminum and like alloys	2% (= 20 g/liter)	10 mins. Do not use in presence of aluminum
b. Sodium carbonate anhydrous (washing soda) (Na2CO3. 10 H20)	4% (=40 g/liter) from powder 100 g/l from crystals	10 mins. Recommended for use in presence of organic materials as above. 30 mins
4. Acids		
a. Hydrochloric	2% (20 ml/liter)	Corrosive, use only when better not available.
b. Citric	0.2% (2 g/l)	30 mins, safe for clothes and body decontamination

c. Formaldehyde gas	Special generation required	15-24 hrs. Toxic, only if others cannot be used.

Table 2. Disinfection of specific items (refer in relation to table 1)

Item	Disinfectant/chemical/procedure
Animal housing/equipment/cages	1, 2a, 2b, 2c, 3 (also, burn)
Humans	1
Electrical equipment	5c
Water tanks	Drain to pasture if possible
Ponds used by poultry/ducks	Drain to pasture if possible
Feed	Compost, bury or burn
Effluent, manure	Compost, bury or burn, 4, 3
Human housing	1, 2a, 2b, 2c
Machinery, vehicles	1,3
Clothing	1,2a,2b,2c,3

15.6 Inspection

The aim of the inspection is to ensure that all tasks detailed on the premises assessment have been performed. The premises are to be inspected by the District VS together with the VS in-charge of the decontamination team.

The inspection should determine whether:

- all contaminated woodwork not able to be cleaned and disinfected has been completely disposed of;
- all fixtures and fittings have been dismantled, where appropriate, so that no organic material is left behind them;
- there are no observable encrustations on any exposed surface;
- all contaminated feedstuff has been destroyed, and remaining material made safe;
- all grossly contaminated sites (slaughter and disposal) have been properly sealed and effectively cleaned and disinfected;
- all vehicles and machinery properly disinfected and action for tracing of vehicles that entered or left the IP during the past 21 days carried out
- all fluid that has been disinfected has been released into drains or a septic tank; and
- the conditions of quarantine, especially at exit/entry points, and warning notices are being maintained.

16 Standard Operating Procedures - Restocking of Affected Places including the use of Sentinel Birds

16.1 Policy on restocking

The agreed policy in Sri Lanka is to allow restocking in the Restricted Area only after 90 days of freedom from the disease based on active surveillance of sentinel birds for any signs of recurrence of the disease. Considering the conditions in Sri Lanka, where large numbers of smallholders are interspersed among the industrial producers, these strict conditions may be justified.

It may be mentioned, however, that there are reports of countries allowing restocking 21 days after satisfactory decontamination and the outbreak has been brought under control. Some countries are monitoring sentinel birds placed in the buildings from the time of depopulation and decontamination to the time of restocking to determine the virus free status. It has been suggested that sampling of dead birds in the repopulated sheds is more efficient than the use of sentinel birds.

Sri Lanka's current policy is not to use vaccination against AI. If a decision is made later on to restock with vaccinated birds, sentinel birds may have to be used among them to ensure that the vaccinated birds do not act as carriers of the virus.

16.2 Surveillance

According to the OIE, if infection from HPAI or LPAI has occurred in a previously free country, the AI free status can be regained three months after a stamping out policy and decontamination of all affected premises, provided that surveillance in accordance with OIE guidelines is carried out during that three month period. Accordingly, a country declaring AI free status should report results of an active surveillance program in which NAI or HPAI susceptible poultry populations are subjected to a well-designed surveillance program based on the OIE guidelines under Articles 10.4.28 to 10.4.34.

The OIE acknowledges that the impact and epidemiology of NAI differ widely in different regions of the world and therefore it is impossible to provide specific recommendations for all situations. However it states that surveillance strategies employed for demonstrating freedom from NAI at an acceptable level of confidence will need to be adapted to the local situation. Variables such as the frequency of contacts of poultry with wild birds, different biosecurity levels and production systems and the commingling of different susceptible species including domestic waterfowl require specific surveillance strategies to address each specific situation. It is therefore necessary that the DAPH provide scientific data that explains the epidemiology of NAI in Sri Lanka and also demonstrates how all the risk factors are managed. There is therefore considerable latitude available to provide a well-reasoned argument to prove that absence of NAI virus (NAIV) infection is assured at an acceptable level of confidence.

A surveillance system considering all the epidemiological factors specific to the particular region of Sri Lanka affected by an AI outbreak will be designed scientifically and

implemented accordingly to demonstrate the confidence that can be given by a randomized representative sample of the population at risk.

16.3 Release of Control of Restrictions Imposed after an Outbreak of AI

The control measures imposed in the restricted area and the control area will be maintained until at least 21 days have elapsed since the decontamination of the confirmed infected places and negative results of surveillance activities in both areas. At the end of 21 days the restricted area and the control area will be merged and measures applied in the control area will be maintained for a further 14 days period before the restrictions imposed are revoked.

16.4 Declaration of Freedom from AI

In accordance with the OIE Terrestrial Animal Health guidelines, Sri Lanka will make an application to the OIE after meeting requirements for proof of freedom from AI. The reinstatement of AI free status will require the submission of Sri Lanka policy on AI, the eradication procedures, the veterinary infrastructure and the PVS, veterinary service delivery and animal disease surveillance systems in place, and the organization of the industry. Acceptance of the freedom from disease status may also involve inspection by the international panel of experts to review the eradication program and available data to verify the freedom from disease.