SEA/CD/171

Guidelines on Laboratory Diagnosis of Avian Influenza

World Health Organization
Regional Office for South-East Asia

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CONTENTS

Acknowledgements

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1 Introduction

Avian influenza (AI), also known as "bird flu", is an infection caused by avian viruses. Avian influenza viruses are comprised of type A influenza viruses, which primarily infect avian species, though infections with these viruses can occur in humans. Recently, highly pathogenic avian influenza (HPAI) viruses have caused outbreaks in poultry in some countries of South-East Asia such as Bangladesh, Indonesia, Myanmar and Thailand where the virus has become endemic in many poultry populations. The potential for avian influenza to cause human diseases was established during outbreaks of avian influenza among poultry-handlers in 1997 in Hong Kong. Subsequently, clusters of human infections have been reported from Indonesia and Thailand. Currently, the predominant avian influenza virus infecting human has been of the H5N1 subtype, although other strains of lesser pathogenicity such as H7N1 have also been detected.

It is important to accurately diagnose infections of humans caused by an avian influenza virus; the purpose of this guideline is to provide information on the laboratory aspects to the public health practitioners and other health-care workers. The information pertains to the collection of the appropriate specimens and ordering and interpretation of laboratory results.

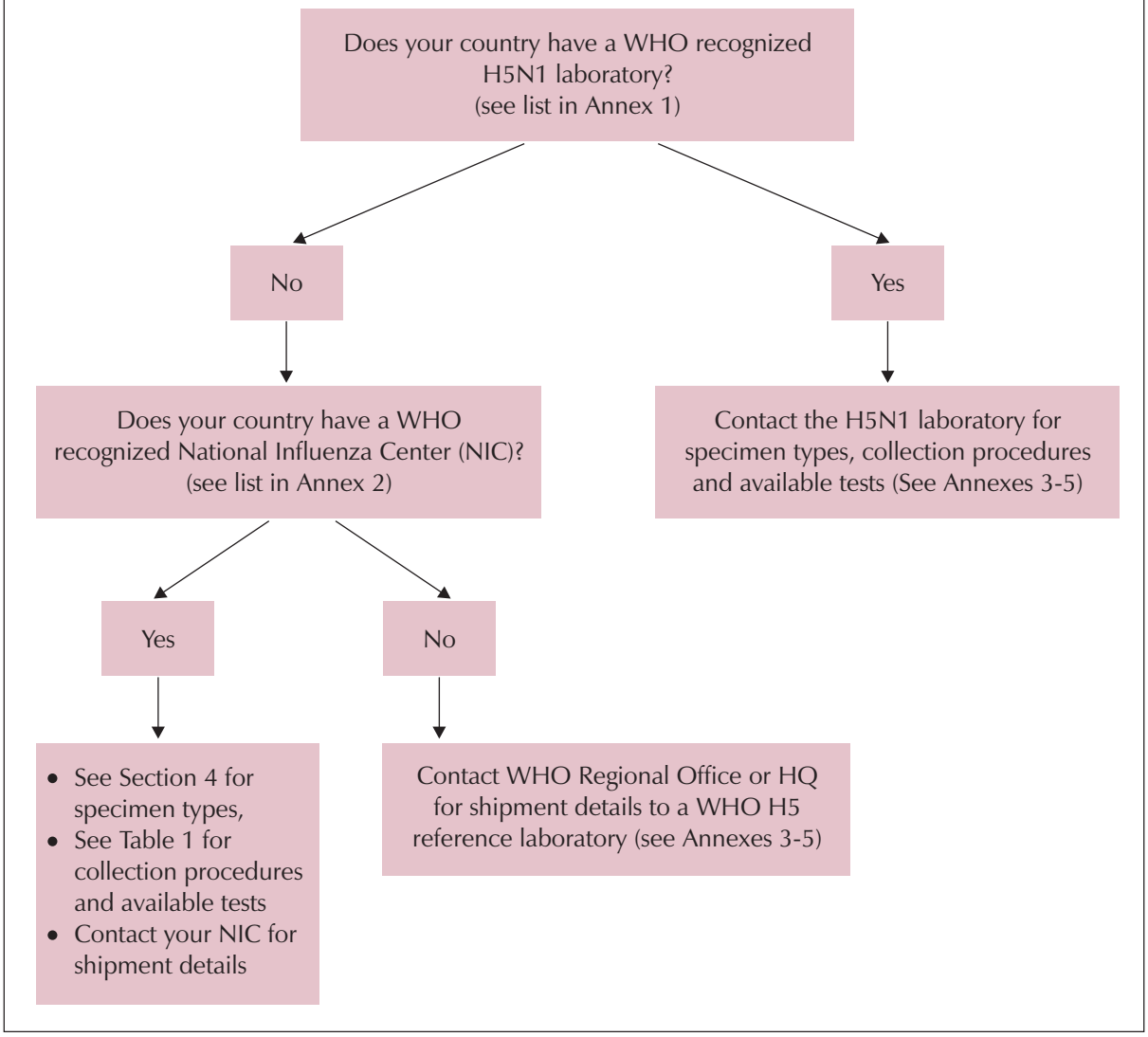
2 Need for laboratory diagnosis of AI

Observation of the avian influenza epidemic in South-East Asia shows that it is not always possible to clinically distinguish human influenza caused by a seasonal influenza virus from those cases due to avian influenza infections, especially in the early phase of the infection. Therefore, laboratory confirmation is required to establish the etiology of clinical influenza. Furthermore, laboratory diagnosis will also contribute to the early detection and characterization of viruses that have undergone genetic changes favourable to the emergence of a pandemic strain. Hence, if the laboratory diagnosis is not performed, there is a risk of failing to detect and warn the global community of potential pandemic strains of avian influenza and to initiate timely public health interventions to avert such an epidemic.

3 Laboratories performing H5N1 testing

Only results confirmed by a WHO H5 reference laboratory, or a WHO recognized laboratory for H5 testing, are accepted by WHO for reporting of human avian influenza cases. Therefore it is important that testing for H5N1 virus be done in a recognized laboratory; flow chart in figure 1 is a guide to selecting a laboratory that can perform H5N1 testing. Countries that do not have laboratory capacity are requested to send the suspected specimens to one of the WHO H5 reference laboratories listed in Annex 1. For H5 laboratories in Member States of the South-East Asia Region, see Annex 2. In countries where there is a WHO National Influenza Center (NIC), the shipment should be coordinated by the NIC (See Annexs 3-5). For countries without NIC, the shipment can be coordinated by headquarters in Geneva (AIResponse@who.int or Dr Wenqing Zhang, zhangw@who.int) or the Regional Office for South-East Asia (Dr Deoraj Caussy, caussyd@searo.who.int). The shipment and packing must conform to the international guidelines given in Annex 3.

Figure 1: Decision tree for selecting a laboratory



4 Specimen collection and handling

4.1 Types of specimens

A variety of specimens are suitable for the diagnosis of avian influenza virus infections. Unlike human influenza viruses which primarily infect the upper respiratory tract, the avian influenza viruses tend to infect the lower respiratory tracts. In the case of mechanically ventilated patients, the best specimens from the respiratory tract are throat, nasal-cavity, bronchioalveolar lavage and endo-tracheal aspirates. For non-ventilated patients, throat and nasal swabs should be collected. To increase the chance of virus isolation it is recommended to collect specimens from different respiratory sites from the same patient on multiple days. When swabs will be used, the preferred choice is Dacron swabs with a plastic shaft, not cotton swab with wooden shaft.

In general, the choice of the type of specimens depends on the disease stage at which the patient is seen and the type of laboratory facilities that are available in the country. Acute phase specimens for virus isolation should be collected during the first three to seven days after the onset of the illness. Generally, convalescent phase specimens such as blood are of limited use unless used in conjunction with acute phase blood specimens. Table 1 lists the types of specimens and their suitability for diagnosing avian influenza virus, including their proper shipment and storage.

4.2 Safety precautions during specimen collection

The collection of specimens from acutely ill or suspected human cases of avian influenza poses a safety risk for health-care workers. Epidemiological observations suggest human-to-human transmission of the virus by very close contact (e.g. face-to-face) with infected individuals may have occurred on some occasions. Avian (H5N1) infection is acquired by inhalation of infectious droplets or droplet nuclei or by direct, and possibly indirect, contact and self-inoculation of infectious virus into the nose, eye or possibly mouth. The H5N1 virus can survive for weeks in a moist environment protected from direct sunlight. The use of personal protective equipment (PPE) is therefore mandatory if direct contact with a patient is anticipated during specimen collection, or when entering a room where aerosol-producing procedures in AI-infected patients are being performed. PPE includes: respiratory protection such as US National Institute of Occupational Safety and Health NIOSH-certified N95 or EU-certified FFP2; non-sterile latex gloves; goggle or face shield; gown, head-covering and in some cases impermeable apron and suitable rubber boots. However, the level of PPE to be employed is determined by the exposure risk. The use of PPE should not be a substitute for recommended infection control precautions, including personal hygiene measures and the correct use of disinfectants.

4.3 Specific specimen collection procedures

4.3.1 Nasal swabs or nasal wash

Nasal swabs or nasal wash are good for virus isolation and other rapid diagnostic tests. To collect a nasal swab, insert the dry swab past the nares until the tip reaches the area below the inferior turbinate; allow the swab to remain there for 5-15 seconds to absorb secretion; rotate the swab gently two to three times and withdraw. Break the tip of the swab by squeezing it in the transport medium containing balanced salt solution, bovine serum albumin and antibiotics. If this virus transport medium cannot be locally made, it can be purchased ready-made from a variety of commercial sources.

Label the container with the patient name, date of collection and type of specimen. If only Polymerase Chain Reaction PCR test is to be performed, the specimens should be collected in a non-phosphate-based medium, either one commercially available or consisting of ethanol (see Annex 4).

Table 1 provides details of storage. Specimens for direct detection of viral antigens by immunofluorescence staining of infected cells should be refrigerated and processed within one to two hours after collection. Specimens for use with commercial near-patient tests should be stored in accordance with the manufacturer's instructions. Specimens for virus isolation should be refrigerated immediately after collection and shipped to the laboratory as soon as possible. Similarly, specimens for PCR test should be kept in either ethanol or lysis buffer at 4°C, available from most virology laboratories or commercial sources. If specimens cannot be processed within 48-72 hours, they should be kept frozen at or below -70°C.

4.3.2 Throat swabs or throat wash

Throat swabs or throat wash are good for virus isolation, PCR and other rapid diagnostic tests. To collect a throat swab, depress the tongue with a tongue blade and gently swab the posterior pharynx up and down several times. Break the tip of the swab by squeezing it in the transport medium containing balanced salt solution, bovine serum albumin and antibiotics. If this virus transport medium cannot be locally made, it can be purchased ready made from a variety of commercial sources. Label the container with the patient name, date of collection, and type of specimen. If only PCR test is to be performed, the specimens should be collected in a non-phosphate-based medium.

The precautions for storage and shipment of specimens are the same as for the nasal specimens. See Table 1 for details of storage.

Table 1: Summary of types of specimens

Types of specimens	**Method of transport/Storage**	**Suitable tests**
Early (3 days) after onset		
Nasal swab or wash or aspirate	In viral transport medium* at 4^{0}C or frozen at -70^0 C	1. Virus isolation 2. Rapid antigen detection by IF* or kits 3. PCR*
Nasopharyngeal aspirate	In viral transport medium* at 4^{0}C or frozen at -70^{0}C	1. Virus isolation 2. Rapid antigen detection by IF or kits 3. PCR
Throat swab or wash	In viral transport medium* at 4^{0}C or frozen at -70^{0}C	1. Virus isolation 2. Rapid antigen detection by IF or kits 3. PCR
Tracheal aspirate or bronchoalveolar lavage	In viral transport medium* at 4^{0}C or frozen at -70^0 C	1. Virus isolation 2. Rapid antigen detection by IF or kits 3. PCR
Acute phase whole blood	At 4^{0}C for whole blood, or -20^{0}C for separated serum	Serology by HA* or MN*
Convalescent phase (14 days post-disease onset)		
Convalescent whole blood	At 4^{0}C for whole blood, or at -20^{0}C for separated serum	Serology by HA or MN
Post mortem specimens		
Biopsy of lungs or trachea		1. Virus isolation 2. Rapid antigen detection by IF or kits 3. PCR

* PCR=polymerase chain reaction; HA=hemagglutination reaction; MN=micro-neutralization; IF= immunofluorescence.

4.3.3 Nasopharyngeal aspirate

Nasopharyngeal secretions are aspirated through a catheter connected to a mucus trap and fitted to a vacuum source. The catheter is inserted into the nostril parallel to the palate. The vacuum is applied and the catheter is slowly withdrawn with a rotating motion. Mucus from the other nostril is collected with the same catheter in a similar manner. After mucus has been collected from both nostrils, the catheter is flushed with 3 ml of transport medium. If only a rapid PCR test is to be performed, the specimens should be collected in a non-phosphate-based medium. The precautions for storage and shipment of specimens are the same as for the nasal specimens. See Table 1 for details of storage.

4.3.4 Nasal wash

The patient should sit in a comfortable position with the head tilted slightly backward, and is advised to keep the pharynx closed by saying "K" while the washing fluid (usually physiological saline) is applied to the nostril. With a transfer pipette, 1-1.5 ml of washing fluid is instilled into one nostril at a time. The patient then tilts the head forward and lets the washing fluid flow into a specimen cup or a Petri dish. The process is repeated with alternate nostrils until a total of 10-15 ml of washing fluid has been used. Dilute approximately 3 ml of washing fluid (1:2) in the transport medium. The precautions for storage and shipment of specimens are the same as for the nasal specimens. See Table 1 for details of storage.

4.3.5 Blood samples

Acute and convalescent serum samples are collected as 2-3 ml of whole blood and may be stored at 4^0C for a maximum of two days before testing; otherwise, the serum should be separated and stored at -20^0C until tested. See Table 1 for details of storage.

5 Clinical and epidemiological information: Laboratory Request Form

The laboratory requires a certain amount of information regarding both the specimens and the patients. The following information should always be on the request form accompanying the specimens (see also Annex 5).

Table 2: Sample request form

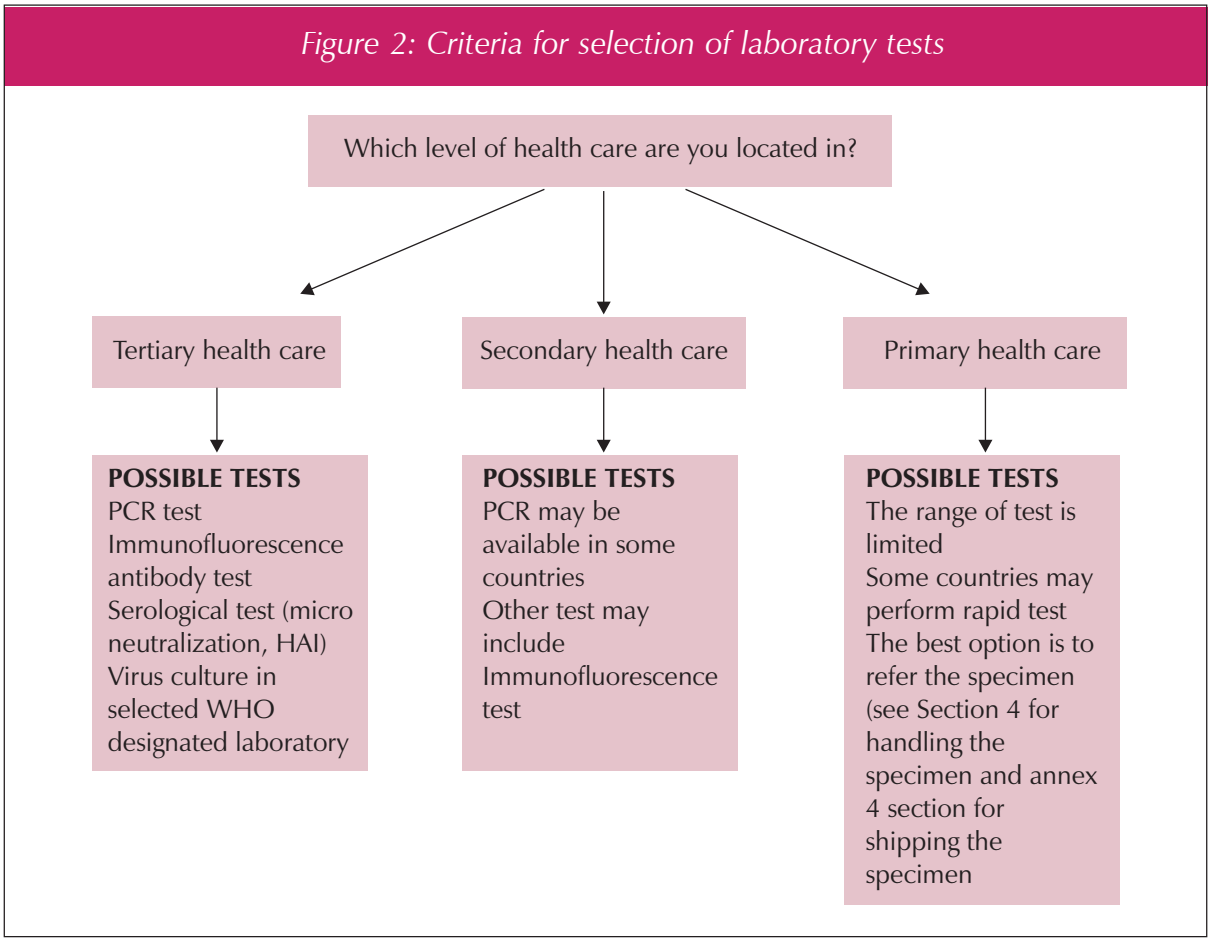
1. Full name and address	2. occupation
3. sex	4. age
5. clinical diagnosis	6. Date of onset of symptoms and duration
7. If patient belongs to a cluster	8. Type of specimens
9. Name and address of person sending the specimens	10. Name and address where results should be sent

6 Application and interpretation of diagnostic tests

The range of tests of laboratory diagnosis of AI can be broadly grouped into those that directly detect the virus or the viral antigens, and those that detect antibodies to the virus. The direct methods include the following: 1) virus isolation; 2) detection of viral nucleic acid by polymerase chain reactions (PCR); or 3) the detection of viral antigens by either immunofluorescence (IFA) tests or rapid antigen detection kit. The serological methods for detecting the viral antibodies include the hemagglutination inhibition test (HAI) and the micro-neutralization tests (MT).

A number of factors have to be considered in choosing which tests to use for the diagnosis of avian influenza viruses, such as: sensitivity and specificity of the tests, turn-around time, availability of the test in the country, ease of performance and cost. Flow chart in figure 2 summarizes the types of test that may be available at the three health care delivery levels of a given country.

Figure 2: Criteria for selection of laboratory tests



6.1 Direct detection of virus or viral antigens

Tissue culture for isolation of avian influenza virus: This is a highly sensitive technique for detecting live avian influenza from clinical specimens. The clinical specimens are inoculated in tissue culture, where they produce a characteristic change in the cell culture called a cytopathic effect (CPE) within one week. The CPE is then confirmed as being an avian influenza virus by using H5N1-specific typing reagents by one of the following methods: 1) polymerase chain reaction (PCR) using

H5 and N1 specific primers; 2) immunofluorescence tests using specific monoclonal antibodies against H5 and N1; or 3) hemagglutination inhibition tests (HAI) using monoclonal antibodies against H5 and N1.

One disadvantage of virus isolation is that it requires a diagnostic laboratory of biosafety level 3 capacities (BSL-3), specialized equipment and trained personnel. These factors may impede virus isolation in all peripheral laboratories or countries with limited diagnostic facilities.

Interpretation: virus isolation is the standard reference method ("gold standard") for avian influenza virus diagnosis and by definition it has a sensitivity and specificity of 100%. However, it must be remembered that virus isolation only detects the presence of whole infectious virus, so specimens that were not properly stored or shipped may yield a false negative result due to loss of viability of the virus. Results of virus isolation and typing may be obtained in five to seven days.

Immunofluorescence tests for avian influenza antigens: The immunofluorescence test is a rapid and sensitive method for directly detecting the presence of avian influenza antigens in clinical samples. The infected cells from the clinical samples are fixed to a slide and the viral antigen revealed by combining with avian-specific monoclonal antibodies that may be either directly tagged with a fluorescent dye (direct test) or may be made to interact with a second anti-antibodies tagged with the fluorescent dye (indirect test). The fluorescence is visualized under a fluorescent microscope. The test must be performed according to the instructions provided in the WHO or commercial kit.

Interpretation: Specific positive immunofluorescence staining is characterized by intense intracellular apple-green fluorescence, in one or more intact cells. When compared to the cell culture, the immunofluorescence test has a sensitivity of 70-100% and specificity between 80 and 100%. Immunofluorescence is the test of choice when fairly rapid results are required as the turn-around time for immunofluorescence results is within a day.

Rapid detection of viral antigens: The rapid tests have been designed to be performed under field conditions, point-of-care of patients or at the bedside by non-laboratory-trained persons. Usually, these test kits consist of a colorimetric strip, which upon incubation with a color-generating substrate displays the presence of the viral antigens as a specific color band. These tests may be directly detected from infected cells shed in patients' specimens, including throat swabs, nasal swabs and nasal aspirates.

Interpretation: The rapid tests take from one to two hours to perform and have the advantage of not requiring BSL-2 or BSL-3 facilities. Currently, rapid tests have been used for the diagnosis of human influenza viruses. These tests can detect either influenza A or B virus without distinguishing the type, or they can detect the joint presence of both influenza A and B without distinguishing the type of influenza virus. None of these tests can differentiate between sub-types of human (H1N1 and H3N2) or avian subtypes (H5N1, H7N1 etc.). The rapid methods for avian influenza have only a limited sensitivity for detection of human cases of avian influenza and may result in false positive and negative results; **therefore they are not recommended for the routine detection of avian influenza viruses.**

Molecular technique of Polymerase Chain Reaction (PCR): The PCR is a method for detecting avian influenza genetic materials from samples that may contain even a single virus particle. This is possible because the PCR has the inherent biochemical capacity to multiply the specific genetic part of the avian influenza by using an enzyme called polymerase. There are two main types of PCR that are commonly used: the conventional Reverse Transcriptase RT-PCR and the real-time rt-PCR. Both types of PCRs can increase the number of copies of avian influenza genome but are multi-step processes requiring an expensive and sophisticated apparatus called a thermocycler and specialized biochemical reagents.

Another requirement for PCR is a pair of oligonucleotide primers, which is a small fragment of nucleic acid that can bind to a specific part of the viral genome and initiate the amplification process. A variety of primers have been used for avian H5N1. Because genetic sequences differ among the various types and subtypes of influenza viruses, it is possible to design PCR primers that will specifically detect only one influenza type or subtype. The M gene is used to detect all influenza A viruses because it is the least variable of the influenza virus genes, whereas subtypes of influenza are determined by primers against the HA and NA genes, which vary according to the subtypes. These primer pairs are designed on the basis of the known HA and NA sequences of H5N1 and will specifically amplify RNA of only one subtype. Primers specific for H5N1 avian influenza viruses have been constructed and are available to the national influenza centers from WHO collaborating centres.

Depending on the types of primers used, the PCR generates a series of viral products of different sizes that have to be definitely identified as being avian influenza virus genome. The main difference between conventional and real-time PCR is in the way the final products are identified. In a conventional PCR, the size of amplified product is estimated by visualization after separation by the technique of gel electrophoresis. Alternatively, the products can be sequenced by further molecular techniques. In real-time PCR, an internal fluorescent probe is used to monitor the PCR product directly on a computer monitor.

This technique has been used for the detection of influenza viruses in original respiratory samples taken from patients with influenza-like illness, or for the characterization of viruses grown in tissue culture. However, there are special technical considerations for using PCR. First of all is the need for specific primers that are only available from the WHO influenza reference and collaborating centres, supplied to the national influenza centres globally. Another common problem is laboratory contamination during specimen processing. Finally, one also needs to use a BSL-2 level laboratory facility for performing PCR on directly collected clinical specimens.

Interpretation of results: PCR produces a fragment of nucleic acid of measurable size in base-pairs or bp. Different sizes of PCR products that are specific for H5 or N1 can be obtained with many different primer pairs. If one uses the current WHO-specific primers (published in http://www.who.int/csr/disease/avian_influenza/guidelines/labtestsMarch07web.pdf) then the PCR products for influenza A/H5 gene is 358 bp in length and the PCR product for the N1 gene is 615 bp in length. In a conventional PCR, these are identified by the technique of gel electrophoresis. The size cannot be measured in the real-time PCR, but the PCR products can be directly quantified by the intensity of the fluorochrome dye emitted by an internal probe.

The absence of the correct PCR products (i.e., a negative result) does not rule out the absence of influenza A (H5N1). Results should be interpreted together with the available clinical and epidemiological information. Specimens from patients with a high probability of infection with influenza A/H5 should be tested by other methods (IFA, virus culture or serology) to rule out influenza A (H5N1) infection. The quality of the clinical samples has a direct impact on the results: if the specimens are contaminated by bacteria or through handling by bare hands, the results will not be reliable. The specimen should be collected in drayon, rayon or polyester-fiber swabs and not calcium alginates or cotton swabs or with wooden sticks that inhibit the PCR. The viral transport medium should not be a phosphate-based medium, which also interferes with the PCR.

The results of all PCRs can be obtained within less than five hours in a functioning diagnostic laboratory and hence the technique is extremely useful for rapid diagnosis, especially during an outbreak. The PCR can measure influenza virus genome in both live and dead viruses, hence its sensitivity is 2%-13% superior to that of cell culture since cell culture can detect only live viruses.

Genetic characterization of avian influenza viruses: Specialized laboratories can perform genetic characterization of the influenza viruses. Genetic characterization has been used to trace the movement and evolution of the H5N1 avian influenza viruses among poultry from South and East Asia, as well to monitor the genetic nature of the avian H5N1 viruses that have been isolated from human infections.

Interpretation of the genetic characterization: Genetic characterization involves many steps and the results may take from 10 to 12 days; therefore, only specialized laboratories with BSL-3 facilities can undertake such testing. The final steps of genetic characterization display the results as a dendogram, which is a computer-generated genetic tree comparing the degree of similarity and differences among the genetic make-up of the different viruses. The dendogram groups the genotypes of viruses sharing a high degree of similarity as families; however, families can still be further sub-grouped into clades. This technique has been used to show that the viruses circulating in Thailand belong to clade 1, whereas viruses circulating in Indonesia belong to the 2A clade.

Testing avian influenza viruses for drug resistance: Genetic characterization is also important to monitor the viral resistance to antiviral drug such as oseltamivir. This is done through either sequencing specific parts of the neuraminidase gene or using a direct real-time PCR. Other methods to test for drug resistance include the immunological technique of enzyme inhibition assay.

6.2 Serological methods for detection of antibodies to avian influenza

Influenza virus diagnosis by virus isolation definitively identifies the infecting strain and is usually more rapid than serologic diagnosis. However, serologic diagnosis is an important approach when clinical specimens for virus isolation are unobtainable or when the laboratory does not have such resources. Two common serological tests for the diagnosis of avian influenza are the hemagglutination inhibition test (HAI) and the micro- neutralization test (NT).

Hemagglutination inhibition test: The hemagglutinin (HA) protein of avian influenza has the property to agglutinate erythrocytes from a number of species including horses. A specific antibody to the antigenic sites on the avian influenza HA molecule prevents or inhibits the hemagglutination reaction. The hemagglutination inhibition (HAI) test can thus be used to type the patient antibodies to avian influenza virus when standard avian influenza antigen is available as reference material.

Interpretation of HAI results: An HAI test is considered positive for avian influenza when paired acute and convalescent sera show an increase in H5 antibody titre of at least fourfold. For this reason, only paired sera should be used for individual diagnosis. However, in an outbreak situation a single serum sample may yield epidemiologic information for presumptive diagnosis. This is because currently, antibody to avian influenza subtypes is presumably low or absent in most human populations. If a statistically significant number of sera from individuals in the acute phase of illness and an equal number matched according to age are collected from individuals in the convalescent phase of illness, then the sera can be tested simultaneously for antibody.

Results of HAI can be available within two to three days, making the test suitable for most epidemiological investigations.

Micro-neutralization test: The micro-neutralization test is based on the fact that when the avian influenza virus is mixed with specific antibody, the avian influenza virus loses all or most of its power to infect--i.e. it is neutralized. The test is called micro-neutralization because it requires a small amount of serum and can be performed in small laboratory plates called microtitre plates. The micro-neutralization test is a sensitive and specific assay for detecting virus-specific antibody to avian influenza A (H5N1) virus in human serum, and potentially for detecting antibody to other

avian subtypes. Under field conditions, the micro-neutralization test has detected H5-specific antibody in human serum at titres that could not be detected by the conventional hemagglutination inhibition assay, the traditional test used for the detection of antibodies to human influenza A and B viruses.

Interpretation of micro-neutralization test: The micro-neutralization test gives the most precise answer to the question of whether or not an individual has antibodies that can neutralize infectivity of a given virus strain. The interpretation of the result of the neutralization test is very similar to that of the hemagglutination tests: a micro-neutralization test is considered positive for avian influenza when paired acute and convalescent sera show an increase in H5 antibody titre of at least fourfold. The results of neutralization may be obtained within three days. However, because of the live nature of the influenza virus that is used, only a laboratory with BSL-3 with cell culture facilities can undertake such testing.

7 Summary of interpretation of laboratory results

In view of the battery of tests available, the following flow chart summarizes how to interpret laboratory results for avian influenza.

Figure 3: Interpretation of laboratory tests

Tests	Interpretation
ONLY rapid commercial test on direct patients samples →	• Cannot confirm or reject Avian Influenza • A positive or negative result needs confirmation by PCR, IF, HAI or micro-neutralization, depending on local availability
Conventional or real time PCR →	• Positive results with two target genes (M, H or N) confirm Avian Influenza • Negative may not rule out AI and may require micro-neutralization test if epidemiological data suspects AI case
Immunofluorescence tests →	• Positive result, confirmed by a second test, constitutes a positive Avian Influenza • Negative may not rule out AI may require MN if epidemiological data suspects AI case
Hemagglutination inhibition or micro-neutralization tests →	• Positive result confirmed by a second test, constitutes a positive Avian influenza, confirm Avian Influenza
Virus isolation and typing by tissue culture →	• Positive result confirm Avian Influenza

8 Further reference materials

Books

(1) WHO-CDC-USAID. H5N1/Avian Influenza Essentials, 2005.

(2) WHO Guide to the collection and transport of Virological specimens for virus. CR Medley, ed. Geneva, 1977.

(3) Diagnostic Procedures for Viral and Rickettsial Infections Lenette and Schmidt, eds. American Public Health Association, 4th ed., 1969.

Links to key documents

For WHO guidelines for the storage and transport of human and animal specimens for laboratory diagnosis of influenza A/H5 infection see:
http://www.who.int/csr/disease/avian_influenza/guidelines/transport/en/

For WHO reference laboratories for diagnosis for influenza A/H5 infection see:
http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/.

For forms to inform WHO Regional Office that a specimen is being shipped visit:
http://www.who.int/csr/disease/avian_influenza/guidelines/humanspecimens/en/index.html
http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf
http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/index.html
http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Annex 1

Laboratories for diagnosis of H5N1 infections

1. **Department of Microbiology**
   Faculty of Medicine
   University of Hong Kong
   University Pathology Building
   Queen Mary Hospital
   Hong Kong Special Administrative Region of China
   Fax: + 852 2855 1241

2. **National Influenza Centre**
   Government Virus Unit
   382 Nam Cheong Street
   Shek Kip Mei
   Kowloon
   Hong Kong Special Administrative Region of China
   Fax: +852 2319 5989

3. **Unité de Génétique Moléculaire des Virus Respiratoires**
   Institut Pasteur
   25 rue du Docteur Roux
   75724 Paris Cedex 15, France
   Fax: +33 1 4061 324

4. ***WHO Collaborating Centre for Reference and Research on Influenza**
   National Institute of Infectious Diseases
   Gakuen 4-7-1, Musashi-Murayama
   Tokyo 208-0011, Japan
   Fax: +81 42 561 0812 or
   +81 42 565 2498

5. ***WHO Collaborating Centre for Reference and Research on Influenza**
   National Institute for Medical Research
   The Ridgeway
   Mill Hill
   London NW7 1AA, United Kingdom
   Fax: +44 208 906 4477

6. ***WHO Collaborating Center for Studies on the Ecology of Influenza in Animals**
   Virology Division
   Department of Infectious Disease
   St. Jude Children's Research Hospital
   332 North Lauderdale Street
   Memphis TN 38105-2794, United States of America
   Fax: +1 901 523 2622

7. ***WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza**
   Centers for Disease Control and Prevention
   1600 Clifton Road, Mail Stop G16
   Atlanta GA 30333, United States of America
   Fax: +1 404 639 2334

8. ***WHO Collaborating Centre for Reference and Research on Influenza**
   45 Poplar Road
   Parkville, Victoria 3052, Australia
   Tel: +61 3 9389 1340
   Fax:+61 3 9389 1881
   http://www.influenzacentre.org

9. **NAMRU-3, Adjacent to Abbassia Fever Hospital**
   Extension of Ramses Street
   Abbassia, Cairo, Egypt 11517
   Tel +2 02-2-342-1375
   FAX: +2 02-2-342-1382

* Also WHO CC for research on influenza

Annex 2

SEA Region National Influenza Centres (NIC) laboratory network

Country	Laboratory	Status as NIC	Diagnostic Facility
Bangladesh	Institute of Epidemiology Disease Control and Research (IEDCR)	Under consideration	PCR
	Institute of Cholera and Diarrhoeal Disease Research (Bangladesh)ICDDR(B)	no	Virus culture for seasonal influenza
Bhutan	National Health Laboratory (NHL)	no	Can only do rapid tests
DPRK	Central Hygiene and Anti Epidemic Institute (CHAEI)	yes	PCR
India	National Insitute of Virology (NIV), Pune	yes	PCR, Culture
	National Institute of Communicable Diseases (NICD)	no	PCR, Culture
Indonesia	National Institute of Health Research and Development) NIHRD*	yes	PCR
	Eijkman Laboratory	no	PCR
Maldives	Public Health Laboratory	no	Rapid tests
Myanmar	National Health Laboratory (NHL)	no	PCR
Nepal	National Health Laboratory (NHL)	no	no
Sri Lanka	Medical Research Institute (MRI)	yes	PCR, virus culture for seasonal influenza
Timor Leste	National laboratory	no	Rapid tests
Thailand	National Institutes of Health (NIH)	yes	PCR, Culture

Annex 3

Checklist for specimen handling and shipment

Note: This form is not an official document but is intended to assist specimen tracking and quality control.

Name(s) of person(s) taking and handling specimens			
Date specimen taken			
Place specimen(s) taken			
Type(s) of specimen(s) (blood, tissue, swab) and storage conditions			
Origin of specimen(s) (human, animal)			
Action	**Yes**	**No**	**Comments**
Safety			PPE used correctly? Any safety problems recorded?
Any shortages of equipment/reagents noted?			
Samples labeled according to protocol?			
Duplicate sample(s) taken?			
Aliquots of specimens taken at local laboratory?			If Yes, was this done before freezing?
Any other manipulations performed on sample at local level?			If Yes, what?
Specimens stored at local laboratory?			If Yes, under what conditions?
Specimens shipped to national laboratory?			If Yes: - Which laboratory? - To whom? - Under what conditions?
Specimens sub-sampled at national laboratory?			If Yes, by whom?

Action	Yes	No	Comments
Specimens tested at local laboratory?			If Yes: - What test(s)? - What result? - Who was informed?
Specimens stored at national laboratory?			If Yes, under what conditions?
National government/MoH approval required for specimen shipment?			If Yes, has it been obtained?
Permission obtained for import of specimens by recipient nation?			
Shipper contacted?			If Yes, which shipper, and who was contacted?
AI response contacted?			If Yes, who was contacted?
Specimens packed?			Notes:
Specimens shipped?			Notes:
Arrival of specimens at reference laboratory?			Date (dd / mm / yy)
Results received from reference laboratory?			Date (dd / mm / yy)
Notes:			

Annex 4

Shipment of infectious substances under the WHO shipment funds project

Guidance for National Influenza Centres

In order to expedite the shipment of influenza specimens and isolates from the national influenza centres (NICs) to the WHO collaborating centres on influenza and the WHO H5 reference laboratories, WHO has entered into an agreement with World Courier, Switzerland to provide shipping services for NICs and other influenza laboratories under the terms of the WHO Shipment Funds Project. Under this agreement, the costs incurred by the courier company will be covered by WHO and not by the NIC.

This document outlines the procedures to be followed by NICs for the shipment of infectious substances and specimens of influenza viruses under the terms of the WHO Shipment Funds Project. NICs are requested to follow the step-by-step instructions below whenever specimens or isolates are shipped.

Procedure and documentation for shipment

(1) For each shipment, NICs are requested to complete the attached booking form and forward by email or fax to World Courier, Switzerland and to WHO Global Influenza Programme at the addresses listed on the booking form given in Annex 5 and can be accessed at http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/

(2) A World Courier local agent will then contact the NIC concerned to arrange collection of the shipment within a maximum period of one week. The World Courier agent will provide all relevant packaging, labeling and paperwork according required to comply with international regulations (see Infectious Substance Category[1]). Dry ice will also be provided should the NIC request "Frozen" shipment on the Booking Form.

(3) The NIC will be required to complete the following paperwork before the World Courier agent can accept the package for shipment:

i. A House Airway Bill (HWB) (see attached example in Annex 5 NB: "Shipper" is the name of the NIC sending the sample or isolate.

ii. A Declaration of Dangerous Goods[2] (if relevant for category of substance).

iii. An export permit for the originating country as relevant.

iv. An import permit for the recipient country as relevant.

v. A packing list/invoice indicating the recipient's address, number of packages, detail of contents including weight and value (see attached sample in Annex 5. NB: for international transport, a minimal value is required even if the items are being provided free of charge.

The World Courier local agent will be able to advise the NIC on any of the above administrative requirements.

1 http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf

2 If the shipment contains an infectious substance (UN2814 or UN2900), a completed Declaration of Dangerous Goods (DGs) is also required. World Courier local shipping agent will be able to provide assistance concerning export documentation upon request.

(4) As soon as the shipment has been dispatched, the NIC is requested to forward shipment details to WHO by entering the details into the password protected database, FluNet http://www.who.int/flunet, following the procedure below.

i. Select "FluNet" under "Data Entry" in the left frame of the screen.

ii. Select "Shipment data".

iii. Choose the year and week number when the shipment is made, then select "Go".

iv. On the new screen, select "Insert" to enter new data, "Update" to revise existing data, or "Delete" to delete existing data.

v. On the data entry/revision screen, inside box "Using WHO shipment funds", select "yes", "no" or "unknown" and enter the costs and all other required information concerning the contents of the shipment.

In the event that a new user account and password for data entry to FluNet are required, the NIC is requested to contact the WHO Global Influenza Programme by email at whoinfluenza@who.int.

Please note:

Costs of shipments will only be covered by WHO when done strictly in accordance with the above instructions. Payment can only be made by WHO directly to World Courier. WHO is not able to accept or reimburse costs or invoices from the NIC.

All NICs are encouraged to plan their shipments of seasonal influenza samples, taking into consideration the timing of the WHO annual vaccine composition consultations in mid-February and mid-September and that WHOCCs require at least one month in order to conduct the analysis of the virus isolates to be included in the WHO annual consultations.

Enquiries and contact information

(1) For standard shipping arrangements, please contact World Courier at opsgva@worldcourier.ch or fax +41 22 8273070.

(2) For any specific queries surrounding logistics and shipping, please contact WHO Shipping and Logistics Support, Mr Christian Fuster (email fusterc@who.int or fax +41 22 7914878) indicating that it is related to the WHO Influenza Shipment Fund Project in the subject heading.

More information about regulations for transport of IS may be found at the following link to the WHO web site:

http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html

Annex 5

World Courier Booking Form

WHO Influenza Shipment Fund Project

BOOKING FORM

(One form per one shipment)

PLEASE SEND TO THE WORLD COURIER GENEVA AT THE CONTACT ADDRESS BELOW BY EMAIL AND/OR FAX TO ARRANGE PICK-UP

!PLEASE FILL IN THIS FORM CAREFULLY!

Information of Booking Form Sending

Date (dd/mm/yyyy):
Pages :
From :

By : ☐ Email ☐ Fax (please click and cross the box(es) you choose)

TO : World Courier Geneva (Switzerland) SA
Email : opsgva@worldcourier.ch
Fax : + 41-22-827.30.70

CC : World Health Organization
Global Influenza Programme
Email : whoinfluenza@who.int
Shipping and Logistics Support (attn. Mrs Paturaux)
Email : paturauxs@who.int
Fax: + 41-22-791.48.37

Requested Date of Pick-up (dd/mm/yyyy) :

PERSON TO BE CONTACTED FOR THE PICK-UP :

NAME : **PHONE :**
E-MAIL :

PLACE OF PICK-UP

Company :
Street :
Dept :
City / Zip Code :
Country :
Name :
Phone :

PLACE OF DELIVERY
(please click and cross one box you choose)

WHO Collaborating Centre in
☐ Atlanta
☐ Melbourne
☐ Tokyo
☐ London

WHO Influenza Shipment Fund Project

DETAILS OF SHIPMENT :

WHO ACCOUNT : **#696002** STUDY / PROTOCOL : WHO

Please click and cross the box(es) you choose:

- ☐ DIAGNOSTIC SPECIMENS FROZEN (UN 3373)
- ☐ INFECTIOUS SUBSTANCES FROZEN (UN 2814)
- ☐ OTHER : _____

NUMBER OF VIALS AND MLS: _____

NUMBER OF INNER PACKAGING AND SIZE (IF AVAILABLE) : _____

NOTE: LOCAL WORLD COURIER OFFICE OR HIS AGENT WILL PROVIDE DRY ICE, ADEQUATE PACKAGING MATERIALS AND PAPER WORKS (House Air Waybill, DG forms) FOR YOUR SHIPMENT.

KIND REGARDS

Annex 6

Composition of virus transport media (VTM)

Virus transport medium for isolation of viruses can be obtained fromWHO HQ (COPAN Universal Transport Medium), or commercially.

Viral transport medium for collection of throat and nasal swabs can be made as follows:

- Add 10g veal infusion broth and 2g bovine albumin fraction V to sterile distilled water (to 400 ml).
- Add 0.8 ml gentamicin sulfate solution (50 mg/ml) and 3,2 ml amphotericin B (250 μg/ml)
- Sterilize by filtration.
- Dispense in 2-5 ml sterile container and store at 4 degrees C until used.

Virus transport medium for PCR work

The medium should not contain any phosphate. Specimens can be collected directly in lysis buffer available commercially, or in alcohols.

Annex 7

A model field data collection form

General patient information **tracking record number**

Name: Date of Birth (dd/mm/yyyy):

Address: Sex: M [ ] F [ ]

Country: Nationality:

County: Occupation:

City/town/village:

Date of onset of illness (dd/mm/yyyy):

Clinical specimens

Unique ID No.	Type	Date of collection	Clinical diagnosis	Health status when specimens collected	Remarks

Post-mortem specimens

Date of death (dd/mm/yyyy): ___/___/___

Name of person completing form: _____

Institutional affiliation: _____

Contact details: _____

Date(dd/mm/yyyy): ___/____/____