Report of the Expert Group Meeting to Standardize Avian Influenza Diagnosis in Referral Laboratories in India

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and
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Executive Summary

An expert group meeting was organized at Pune from 7 to 8th April 2006 to strengthen laboratory support for Avian Influenza surveillance and diagnosis through standardization of laboratory testing protocol and networking among referral laboratories in India. The meeting was convened by National Institute of Virology with technical and financial support from WHO India.

The meeting was attended by laboratory experts in the field of influenza diagnosis and epidemiologists from ICMR HQ, National Institute of Virology (Pune), All India Institute of Medical Sciences (Delhi), King's Institute of Preventive Medicine (Chennai), Regional Medical Research Centre (Dibrugarh), Virus Research Unit of National Institute of Cholera and Enteric Diseases (Kolkata), Indian Veterinary Research Institute (Bareilly), National Institute of Communicable Diseases (Delhi), High Security Animal Disease Laboratory (Bhopal), Institute of Animal Health & Veterinary Biologicals (Kolkata), Regional Disease Diagnostic Laboratory (Bangalore), Regional Disease Diagnostic Laboratory (Pune), Armed Forces Medical College (Pune), WHO India and WHO H5 Global Reference Laboratory at University of Hong Kong (Hong Kong).

Discussions were held on the current situation at the global, SEAR and India level with special reference to the recent outbreaks and associated public health concerns. Details of laboratory preparedness, experiences from the current outbreak and the current international trends in avian and human influenza diagnosis were deliberated.

Problems associated with avian influenza diagnosis in the Indian context was then discussed within the expert group. Recommendations were made by the expert group to finalize the overall protocol for AI diagnosis in humans and animals followed by finalization of protocol, standard operative procedures and interpretative criteria for individual steps in laboratory diagnosis which included specimen management, direct antigen detection methods, virus isolation, molecular diagnosis, serology in diagnosis and surveillance and animal disease diagnosis and surveillance.

WHO's role in facilitating international collaborations and providing technical support for improving coordination was identified.

Background

Expert Group Meeting to Standardize Avian Influenza Diagnosis in Referral Laboratories in India

National Institute of Virology, Pune

7–8th April 2006

The present epidemics of highly pathogenic avian influenza (HPAI) caused by avian influenza virus A (H5N1), which was first detected in December 2003 in the Republic of Korea have now been reported from as many as 33 countries (including poultry and wild birds) like Vietnam, Japan, Thailand, Cambodia, China, Iraq, Turkey, Laos, Indonesia, and very recently from Maharashtra, Gujarat and Madhya Pradesh in India. Vietnam, Thailand and China have been the worst affected countries. Human cases due to the deadly virus have been reported from 8 countries in Asia and Europe.

The widespread poultry outbreaks due to avian influenza A (H5N1) have only increased the chances of the avian virus coming into close contact with the human influenza viruses. Such encounters could trigger the emergence of the pandemic influenza virus strain. Prevention of this reassortment of the avian and human strains of influenza through good veterinary and human surveillance for early detection is one of the frontline prevention and control strategies. Early detection, however, is made difficult by similar illnesses, especially in poultry, which cause a delay in response. Public health response, therefore, is greatly supported by a reliable and prompt laboratory diagnosis of avian influenza (H5N1), both in the poultry and humans. Laboratory support is also the cornerstone for outbreak investigation and rapid containment of the virus in human and poultry populations. The importance of economic and social implications in this context cannot be overemphasized.

This expert group meeting is proposed with the objective of standardizing laboratory testing protocol for referral laboratories in India. This will ensure uniform testing and reliable laboratory results in the quickest possible time for appropriate and effective public health action.

The Meeting

An expert group meeting was organized by WHO at NIV Pune with the objective of strengthening laboratory support for Avian Influenza surveillance and diagnosis through standardization of testing and networking among referral laboratories in India. Specific meeting objectives were:

1. To review the existing laboratory testing protocols in referral laboratories designated for human and avian influenza diagnosis.

2. To define and finalize a standard laboratory testing protocol for avian influenza diagnosis for both human and veterinary referral laboratories.
3. To define a functional coordination between the human and veterinary referral laboratories.

The meeting was attended by laboratory experts in the field of influenza diagnosis and epidemiologists from ICMR HQ, National Institute of Virology (Pune), All India Institute of Medical Sciences (Delhi), King's Institute of Preventive Medicine (Chennai), Regional Medical Research Centre (Dibrugarh), Virus Research Unit of National Institute of Cholera and Enteric Diseases (Kolkata), Indian Veterinary Research Institute (Bareilly), National Institute of Communicable Diseases (Delhi), High Security Animal Disease Laboratory (Bhopal), Institute of Animal Health & Veterinary Biologicals (Kolkata), Regional Disease Diagnostic Laboratory (Bangalore), Regional Disease Diagnostic Laboratory (Pune), Armed Forces Medical College (Pune), WHO India and WHO H5 Global Reference Laboratory at University of Hong Kong (Hong Kong).

Delegates to the meeting were welcomed by **Dr. A. C. Mishra**, Director NIV Pune. This was followed by remarks from **Dr. Lalit Kant**, Senior DDG, ICMR HQ on the importance of laboratory diagnosis, preparedness and networking in the face of the emerging threat of avian influenza and pandemic influenza. **Dr. Manish Kakkar,** WHO National Consultant (Laboratory Surveillance) then explained the objectives and the expected outputs at the end of the expert group meeting. **Dr. Sampath Krishnan**, CDS Coordinator WHO India, then briefed the group about the current situation at the global, SEAR and India level with special reference to the recent outbreaks and associated public health concerns.

An overview on laboratory diagnosis of avian influenza H5N1 in humans and animals was then presented by **Dr. J. S. M. Peiris**, Professor, Department of Microbiology, University of Hong Kong, a WHO Global H5 Reference Laboratory.

Details of laboratory preparedness in terms of available infrastructure at each of participating laboratories were presented by the laboratory representatives who also shared their experiences during the current outbreak in India. An inventory of laboratory diagnostic facilities in referral laboratories for influenza A (H5N1) diagnosis in India is given in Table 1.

A problem based-approach to the avian influenza diagnosis in the Indian context was then discussed within the expert group. The experts thereafter finalized the overall protocol for AI diagnosis in humans and animals, followed by finalization of protocol, standard operative procedures and interpretative criteria for individual diagnostic tests. These included:

- Specimen management
- Direct antigen detection methods
- Virus isolation
- Molecular diagnosis
- Serology in diagnosis and surveillance
- Animal disease diagnosis and surveillance

Recommendations were also made on various aspects of AI testing in humans and animals in the Indian context.

The expert group was briefed on support provided to seasonal influenza surveillance as well as avian influenza A (H5N1) diagnosis and surveillance by the WHO Global Influenza programme. Detailed discussions were also held within the group on the evolution of the emerging H5N1 virus and the role of mutations in its virulence and

transmission characteristics. The group then deliberated on the collaboration between the human and veterinary laboratories in the light of the Hong Kong experience.

Recommendations

Recommendations for specimen management

- Various studies and experience of the H5 reference laboratories have demonstrated a higher yield of the virus when different types of respiratory specimen are collected from the same patient.
- Respiratory specimen should be collected as early as possible during first 4–5 days of onset of symptoms to get the maximum yield of virus and genetic material of the virus.
- Both respiratory and serum specimens should be collected from all clinically suspect cases for complete laboratory work-up.
- Nasopharyngeal aspirate is the first choice specimen in the case of secretions being present.
- Nasal swab and throat swab from the same patient should be collected and the specimens should be pooled in the same container.
- Upper respiratory specimen and lower respiratory specimens, when available, should be processed separately as a group. Specimen within the group should then be processed separately within the group, with the exception of nasal and throat swabs which need to be pooled as mentioned above.
- Tissue specimen following autopsy must be collected from all organs, whenever possible, after consent. In case autopsy samples are not possible, deep lung biopsy using a TRUCUT Biopsy needle should be collected for histopathology, virus isolation, antigen detection and RT-PCR. When tissue samples are available, then process as in Figure 1.
- Standard procedures for specimen collection, storage, handling and safe transport are given in Annex 2.

Various studies and experience of the H5 reference laboratories have demonstrated a higher yield of the virus when different types of respiratory specimen are collected from the same patient

Figure 1: Protocol for processing tissue specimen for human avian influenza diagnosis

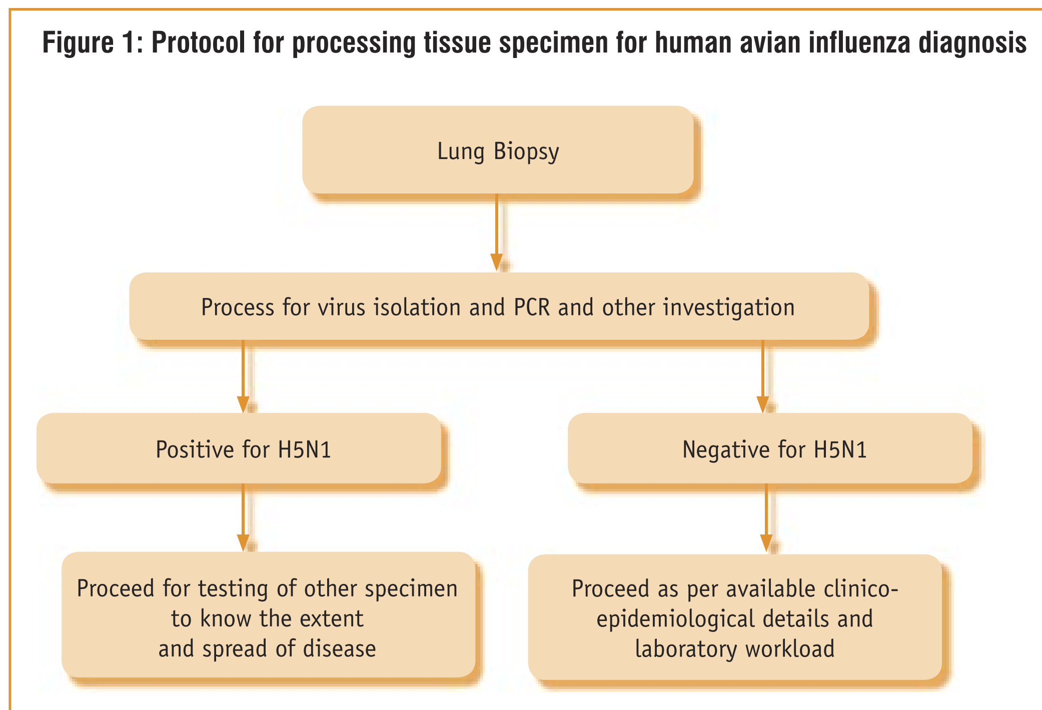


Figure 2: Protocol for processing respiratory specimen for human avian influenza diagnosis by immunoflourescence

Immunoflourescence

↓

Stain using pool for RSV, Inf A & B, adenovirus, Parainfluenza viruses 1,2,3

↓ Pool+
then test for complete panel of RSV+ Inf A Pool+ Inf B pool+ Adeno +PIV 1,2,3 +H5 monoclonal

- Inf A + / Inf B – / H5 + → Presumptive H5
- Inf A + / Inf B – / H5 – → Look for H3 & H1
- Inf A – / Inf B + / H5 – → Influenza B
- Others + → Decide on other viruses in the panel; consider mixed infection if more than positive

↓ Pool–

Do not proceed further

Recommendations for antigen detection tests for detection of influenza A H5N1 virus (commercial kits)

- The performance characteristics (sensitivity, specificity, PPV and NPV) of all the currently available commercial antigen detection tests are based on their performance in human seasonal influenza diagnosis. Data on their performance in diagnosis of H5N1 is limited and indicates that they are not sensitive enough for detection of H5N1 infection in human respiratory specimen. In the scenario of very low prevalence of human cases due to H5N1, the positive predictive value of these tests is not in favour of their use as frontline tests.
- Currently available antigen detection tests utilize up to 200 µl of the clinical specimen, a quantity that might not be always available. Their use should, therefore, be strictly determined by the availability of sufficient quantity of specimen and preferably only if the sample is positive for PCR. Positive result only adds to the strength of the diagnosis and is not a means of confirmation of PCR results. Negative result before or after a positive PCR does not rule out H5N1 infection.

- Rapid antigen detection tests can be used for culture identification; these tests should perform better for this purpose as the virus copy number is higher.
- Immunoflourescence test on clinical specimen (using the commercial kits or the WHO reagents) is a preferred method for antigen detection over the rapid tests. It should be performed simultaneously in preliminary processing of the respiratory specimens along with PCR and culture in case clinical material is in sufficient quantity.
- Immunoflourescence is best performed on respiratory specimen of good quality in terms of cells present, e.g. nasopharyngeal aspirate, broncho-alveolar lavage following the protocol mentioned in Figure 2.
- Standard procedures for immunoflourescence test are given in at Annex 3.

Recommendations for Influenza A (H5N1) virus isolation

- Virus isolation is the gold standard for avian influenza H5N1 diagnosis and should be attempted in all suspected and probable cases.
- Specimen inoculation for virus isolation should be attempted preferably in embryonated eggs which gives a better isolation rate for the purely avian virus.
- Additionally, MDCK cell lines should also be inoculated. Since the availability of embryonated eggs may be a limiting issue, laboratories with limited or no access to eggs may use MDCK cell line alone.
- Routine recommended method (s) for virus identification—HA/HAI and immunoflourescence should be used. RT-PCR should be used for detection and identification of the virus in culture in case the clinical sample is positive by RT-PCR and presence of virus in culture is negative by HA/HAI and IF.
- Commercial rapid antigen detection kits can be used as an adjunct for virus identification from culture.
- Standard procedures for virus isolation and identification methods are given in Annex 4.

Recommendations for serology in diagnosis of human avian influenza H5N1

- Utility of serology for identifying outbreaks is limited. Serological responses may not appear till the 3rd week of illness. However, it helps in final exclusion of infection, or to maximize the case ascertainment rate, especially for discrepant results.
- Serology is best done demonstrating a rise in antibody between paired sera. Available data and experience indicates that traditional hemagglutinion inhibition (HAI) is not suitable for sero-diagnosis of human H5N1 infection. Modified HAI test for H5 using horse RBC is currently under evaluation but is not a confirmatory test by itself.
- Micro-neutralization tests are the method of choice and are best carried out in reference laboratories with adequate BSL-3 containment facilities and experience.
- Local strain of H5N1 virus (human or animal) is preferable for micro-neutralization assay. Animal/poultry strain can be used for human serology, provided adequate laboratory containment can be assured.
- Alternatively, a homologous strain from WHO Global H5 reference laboratory may be used.
- Standard procedure for serodiagnosis

Virus isolation is the gold standard for avian influenza H5N1 diagnosis and should be attempted in all suspected and probable cases

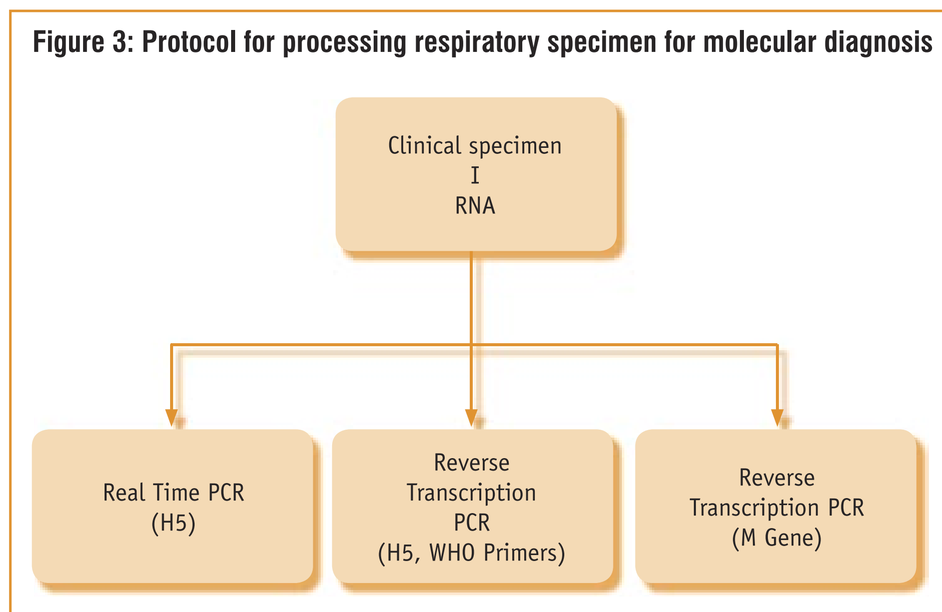
RT-PCR (real time or conventional) will be the frontline test for diagnosis of human avian influenza H5N1 due to its rapidity and high sensitivity and specificity

by neutralization test in animal specimen is given in Annex 7.

Recommendations for molecular diagnosis of human avian influenza H5N1

- RT-PCR (real time or conventional) will be the frontline test for diagnosis of human avian influenza H5N1 due to its rapidity and high sensitivity and specificity.
- Respiratory sample upon receipt for molecular diagnostics will be processed as in Figure 3.
- Sample positive for H5 product should only be processed for N1 PCR.
- A PCR positive result implies:
  - Positive for M gene, H5 gene and N1 gene AND
  - Sequence match of H5 & N1 product with the available database.
- A sample will be considered positive for H5N1 infection by PCR when it has tested positive with another set of PCR primers (particularly H5 which have been used by other international and reference laboratories of repute and published as scientific data) or in a different laboratory with same set of primers or with two different set of RT-PCR protocols (conventional and real time). Both PCRs should start with extraction of RNA from the specimen to confirm the findings. This is to improve the **PPV of a positive laboratory test for a disease with very low prevalence.**
- The first RT-PCR assay should include a positive control; positive control should be avoided in the second test to avoid any carryover contamination; a laboratory can include positive control in the second assay in case non-contamination can be ensured.
- Avoid multiplex testing by RT-PCR. This may give false positive or negatives.
- **PCR results need to be confirmed from a WHO H5 Reference laboratory**. It may also be advisable to **confirm some of the negative results in case there is a strong clinico-epidemiological suspicion** and test results are not positive. Also, results should be correlated with culture and serology findings.

Figure 3: Protocol for processing respiratory specimen for molecular diagnosis



- In the situation of an established outbreak, a sample positive for M gene and H5 gene may be considered as a positive PCR result.
- Real time or conventional RT PCR have equal value in detecting the H5N1 targets. Positive results of either PCR should be confirmed by the other.
- WHO protocols to be followed by all centres for uniform results; suitable change in the protocols may be made as and when these are made available by WHO.
- Standard procedures for molecular diagnosis are given in Annex 5.

Recommendations for animal surveillance

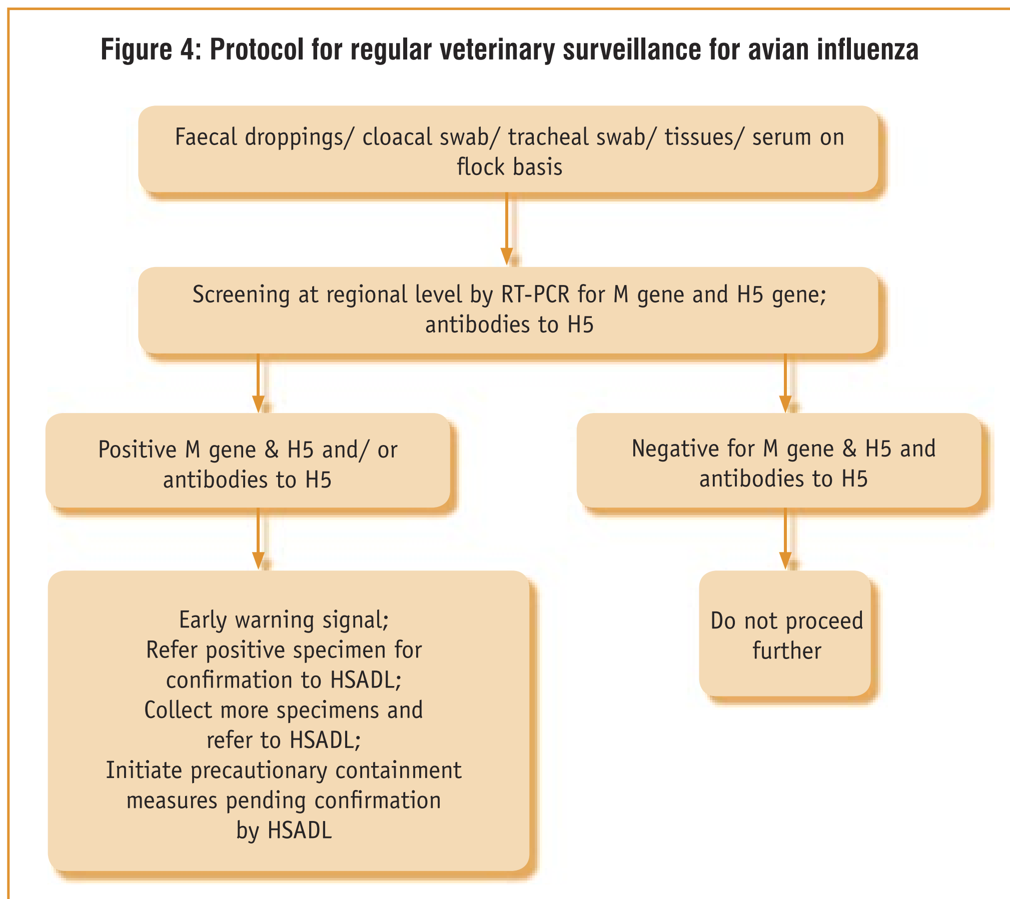
Regular surveillance

- Under the current circumstances, majority efforts should be focused on containing the virus and outbreaks due to the virus in the poultry and birds through efficient country-wide veterinary surveillance to minimize its spread and contact with humans. Standard procedures for animal influenza surveillance and diagnosis are given in Annex 6.
- Regular veterinary surveillance will be carried out in live poultry as well as in case of suspected bird deaths.
- Surveillance will be clinical, serological (using Agar Gel Immunodiffusion) and virological (Figure 4).
- Serological surveillance will provide additional information on activity of other influenza viruses.
- Virological surveillance (Fecal droppings/cloacal swab/tracheal swab/tissues) will be done as a 2-tier system:
    - Screening of clinical samples at regional level using RT-PCR for M gene and H5 gene and serology
    - Confirmation of results should be done at HSADL
    - Virus isolation should not be attempted at the regional level till the time BSL-3 facilities are available
    - Number of specimen and frequency of testing can be decided using appropriate models.

Outbreak window period

- The window period between reporting of the outbreak/unusual mortality in poultry and reporting of confirmatory diagnosis by the HSADL Bhopal has been extended up to 15 days, which in turn has led to delayed epidemiological response. This has been due to the increased specimen load and other logistics issues.
- Laboratory turn-around time can be shortened by a 2-tier approach which will also help the HSADL to prioritize positive specimens for confirmatory diagnosis (Figure 5).
    - Screening at regional level by antigen detection (using rapid kits) and RT-PCR for M gene and H5 gene
    - Confirmation at HSADL.
- Results in either scenario to be communicated following confirmation by HSADL.
- Public health action can be considered even at regional-level diagnosis, keeping in consideration the epidemiological information.

Real time or conventional RT PCR have equal value in detecting the H5N1 targets. Positive results of either PCR should be confirmed by the other



Serology by neutralization is the method of choice for human samples, preferably using the homologous strain

Recommendations for interpretation of test results/ value of each laboratory tool:

- **Rapid tests (Rapid antigen detection/ IF):**
  - Give only presumptive diagnosis of influenza A &/or B virus infection
  - Negative or positive results require confirmation by other tests
  - Use of rapid antigen detection tests should be determined by the quantity of specimen available.
- **Virus isolation:**
  - Virus isolation is the gold standard for confirmatory diagnosis of human avian influenza A (H5N1), especially when declaring the first case in a country or a large geographic area.
  - Virus isolation is a sensitive technique with the advantage that virus is available both for identification and for further antigenic and genetic characterization, drug susceptibility testing, and vaccine preparation.
- **Serology:**
  - Serology by neutralization is the method of choice for human samples, preferably using the homologous strain.
  - Serology is diagnostic when sero-conversion or a 4-fold rise in antibody titer can be demonstrated in paired samples (collected usually at least 15 days apart).
  - Single serum sample positive for antibody response to influenza A H5 virus indicates exposure to virus but cannot be considered diagnostic.

Figure 5: Protocol for presumptive veterinary diagnosis for avian influenza during investigation outbreak window period

Outbreak/ unusual mortality in poultry/ wild bird

↓

Collect fecal droppings/ cloacal swab/ tracheal swab/ tissues/ serum on flock basis

↓

Screening at regional level by
- Rapid antigen detection test AND
- RT-PCR for M gene and H5 gene
- antibodies to H5 not a priority; to be tested at HSADL

↓

Positive for Influenza A antigen and/ or M gene & H5 by RT PCR

↓

Early warning signal

↓

A newly identified area:
- refer positive specimen for confirmation to HSADL;
- Collect more specimens and refer to HSADL;
- Initiate precautionary containment measures pending confirmation by HSADL

Area contiguous to confirmed outbreak area (within 3-10 km or outside 10 km):
- refer positive specimen for confirmation to HSADL;
- Collect more specimens and refer to HSADL;
- Consider initiating culling operations for early containment & minimize exposure to humans

Negative for Influenza A antigen and/ or M gene & H5 by RT PCR

↓

Do not proceed further

- Serology can be used in establishing the final diagnosis, e.g. suspect/probable case with positive PCR and negative culture results and as given below.
- **RT-PCR/Real-time PCR:**
  - Both real-time and conventional PCRs have equal value as PCR protocols.
  - The results of PCR confirm the diagnosis, only if positive twice on the same patient; each PCR to start from the step of extraction of RNA from specimen.
  - The absence of the correct PCR products (i.e. a negative result) does not rule out the presence of influenza virus. 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 or H9 should be tested by other methods (IFA, virus culture or serology) to rule out influenza A (A/H5) infection.
- **For declaring the first case in a country/large geographic area:**
  - In a suspect or probable case, any of the following:
    - Positive PCR* + serology (4-fold rise)
    - Positive PCR* + culture
    - Positive Culture
    - Positive serology (4 fold rise).

The results of PCR confirm the diagnosis, only if positive twice on the same patient; each PCR to start from the step of extraction of RNA from specimen

Recommendations for laboratory biosafety

General recommendations:

- Good microbiological technique is fundamental to laboratory safety. The use of safety equipment, combined with good procedures and practices, will help to reduce the risks involved in dealing with biosafety hazards. The most important concepts for ensuring laboratory biosafety are as follows:
- Standard precautions should always be followed; barrier protection (gowns, gloves) should be used whenever samples are obtained from patients. In addition to these standard precautions, eyes should be protected.
- Basic containment – Biosafety Level 2 (BSL2) – practices and procedures should be the minimum requirement for handling specimens.
- Examples of routine laboratory procedures that require BSL2 include:
  - routine diagnostic testing of serum and blood samples (including haematology and clinical chemistry);
  - manipulations involving neutralized or inactivated (lysed, fixed, or otherwise treated) virus particles and/or incomplete, non-infectious portions of the viral genome;
  - final packaging of specimens for transport to diagnostic laboratories for additional testing;specimensshouldalready be in a sealed, decontaminated primary container.
- Good laboratory practices should be followed, i.e. eating, drinking, smoking, applying cosmetics, and handling contact lenses should be prohibited in the laboratory working areas.
- Personal protective equipment (gown, gloves, eye protection) should be worn in the laboratory when handling and processing specimens and performing diagnostic testing.
- All technical procedures should

be performed in a way that minimizes the formation of aerosols and droplets.

- Biological safety cabinets or other physical containment devices should be used for all manipulations that may cause splashes, droplets, or aerosols of infectious materials (e.g. centrifugation, grinding, blending, vigorous shaking or mixing, sonic disruption, opening of containers of infectious materials whose internal pressure may be different from the ambient pressure).
- The use of hypodermic needles and syringes should be limited. They must not be used as substitutes for pipetting devices or for any purpose other than parenteral injection or aspiration of fluids from laboratory animals. Mouth pipetting must be strictly forbidden.
- Adequate and conveniently located biohazard containers should be available for disposal of contaminated materials.
- Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day. Generally, 5% bleach solutions are appropriate for dealing with bio-hazardous spillage.
- Personnel must wash their hands often — especially after handling infectious materials and animals, before leaving the laboratory working areas, and before eating.
- Personal protective equipment must be removed before leaving the laboratory.

Specific biosafety guidelines for handling specimens that may contain avian influenza A (H5N1) virus

- Laboratories must meet basic *BSL2 standards and use BSL3 work practices* to be able to safely
  - Aliquot and/or dilute specimens
  - Perform diagnostic testing that does not involve propagation of viral agents in vitro or in vivo
  - Perform nucleic acid extractions that involve untreated specimens
  - Prepare smears using heat or chemical fixation.
- BSL3 practices cover the following areas:
  - Any procedure that may generate aerosols or droplets should be performed in a biological safety cabinet (e.g. sonication, vortexing).
  - Laboratory workers should wear protective equipment, including disposable gloves, solid-front or wrap-around gowns, scrub suits, or coveralls with sleeves that fully cover the forearms, head coverings and, where appropriate, shoe covers or dedicated shoes, eye protection and a surgical mask, or full-face shield, because of the risk of aerosol or droplet exposure when performing specific manipulations.
  - Centrifugation of specimens should be performed using sealed centrifuge rotors or sample cups. These rotors or cups should be unloaded in a biological safety cabinet.
  - Work surfaces and equipment should be decontaminated after specimens are processed. Standard decontamination agents that are effective

Adequate and conveniently located biohazard containers should be available for disposal of contaminated materials

Biological waste contaminated with suspect or confirmed influenza A/H5 specimens, should be treated appropriately

against non-enveloped viruses should be adequate if used according to the manufacturer's recommendations. Generally, 5% bleach solutions are appropriate for dealing with biohazardous spillage.

- Biological waste contaminated with suspect or confirmed influenza A/H5 specimens, should be treated appropriately.
- When a procedure or process cannot be conducted within a biological safety cabinet, appropriate combinations of personal protective equipment (e.g. respirators, face shields) and physical containment devices (e.g. centrifuge safety cups or sealed rotors) must be used.
- It is strongly recommended that BSL3 precautions described above are adopted and followed for work in BSL2 laboratories with influenza A/H5 virus specimens.
- Where laboratory facilities do not meet at least basic BSL2 containment conditions, specimens should be referred to suitably equipped reference laboratories for primary diagnostic tests.

- For laboratories that meet BSL3 containment standards and are operated by staff trained in the use of appropriate BSL3 work practices, the following procedures can be undertaken:
  - Diagnostic tests that involve propagation of viral agents in vitro or in vivo.
  - Work involving the replication of influenza A/H5 virus in cell culture and/or storage of cell culture isolates.
  - Recovery of viral agents from cultures of influenza A/H5 specimens.
  - Manipulations involving growth or concentration of influenza A/H5 virus.

Recommendations for laboratory confirmation of results

- All laboratory results for influenza A/H5, H7 or H9 during Interpandemic and Pandemic Alert periods of the WHO Global Influenza Preparedness Plan (http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_5/en/index.html) should be confirmed by a WHO H5 Reference Laboratory or by a WHO recommended laboratory. Influenza A/H5, H7 or H9-positive materials, including human specimens, RNA extracts from human specimens, and influenza A/H5, H7 or H9 virus in cell-culture fluid or egg allantoic fluid, should be forwarded to a WHO H5 Reference Laboratory ii or a WHO recommended laboratory. Communication and publication of analysis results should be according to the WHO Guidance for the timely sharing of influenza viruses/specimens with potential to cause human influenza pandemics (http://www.who.int/csr/disease/avian_influenza/guidelines/Guidance_sharing_viruses_specimens/en/ind ex.html)
- A comprehensive decision-making protocol for laboratory diagnosis of human avian influenza A H5N1 is given in Annex 1.
- There is a need for initiation of an external quality assurance program for molecular diagnosis of avian influenza A (H5N1) for 4 major laboratories, i.e. NIV, NICD, HSADL and AIIMS that can be organized by

Hong Kong laboratory and supported by WHO India. This will promote further improvement in testing in referral labs in India and networking at the national and international level.

Recommendations for networking between human and veterinary laboratories for surveillance of avian influenza A (H5N1)

- The veterinary and human laboratories should share laboratory surveillance data, particularly information on the nature and genetic characteristics of the virus strains isolates, on a regular basis to guide surveillance and diagnosis on both sides.
- The results of the poultry virus cannot be confirmed in the laboratory testing human samples (as per the recommendations) and therefore the results could be confirmed in a third laboratory with PCR and sequencing facilities using the kits from the human side laboratory. Alternatively, local strain (human or animal) can be handled in human laboratory or vice versa, provided adequate laboratory containment can be assured. The theoretical risk of re-assortment within the laboratory under adequate containment is very low.
- The following materials from veterinary laboratories can be shared and handled in human laboratories and vice versa, in case adequate containment for live virus is not guaranteed:
    - Total cDNA of H5N1 AIV
    - PCR amplicons of HA gene (WHO, OIE)
    - Plasmid containing 1430 bp HA insert
    - Formalin-killed virus.

Role of WHO

- Providing technical support for improving coordination between stakeholders and development of strategies, protocols, guidelines and manpower, especially to cater to the priority issues as identified.
- Facilitating international collaborations through provision and exchange of samples and reference materials and exchange of expertise through visits of scientists.

Table 1: Inventory of referral laboratories for influenza A (H5N1) diagnosis in India

Name of the laboratory	Type of lab	Lab containment facility	Diagnostic facilities				IDSP level of the lab	International collaboration
			Direct detection	Virus isolation	Serology	Molecular diagnosis		
National Institute of Virology, Pune	Human	BSL-3	-Rapid kits -IF	-MDCK -Egg		-RT-PCR -Real time PCR -Sequencing	Level 5	National Influenza Center of WHO GIP*
National Institute of Communicable Diseases, Delhi	Human	BSL-2 with BSL-3 precautions	-Rapid kits -IF	-MDCK$	-HA/ HAI for H5	-RT-PCR	Level 4	-
All India Institute of Medical Sciences	Human	BSL-2 with BSL-3 precautions	-Rapid kits -IF	-$		-RT-PCR -Real time PCR -Sequencing	Level 4	-
King's Institute of Preventive Medicine, Chennai	Human	BSL-2	-Rapid kits -IF	-$		-RT-PCR	Level 4	-
Virus Research Unit, National Institute of Cholera & other Enteric, Kolkata Diseases	Human	BSL-2	-Rapid kits -IF	-$		-RT-PCR	Level 4	-
Regional Medical Research Centre, Dibrugarh	Human	BSL-2	-Rapid kits -IF	-			-	-
High Security Animal Disease Laboratory, Bhopal	Veterinary	BSL-4	-Rapid kits –IF -AGID	-MDCK -Eggs -Pathoge-necity testing by IVPI & multiple basic aa cleavage site of HA gene	-HA/ HAI for H5 -AGID -ELISA -NA subtyping -Western blot	-RT-PCR -Real time PCR -Sequencing	Level 5	-
Central Disease Diagnostics Lab, Indian Veterinary Research Institute, Bareilly	Veterinary	BSL-2 with BSL-3 precautions	-Rapid kits -IF	-$	-HA/ HAI for H5 -AGID -ELISA	-RT-PCR -Real-time PCR -Sequencing	Level 4	-
Regional Disease Diagnostic Lab, Bangalore	Veterinary	BSL-2	-Rapid kits -IF	-$	-HA/ HAI for H5	-RT-PCR	-	-
Regional Disease Diagnostic Lab, Pune	Veterinary	BSL-2	-Rapid kits -IF	-$	-HA/ HAI for H5	-RT-PCR	-	-

*WHO Global Influenza Surveillance Programme.

$ These laboratories undertake virus isolation in MDCK cell lines and embryonated eggs for seasonal influenza viruses but not for H5N1 due to lack of adequate containment facilities.

Annexures

Annex 1

Clinically suspect case of Human avian influenza H5N1

Collect clinical specimen & aliqouting

* Serum specimen (paired samples at least 14 days apart)

Respiratory specimen (Preferably more than one type of specimen from each patient)

IFA by polyclonal & monoclonal antibodies to respiratory panel

1

RT-PCR for M gene (Flu A) + H5 N1

2

Virus culture in Egg (preferable) and MDCK cells

Four-fold rise to H5 Present

Four-fold rise to H5 absent

Negative/ Positive

Negative/

Positive

Positive for H5N1

Negative for H5N1

Positive

Negative; correlate with other findings

Correlate with RT-PCR. If discrepant, look into QC

Look into QC; Correlate with culture

Repeat RNA extraction and RT-PCR

Refer pt. sample/Virus culture/RNA extract, to H5 reference lab

Confirm results by RT-PCR & culture; establish alternative diagnosis

Negative for H5N1

Positive for H5N1

Positive

Negative

RT-PCR Negative

2 RT-PCR Positive

Positive

Look into QC; Correlate with culture

Confirmed H5N1 infection

$Refer pt. sample/ Virus culture/ RNA extract to H5 reference lab

Negative; No H5N1 infection

Positive

Negative No H5N1 infection

Culture Negative for H5N1

Culture Positive for H5N1

Refer pt. sample/Virus culture/RNA extract, to H5 reference lab

Refer pt. sample/Virus culture/RNA extract, to H5 reference lab

Negative; No H5N1 infection

Confirmed H5N1 infection

Refer pt. sample, Virus culture, RNA extract, to H5 reference lab

* Utility of serology for identifying outbreaks is limited. Serological responses may not appear till the 3rd week of illness. However it helps in final exclusion of infection, or to maximize the case ascertainment rate in cases, especially for discrepant results Serology is best done demonstrating a rise in antibody between paired sera. Traditional haemagglutinion inhibition (HAI) is not suitable for sero-diagnosis of human H5N1 infection. Modified HAI tests for H5 using horse RBC is currently under evaluation but is not a confirmatory test by itself. Microneutralization tests are the method of choice and is best carried out in reference laboratories with adequate BSL-3 containment facilities and experience.

$To report first case in the country or a large geographic area, in a suspect or probable case, any of the following should be positive: Positive PCR* + serology (4 fold rise)/ Positive PCR* + culture/ Positive Culture/ Positive serology (4 fold rise)

1,2: in the hypothetical situation of very limited quantity of respiratory specimen for only single investigation, preference should be given to RT-PCR followed by virus isolation

Annex 2

STANDARD OPERATIVE GUIDELINES FOR HUMAN AND ANIMAL SPECIMEN COLLECTION, STORAGE, HANDLING AND SAFE TRANSPORT

Human Specimen

General information

Respiratory virus diagnosis depends on the collection of high-quality specimens, their rapid transport to the laboratory and appropriate storage before laboratory testing. Virus is best detected in specimens containing infected cells and secretions. Specimens for the direct detection of viral antigens or nucleic acids and virus isolation in cell cultures should be taken preferably during the first 3 days after onset of clinical symptoms.

Type of specimens

A variety of specimens is suitable for the diagnosis of virus infections of the **upper respiratory tract:**

- nasal swab
- nasopharyngeal swab
- nasopharyngeal aspirate
- nasal wash
- throat swab

In addition to swabs from the upper respiratory tract, invasive procedures can be performed for the diagnosis of virus infections of the lower respiratory tract where clinically indicated:

- transtracheal aspirate
- bronchoalveolar lavage
- lung biopsy
- post-mortem lung or tracheal tissue

Specimens for the laboratory diagnosis of avian influenza A should be collected in the following order of priority

1. nasopharyngeal aspirate
2. acute serum
3. convalescent serum

Specimens for direct detection of viral antigens by immunofluorescence staining of infected cells should be refrigerated and processed within 1–2 hours. 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 inoculated into susceptible cell cultures as soon as possible. If specimens cannot be processed within 48–72 hours, they should be kept frozen at or below –70°C.

Respiratory specimens should be collected and transported in virus transport media. A number of media that are satisfactory for the recovery of a wide variety of viruses are commercially available.

Procedures for specimen collection

Materials required

- Sputum/mucus trap
- Polyester fibre-tipped applicator
- Plastic vials
- Tongue depressor
- 15ml conical centrifuge tubes
- Specimen collection cup or Petri dishes
- Transfer pipettes

Virus transport medium

(A) Virus transportation medium for use in collecting throat and nasal swabs

1. Add 10g veal infusion broth and 2g bovine albumin fraction V to sterile distilled water (to 400ml).
2. Add 0.8ml gentamicin sulfate solution (50mg/ml) and 3.2ml amphotericin B (250µg/ml).
3. Sterilize by filtration.

Hanks BSS with BSA and antibiotics can also be used

(B) Nasal wash medium

1. Sterile saline (0.85% NaCl).

Preparing to collect specimens

Clinical specimens should be collected as described below and added to transport medium. Nasal or nasopharyngeal swabs can be combined in the same vial of virus transport medium. When possible, the following information should be recorded on the **Field Data Collection Form:** general patient information, type of specimens, date of collection, and contact information of person completing the form, etc.

Standard precautions should always be followed, and barrier protections applied whenever samples are obtained from patients.

Nasal swab

A dry polyester swab is inserted into the nostril, parallel to the palate, and left in place for a few seconds. It is then slowly withdrawn with a rotating motion. Specimens from both nostrils are obtained with the same swab. The tip of the swab is put into a plastic vial containing 2–3ml of virus transport medium and the applicator stick is broken off.

Nasopharyngeal swab

A flexible, fine-shafted polyester swab is inserted into the nostril and back to the nasopharynx and left in place for a few seconds. It is then slowly withdrawn with a rotating motion. A second swab should be used for the second nostril. The tip of the swab is put into a vial containing 2–3ml of virus transport medium and the shaft cut.

Nasopharyngeal aspirate

Nasopharyngeal secretions are aspirated through a catheter connected to a mucous 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. Mucous from the other nostril is collected with

the same catheter in a similar manner. After mucous has been collected from both nostrils, the catheter is flushed with 3ml of transport medium.

Nasal wash

The patient sits in a comfortable position with the head slightly tilted 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, 1–1.5ml 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 10–15ml of washing fluid has been used. Dilute approximately 3ml of washing fluid 1:2 in transport medium.

Throat swab

Both tonsils and the posterior pharynx are swabbed vigorously, and the swab is placed in transport medium as described above.

Sera collection for influenza diagnosis

An acute-phase serum specimen (3–5ml of whole blood) should be taken soon after onset of clinical symptoms and not later than 7 days after onset. A convalescent-phase serum specimen should be collected 14 days after the onset of symptoms. Where patients are near death, a second ante-mortem specimen should be collected.

Although single serum specimens may not provide conclusive evidence in support of an individual diagnosis, when taken more than 2 weeks after the onset of symptoms they can be useful for detecting antibodies against avian influenza viruses in a neutralization test.

Animal Specimen

General information

The success of virus diagnosis depends largely on the quality of the specimen and the conditions under which the specimen is transported and stored before it is processed in the laboratory. Specimens for isolation of respiratory viruses in cell cultures or embryonated chicken eggs and for the direct detection of viral antigen or nucleic acids should generally be taken during the first 3 days after onset of clinical symptoms of influenza. In mammals, including humans, pigs and horses, influenza is primarily a respiratory tract infection while in avian species it can be an infection of both the respiratory tract and the large intestinal tract.

Type of specimens

A variety of specimens from ammmals and birds is suitable for the diagnosis of virus infections of the **upper respiratory tract:**

- nasal swab
- throat swab
- tracheal swab

In addition to swabs from the upper respiratory tract, **sampling of avian species** for influenza infection should include:

- cloacal swab
- faecal specimen

Whenever possible, cloacal swabs should be collected from live or freshly killed birds. Faecal specimens collected from cages or from the environment are often the only specimens that are available and cannot be assigned with total certainty to the species of origin.

If dead animals are found as part of the investigation, highly pathogenic avian influenza virus should be suspected, and representative internal organs, including brain, spleen, heart, lung, pancreas, liver and kidney, should be sampled together with sampling of the respiratory and intestinal tracts. For flock diagnosis, tissue samples from 3–5 birds should be collected, while for swabs 20–30 samples (pooled 5/ tube)

Specimens for the laboratory diagnosis of influenza infection should be collected in the following order of priority

- ***From live animals***
  1. Tracheal swab
  2. Throat/nasal swab
  3. Cloacal swab
  4. Faeces (environmental)
  5. Drinking water

- ***From dead animals***
  1. Lung
  2. Pooled tissue (including trachea and lung)
  3. Feces (environmental)
  4. Cloacal
  5. Drinking water

Note

- *Do not pool tracheal and lung samples with faecal material*
- *Do not pool tissues from different birds.*

Procedures for specimen collection

Materials required

- 2–4ml screw-cap plastic tubes
- Polyester fibre-tipped swabs
- Viral transport medium
- Instruments for post-mortem examination

Virus transport medium

(A) Transport medium 199

1. Tissue culture medium 199 containing 1.0% bovine serum albumin (BSA)
2. To 1 litre of 1.0% BSA add:
   - benzylpenicillin (2 x 10^6 IU/litre)
   - streptomycin (200mg/litre)
   - polymyxin B (2 x 10^6 IU/litre)
   - gentamicin (250mg/litre)
   - nystatin (0.5 x 10^6 IU/litre)
   - ofloxacin hydrochloride (60mg/litre), and
   - sulfamethoxazole (0.2g/litre)

3. Sterilize by filtration and distribute in 1.0–2.0ml volumes in screw-capped tubes.

Note: With increasing use of antibiotics in animal husbandry it has become necessary to use high concentrations of antibacterial and antifungal agents.

- OR -

(B) Glycerol transport medium

1. Phosphate-buffered saline (PBS):

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
Distilled water	to make 1 litre

2. Autoclave PBS and mix 1:1 with sterile glycerol to make 1 litre
3. To 1 litre PBS/glycerol add:
   - benzylpenicillin (2 x 10^6 IU/litre)
   - streptomycin (200mg/litre)
   - polymyxin B (2 x 10^6 IU/litre)
   - gentamicin (250mg/litre)
   - nystatin (0.5 x 10^6 IU/litre)
   - ofloxacin hydrochloride (60mg/litre), and
   - sulfamethoxazole (0.2g/litre)

For the appropriate choice of medium see "Preparing to take specimens" section below.

Preparing specimen collection vials

To sterile plastic screw-cap vials dispense 1.0–2.0ml of transport medium. It is preferable to store these vials at –20°C until use. However, they can be stored at 4°C for 48–96 hours (optimally less than 48 hours) or at room temperature (22°C) for short periods of 1–2 days. For virus isolation, tissues/ swabs should not be kept at –20°C.

Preparing to collect specimens

Vials should be assigned a number that corresponds to that on the **Field Data Collection Sheet**. When possible, the following information should be recorded on the Field Data Collection Sheet: type of animal sampled, species, type of specimen, date of collection, and geographical location of specimen collection, etc.

Tissue culture medium (A) is widely used for collection and transport of clinical specimens from all species. The glycerol-based medium (B) provides longer-term stability of specimens where cooling is not immediately possible; it is suitable for egg inoculation but not suited for tissue culture inoculation.

Clinical specimens should be collected as described below and added to transport medium. All specimens should be kept on ice or at 4°C.

Key points to remember in collection, preservation and transport of animal specimens
Sample for antibody detection
• Serum—1–2ml • Plasma—1–2ml • Yolk—whole egg or 1ml of yolk with 1ml of PBS **Note:** *There is usually a negligible chance of detection of antibody in case of H5N1 due to high mortality*
Samples for virus isolation
Swabs: Swab the tracheal /oropharynx/cloaca with sterile Dacron swab Dispose swab contents in media and remove the swab Can pool 5 tracheal /oropharyngeal or cloacal swab ina single tube **Tissues:** Respiratory: Lungs, Trachea, air sac (posterior) Digestive: Liver, pancreas, small intestine, caecum, proventriculus, large intestine Urinary: kidney Lympho-reticular: spleen, bursa Cardiovascular: Heart Reproductive: ovary, oviduct Nervous system: brain

Dos of tissue collection	Don'ts of tissue collection
Pool tissues from an organ system Heart and spleen can be pooled Lungs and spleen can be pooled Liver and kidney can be pooled	Do not pool tissues from more than one bird Do not pool brain with any organ Do not pool digestive organ tissues with other organ tissues
Dos of swab collection	**Don'ts for swab collection**
• Trachea (tr) and oropharyngeal (op) swabs can be poled • Cloacal swab/faeces can be collected in one tube • Samples from 5 birds from the same flock can be pooled	• Do not pool tracheal/ oropharyngeal swabs with cloacal swabs
Preservatives for virus isolation	
• Tris-buffered tryptose broth	• Cell culture medium with 1.0% BSA (ph 7.2–7.4)
• Phospharte buffer saline (pH 7.2–7.4)	• Nutrient broth
• Peptone broth	• For swabs—2–4ml/tube
• For tissues—1:10 (V/V) ***Note:*** media should be sterile	
Sample preservation	
Serum: • Short term 4°C • Long term –20°C	Tissues/swabs: • At 4°C upto 72–96 hrs • At –70°C long-term storage • At –20°C not recommended
Stability of H5 N1 in faeces	
• Dry faeces at 25°C—1 day	• Wet faeces at 35°C—2 days • Wet faeces at 4°C—40 days

(Contd.......)

(Contd.......)

• Wet faeces at 25°C—8 days	
Number of samples to be collected:	
• Serum—20–30 samples/flock • Tissues—From 3–5 birds/flock • Swabs—20–30 (pooled 5/tube)	
Container and packing	
• Leak-proof primary container • Leak-proof secondary container • Sufficient absorbent matter(cotton) • Thermos flask • Thermocol box with ice or ice packs • Infectious materials in dry ice	
Precautions for sample collection	
• Personal protection equipment—gloves, mask, apron, shoe cover • Sterile tubes and media • Postmortem set. ***Note:*** *In case of strong suspicion for HPAI (H5N1), do not open birds. Pack in double- layered polythene bags and transport in thermocol box with ice or ice packs.*	

Information to accompany samples	
• Name and address	• Nature of farm-backyard/organized
• Age and sex of birds	• Population size
• History of vaccination	• Clinical signs
• Rate and pattern of mortality	• Type of sample

Standard precautions should always be followed, and barrier protections applied whenever samples are obtained from patients.

Nasal swab

A dry polyester swab is inserted into the nostril, parallel to the palate, and left in place for a few seconds. It is then slowly withdrawn, with a rotating motion, down the inside of the nose. Specimens from both nostrils are obtained with the same swab. The tip of the swab is put into a vial containing 2–3ml of transport medium and the applicator stick is broken off.

Throat swab

The posterior pharynx is swabbed vigorously, and the swab is placed in transport medium as described above.

Tracheal swab

The trachea of live birds is swabbed by inserting a polyester swab into the trachea and gently swabbing the wall. The swab is then placed in transport medium as described above.

Tracheal swabs from dead animals, including pigs at slaughterhouses, can be taken after removal of the lungs and trachea from the carcass. The trachea is held in a gloved hand and the swab inserted to its maximal length with vigorous swabbing of the wall. The swab is then placed in transport medium as above.

Cloacal swab

A cloacal swab from a live bird is taken by inserting a swab deeply into the vent and vigorously swabbing the wall. The swab should be deeply stained with faecal material. The swab is then placed in transport medium as above.

Faecal specimens

Faecal specimens from the cages of live poultry in bird markets or from wild birds in the field are collected from freshly deposited wet faeces; the swab should be heavily coated with faeces. The swab is then placed in transport medium as above.

Tissue specimens

Tissue specimens should ideally be frozen immediately, without transport medium, and later ground in transport medium before inoculation of eggs or tissue culture.

For diagnosis, an acute-phase serum specimen (3–5ml of whole blood) should be taken soon after onset of clinical symptoms, and not later than 7 days after onset. A convalescent-phase serum specimen should be collected 2–4 weeks later. In serological surveillance studies at slaughterhouses or of free-flying wild birds that are bled and released, a single sample of serum is collected. The blood is allowed to clot, then centrifuged at 2500 rpm for 15 minutes to separate red blood cells and serum. The serum should be pipetted off, and the red cells may be discarded. Serum samples are stored at –20°C.

Specimen storage

Specimens in viral transport medium for viral isolation should be kept at 4°C and transported to the laboratory promptly. If specimens are transported to the laboratory within 2 days, they may be kept at 4°C; otherwise they should be frozen at or below –70°C until they can be transported to the laboratory. Repeated freezing and thawing must be avoided to prevent loss of infectivity. Sera may be stored at 4°C for approximately one week, but thereafter should be frozen at –20°C.

Specimens should be collected and transported in a suitable transport medium on ice or in liquid nitrogen. Standard precautions should always be followed, and barrier protections applied whenever samples are obtained from patients. Specimens for influenza should not be stored or shipped in dry ice (solid carbon dioxide) unless they are sealed in glass or sealed, taped and double plastic-bagged. Carbon dioxide can rapidly inactivate influenza viruses if it gains access to the specimens through shrinkage of tubes during freezing.

Specimen transport

Transport of specimens should comply with *WHO guidelines for the safe transport of infectious substances and diagnostic specimens (WHO, 1997)*.

The receiving laboratory should be notified before shipment of specimens in order to arrange for an import licence for the specimens.

Transport of specimens within national borders should comply with the procedures detailed within each country's regulations.

International air transport of human specimens known or suspected to contain the avian influenza agent, or of specimens from avian influenza infected animals must follow the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulations.

- *Dangerous Goods Index*
- *Consignment of Diagnostic Specimens, 2003*.

The IATA Regulations, Consignment of Diagnostic Specimens, 2003 allow specimens known or suspected to contain the avian influenza agent to be transported as UN 3373 "diagnostic specimens" when they are transported for diagnostic or investigational purposes.

Specimens transported for any other purposes, and cultures (as defined in the IATA Regulations) prepared for the deliberate generation of pathogens, must be transported as UN 2814 or UN 2900, as appropriate.

All specimens to be transported (UN 3373, UN 2900, or UN 2814) must be packaged in triple packaging consisting of three packaging layers as indicated in the *Dangerous Goods Index*.

UN 3373, Diagnostic Specimens, shall be packed in good quality packaging, which shall be strong enough to withstand the shocks and loads normally encountered during transport. Packaging shall be constructed and closed so as to prevent any loss of contents that might be caused under normal conditions of transport, by vibration or by changes in temperature, humidity or pressure.

Primary receptacles shall be packed in secondary packaging in such a way that, under normal conditions of transport, they cannot break, be punctured or leak their contents into the secondary packaging. Secondary packaging shall be placed in a final outer package with suitable cushioning material. Any leakage of the contents shall not substantially impair the protective properties of the cushioning material or of the outer packaging.

For liquids

The primary receptacle(s) shall be leakproof and shall not contain more than 500ml. There shall be absorbent material placed between the primary receptacle and the secondary packaging; if several fragile primary receptacles are placed in a single secondary packaging, they shall be either individually wrapped or separated so as to prevent contact between them. The absorbent material shall be in sufficient quantity to absorb the entire contents of the primary receptacles and there shall be a secondary packaging that shall be leakproof. The primary receptacle or the secondary packaging shall be capable of withstanding without leakage an internal pressure producing a pressure differential of not less than 95 kPa (0.95 bar). The outer packaging shall not contain more than 4 litres.

For solids

The primary receptacle(s) shall be sift-proof and shall not contain more than 500g. If several fragile primary receptacles are placed in a single secondary packaging, they shall be either individually wrapped or separated so as to prevent contact between them and there shall be a secondary packaging which shall be leak proof. The outer packaging shall not contain more than 4kg.

For air transport, the smallest overall external dimension of a completed package must be at least 10cm.

Packaging must conform to certain performance standards.

For further information about definitions, packaging requirements, markings and labels, accompanying documentation, and refrigerants, please refer to the competent authority, current IATA shipping guidelines, commercial packaging suppliers, or available courier companies.

Processing and Storage of Specimens

Objectives

Procedure to be followed for processing and storing specimens of patients with acute respiratory tract infections for isolation of influenza viruses.

Requirements

Equipment	Consumables	Registers
Vortex mixer Laminar flow BSL-2 Centrifuge –70°C ultra freezer Refrigerator	Tissue paper, cotton wool, gloves, labels, mask, storage vials	Nominal register Requisition file Storage cards

Check the equipment before starting the processing — Safety cabinet, Vortex mixer, Centrifuge.

On receipt of the specimen
(Always wear lab coat, mask and gloves during sample processing)

- Open the icebox and confirm presence of ice in the box.
- Remove specimen collection vial with the specimen from the icebox and place on the paper towel.
- Wipe the collection vial with spirit swab.
- Sample is good if there is no leakage and cold chain is maintained. Arrange samples in a wire rack in an ice pan. (In case of leakage, transfer that sample to another container, in a BSL II cabinet. Pour ice and ice water in a boiling pan and boil the pan. Wipe the exterior surface of all the other collection vials and ice box with 1% hypo/spirit swab/Lysol and transfer the samples to the receiving carrier. Inform the Laboratory head).
- Check if the "Label" on the specimen collection vials bears the following details:
  - Field number
  - Date of collection
- Check that all mandatory columns in the consent form and clinical data sheet are properly filled.
- Assign lab number (based on current running number) on the clinical data sheet, consent form and specimen collection vial. Make specimen storage cards for each sample.
- Arrange 1.5ml storage vials on ice. Label each vial with lab number and field number (3 vials per samples).
- Switch on laminar UV for 15 min before starting the work.
- Vigorously agitate the sample vial with swab on vortex mixer. Express the fluid by squeezing the swab on the inner wall of the vial. Remove the swab from the collection vial and discard it in 1% hypochlorite solution/ boiling pan. Wipe gloves with spirit before handling the next sample. Change gloves if found contaminated while processing the samples.
- Repeat the same for all the specimens. Maintain cold chain while processing.
- Balance the tubes and place them in a Centrifuge. Centrifuge at 1000 rpm for 5 minutes.
- Switch off UV light from laminar flow and transfer material to laminar flow.
- Remove the supernatant using a 5.0ml pipette and transfer 1.0ml to each storage vial with respective lab number. Wipe gloves with spirit before handling the next sample Change gloves if found contaminated while processing the samples

- Repeat the same for all the specimens. Arrange the storage vials in storage box and store in –70°C freezer.
- Switch on laminar UV for 15 minutes after completion of work.
- Make entries in the specimen storage cards.
- Enter complete information from clinical data sheet and storage location in the computerized database.
- Discard the sample vials, tissue paper, cotton, pipettes in 1% Lysol solution and boil at the end of the day.

Safety condition to be followed while processing

- Handle fluids slowly and gently to avoid creating aerosols.
- At any stage — do not pour or decant — only pipettes are to be used.
- Do not overcrowd the cabinet and never obstruct the front opening. Organize the work area so that sterile reagents and samples do not come into contact with each other (pots for liquid waste to the left, and sterile reagents to the right with samples handled centrally).
- Clean and decontaminate the inner surfaces of the cabinet with 1% hypochlorite/spirit swab/Lysol after every working session.
- In case of spill during transfer, shaking or centrifuging, wipe the spot thoroughly with cotton dipped in 1% hypochlorite/spirit swab/Lysol and wipe dry.
- In case of breakage/leak into the centrifuge: Remove the centrifuge bucket, place in 1% hypochlorite for half an hour, wash thoroughly in running water, dry and replace. Inform the Laboratory head.

Source

- WHO Reference Laboratory for influenza A/H5 infection (Hong Kong);
- http://www.who.int/csr/disease/avian_influenza/guidelines/humanspecimens/en/index.html
- http://www.who.int/csr/disease/avian_influenza/guidelines/animalspecimens/en/index.htmlhttp://www.who.int/csr/disease/avian_influenza/guidelines/transport/en/index.html
- http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/index.html
- http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf;
- WHO Manual on animal influenza diagnosis and surveillance, September 2004 (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf)
- National Institute of Virology (Pune), Indian Council of Medical Research
- High Security Animal Disease Laboratory (Bhopal), Indian Council of Agricultural Research

Annex 3

STANDARD OPERATIVE GUIDELINES FOR IMMUNOFLOURESCENCE TEST FOR INFLUENZA A (H5N1)

Identification of Respiratory Virus Infection by Immunofluorescence Test

Introduction

Respiratory virus infections are associated with major outbreaks of respiratory diseases throughout the world which have significant impact on world health. Viruses cause disease in all age groups but infections are most severe in infant, elderly and immunocompromised populations leading to hospitalization of patients. Nosocomial outbreaks can occur in paediatric and geriatric wards resulting in extended hospitalization, prolonged patient management and increased morbidity and mortality. Respiratory virus infections in infants may cause obstruction of airway and lead to respiratory distress.

The methods used for diagnosis of acute respiratory virus infections include direct testing of specimens such as nasopharyngeal aspirates by immunofluorescence and/or enzyme immunoassays for detection of viral proteins, isolation and identification of virus in cell culture mono-layers or detection of immune response by serological techniques. Isolation of respiratory viruses by traditional culture method takes a few days to provide result. Immunofluorescence (IF) screening test utilizing specific monoclonal antibodies to detect viral antigens in clinical specimen directly and hence provides a rapid and less time consuming method for diagnosis of respiratory infection.

Principle

Immunofluorescence (IF) allows the visual localization of viral antigens on or within infected cells and is an essential tool in the diagnostic virology laboratory for the identification of viral isolates and the detection of viral antigens in clinical samples. The IF assay may be either direct or indirect. With direct IF, virus-infected cells are overlaid with a fluorochrome-labeled (conjugated) antiviral antibody. After a single incubation and washing step, the cells are ready for observation. In the indirect format, the cells are first overlaid with an unlabelled antiviral antibody. After incubation and washing step, the cells are re-incubated with a fluorochrome-labeled antibody directed against the immunoglobulins of the species supplying the primary antibody. A counterstain is usually included to diminish nonspecific staining and to give good contrast to negative cells. The “stained” slides are then examined under a fluorescence microscope at a wavelength specified by the fluorochrome.

Clinical significance

A. Respiratory Syncytial Virus (RSV)

Respiratory Syncytial Virus (RSV) is an enveloped, spherical, RNA virus of the family *Paramyxoviridae* and classified in the genus *Pneumovirus*. It is the most common viral agent causing lower respiratory tract illness in infancy. Virus transmission has been documented to occur most commonly through contact with infected droplets, most often in fomites.

All age groups are susceptible to RSV and the common syndromes include bronchiolitis and pneumonia. The former is a wheezing-associated illness preceded by 3–5 days of upper respiratory tract symptoms. Wheezing and thoracic hyperinflation are prominent. Patients with pneumonia also present after several days of upper respiratory tract symptoms, and crackles are heard on auscultation of the chest. Frequently, the two syndromes overlap. Significant morbidity and mortality may occur in RSV infection of the elderly. Infants with underlying complications such as congenital heart disease, bronchopulmonary dysplasia and congenital or other immunodeficiencies may be susceptible to severe, sometimes life-threatening.

B. Adenovirus (Ad)

Adenoviruses (Ad) are non-enveloped DNA viruses and classified under the family of *Adenoviridae*, genus Mastadenovirus. At least 47 known serotypes of human *adenovirus* have been identified. *Adenoviruses* illnesses are endemic throughout the year and occur in all age groups. *Adenoviruses* cause localized outbreaks of respiratory disease, swimming pool associated pharyngoconjunctival fever (PCF), and epidemic keratoconjunctivitis (EKC) associated with industrial eye trauma or ophthalmologic procedures.

Upper respiratory illness caused by adenoviruses can take the form of pharyngitis or tonsillitis and occurs chiefly in infants and young children. It is associated primarily with types 1 to 7. Findings include coryza, fever, coughing, exudates on the pharyngeal walls, a granular appearance of mucosa, and tender, enlarged cervical nodes. Lower respiratory illness, including bronchitis, bronchiolitis, and pneumonia, often complicates adenovirus infection. Extrapulmonary signs such as kidney and liver involvement and encephalomeningitis can occur, especially in infants and immunocompromised patients, and in such patients, the generalized disease usually has a fatal outcome.

C. Parainfluenza viruses (Pa)

Parainfluenza viruses (Pa) belong to the family *Paramyxoviridae*. The four parainfluenza viruses of humans cause upper respiratory disease in children and adults. PIVs are transmitted via infected respiratory secretions through close person-to-person contact and aerosols. Parainfluenza type 1, 2 and 3 (P1, P2 and P3) are the causative agent for laryngotracheobronchitis while Parainfluenza type 4 (P4) may also cause mild upper respiratory disease but rarely encountered. P1 and P2 occurs in children less than 5 years of age. Type 3 infections produces the most severe illness in infants less than 1 year old. P3 is second only to Respiratory Syncytial virus (RSV) in importance as a cause of bronchiolitis and pneumonia in infants.

D. Influenza A (A) and Influenza B (B) viruses

Influenza is an acute respiratory disease, may spread rapidly and pervasively through a population. Influenza viruses are classified under the family of *Orthomyxoviridae*, and subdivided into types A (A), type B (B) and type C (C) according to the antigenic differences in the nucleoprotein and the matrix protein. Influenza A viruses are further classified into subtypes according to the properties of their major membrane glycoprotein, the haemagglutinin and the neuraminidase.

Transmission of influenza virus infection occurs through inhalation of virus-laden droplets from respiratory secretions of symptomatic or asymptomatic carriers. Environmental conditions such as crowding enhance transmission of infection.

Influenza virus replicates in the columnar epithelial cells of the respiratory tract resulting in necrosis and sloughing of cells. From there, the virus gains access to respiratory secretions and is spread by

small-particle aerosols expelled into the air during sneezing, coughing, and speaking. Transmission may also occur by direct contact.

Influenza often begins after an incubation period of 1 to 4 days with sudden onset of fever that may be accompanied by sore throat, dry cough, headache, myalgia, malaise or anorexia. The spectrum of illness ranges from asymptomatic infection or mild pharyngitis to pneumonia with fatal outcome. Otitis media is a common complication of influenza in children. The most severe complications contributing to the mortality associated with influenza are lower respiratory tract infections, which may present as primary viral pneumonia, mixed viral and bacterial pneumonia, or secondary bacterial pneumonia.

If a laboratory result is available early during the course of infection, the patient may benefit from specific antiviral treatment with amantadine or rimantadine. A diagnostic result obtained soon after the onset of illness also may help prevent spread of the virus to contacts.

Specimen

A. Specimen type and specimen collection

1. **Nasopharyngeal wash (NPW); Nasopharyngeal aspirate (NPA) or Nasopharyngeal swab (NPS)**

   NPW are obtained by placing a 1-oz tapered rubber bulb syringe containing 5-7ml of sterile saline solution into and occluding the nostril and then rapidly squeezing and releasing the bulb. Fluid recovered in this bulb is emptied into a container containing viral transport medium (VTM, **Appendix A**).

   NPA are obtained by attaching a size 5 or 8 French polyethylene paediatric feeding tube to a DeLee collection trap, passing the tube into the nasopharynx, and applying suction by wall suction or with a manual suction pump. Recovery of secretion is greater if transport medium is rinsed through the feeding tube into the specimen trap. A curved wire or straight stick swab containing a dry tip is inserted into one or both nostrils to the nasopharyngeal area and pressed against the nasal wall. After a moment to permit the swab to absorb secretions, the swab is rotated two to three times and withdrawn. The NPS is then placed in VTM, the shaft is broken, and the vial is capped.

2. **Sputum**

   Instruct patient to cough deeply to produce a lower respiratory specimen. Collect in a sterile container. VTM is then added.

3. **Bronchial alveolar Larvage (BAL), Tracheal aspirate (TA), Endotracheal aspirate (ETA) or Endotracheal Tube (ETT)**

   Collect aspirate with a sterile disposable tracheal suction kit, and add VTM.

   Suitability of specimen The quality of each specimen must be assessed as it is of paramount importance that the cells present are of the type required and that they are present in sufficient numbers for the specimen to be suitable for DIF diagnosis.

4. **NPA, NPS and NPW**

   The cells which show virus infection are respiratory nasopharyngeal epithelial cells **(NPCs)** or ciliated cells (CILs) if present. The NPA should contain a sufficient number of NPCs or CILs. If mostly (or all) squamous cells **(SQCs)** are seen the DIA diagnosis is compromised, as the specimen has not come from the nasopharynx. An assessment is made as to whether a reasonable number

of RECs or CILs is also present or not. If the latter this is recorded, e.g. "probably negative but all squamous cells".

5. **Sputum**
   It may contain either a mixture of NPCs and SQCs or only the latter. Assess suitability as in NPA.

6. **BAL, TA, ETA or ETT**
   These specimens will usually contain the cells suitable for diagnosis, assess whether in sufficient numbers for DIT diagnosis. The presence of debris, red blood cells, fluorescing bacteria, hyphae, yeast, etc, or unusual findings in any specimen are always recorded.

B. Specimen transportation

Specimens taken from respiratory tract or vesicle should be delivered to the laboratory as soon as possible. If storage is necessary, they should be kept at 4°C. Smears of cellular material from skin lesion, vesicle fluid or blister fluid should be hand carried to the laboratory immediately for processing. If the slides cannot be delivered immediately, it should be air dried and then flooded with 0.5ml of acetone. After the acetone evaporates, the slide can be refrigerated at 2–8°C for storage. For longer holding periods, freezing at –70°C is required. For best results, the refrigeration time should be minimized.

C. Specimen handling

Spare specimens are kept at cold room, 4°C, for 2 months before discard.

Spillage and contamination of laboratory surface must be decontaminated immediately. The standard disinfectant recommended for decontaminated surface is 1 in 10 diluted Clorox.

If stained slides are not read immediately, store at 2–4°C in the dark for up to 24 hours. All slides are kept in slide folder at room temperature for approximately two months before disposal.

Workflow

Workflow for respiratory specimen processing

For detection of respiratory virus antigen, do **indirect IF** using **Imagen™ Respiratory screen reagent (Dako Pool) monoclonal antibodies** against seven viruses: RSV, Adenovirus, Parainfluenza 1, 2, and 3, Influenza A and B. If Dako pool is positive or quantity insufficient (QI), do individual viral typing by **direct IF** using **Imagen™ typing reagents.** If results are indeterminate, proceed to Chemicon staining. If specimen consistently quantity insufficient, report quantity insufficient on each antigen.

Respiratory virus panel includes 7 viruses: Respiratory Syncytial virus (RSV), Adenovirus (Ad), Influenza A virus (A), Influenza B virus (B), Parainfluenza virus type 1 (P1), 2 (P2) and 3 (P3).

Reagents and materials

A. Reagents

- Viral Transport Medium (VTM)
- 1 in 10 diluted Clorox. Freshly prepared every day; for biohazardous material disposal and specimen spillage decontamination.
- Acetone.

- Water (milli-Q purified); sterilize by autoclave at 121°C for 15 minutes. Kept at room temperature. Shelf life is 1 year. Prepared by IF staff.
- pH 7.5 1X Phosphate buffer solution (PBS): Prepared 10X PBS by dissolving 100 phosphate tablets in 1L water, autoclaving at 112°C for 15 minutes. Dilute 1 in 10 of the 10X PBS with water. Shelf life is 1 year. Kept at room temperature. Prepared by IF staff, ask medium staff to generate one lot number.

B. Materials

1. Gloves
2. Plastic jar with 1 in 10 dilution clorox solution for disposal of infectious materials
3. Plastic jar with plastic bags for disposal of plastic disposable pipette
4. 1ml sterile disposable pipette for specimen disaggregate
5. 1ml non sterile disposable pipette for specimen processing
6. 15ml falcon tubes
7. 10 wells nonfluorescent Telfon-coated slide for making smears
8. Centrifuge
9. Coplin jar
10. Pipette
11. Pipette tips
12. Bijou bottles
13. Plastic moist chamber
14. Slide holder
15. Coverslips (18X18mm or 18X24mm)
16. Control smear included in each kit

C. Equipments

1. Class I biological safety cabinet
2. Centrifuge
3. 37°C incubator
4. Hot plate for drying smear
5. 4°C refrigerator
6. –70°C refrigerator
7. Fluorescence microscope with 10X and 40X objective

Required reagents

A. Staining reagents

All staining reagents should be stored at 4°C, and left at room temperature (15–30°C) for 5 minutes before use.

1. Imagen™ Respiratory Screen (Dako Pool) kit [catalogue number: Dako K6120]
2. Imagen™ Respiratory Syncytial Virus (RSV) [catalogue number: Dako K6102]
3. Imagen™ Adenovirus (Ad) [catalogue number: Dako K6100]
4. Imagen™ Parainfluenza Virus group (Pa) [catalogue number: Dako K6103]
5. Imagen™ Parainfluenza Virus typing (P1, P2 and P3) [catalogue number: Dako K6104]
6. Imagen™ Influenza A (A) [catalogue number: Dako K6105]
7. Imagen™ Influenza B (B) [catalogue number: Dako K6105]
8. Light Diagnostic® RSV (direct) [catalogue number: Chemicon: 5022]
9. Light Diagnostic® Adenovirus (direct) [catalogue number: Chemicon: 5000]

10. Light Diagnostic® Parainfluenza type 1 [catalogue number: Chemicon: 5019]
11. Light Diagnostic® Parainfluenza type 2 [catalogue number: Chemicon: 5020]
12. Light Diagnostic® Parainfluenza type 3 [catalogue number: Chemicon: 5021]
13. Light Diagnostic® Influenza A (direct) [catalogue number: Chemicon: 5017]
14. Light Diagnostic® Influenza B (direct) [catalogue number: Chemicon: 5018]
15. Dako Mouse immunoglobulin/FITC [catalogue number: Dako F0313]

Mounting fluid

All mounting fluids should stored at 4°C refrigerator, and left at room temperature (15-30°C) for 5 minutes before use.

16. Imagen ImagenTM Mounting fluid [catalogue number: Dako K6120]
17. Adenovirus mounting fluid [catalogue number: Dako 6100]
18. DAKO mounting medium [catalogue number: Dako S3023]
19. Chemicon Mounting fluid

B. Directingen Flu A detection kit

[catalogue number: Becton Dickinson 8560-20]. Stored at room temperature. (Appendix B)

Quality control

A. Quality control of respiratory reagents

1. External quality control program of the bench

- Perform CAP QC program, two to three times per year. Both Dako and Chemicon reagents are tested
- Record the results in the Viral antigen detection worksheet (Appendix C), the worksheet is kept in the IF worksheet box file.
- Any trouble shooting and remedy actions should also be recorded.

2. External quality control of reagent

- Whenever a new lot number of reagent or a new shipment of reagent (Dako Pool, Dako typing or Chemicon reagent) is received, record the number of reagent received, lot number of the reagent, receiving date and expiry date in the Dako/Chemicon Reagent stock-list and quality control worksheet (Appendix D), fill in the appropriate column.
- Perform quality control of the new shipment/lot number reagents by staining an in-house external control smear. (Preparation of in-house control smear is described in Appendix E).
- Record the result in Dak/Chemicon Reagent stock-list and quality control worksheet Appendix D.

3. Daily quality control of reagents

- Perform QC daily by using either internal or external control slides. Perform QC when Chemicon reagent is used (may not be daily).
- Each Dako Pool reagent kit provides five control slides coated with infected cells and non-infected cell for Dako respiratory screening reagent. Each reagent kit provides three control slides coated with the positive antigens
- Dako Control slides should be left in its foil wrapper for 5 minutes at room temperature should. If external in-house QC slides are used, take it out from –70°C refrigerator, let it reach room temperature.
- Perform QC parallel with other specimens.

- Record the results in Daily quality control worksheet (Appendix F).
- Any trouble shooting and remedy action should also be recorded.
- Precaution and Performance limitation are listed in Appendix G.

Procedure

A. Preparation of smear

1. **From respiratory tract specimen**
   - In Class I safety cabinet, use sterile disposable pipette to add in VTM to make up volume to 5ml if specimen volume <5ml in the aspirator.
   - Using a 1ml sterile disposable plastic pipette, disaggregate aspirated cells by pipetting the specimen up and down several times.
   - Label 15ml falcon tube with appropriated lab number. Aspirate half volume of specimen into a 15ml falcon tube.
   - From the aspirator, transfer about 1ml specimens to bijou bottle for keeping (done in Class I safety cabinet). The remaining specimen 1ml is then matched with the original form and send to DH Government Virus Unit for viral culture as soon as possible.
   - Fill up the falcon tube to 5ml with PBS, pipette up and down several times by non-sterile disposable pipette.
   - If mucous is not observed, jump to step (9) in this section.
   - If mucous is present, stand for 2 minutes to settle down the large aggregates or clumps.
   - Transfer supernatant to another falcon tube without disturbing the loose aggregates. Discard the original falcon tube.
   - Centrifuge at 2,000 rpm for 10 minutes at room temperature.
   - Discard supernatant in diluted clorox and resuspend the cell deposit in sufficient PBS (usually 0.1–0.5ml) to dilute any remaining mucous while at the same time maintaining a high cell density. If mucous is still observed, repeat steps (5) to (9) in this section.
   - Label the slides with appropriate LN/LNs.
   - Coat 1 drop (~25µL or according to the density of cells in the sediment) of re-suspended cells onto a well of Teflon-coated slides. At the same time, coat all the wells with cell sediment in another slide for typing.
   - For BAL, TA, ETA or ETT, coat 1 more well on another slide for HSV direct IF test.
   - After put up, the cell sediments and spare specimens are kept at cold room at 4°C. Cell sediments will discard every alternative day and spare specimen will discard two months later.

2. **Fixation of smears**
   - All the smears are allowed to dry up on a 30°C heating block. Transfer smears to Coplin jar, cover coplin jar with glass cover. Transfer the smears to cup cupboard.
   - Fix in fresh acetone for 10 minutes at room temperature. Acetone is always put inside fume cupboard.
   - Take out the slides from acetone; let it dry up for a while inside the fume cupboard. The slides are ready for staining.
   - If the slides are not stained immediately, store at 4°C overnight or –70°C for longer storage period.
   - Put unstained spare smears inside plastic slide holders, kept at –70°C for two months before disposal.

B. Staining of antigens

1. **Indirect Immunofluorescence Test (IDIT) by Dako Imagen Respiratory Screen**
    - Put all the slides inside a plastic moist chamber (Add small amount of water to the paper towel inside). For each well, add 10μL of Screening Reagent.
    - Incubate at 37°C for 20 minutes. Do not allow the reagents to dry on the specimen, as this will cause the appearance of non-specific staining.
    - Take out the slide from the moist chamber and dip in PBS twice. Immerse the slide into another Coplin jar with PBS for 2 minutes.
    - Drain off excessive PBS.
    - Allow it to dry on a 30°C heating block.
    - Put the slide back into the plastic moist chamber. Counter stain each well with 10μL Imagen conjugate FITC.
    - Incubate at 37°C for 15 minutes. Do not allow the reagents to dry on the specimen, as this will cause the appearance of non-specific staining.
    - Repeat washing step (c) to (e) as above.

2. **Direct Immunofluorescence Test (DIT) by Imagen typing reagents**
    - Place the slides inside a plastic moist chamber (Add small amount of water to the paper towel inside). Apply 10μl of each DAKO FITC-conjugated monoclonal antibodies (include RSV, Ad, A, B, Pa or P1, P2 and P3 respectively) to 5 or 7 wells.
    - Incubate at 37°C for 15 minutes. Do not allow the reagents to dry on the specimen, as this will cause the appearance of non-specific staining.
    - Take out the slide from the moist chamber and dip in PBS twice. Immerse the slide into another Coplin jar with PBS for 2 minutes.
    - Drain off excess buffer from the slides.
    - Allow the slide to dry on a 30°C heating block.

3. **Direct Immunofluorescence Test (DIT) by Chemicon typing reagents**
    - Place the slide inside a plastic moist chamber (Add small amount of water to the paper towel inside). Apply 10μL of each Chemicon FITC-conjugated monoclonal antibodies (including RSV, Ad, P1, P2, P3, A and B) to seven wells respectively.
    - Incubate at 37°C for 30 minutes. Do not allow the reagents to dry on the specimen, as this will cause the appearance of non-specific staining.
    - Take out the slide and dip in PBS twice, and then immerse the slide into another Coplin jar with PBS for 5 minutes.
    - Drain off excessive PBS from the slide.
    - Allow the slide to dry on a 30°C heating block.

C. Examination of smear

- For best results, slides should be read immediately after staining, but slides can be stored at 2–8°C in the dark for up to 24 hours.
- Add a drop of appropriate mounting fluid (according to Table 1) to each spot and lay a coverslip on the top.

Table 1. Appropriate mounting fluid use for IF

Staining method	Mounting fluid
IDIT by Dako Pool reagents	ImagenTM mounting fluid
DIT by Dako typing reagents RSV, Pa, P1, P2, P3, A and B	ImagenTM mounting fluid
DIT by Dako Ad typing reagent	ImagenTM Adeno mounting fluid
DIT by Chemicon typing reagent	Chemicon mounting fluid

- Wait 5 minutes and examine under an UV Epi-fluorescence microscope.
- Use a 10X objective to focus the cells first and then screen the whole well of each spot under a 40X objective.
- Record the number of different cell types found especially columnar nasopharyngeal epithelial cells (NPs); squamous cells (SQs); any positive IF staining cells; density of positive IF cells (+ve cells); intensity of fluorescence (IF), following up and results on the Viral antigen detection worksheet (Appendix C).
- After examination, the slides are kept inside slides folder at room temperature for about 2 months before disposal.

Interpretation and recording results

A. Interpretation of result

1. **Respiratory viral antigen detection**
    - Intracellular nuclear and/or cytoplasmic granular apple-green fluorescence is seen in epithelial cells. They are regard as positive cells. Negative cells stained red with the Evan's blue counterstain.
    - Differentiation of positive cells:
2. **Dako Pool**
    - Positive cells are either fine granular cytoplasmic fluorescent particles or both nuclear and cytoplasmic particles can be seen.
3. **Respiratory Syncytial Viruses (RSV)**
    - Positive cell is cytoplasmic usually large inclusion like bodies and fine particulate fluorescence. Positive cells vary in size from large (sometimes syncytia) to small.
4. **Influenza A (A)**
    - Both nuclear and cytoplasmic fluorescent particles are seen. Frequently the nucleus is more densely stained, sometimes only nuclear fluorescence is seen.
5. **Influenza B (B)**
    - Appearance is very similar to Influenza A. That is both nuclear and cytoplasmic fluorescent particles are seen and the nucleus is often more densely stained.
6. **Adenovirus (Ad)**
    - Both nuclear often coarse and cytoplasmic fluorescent particles are seen and the nucleus invariably more densely stained.
7. **Parainfluenza group (Pa); Parainfluenza 1, 2 and 3 (P1, P2 and P3)**
    - Positive cells are cytoplasmic often similar in appearance with RSV (particularly P3) with large inclusion-like bodies and fine particulate fluorescence.

B. Recording results

1. **Grading of cell density**
   - **Grading given to respiratory specimen**
     - Results are record in viral antigen detection worksheet. Lot number for all reagents are refer to the Dako/Chemicon reagent stock-list and quality control worksheet (Appendix D).
     - Record number of squamous epithelial cells (SQCs) according to Table 2.

Table 2. Grading of Squamous epithelial cells (SQCs)

Number of SQCs seen per field under a 40X objective	Grading given
< 10	Scanty (S)
10–19	+
20–29	++
30	+++
>30	>+++

- Record number of nasopharyngeal columnar cells according to Table 3.

Table 3. Grading of respiratory nasopharyngeal columnar cells (NPCs).

Number of NPCs seen per field under a 40X objective	Grading given	Interpretation
< 1 (total cells <20 cells in whole well)	QI	Quantity insufficient
<10 (total cells ≥20 cells in whole well)	S	Scanty
10 –19	+	--
20–29	++	--
≥30	+++	--
If only squamous cells seen and cells number is ≤20 cells	NPR	Mainly squamous epithelial cells seen

- If the total number of respiratory nasopharyngeal columnar epithelial cell (NPCs) in a negative smears is less than 20, the specimen is graded as quantity insufficient (QI)
- At least 20 uninfected NPCs must be obtained in the whole well before a negative result is reported.
- For other specimens, e.g. sputum, it is recommended that at least 50 uninfected NPCs are observed before a negative result is reported.
- If only squamous cells (SQCs) seen and not enough NPCs, report probably negative (NPR).

2. **Grading of positive cells (PCs)**
   - Record number of positive cells (PCs) according to Table 4.

Table 4. Grading of positive cells (PCs)

Number of positive cells (PCs) per field under a 40 X objective	Grading given
<1	<+
1	+
2	++
≥3	+++

3. **Grading of intensity of fluorescence (IF)**
   - Record intensity of fluorescence (IF) according to Table 5.

Table 5. Grading of intensity of fluorescence (IF)

Intensity of fluorescence (IF)	Grading given
Weak	<+
Easily seen	+
Strong	++
Very strong	+++

C. Recording results and following up

1. **Recording results and follow up of respiratory tract specimen**
   - Following up of Dako Pool typing
   - Follow-up after Dako Pool staining according to Table 6.

Table 6. Follow-up after Dako pool staining

Staining	IF result	Equivalent result	Following up
Dako Pool	-	Negative	Ready to report
Dako Pool	+	Positive	Proceed to Dako typing
Dako Pool	?	Doubtful	Proceed to Dako typing

   - Following-up of Dako typing
   - Follow-up after Dako typing according to Table 7.

Table 7. Follow-up after Dako typing

Staining	IF result	Equivalent result	Following up
Dako typing	-	Negative	Ready to report
Dako typing	+	Positive	Ready to report
Dako typing	?	Doubtful	Proceed to Chemicon typing

   - Following-up of Chemicon typing
   - Follow-up of after Chemicon typing according to Table 8.

Table 8. Follow up after Chemicon typing

Staining	IF results	Equivalent result	Following up
Chemicon typing	-	Negative	Ready to report
Chemicon typing	+	Positive	Ready to report
Chemicon typing	?	Doubtful	Report equivocal, E

Workflow shortcut

Workflow shortcut for respiratory tract viruses detection

Specimen collection
↓
Preparation of smears
↓
Fixation of smears
↓
Staining Dako Pool
↓
Examination of smears
↓
Record results → (-ve) → Report negative
↓ +ve
Staining typing reagent
↓
Examination of smears
↓
Record results → (+ve) → Report positive
↓ doubtful
Staining Chemicon reagent
↓
Examination of smears
↓
Record results
↓
Report positive or negative result

Viral antigen detection worksheet

Date: ___/___/______

Write down “P” for positive and “N” for negative result in the result column.

Please refer to Tables 4–8 for format of recording result.

Performed by: ______________________ Checked by: ____________________

LN	Dako Pool					Follow up	Dako typing				Positive Virus(es)	Remarks
	Grading of				Result		Grading of					
	SOCs	NPCs	PCs	IF			SOCs	NPCs	PCs	IF		
1						RSV— A—B— Ad—Pa P1—P2 —P3						
2						RSV—A— B—Ad—Pa P1—P2— P3						
3						RSV—A— B—Ad—Pa P1—P2— P3						
4						RSV—A— B—Ad—Pa P1—P2— P3						
5						RSV—A— B—Ad—Pa P1—P2— P3						
6						RSV—A— B—Ad—Pa P1—P2— P3						
7						RSV—A— B—Ad—Pa P1—P2— P3						
8						RSV—A— B—Ad—Pa P1—P2— P3						

Dako Reagent / Chemicon reagent stock-list and QC worksheet

Please write down "P" for positive and "N" for negative result in QC result column and ignore inappropriate column

Reagent: ______________________ Catalogue number: ____________________

Receiving Date	Received by	Lot No. on the box	Lot No. on the bottle	Expiry date on the bottle	Opening date	External Control smears Lot No.	Opened and performed QC by	QC result	Trouble shooting and remedy action

Preparation of in-house external quality control slides

1. Passage of cell lines

- **Media, reagents, instruments and materials**
  1. Details of preparation and quality control of reagents and media are illustrated in the Rapid Viral Culture bench book.
  2. MDCK, HEL, and HEP-2C cell lines
  3. 10% Growth medium (MEM with 10% FCS & antibiotics). Stored at 4°C
  4. 1% Maintenance medium (MEM with 1% FCS & antibiotics). Stored at 4°C.
  5. Trypsin – Versene (0.125% trypsin + 0.02% EDTA). Stored at –20°C
  6. 1X PBS
  7. Sterile disposable 10ml Pippette
  8. Class II safety cabinet
  9. Inverted microscope
  10. 75cm^2 Greniner sterile plastic bottles for cell culture.

- **Procedures**
  1. Examine the culture flasks with confluent monolayer absence of bacterial contamination using an inverted microscope prior use.
  2. Warm up medium and reagents to 37°C before use.
  3. Remove medium from the flask by aspiration, and wash the cell layer twice with 10ml PBS. Gently rotate the flask back and forth to wash away the residual medium from the cells, because medium can inactivate the action of trypsin-EDTA.
  4. Aspirate out PBS from the flask.
  5. Add 2ml trypsin-EDTA to the flask to completely cover the cell monolayer, and incubate at 37°C for 10 minutes. (For MDCK cell line, extend the incubation time up to 20 to 30 minutes)
  6. Observe the cells under inverted microscope. When the cells begin detaching from the surface of the flask, hold the flask and slap the side of the flask with hand to dislodge the cells completely. Do not trypsinize cells for longer than needed. Make sure all cells are loose.
  7. Add 10ml 10% FCS MEM to the cell suspension to inhibit the action of trypsin-EDTA.
  8. Gently pipette up and down to produce an even suspension. Avoid too many bubbles.
  9. Transfer 1ml of the resuspended cells into a new flask, add 14ml of 10% FCS MEM.
  10. Label the preparation date, name of cell line, cell line passage number on the side of the flask.
  11. Incubate the flask at 37°C, 5% CO_2 incubator in a flat position.Observe the cells at 24 hour for growth and any contamination.
  12. Observe the cells at 24 hours for growth and any contamination.
  13. Change with 1% maintenance medium while the flask with confluent monolayer of cells.

2. **Preparation of in-house viral isolates**
   - **Media, reagents, instrument and materials**
     1. Positive specimen or viral infected cell lysate of 7 respiratory virus. (RSV, Adenoviruses, Influenza A, Influenza B, Parainfluenza 1, 2 and 3). These wild strains are confirmed by conventional viral culture and immunofluorescence staining method.
     2. MDCK, HEL, and HEP-2C cell lines
     3. 10% Growth medium (MEM with 10% FCS and antibiotics). Stored at 4°C.
     4. 1% Maintenance medium (MEM with 1% FCS and antibiotics).
     5. 0% Maintenance medium for Flu A and B (MEM with 0% FCS with antibiotics). Stored at 4°C.
     6. Trypsin – Versene (0.125% trypsin + 0.02% EDTA). Stored at –20°C.
     7. 1X PBS.
     8. Sterile disposable 10ml Pippette.
     9. Class II safety cabinet.
     10. Inverted microscope.
     11. Acetone.
     12. 10 well Teflon-coated slides.
     13. 75cm 2 Greniner sterile plastic flasks for cell culture.
     14. Heating block (<30°C)
     15. TPCK Trypsin solution in PBS (Sigma T-8802, 5mg resuspended in 5ml PBS, TPCK treated, from Bovine Pancreas). Stored at –20°C.
     16. 0% Maintenance medium for Flu A and B supplemented with TPCK – Trypsin solution. – 100ml 0% Maintenance medium for Flu A and B added with 0.2ml TPCK-Trypsin solution. {0.2% TPCK-trypsin Serum Free Medium (SFM)}
     17. 0% Maintenance medium for Parainfluenza 1, 2 and 3 supplemented with TPCK-Trypsin solution – 100ml for 0% Maintenance medium added with 0.025ml TPCK-Trypsin solution {0.025% TPCK-trypsin serum Free Medium (SFM)}

- **Procedures**
  1. Examine the culture flasks with confluent monolayer absence of bacterial contamination using an inverted microscope prior use.
  2. Remove medium from the flask by aspiration, and wash the cell layer twice with 10ml PBS. Gently rotate the flask back and forth to wash away the residual medium from the cells.
  3. Aspirate out PBS from the flask.
  4. Infect cell lines with different viruses according to the following table.

Cell lines	Infected by
MDCK	Flu A, Flu B, P1, P2 and P3
HEL	Adenoviruses
HEP-2C	RSV

  5. For establishment of viral isolate, inoculate 0.5ml conventional culture positive specimen into appropriated flask; whereas for expansion of viral isolate, inoculate 0.5ml viral-infected cell lysate, incubate at 37°C, 5% CO_2 incubator for 1 hour.
  6. Add 14.5ml of maintenance medium to each flask according to the following table. For RSV, Adeno, P1, P2 and P3, add 14.5ml 1% FCS MEM to each flask. For Flu A, and Flu B, add 0% FCS MEM supplemented with 2ug/ml of TPCK-trypsin to enhance infection.

RSV, Adeno	1% FCS MEM
Parainfluenza 1, 2 and 3	0.025% trypsin SFM
Influenza A and B	0.2% trypsin SFM

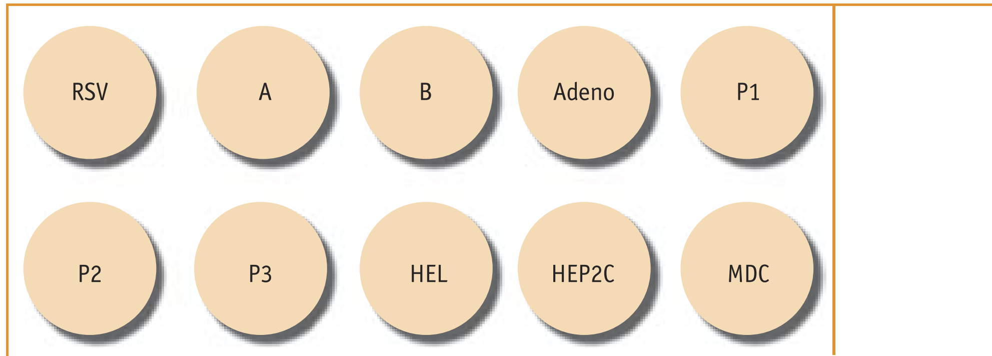
  7. Incubate the flask at 37°C, 5% CO_2 for 7 days or >30% CPE seen. (Change maintenance medium every week if necessary).
  8. Check the infection of the cells by IF as follows for positive control smears. If infection >30% (% of IF positive cells in the smear), freeze the flask and thaw once, aliquot the viral-infected cell lysate 0.5ml each in eppendorf (viral isolate for preparing positive control smear) and keep at –70°C.

3. Preparation of in-house external positive and negative control slides

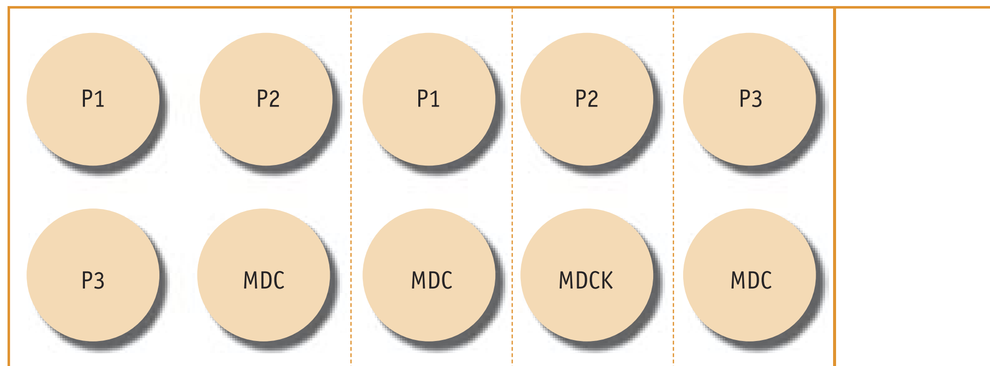
- Follow up the procedure as above 2(b)7, once incubated for 7 days or >30% CPE found, remove medium from the flask by aspiration.
- Scrap some of the cells out and add 10uL of cell suspension into one well of 10-welled Teflon-coated slides.
- Fix in acetone for 10 minutes.
- Test the presence of appropriated virus in the cell line by staining with Dako typing reagent. If virus-infected cells are >10% by IF, large-scale positive control smear preparation can be proceed as follow. IF not, add fresh maintenance medium to the flask and allow it to grow for 7 days or >30% CPE seen. (For preparation of uninfected cell smears, start with uninfected confluent cell monolayer flask and start proceeding from this step.)
- Wash the cell layer twice with 10ml PBS. Add 1ml Trypsin-Versene into the flask.
- Incubate at 37°C for 6 minutes.
- Tap the flask to loosen the cells from the flask.
- Add 9ml PBS to resuspend cells.
- Centrifuge at 1200 rpm for 5 minutes.

- Discard supernatant.
- Add 3ml PBS to resuspend cells and transfer to a bijou bottle.
- Add 10ul of cell suspension into one well of a 10-welled Teflon coated slides according to the following illustration. Air dry. Distribution of cell suspension is made according to slide 1, 2 and 3 listed below:

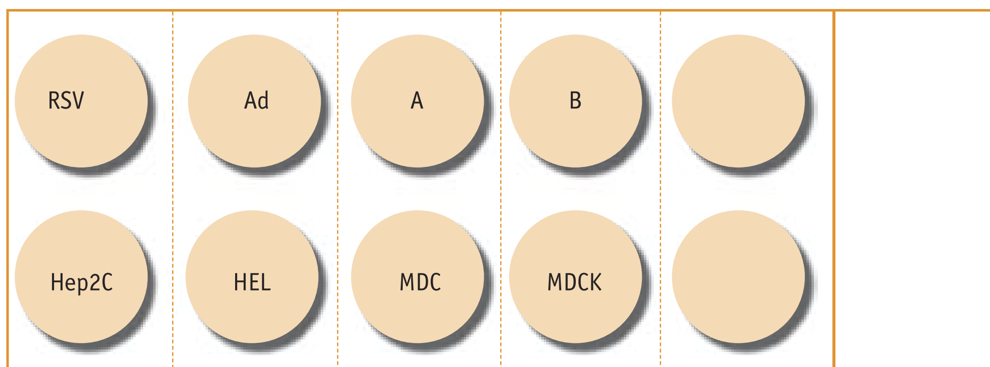
Slide 1: Purpose: QC of Dako screening reagents
Procedure: stain all wells with Dako screening reagents



Slide 2: Purpose: QC of Paragroup and Parainfluenza 1, 2, and 3 typing reagaent.
Procedure: (1) stain the left 4 wells with Paragroup reagent
(2) Stain P1 & MDCK with Parainfluenza 1 typing reagent
(3) As above step for P2 and P3 typing reagent



Slide 3: Purpose: QC for RSV, Adeno, Influeza A and B and typing reagent
Procedure: As slide 2, stain the positive and negative control wells with appropriate typing reagent.



- Fix cells in acetone for 10 minutes.
- Label the slide
- Test the smear with Dako screening and typing reagents
- Store slide at –70°C and ready for use
- Record the result on the External QC slides record for Dako Pool and typing reagent (Appendix E4)

Record for preparation of in-house external control slides for respiratory viruses IF detection

Lot No.: _______ Preparation date: ___/___/_____ Expiry date: ___/___/_____

Please write down "P" for positive and "N" for negative QC result

Well No.	Virus/Negative control	Strain	Cell line	QC result	Done by	Remarks
Slide 1						Number of slide:
1.	RSV					
2	A					
3.	B					
4.	Adeno					
5.	P1					
6.	P2					
7.	P3					
8.	HEL					
9.	Hep-2C					
10.	MDCK					
Slide 2:						Number of slide:
1.	P1					
2.	P2					
3.	P1					
4.	P2					
5.	P3					
6.	P3					
7.	MDCK					
8.	MDCK					
9.	MDCK					
10.	MDCK					
Slide 3						Number of slide:
1.	RSV					
2.	Adeno					
3.	A					
4.	B					
5.	—					
6.	Hep 2C					
7.	HEL					
8.	MDCK					
9.	MDCK					
10.	—					

Daily QC record for respiratory tract virus detection

Year/Month:____________________

Please write down “P” for positive and “N” for negative result in the appropriate column

Date	Control smear lot No. E for external QC & I for internal QC	Dako Pool Positive/Negative well							ParaPool			Typing reagent Positive /Negative wells							Done by	Checked by	Remarks (Write down C for Chemicon reagent)
		RSV	A	B	Ad	P1	P2	P3	P1	P2	P3	RSV	A	B	Ad	P1	P2	P3			

Precaution and limitation of Dako reagent

- The Imagen reagent contains 15mmol/ml sodium azide which is a poison. Sodium azide may react with copper and lead plumbing systems to form explosive metal azides. Always dispose of materials containing azide by flushing with large quantities of water.
- The positive control slides have been shown to be non-infectious in cell culture; however, the slide should be handled and disposed of as though potentially infectious.
- Evans blue dye is present in the reagent. This may be carcinogenic and contact with the skin should be avoided.
- Care should be taken when using the mounting fluid as it may cause skin irritation. Skin should be flushed with water if contact occurs.
- Dispose of all clinical samples in accordance with HK legislation.
- Reagents must not be used after the expiry date printed on the labels.
- Reagents are provided at fixed working concentrations. Sensitivity may be affected if the reagents are modified in any way or not stored under recommended conditions.
- Do not mix different batch lots of reagents.
- Avoid microbial contamination of reagents.
- Cross-contamination of reagents must be avoided.
- Do not freeze the reagents.
- The visual appearance of the fluorescence image obtained may vary due to the type of microscope and light source used.
- Failure to detect viruses may be a result of factors such as collection of specimen at an inappropriate time of the disease, improper sampling and/or handling of specimen, etc. A negative result does not exclude the possibility of viral infection.
- The presence of virus in nasopharyngeal secretions does not necessarily exclude the possibility of concomitant infection available from epidemiological studies, clinical diagnosis of the patient and other diagnostic procedures.
- Non-specific staining is sometimes observed as an artifact in immuno-chemical test due to binding between antibody Fc regions and protein A antigen found in the cell wall of some strains of Staphylococcus aureus.
- Test results should be interpreted in conjunction with information available from epidemiological studies, clinical assessment of the patient and other diagnosis procedures.

Source

- WHO Reference Laboratory for influenza A/H5 infection (Hong Kong);
- http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf;
- National Institute of Virology, Indian Council of Medical Research

Annex 4

STANDARD OPERATIVE GUIDELINES FOR INFLUENZA A (H5N1) VIRUS ISOLATION AND IDENTIFICATION

Maintenance of cell culture

Principle

The quality of cell cultures used in virological study is important for virus isolation and identification. It is vital to pay strict attention to three characteristics that are fundamental to the quality of cell culture assays: purity, authenticity and stability.

Purity: Contamination with micro-organisms such as bacteria and fungi will normally kill the cells and put other cultures in the laboratory at risk. Mycoplasma contamination can have serious effects on a cell culture without inhibiting cell growth and, furthermore, the presence of such contamination will rarely be apparent even under microscopic observation. This is due to the extremely small size of mycoplasma organisms that can enable them to pass through sub-micron filters. Whilst viral contamination typically produces a cytopathic effect in cell cultures, persistent non-cytopathic infections may arise that can influence virological investigations and may represent a hazard to laboratory workers (e.g. Epstein Barr Virus expressed by B95-8 and B95a cells). Screening for viral contamination can be extremely costly and time consuming. However, routine checks for bacteria, fungi and mycoplasma are relatively easy to establish and will provide confidence in the quality of cell culture results.

Authenticity: Accidental switching of cell lines or cross-contamination between cultures can result in erroneous or misleading data. Obtaining documentary evidence for the authenticity of new cell lines is therefore very important. Cell lines should be obtained through World Health Organization, American Type Culture Collection or other authorized institutes.

Stability: Cell cultures serially passaged over an extended period of time will invariably show some signs of variation in genetic or phenotypic characteristics. The susceptibility to such variation will differ between cell lines. To minimize the effects of cell line deterioration it is strongly recommended that all cell lines used routinely are replaced after a maximum of 15 sequential passages.

Maintenance of trouble-free cell cultures depends on careful attention to culture conditions and passage procedures. The passage procedure involves routine maintenance, cell dispersal and quantitation.

Routine maintenance of cell culture flasks requires a periodic medium change for proliferating cells. Intervals between medium changes and between subcultures vary from one cell line to another, depending on the rate of growth and metabolism. Four factors indicate the need for the replacement of culture medium.

1. A drop in pH — Most cells will stop growing as pH falls from pH 7.0 to pH 6.5 and will start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed.

2. Cell concentration — Cultures at a high cell concentration will exhaust the medium faster than at a low concentration. This is usually evident in the rate of pH change but not always.

3. Cell type — Normal cells, e.g. diploid fibroblasts will usually stop dividing at a high cell density due to growth factor depletion and other factors. The cells deteriorate very little even if left for 2 weeks or longer. Transformed cells and continuous cell lines, however, will deteriorate rapidly at high cell densities unless the medium is changed or they are passaged.

4. Cell morphology — When checking a culture for routine maintenance, be alert to signs of morphological deterioration: granularity around the nucleus, cytoplasmic vacuolation, rounding up or detachment of the cells. This may imply that the culture requires a medium change, or may indicate a more serious problem, e.g. inadequate or toxic medium or serum, microbiological contamination, or senescence of the cell line. During routine maintenance, the medium change or passage frequency should prevent such deterioration.

Cell dispersal can be accomplished by treatment with trypsin and Versene,ethylene-diamine-tetraacetic acid (EDTA) used either separately or combined. The proteolytic enzyme trypsin is particularly suitable for the digestion of cells from whole organs. It is also used for the removal of cells from glass or plastic, but the chelating agent Versene is probably as good. Solutions of trypsin and/or Versene are prepared in PBS free of calcium and magnesium, as the presence of these ions increases the stability of the intercellular matrix thereby making detachment of the cells from the glass/plastic difficult. The washed monolayer is covered with the dispersal agent and left in contact with the cells until they are separated. Incubation at 37°C facilitates cell separation.

The cell concentration of the suspended cells is determined by cell counting. In passaging cell culture, the split ratio method is commonly used. For example, with a culture having a split ratio of 1:6, six new cultures of the same size can be obtained from each monolayer that is dispersed. The cells are suspended in growth medium at the desired concentration. Concentrations generally range from $1x10^5$ to $2.5x10^5$ cells per ml. Diploid cells are usually seeded at higher concentration than heteroploid cells.

Scope of application

To supply cell cultures in T25 flasks for isolation of virus from clinical specimens.

Precaution

- All glassware for handling cell cultures and media should be sterilized.
- Avoid splashes, spills and aerosols.
- Never process different cell lines simultaneously to avoid cross contamination. All working areas should be thoroughly cleaned between preparations of different cell types.
- Never use the same pipette to transfer medium to different flasks to avoid serial contamination.
- Never touch the neck of the culture flasks with the bottle containing the medium.
- Never add reagents or media directly to the surface of cell layers. Dispense towards another side of the flask gently.
- Minimize exposure of sterile media and cell cultures to open air (even within the BSC II).
- Wear a clean laboratory gown which is reserved solely for cell culture work.

- Wash hands or change gloves between cell culture procedures.
- Do not handle virus, mycoplasma and chlamydia in the Tissue Culture Laboratory.
- Do not attempt to treat contaminated cell cultures. Remove them from the laboratory and destroy by autoclaving.
- Disinfect working area with 75% ethanol before and after each use.
- Decant medium into discard jar containing 1:25 Clorox. Approximately equal volume of medium is added to result in a final 1,000ppm sodium hypochlorite.
- 37°C incubator must be leveled before use to ensure even distribution of seeded cells in flasks.
- During cell dissociation step, use minimum concentration of trypsin/versene and duration to give monodispersed cells.
- Media without antibiotics should be used for cell cultures in flasks to prevent passage of low-grade contamination.
- Media with antibiotics should be used for cell cultures in flasks/tubes/vials to inhibit growth of any microorganism in the specimens for viral cultures.
- Entry to Tissue Culture Laboratory is controlled by locking the door facing the working bench to minimize contamination due to air current.

Store all quality control viruses in –70°C freezer in Quality Control Laboratory.

Definitions

MDCK refers to Madin Darby Canine Kidney cell line sensitive to Influenza virus.

Requirements

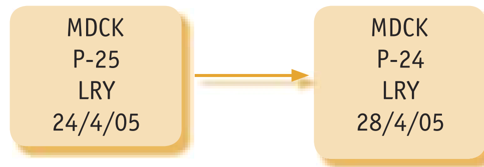
Equipment	Consumables
Inverted Microscope Incubator Laminar flow 37°C waterbath	T25 flasks, pipettes, EMEM, TPVG, FCS, P&S, glutamine vial (5.0ml, 3%), spirit Cotton, discarding bowl, gloves, mask, 100ml sterile bottle.

Procedure

- Observe the Tissue culture bottles for growth, cell degeneration, pH and microbial contamination. Contaminated and sheet torn bottles are to be discarded.
- Select the TC bottles for work according to the days work schedule.
- Switch on laminar UV for 15 min before starting the work
- Remove EMEM, TPVG and FCS from fridge and stand at 37°C waterbath for 30 minutes.
- Switch off UV lamp after 15 minutes and switch on laminar flow and light. Allow laminar flow to run for 5 minutes before starting work.
- Wash hands with soap. Wear gloves and mask.
- Remove reagents from waterbath and wipe with spirit swab.
- When a new bottle of EMEM in taken add 5.0ml of glutamine and 1.0ml of P&S. Mark on the bottle.
- To prepare required amount of growth medium add inactivates FCS (final concentration 10%) to EMEM containing P&S and glutamine in 100ml sterile bottle.
- On the laminar flow table arrange selected TC bottles in standing position (dead cells settle at the bottom)
- Remove/decant the medium from the bottle.
- Add 2ml of TPVG on the side opposite to the cell sheet, wash cell layer and discard.
- Add 5ml of TPVG solution on the side opposite to the cell sheet. Disperse evenly on to the monolayer. Lay the bottle flat with the cell surface side down on the table. Observe cells after every 15–20

seconds for signs of cells dislodging. When first signs of dislodging is observed (generally after 2–3 minutes) immediately remove the TPVG and place the flask in incubator until all the cells detach from the surface (check under microscope).

- Resuspend the cells in 5ml (per T25 flask) of growth medium. Aspirate the suspension using a pasteur pipette few times to break cell clumps.



- Determine the cell concentration per ml by counting the cells in haemocytometer (See cell counting). Dilute cell to prepare a suspension of 3 X 10^5 cell/ml. Seed 5.0 ml of diluted suspension to T25 flask (1.5 X 10^6 cells/flask). Usually 1:4 split ratio gives uniform monolayer on 3rd day.
- Label each flask with cell name, passage number, date of passage and initials of person preparing the culture.
- Incubate the flasks at 37°C.
- The flask is sub cultured every 3–4 days.

Precautions

- If some other cell line was handled in the cabinet, the cabinet should be swabbed with 1% hypochlorite/ spirit, airflow run for 10 minutes and UV switched on for 30 minutes before introduction of another cell line.
- Observe sub-cultured flask every day for cell growth and contamination. In case any irregularity in growth is observed sub-culturing of stand-by flasks should be done after confirming quality of cells in those flasks.

Cell counting

- Dilute 0.1ml of the cell suspension in 0.9ml of trypan blue (0.05% Trypan Blue)
- Mix well with pipette and aspirate sufficient volume to fill haemocytometer.
- Count cells in each of the four corners of the chambers. Do not count cells lying on the left hand and upper lines
- If cell clumping is observed, discard and resuspend original cell suspension for recounting.

Calculation of Viable Cell concentration per ml.

$$\text{Cell concentration per ml} = \frac{\text{total cell count of 4 squares}}{4} \times \text{dilution factor} \times 10^4$$

Example

Step 1: Cells from one T25 flask is resuspended in 5ml growth medium

Step 2: 0.1ml of this suspension is mixed with 0.9ml of trypan blue
(hence diluted factor will be1:10)

Step 3: 1:10 diluted material is loaded in a hemocytometer and cell from the four squares
counted = 10+12+9+17 = 48

Step 4: Cell concentration = $(48/4) \times (10) \times 10^4 = 12 \times 10^5$ per ml

Step 5: Total number of cell in 5ml = $5 \times 12 \times 10^5 = 60 \times 10^5$ i.e. 6×10^6
(amount in which total cells from a T25 flask were resuspended)

Step 6: To make a suspension of 3×10^5 cells per ml;
6×10^6 stock is resuspended in $(6 \times 10^6)/(3 \times 10^5)$
= 20ml growth medium

NOTE
Do not passage the cells beyond 25-passage level.

Sensitivity test of MDCK cell line (SOP# 8) should be performed at revival, 10th and 20th subculture levels. Besides it is performed when a new batch of medium/FCS/Cell line is procured.

Virus isolation

Principles

- Viruses cultivable using the MDCK cell lines include orthomyxoviruses (influenza), paramyxoviruses (parainfluenza and RSV) and occasionally adenovirus and herpes simplex viruses.
- The presence of influenza virus in the cell culture tube may be indicated by degeneration of the cell sheet, specific cytopathic effect or a positive haemadsorption test. The viruses are identified by the IF test and subtyping of influenza isolates is by the haemagglutination inhibition (HAI) test.

Objective
Describes the procedure for isolation of Influenza viruses from specimen.

Requirements

Equipment	Consumables	Registers
Laminar BSL II Cabinet Inverted Microscope –80°C deep freezer Refrigerator	T_{25} flask of MDCK cell line EMEM, TPCK trypsin vials (0.2ml per vials, 0.5 mg/ml), Nystatin vials (0.2ml per vial, 1.25×10^4 units/ml), pipette, 100ml sterile bottle, mask, gloves, Spirit swab, discarding bowl	Storage cards K-cards K register

Procedure
- Microscopically examine 48–72 hours T25 flask of MDCK cell line for uniform monolayer formation and make sure that the cells are healthy, confluent and free from microbial contamination.

If flasks are NOT OK — DO NOT USE
- Remove EMEM from the fridge and TPCK trypsin vials and Nystatin vials from –20°C freezer.
- Prepare K cards and enter details of sample on K cards and K register. Mark T25 flask (one flask per sample plus one flask for control) with K number, sample number and date of inoculation.
- Wash hands, wear lab coat/mask before entering the cubicle. Put on the gloves and mask.
- Switch on the laminar UV for 15 min before starting the work. After 15 minutes switch off laminar UV and switch on laminar flow, light and exhaust, let the flow run for 5 minutes before starting the work.
- Remove the throat and nasal swab specimens from –80°C freezer, thaw quickly at room temperature and place on wet ice. Enter use of vial in storage card.

- Wipe media bottle and vials with spirit swab.
- Prepare virus growth medium by adding TPCK trypsin (one 0.2ml vial to 50ml medium, final concentration 2ug/ml), Nystatin (one 0.2ml vial to 50ml medium final concentration is 50units/ml) in sterile 100 ml bottle.
- Discard growth medium from T25 flask with monolayer MDCK culture, add 2.0ml of virus growth medium, rinse cell sheet, decant and add 3.0ml virus growth medium. Repeat this procedure for all T25 flasks to be used.
- Arrange T25 flask and corresponding specimens to be inoculated in corresponding order.
- Wipe the mouth of the sample vial with spirit. Decant virus growth medium from flask with matching sample number.
- Using 1ml/5 ml sterile pipette aspirate complete content of specimen vial and add to the T25 flask gently, directly on the cell sheet. Discard pipette and tightly cork the flask.
- Before inoculating the next specimen, wipe the gloves with spirit and proceed. If wetting of the gloves with the specimen is suspected, discard and wear fresh gloves. Proceed in this manner till all the specimens are inoculated.
- Mock inoculate control bottle with transport medium.
- In case of spill on the worktable: Wipe the area thoroughly with spirit swab. Discard gloves and cotton in hypo solution and use fresh gloves.
- Allow the inoculum to adsorb for 30–60 minutes at 37°C with intermittent shaking.
- Add 4ml of virus growth media to each tube at the end of adsorption. Wipe gloves with spirit between flasks.
- Incubate the inoculated flasks at 37°C in incubator.
- Wipe the worktable with spirit swab and switch on the UV for 15 minutes.
- Observe the flask daily for the following:

Observation	Procedure to proceed
Toxicity: Cell cultures show rapid degeneration within 1 or 2 days of inoculation, this may be due to non-specific toxicity of the specimen.	Culture should be subjected to HA test (SOP#005) before discarding. In case sample shows HA activity then subjected to second passage.
Contamination: Cell cultures medium shows high turbidity or fungal culture	Culture should be discarded and second aliquot of samples should be Millipore filtered before inoculation.
Cytopathic effect: Cells become progressively granular, swollen and round. Amount of floating cells increase significantly. The CPE is graded from 1+ to 4+, depending on the percentage of cell sheet affected. (1+ =25%, 2+ = 50%, 3+ = 75% and 4+ = 100%).	At 3+/4+ CPE transfer flask to 4°C refrigerator and perform a HA test. If the HA titre is >/= 1:8 remove 1.0ml and subject supernatant to HI test (SOP#006). To remaining supernatant add 0.2ml of 10% BSA (final concentration is 0.5%). Make 1.0ml aliquots and store at –80°C. Make storage cards and make entry in isolate sheet. If HA titre <1:8 subject to second passage. To remaining supernatant add 0.2ml of 10% BSA (final concentration is 0.5%). Make 1.0ml aliquots and store at –80°C.
No effect: Cells remain healthy.	Harvest supernatant from all flask that do not indicate CPE into sterile 10ml vials and perform a HA test of supernatant from all flasks. Pass the supernatant from all the flask to fresh T25 flask for second passage on 7th day.

- Passage 1 and 2 are performed separately. The procedure is the same as for passage 1. Observe flask daily.
- Perform HA on all flasks showing no CPE on day 7 of passage 2 and report negative if no titre is observed.

Safety conditions

- All safety guidelines to be followed for disposal of waste.
- Biosafety guidelines are adhered to.
- Viral cross-contamination: Utmost care should be taken to avoid viral cross contamination of cultures during inoculation and passage collection. Do not decant the supernatant from inoculated flasks. It should be removed only with a pipette. Take care to avoid aerosols created by vigorous pipetting. Perform Passage 1 & 2 in different sittings. Passage 2 of those found positive in Passage 1 are inoculated after the negatives.
- Spilled droplets should be immediately cleaned with disinfectant. Any spillage of infective material should be brought to the notice of the lab head.

Quality control in tissue culture laboratory

Principle

The quality of cell cultures is important for virus isolation and identification. Routine monitoring of the sensitivity of cell lines is an important component of the laboratory's quality assurance programme. A well-characterized control virus preparation of known history and reproducible titre should be used to evaluate cell line sensitivity whenever fresh cells are resuscitated or received in the laboratory. It is critical to use relatively low passage virus isolates, usually from clinical specimens as challenges. Cell lines are replaced after 15 passages regardless of sensitivity assay, which can be carried out in plate or tube. For L20B and RD-A cells, plate method is used. Tube method is preferred for HEp-2C, Vero, LLC-MK_2, MDCK, E6 and $FRhK_4$ cells.

It is important to note that multiple factors can adversely affect cell line sensitivity including: mycoplasma contamination; quality of media and reagents; passage levels of cell cultures and growth conditions. Strict quality control measures on cell cultures include assay for cell line sensitivity, sterility, toxicity and mycoplasma detection. Animal sera used for media are checked for absence of toxicity to cells.

Cell culture stocks should be propagated in antibiotic free media to prevent passage of low level contaminants. All media and reagents are checked for sterility prior to use. New medium ingredients and equipment are checked for toxicity. Mycoplasma detection by mycotect assay is done every three months.

Scope of application

Cell line sensitivity

To access ability of cell cultures to isolate viruses, this assay is performed

- on retrieval of stock cells from liquid nitrogen for regular replacement of cell lines grown not more than 3 months or 15 passages, whichever is the shorter period
- cell lines at end passage
- upon receipt of cell ampoules from suppliers

- whenever sensitivity problems suspected
- upon introduction of new chemical or physical component for cell cultures.

Sterility testing

- To check reagents and media free from bacterial contamination.
- To rule out contamination in preparation steps of cell culture ampoules and routine cell culture tubes.

Precaution

1. Aseptic technique in cell culture work is essential.
2. Scrupulous preparation of media, reagent and glassware is of utmost importance.
3. Wear disposable gloves when handling quality control standards.
4. Perform laboratory tests involving virus in Quality Control Laboratory.

Reagent

Preparation of quality control standard

1. Cell cultures in maintenance medium
2. Control Virus
3. Trypsin solution (0.05%).

Titration of quality control standard

1. Maintenance medium with 2% foetal bovine serum (2% MEM)
2. Quality control standard
3. Known titreed stock (in calibration experiment)
4. Cell suspension containing 1–2 x 105 cells/ml (plate method)
5. Cell culture tubes with confluent, healthy layer (tube method)
6. Guinea pig red cell suspension (0.75%) (tube method).

Equipment

Preparation of quality control standard

- Pipettor
- Pipette tips
- Tissue culture roller
- Drum for tissue culture tubes
- Microscope
- Graduated pipettes
- Pipette aid
- Centrifuge tubes
- Refrigerated centrifuge
- Sterile vials
- 33°C incubator/ 37°C incubation room
- –70°C freezer

Titration of quality control standard

- Sterile bijou bottles/vials
- 37°C water bath
- Pipettor
- Pipette tips
- Vortex mixer
- Sterile 96-well flat bottomed microtitre plates (plate method)
- Sterile, non-toxic plate sealers (plate method)
- 37°C CO_2 incubator (plate method)
- 33°C incubator/ 37°C incubation room (tube method)
- Microscope

Preparation of quality control standard

Procedure

1. Inoculate 5 susceptible tubes/vials with 0.1ml control virus of known origin and history which are clearly documented. Refer to the quality control standard panel.
2. Incubate tubes/vials at 37°C incubator except LLC-MK2 and MDCK which are kept at 33°C incubator.
3. Examine culture daily for appearance of cytopathic effect (CPE).
4. When 75 to 100% of cells show CPE (3+ to 4+ CPE), transfer tubes to –70°C freezer. Freeze and thaw tubes to ensure that all cells are disrupted.
5. Pool and transfer the contents to a centrifuge tube.
6. Spin for 20 minutes at 1500g in a refrigerated centrifuge.
7. Label each vial with the name of the virus, the name of the cell line, the origin, the volume and the date.
8. Aliquot the supernatant into the labeled vial as quality control standard into single use volume (120–150µL) and store at –70°C freezer.

Quality control standard panel for cell lines

Cell line	Control virus
MDCK	influenza A, current isolate of influenza A, influenza B

Titration of quality control standard

The procedure outlined below is used.

For initial determination and validation of the titre of the quality control standard

Before being accepted for routine use, the titre for the quality control standard must be shown to be reproducible on at least 3 separate occasions, when tested in parallel with the known titreed stock which is used to validate the titre on all 3 occasions. Both titres should not vary by more than +/– 0.5 log10 when tested on the 3 occasions. Once these criteria are met, the expected titre to be assigned to the quality control standard will be the average of the titres obtained on the 3 occasions of testing. The identification of the quality control standard should be confirmed by immunofluorescence, haemagglutination, neutralization or molecular methods. The dilution range depends on the type and growth rate of virus, for example, 10^{-1} to 10^{-4} for influenza A and 10^{-1} to 10^{-9} for Sabin poliovirus.

Subsequent routine monitoring of cell line sensitivity

It is performed when only the quality control standard of established and reproducible titre needs to be used for testing.

Procedure

Plate method

1. Label bijou bottles 10–2 to 10–9.
2. Dispense 2.7ml medium to bijou bottles.
3. Rapidly thaw one aliquot of quality control standard.
4. Add 0.9ml medium to 0.1ml thawed standard and mix. This is the 10^{-1} dilution of the standard.
5. For a calibration experiment, also thaw an aliquot of the known titreed stock in an identical manner.
6. Add 0.3ml virus to the first bijou bottle using a pipettor with sterile tip.

7. Vortex gently.
8. Take another pipette tip, transfer 0.3ml to the second bijou bottle and discard pipette tip.
9. Repeat dilution steps, transferring 0.3ml each time and always changing pipette tips between dilutions.
10. Label the microtitre plate as indicated in Figure 1 in Appendix 1.
11. Add 0.1ml virus dilution (10-3 to 10-9) to wells 1 to 10 in rows A to F (i.e. 10 wells per dilution).
12. Add 0.1ml maintenance medium to wells A11 to F12 in rows A to F for the cell controls.
13. Prepare a suspension of approximately 1-2 x 105 cells/ml,calculating at least 10ml per plate.
14. For newly retrieved cells, run test with routine cells at end passage in parallel.
15. Add 0.1ml cell suspension to all wells in rows A to F.
16. Cover the plate with a plate sealer and incubate at 37°C in a CO_2 incubator.
17. Examine for development of CPE using an inverted microscope and record reading for 4 to 7 days. For a valid test, the cell control should have a complete monolayer of healthy cells.
18. Counter check result by another experienced staff.

Tube method

1. Label sterile vials 10 –1 to 10 –4.
2. Dispense 0.9ml medium to vials 1 to 4.
3. Rapidly thaw one aliquot of quality control standard.
4. Add 0.1ml thawed standard to the first vial using a pipettor with sterile tip and mix. This is the 10 –1 dilution of the standard.
5. For a calibration experiment, also thaw an aliquot of the known titreed stock in an identical manner.
6. Take another pipette tip add 0.1ml virus to the second vial.
7. Vortex gently.
8. Repeat dilution steps, transferring 0.1ml each time and always changing pipette tip between dilutions.
9. Inoculate 0.1ml virus dilution (10 –1 to 10 –4) to 4 culture tubes. Include one uninoculated tube cell control.
10. For newly retrieved cells, run test with routine cell culture at end passage in parallel.
11. Incubate tubes at 37°C except LLC-MK 2 and MDCK which are kept at 33°C.
12. Examine culture daily for appearance of cytopathic effect (CPE) and record reading for 4 to 7 days. Read endpoint by haemadsorption for myxovirus and paramyxovirus. For a valid test, the cell control should have a monolayer of healthy cells.
13. Counter check result by another experienced staff.

Documentation

Preparation of quality control standard

Record should be entered into *record book of control virus* which include

- quality control standard
- control origin
- preparation date
- lot number
- number of vials
- volume per vial
- dilution status
- endpoint in $TCID_{50}$
- titration range
- start date to use

Evaluation of cell line sensitivity

Result should be entered in record sheet.

Interpretation

Once the titre of the quality control standard has been established, the standard can be accepted for routine use. On each occasion that cell sensitivity is evaluated, the titre of the quality control standard is compared to the established reference value. If the titre is the same or ±0.5 log of the expected reference value, it is inferred that there is no decline in cell line sensitivity. If there is evidence of decline in sensitivity, a fresh aliquot of the quality control standard should be titrated again. If the

Figure 1 Microtitre plate layout for virus titration

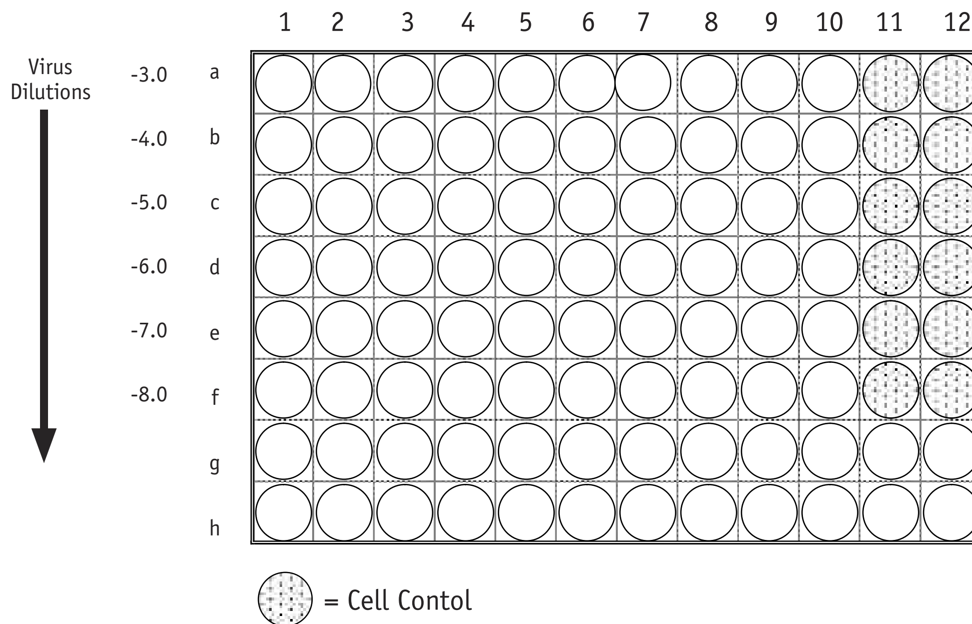
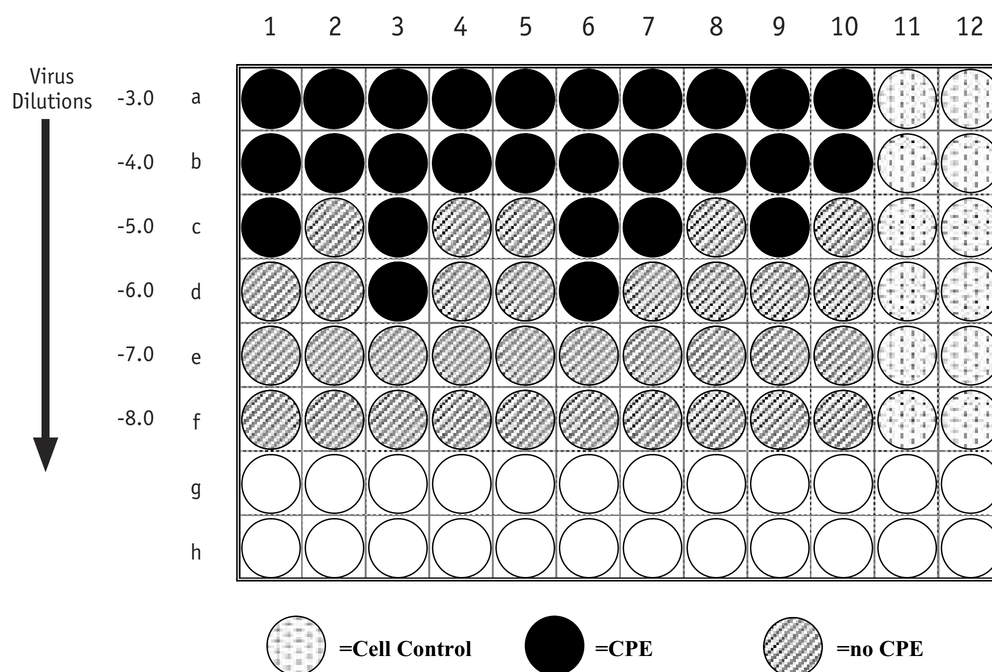


Figure 2 Example of results of titration of quality control standard



titre is reproducibly low, a new cell line should be resuscitated. Sensitivity of new cell line should always be evaluated before discarding old one.

Sterility test

Procedure

1. Inoculate 1ml preparations (medium or reagent) into 10ml nutrient broth (less than 1ml from ampoule or cell culture flask preparations).
2. Incubate at 37°C for 7 days.
3. Observe any bacterial or fungal growth daily.
4. Quarantine preparations until sterility is confirmed.
5. Repeat sterility test with two nutrient broths for any contamination detected.
6. Discard the preparations if one or both broths are turbid.

Documentation

Test results should be entered into record sheet.

Calculation of the virus titre by the Kärber formula

Log $TCID_{50}$ = L – d (S – 0.5),

where: L = log of lowest dilution used in the test;

d = difference between log dilution steps;

S = sum of proportion of "positive" tests (i.e. cultures showing CPE)

In this example:

L = -3.0; d = 1.0; S = 1.0 + 1.0 + 0.5 + 0.2; then:

log $TCID_{50}$ = -5.2; Virus titre = $10^{5.2}$ $TCID_{50}$ / 0.1ml

Result of cell line sensitivity (Tube method)

Virus: ____________________ Date: ___/___/____________

Cell line:____________________ Done by: ________________

Passage number: ____________ Checked by: ______________

Date	Dilution of virus					

Result: $TCID_{50}$ = ____

Virus: ____________________ Date: ___/___/____________

Cell line: ____________________ Done by: ________________

Passage number: ____________ Checked by: ______________

Date	Dilution of virus					

Result: $TCID_{50}$ = ____

____

Interpretation: ____

____

Immunofluorescence test

Immunofluorescence (IF) allows the visual localization of viral antigens on or within infected cells and is an essential tool in the diagnostic virology laboratory for the identification of viral isolates and the detection of viral antigens in clinical samples. The IF assay may be either direct or indirect. With direct IF, virus-infected cells are overlaid with a fluorochrome- labeled (conjugated) antiviral antibody. After a single incubation and washing step, the cells are ready for observation. In the indirect format, the cells are first overlaid with an unlabeled antiviral antibody. After incubation and washing step, the cells are re-incubated with a fluorochrome-labeled antibody directed against the immunoglobulins of the species supplying the primary antibody. A counterstain is usually included to diminish nonspecific staining and to give good contrast to negative cells. The "stained" slides are the examined under a fluorescence microscope at a wavelength specified by the fluorochrome.

Preparation of cell smears for immunofluorescence test

- When CPE occurs or HAd +ve, transfer medium from culture tube to a labeled sterile vial (Freeze and thaw the culture fluid to rupture the RBCs if HAd had been performed as the RBCs might interfere with the HA/HAI result).
- Rinse cell sheet with PBS three times.
- Scrape off cells and add to appropriate well(s) of the slide.
- Routinely 4 wells, 2 for direct IF (FLU A & FLU B) and 2 for indirect IF (H1 & H3), per specimen are required for isolates with Flu A/B CPE
- For specimen which is suspected to be FLU A H5. An extra well for FLUA H5 monoclonal antibodies indirect IF testing must be included.
- Dry smear on slide drying bench and fix in acetone for 10 minutes.

Procedure for direct IF

- Add 10µl/well of the appropriate monoclonal antibody-FITC conjugate (FLU A, FLU B) to the cell smear. (Return the reagents to the refrigerator after use.)
- Incubate in a moist chamber at 37±1°C for 15 minutes
  Note: Do not allow the reagent to dry on the specimen, as this will cause the appearance of non-specific staining.
- Rinse slide with a gentle stream of PBS and then immerse in PBS (5 minutes).
  Note: To avoid cross-contamination, direct PBS stream along the midline of the slide, squirting and tilting first towards the upper wells followed by the lower wells.
- Dry on slide drying bench or blot dry.
- Add mountant and cover slip and examine under the fluorescence microscope using low power magnification.
- If it is not possible to read the slides immediately after staining, store them in the dark at 2–8°C and read within 24 hours for best results.
- All the results are counter-checked by another experienced technical staff.

Procedure for indirect IF

- Add 10µl/well of the appropriate working monoclonal antibody (FLUA H1, FLUA H3 or FLUA H5) to the cell smear.
- Incubate in a moist chamber at 37±1°C for 30 minutes.
- Rinse with a gentle stream of PBS and then immerse in 2 changes of PBS (5 min each).
- Dry on slide drying bench or blot dry.
- Add 10µl/well of working anti-mouse FITC conjugate to all the wells of the cell smear.
- Repeat steps (2) to (4).
- Add mountant and coverslip and examine under the fluorescence microscope using low power magnification.

Worksheet for IF of Influenza

Date: ___/___/______Done by: ______________ Checked by: _____________

+ : apple green fluorescence **W+** : weakly apple green fluorescence

- : no apple green fluorescence

No.	Lab no.	IF results									Remarks
		A	B	H1	H3	H5	Para				
							1	2	3	4	
1											
2											
3											
4											
5											
6											
7											
8											
9											
0											
1											
2											
3											
4											
5											
6											
7											
8											
9											
0											
1											
2											
3											
4											
5											
6											
7											
8											
9											
0											

Haemagglutination test

Objective
Describes the procedure for Haemagglutination (HA) test

Equipment	Consumables	Registers
Laminar Flow Vortex mixer Micro pipette-5–50 and 20–200µl, Multichannel micropipettes, Refrigerator No-6	U bottomed Microtitre plate Ice pack, syringes, needles Guinea pig Alsever's solution, graduated tube, Phosphate buffered saline pH 7.2, Micropipette filter tips Spirit swab, Gloves, Mask	HA work sheet

Definition
HA: Haemagglutination RBC: Red blood cell

Procedure

Preparation of standardized RBCs (0.75% G.Pig)
- Restrain the animal properly. Feel the heart of the animal. Surface sterilize using spirit swab. Take 2–3ml Alsever's solution in a 5ml syringe with 23G needle and withdraw 2.0ml of Guinea pig blood from the heart. Deposit in a vial containing 6.0ml Alsever's solution and mix slowly.
- Monitor the animal and then release in the cage.
- Keep RBCs overnight at 4ºC.
- Remove Alsever's solution using Pasteur pipette.
- Add 10ml PBS, mix thoroughly using Pasteur pipette, centrifuge at 1000 rpm for 10 minutes.
- Aspirate supernatant and repeat step 4 and 5 twice.
- Measure the quantity of cell pack (generally 2.0ml original blood yields 0.5 to 1.0ml pack) Prepare 10% suspension in PBS

  with 0.1ml cell pack make final volume 1.0ml with PBS,

  0.2 to 2.0ml,

  0.3 to 3.0ml and so on
- Resuspend 0.75ml 10% G.Pig RBCs in 10ml PBS.

  1.50 ml to 20ml

  2.25 ml to 30ml

  3.75 ml to 50ml

HA test

1. Label a U-bottom 96-well microtitre plate as shown in the Plate 1.
2. Using a micropipette or 50µl dropper, add 50µl of PBS (pH 7.2) to all wells.
3. Add 50µl of antigen (tissue culture fluid) to the first well of each row.
4. Make twofold dilutions by transferring 50µl from the first well of each column A1-K1 to A2-K2 using multi-channel micropipette. Proceed till the 8th column. Discard remaining 50µl after the 8th column.

PLATE 1

		1	2	3	4	5	6	7	8
	Dilutions→ Sample ↓	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
A	S1								
B	S2								
C	S3								
D	S4								
E	S5								
F	S6								
G	S7								
H	S8								
I	S9								
J	S10								
K	S11								
L	Control								

5. Add 50μl of 0.75% G.pig RBCs to all wells using a 50μl dropper.
6. Mix by manually agitating the plates thoroughly.
7. Incubate the plates at 4°C for 60 minutes. Check cell control for complete settling of RBCs
8. Record results in HA sheets.

Interpretation

Guinea pig RBCs will form a button or a ring at the bottom of the wells and recorded as '0'. If haemagglutination occurs, i.e. RBCs remain in suspension it is recorded using a "+" symbol.

Button formation

Ring

Haemagglutination

The highest dilution of virus that causes complete haemagglutination is considered the end point in HA titration. The HA titre is the reciprocal of the dilution of virus in the last well with complete haemagglutination.

If partial agglutination is seen the titre of the last well showing complete agglutination must be added with the titre of the well showing partial agglutination and divided by 2 to obtain the haemagglutination titre.

Haemagglutination inhibition test

The principle of the HAI test is as follows. When a standardized concentration of virus is mixed with a constant amount of red blood cells (RBCs), agglutination of RBCs will normally occur since RBCs contain numerous receptors for viral haemagglutinin, producing a lattice formation of agglutinated cells. This is inhibited by the presence of specific antibodies against the haemagglutinin.

Objective

Describes the procedure for preparation of standardized antigen, treatment of reference antisera and HI test for identification of Influenza viruses isolated from throat and nasal swabs.

Definition

HA: Haemagglutination
HI: Haemagglutination inhibition
RBC: Red blood cell

Equipment	Consumables	Registers
Laminar Flow, Vortex mixer, Micro pipette and multi-channel 5–50 and 20–200µl, Centrifuge maintained at room temperature, Refrigerator, –20°C deep freezer	Guinea pig RBCs, centrifuge tube, 50ml conical flask, PBS pH 7.2, Pipettes, U bottomed Microtitre plate, Ice pack, WHO influenza reagent Kit, sterile DW, PBS pH 7.2, normal saline, micropipettes tips, spirit, sterile cotton, gloves, discarding jar.	HI protocol worksheet HI Antisera register

1. PREPARATION OF STANDARDIZED ANTIGEN*

- Perform HA test conduct using G.pig RBCs as per the SOP 005 with
  - All homologous viruses provided with the kit
  - All laboratory isolates that are to be typed
- Prepare dilution that contains 4 HA units/25µl or 8 HA units/50µl as follows:
  - Calculate the antigen dilution by dividing the HA titre (obtained in the HA titration), by 8. For example if HA titre is 1:32, divided by 8 is 4. Therefore dilution factor is 4.
  - Add 500µl of antigen/virus isolate to 1500µl of PBS (1part of control antigen or virus isolate to 3 parts of PBS) in a 5ml vial. Mix well by vortexing. This gives the standardized antigen.
- Perform a HA test (back titration) as described below to confirm the HA units.
  - Standardized antigen is defined as the optimum amount of virus that would react with standard antisera to inhibit agglutination of an equal volume of standardized red blood cell suspension. For the HAI test 4 HA units of virus/antigen in 25µl is used.

"Back Titration" of Standardized Antigen

PLATE 1

		1	2	3	4	5	6
	Dilutions→ Sample No↓						
A	A/H3	✦	✦	✦	✦	•	•
B	A/H1	✦	✦	✦	✦	✦	•
C	B/SIC	✦	✦	✦	•	•	•
D	B/H.K	✦	✦	✦	✦	•	•
E	MS-001	✦	✦	✦	✦	•	•
F	MS-002	✦	✦	✦	✦	•	•
G	MS-003	✦	✦	✦	✦	•	•
H	C.C	•	•				

MS – Lab isolate no
Note: The standardized antigen that is prepared is considered as neat and HA test is performed in 2 fold dilutions from this neat antigen

Important: Use U bottomed microtitre plates when using guinea pig RBCs

1. Label a U-shaped 96-well microtitre plate as shown in the Plate 2.
2. Add 50µl of PBS (pH 7.2) to wells between A2–G6 with a micropipette or 50µl dropper, leaving the first column free.
3. Prepare RBC control wells in H1 and H2 by adding 50µl of PBS.
4. Add 100µl of each standardized control antigen or field isolate (diluted according to the calculation described above) to the first well of column 1 (A1- G1) as marked in the plate. This serves as neat.
5. Make twofold dilutions by transferring 50µl from the first well of each column A1-G1 to A2-G2. Proceed till the 6th column. Discard the final 50µl.
6. Add 50µl of RBC suspension to all the wells on the plate with a 50µl dropper.
7. Mix by manually agitating the plates thoroughly.
8. Incubate the plates at 22°C to 25°C in B.O.D. incubator for 1 hour. Check cell control for complete settling of RBCs.
9. Record results.

Interpretation

Standardized antigens must have a HA titre of 4 HA units/25µl.

- Check the number of wells showing haemagglutination.
- If there is hemagglutination in the first four wells of the back titration plate (as illustrated in row A of plate 2), the antigen has a titre of 8 HA units.
- If there is hemagglutination in less than or more than 4 wells the titre must be adjusted accordingly, by adding more antigen to increase units or by diluting to decrease units.
- For example, if complete haemagglutination is present in the fifth well (as illustrated in row B of plate 2), the virus now has a titre of 16 and the test antigen should be diluted twofold.
- Conversely, if haemagglutination is present only up to the third well (as illustrated in row C of plate 2), the antigen has a titre of 4 and an equal volume of virus must be added to the test antigen. This would double the concentration of virus in the test antigen to give a titre of 8.
- Adjust the concentration of antigen and repeat HA until 4 HA units/25µl (8 units/50µl) is obtained.
- Calculate the volume of standardized antigen required for the HAI test. For example, with 1ml of antigen 5 anti sera can be tested, (8 wells/antisera and 25µl of antigen per well). Prepare an additional 1ml volume for “back titration” and wastage.

2. TREATMENT OF REFERENCE ANTISERA

All the reference antisera supplied in the WHO reagent kit are to be treated.

1. Reconstitute the antisera with sterile distilled water to the volume indicated on the label, mix well and distribute in 1ml aliquots and store at –20°C.
2. Reconstitution of receptor destroying enzyme (RDE): Check procedure for receptor destroying enzyme preparation in the insert accompanying the kit. (Standard protocol involves reconstituting RDE in 25ml sterile normal saline (0.85% NaCl)). Distribute in 1ml aliquots and store at –20°C.
3. Treatment of reference antisera:

- Arrange requisite number (depends on the number of antisera supplied in the CDC kit usually five-six) of 5ml vials and label them with name of antisera.
- Add 0.9ml RDE to each of the vials.
- Add 0.3ml of corresponding serum (3 volume of RDE to 1 volume serum) to the labeled vials.
- Incubate overnight in a 37°C waterbath.

- After overnight incubation heat in water bath at 56°C for 30 minutes to inactivate remaining RDE. Allow antisera to cool to room temperature.
- Add 1.8ml (6 volumes to the original volume of serum) of physiological saline (0.85% NaCl). The final dilution of antisera is 1:10.
- The antisera can now be used for further testing. It is sufficient for 50 tests approximately.

Detection of Nonspecific Agglutinins in Treated Sera

Plate 2

		1	2	3	4	5	6	7
	Sera No→ Dilutions↓	A/H3	A/H1	B/Sic	I-4 B/H.K	N.C	C.C	
A	1: 10	•	•	✱	•	•	•	•
B	1: 20	•	•	✱	•	•		
C	1: 40	•	•	•	•	•		
D	1: 80	•	•	•	•	•		
E	1:160	•	•	•	•	•		
F	1:320	•	•	•	•	•		
G	1:640	•	•	•	•	•		
H	1:1280	•	•	•	•	•		

Important: Use U bottomed microtitre plates when using guinea pig RBCs

1. Label a microtitre plate as depicted in Plate 2. Column 1 as A/H3, column 2 as A/H1, column 3 as B/Sic, column 4 as B/H.K and column 5 as negative control. Label the row A as 1: 10, B as 1: 20, and similarly in doubling dilutions till row H (1:1280)
2. To the labelled plate add 25µl of PBS (pH 7.2) to rows B through H (B1-H5) using a 25µl micropipette or dropper.
3. Add 50µl of PBS (pH 7.2) to 2 wells A6 and A7 to serve as RBC control.
4. Add 50µl of corresponding treated antiserum to the first wells of row A (A1 - A5).
5. Using 25µl microdiluters or multichannel pipettes, prepare twofold dilutions of the antisera by transferring25 µl from A1 - 5 to B1 – 5. Proceed in the same manner till H1–5 and discard 25µl from H1–5.
6. Add 25µl of PBS, pH 7.2 to all wells.
7. Add 50µl of 0.75% RBC suspension to all the wells (A1-H5 and A6&A7) using a 50µl dropper.
8. Mix by manually agitating the plates carefully.
9. Incubate the plates at room temperature (22°C to 25°C or 4°C) in B.O.D. incubator for 1 hour.
10. Check the cell control wells. Read the results when cell control wells show complete settling.

Interpretation

- If the RBCs settle completely, the antiserum is free of nonspecific agglutinins and can be used in the HAI test. Such antisera free of nonspecific agglutinins, are aliquoted in 500µl volume and stored at –20°C.
- The presence of nonspecific agglutinins will be evident by haemagglutination of the RBCs as seen in Plate 1 for B/Sichuan. In this case, the antisera must be adsorbed with RBCs according to the alternate serum treatment protocol

Adsorption of Antisera to Remove Nonspecific Agglutinins (For antisera demonstrating prescence of non specific agglutinins)

- In a 2ml Polypropylene vial, mix 50µl of packed RBC with1000µl of antiserum (one volume of packed RBCs to 20 volumes of RDE-treated serum).
- Mix thoroughly and incubate at 4oC in a refrigerator for 1 hr, mixing manually at regular intervals (10 minutes) to resuspend cells.
- Centrifuge at 1200 rpm for 10 minutes.
- Using a 2ml pipette carefully remove the adsorbed serum without disturbing the packed cells. Transfer it to 1.5ml eppendorf tube.
- Check for non-specific agglutinins by procedure described above.
- Repeat adsorption with RBCs until the serum shows no agglutination.

If the sera do not contain any nonspecific agglutinins, it is ready for use. Aliquot in 500µl volume and Store at –20°C.

3. HI TEST

Label appropriate microtitre plates as per plate 3. Two antigens (control antigens/laboratory isolate) can be tested per plate. One extra plate is required for serum control.

1. Add 25µl of PBS to all wells from row B to H.
2. Using the set of treated anti-sera (5 sera) which are already diluted 1:10 Add 50µl of each serum to the first well of the appropriate numbered column
3. Add 50µl of PBS to first well of columns 6 & 7.
4. Make twofold dilutions by transferring 25µl from the first well of numbered column 1–12 to successive wells. Discard the final 25µl.

Plate 3

		1	2	3	4	5	6	7	8	9	10	11	12
		H1	H3	B/ Vict	B/ Yam	Nor	CC	CC	H1	H3	B/ Vict	B/ Yam	Nor
A	1:10												
B	1: 20												
C	1:40												
D	1:80												
E	1:160												
F	1:320												
G	1:640												
H	1:1280												

5. Add 25µl of standardized antigen #1 to all well from column 1 to 6 in rows A to H and antigen #2 to all wells from column 7 to 12 in row A to H.
6. Repeat for all control antigens and laboratory isolates.
7. Mix the content by agitating the plates manually.
8. Keep at room temperature for 30 minutes.

9. Add 50µl of RBCs to all well. Shake manually.
10. Keep at 4°C for 45 minutes. Confirm button formation in cell control wells.
11. Record results as '0' in wells showing button or ring pattern. All other wells are recorded as '+'.

Interpretation of results

- If an antigen/antibody reaction occurs heamagglutination of the RBCs will be inhibited. The HI titre is the reciprocal of the last dilution of antiserum that completely inhibit heamagglutination.
- To verify the specificity of the test one should test control sera not only with homologous antigens but also with heterologous antigens representing other currently prevalent strains. The control tests should show that each antiserum is type-specific and sub-type specific, i.e. antisera prepared with any subtype of influenza A should not react with other influenza A sub-types in the test with control antigens.
- An isolate is identified as a particular type or subtype if the field isolate reacts with one reference antiserum at a fourfold or greater H1 titres than to other antisera.
- To identify a field isolate compare the results of the unknown field isolate to those of the control antigen. An isolate that reacts with a reference serum at a titre equal to that obtained with the homologous virus (plus or minus one-two fold dilution) is considered to be similar to that reference virus.
- An isolate that is inhibited by a reference antiserum at a titre of fourfold less than that for the homologous virus may be considered to be antigenically different from that virus.

Quality control measures

- Susceptibility of the cells to various viruses must be tested.
- Positive controls should be included in everyday culture process.
- All reagents are standardized if necessary (e.g. WHO IF reagents) before use and standardization results are recorded in the appropriate workbook.
- Positive and cell controls should be included in each HAI test run.
- Counter-checking of procedures by another staff should be undertaken for all positive or suspected isolates and before finally issuing laboratory request forms.
- All performing and counter-checking staff should initial against all records.
- Persons who observed the CPE must countersign in the worksheet.

Potential problems and remedy

Some isolates may be untypable. Typing procedures are repeated and the experienced staff notified. If further subtyping still fails to yield result, isolate should be sent overseas to WHO collaborating centre for further investigations.

Worksheet for HAI of Influenza A virus

Date: ___/___/__________ Done by: ______________________ Checked by: ___________________

+ Complete hemagglutination, ± Partial hemagglutination, – No hemagglutination

Starting dilution for H1N1 antiserum:______________ & H3N2 antiserum:_______ RBCs control:______

No	Lab no.	Anti- serum	HAI result	HAI titre	Ag BT	Remark	Ampoule
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					

Worksheet for HAI of Influenza B virus

Date: ___/___/_________________________ Done by: ________________ ___Checked by: ___________

+ Complete hemagglutination, ± Partial hemagglutination, – No hemagglutination

Antiserum: _________________________ and its corresponding control antigen: _____________________

Antiserum: _________________________ and its corresponding control antigen: ________________

Starting dilution for both antiserum: ___________________________ RBCs control: __________________

No	Lab no.	Anti- serum	HAI result	HAI titre	Ag BT	Remark	Ampoule
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					
		H1N1					
		H3N2					

Media and reagent preparation

Objectives

Describes procedure for preparation of media and reagents used for maintenance of MDCK cell line and for virus inoculation. It also describes the procedure for preparation of reagents in the HA and HI tests.

Definitions

DW: Distilled Water
EMEM: Earles Minimum Essential Medium
FCS: Fetal Bovine (calf) Serum

HBSS:	Hanks Balanced Salt Solution
PBS:	Phosphate Buffered Saline
TPVG:	Trypsin Phosphate Buffered Saline, Versene, and Glucose
VTM:	Virus Transport Medium

Procedure

Requirements

Equipment	Consumable	Register
Biosafety cabinet, CO_2 cylinder with connections, –20°C deep freezer, refrigerator, media filtration unit, syringe filters, pH meter, balance	1 and 2 litre flask, measuring bottles, media and reagent distribution bottles, pipettes, weighing paper, sealing tape, storage vials, DW, glass DW, reagents.	Media Preparation register Sterility register

Before commencing media preparation the following preliminary procedures have to be observed.

- Check the media preparation register for stock; decide on the amount of media to be prepared.
- Check if all required sterilized glassware and ingredients are available on the day of preparation. (Check the media sterility register and sterility tubes. Note down the details about the ingredient sterility of previous preparations)
- All glassware is to be sterilized 48 hrs prior to use.
- Millipore double distilled water or glass distilled water (GDW) autoclaved at 10lbs /110°C/10 min on the previous day and cooled to room temperature.
- Two individuals should be involved in media preparation.
- Bio safety guidelines for transfer of chemicals and reagents to be followed.
- Do not overcrowd the cabinet and never obstruct the front opening.
- Manipulate fluids slowly and gently to avoid aerosols.
- Avoid transfer of sterilized glassware and ingredients in and out of media room.
- Arrange CO_2 cylinder with the copper sulphate bottle connection and vacuum pump before work commences.
- General safety guidelines to be followed for disposal of waste.
- Check sterility of the safety cabinets periodically.

On the day of preparation

- Switch on the UV light in the media preparation cubicle and in the Laminar Flow hood for ½ hr before commencement of the work and then switch it off.
- Disposable materials like pipettes, measuring cylinders, are kept under UV light for ½ hrs inside the laminar hood.
- All the ingredients used for media preparation should be brought to room temperature.
- Switch on the airflow of the Laminar hood; allow running for 10 minutes before starting the work.
- Switch on the pH metre, weighing balance 10mts before starting the media and reagents preparation.
- Check the CO_2 cylinder.
- Record the work for the day in the media preparation register and sterility register.

Alsever's solution (pH 6.1) for collection of erythrocytes

1.	Dextrose (D. glucose)	20.5gms
2.	Sodium citrate dihydrate ($Na_3C_6H5O_{7.}2H_2O$)	8.0gms
3.	Sodium chloride (NaCl)	4.2gms
4.	Citric acid (C6H807)	0.6gms

Dissolved in 900ml autoclaved DW. Adjust pH to 6.1+/-0.1 with 1N NaOH or 1N HCl. Make final volume one liter. Autoclave at 110°C, 10lb for 10 min. Store at 4°C.

Amido black solution (0.1%)

Amido black stain	0.5gm
Sodium acetate	6.8g
Acetic acid	30.0ml
D.W	470ml
Store at room temperature	

Amphotrecin B (100ug/ml)

Dissolve 50mg vial of Amphotrecin B in 10ml sterile DW (50X stock).
Distribute 1ml/vial and store at –20°C.
Dilute 1ml 50X stock in 49ml sterile DW.
Distribute 1.0ml per vial and store at –20°C.

Bovine serum albumin (10%)

Dissolve 1.0g of BSA in 10ml DW.

Filter through 0.22 um Millipore filter

Distribute 1.0ml per vial and store at 4°C. Check sterility.

Crystal violet (0.1%)

Crystal violet stain	0.1g
Citric acid	1.92g
Dissolve in 100ml DW and store at RT	

EDTA (0.2%)

Dissolve 200mg of EDTA in 100ml DW
Autoclave at 10lbs/10 minutes/110°C store at 4°C.

EMEM (Autoclavable)

MEM Earles(for1 litre)	1 vial
DDH_2O	928.00ml
Stir & divide this solution into 2 bottles MEM (above)	464.00ml
Fe (NO_3) $9H_2O$ (0.002%)	2.5ml
Autoclave: 10lbs/10 minutes/110°C	
Cool to room temperature (15-20°C)	
Add sterile $NaHCO_3$ (3.5%)	31.4ml
pH 7.2 – 7.4	

Test sterility. Store at 4°C. Before use add Glutamine (3%) 5.00ml, P & S 1.0ml.

$Fe(NO_3)_3.9H_2O$ (0.002%)

Dissolve 2mg of $Fe(NO_3)_3.9H_2O$ in DW

Sterilize by autoclaving at 10lbs/10 minutes/110°C store at 4°C.

Fetal Bovine serum (FCS)

Thaw a 500ml bottle of fetal bovine serum and distribute 50ml each in sterile 100 ml saline bottles. Inactivate at 56°C for 20 min. Check each bottle for sterility and store at 4°C for short term storage or –20°C for storage over a longer period.

Glucose (10%)

Dissolve 10g of glucose in 100ml DW

Autoclave at 10lbs/10 minutes/110°C and store at 4°C.

Glutamine (3%)

Dissolve 3g of L-glutamine in 100ml DW

Filter through millipore membrane filter 0.22micron and distribute in 5ml aliquots.

Store at –20°C. Put up sterility.

Hanks balance salt solutions (A and B)

A. Hank's A (20X) 100ml

NaCl	16.00gms
KCl	0.80
$MgSO_4.7H_2O$	0.40
$CaCl_2$	0.28

Dissolve the above (except $CaCl_2$)in 80ml of DD H_2O. Dissolve $CaCl_2$ separately in 10ml of DD water and add it to the main solution. Bring up the volume to 100ml with DD water. Add 0.2ml of chloroform (preservative). Store at 4°C.

B. Hank's B (20 x) 100ml

$Na_2HPO.2H_2O$	0.12gms
KH_2PO_4	0.12
Glucose	2.00

DD water to make 100ml. Add 0.2ml of chloroform (preservative). Store at 4°C.

For the preparation of Hank's Balance Salt solution

Hank's A (20X)	5.0ml
Hank's B(20X)	5.0ml
GDW autoclaved	88.0ml
Phenol Red (0.5%)	0.2ml

Autoclave at 10lb, 10 min at 110°C and store at 4°C

Normal saline

Dissolve 8.5g NaCl in 100ml DW.

Autoclave at 110°C, 10lb for 10 min store at 4°C.

Nystatin (25000 U/ml)

To a vial containing 5 million unit of nystatin add 10ml sterile distilled water.
Distribute 1.0ml/vial & store at –20°C
To 39ml Sterile DW add l.0ml of resuspended nystatin.
Distribute 0.2ml/vial and store at –20°C.

Penicillin (1,00,000U/ml) & Streptomycin (100mg/ml)

To 2 vials of Penicillin (5,00,000U) and 1 vial of Streptomycin (1g) each add 2ml of sterile DW using a syringe. Dissolve the powder completely and aspirate the solution in a vial to make final volume 10ml. Distribute 1ml per vial and store at –20°C.

Phenol red (0.5%)

Take 500mg of phenol red powder in a mortar pestle and grind to a fine powder. Dissolve the powder in small amount (about 2-4ml) 1N NaOH to get a pH of about 6.5-7.0. Make up the volume to 100ml using sterile DW and filter through filter paper if particles are observed. Autoclave at 110°C, 10lbs for 10 min. store at 4°C.

PBS Phosphate Buffer Saline (0.001M) pH 7.2

Dibasic sodium phosphate Anhydrous (Na_2HPO_4)	5.48gms
Monobasic sodium phosphate monohydrate ($NaH_2PO4.H_2O$)	1.58gms
Sodium chloride	42.5gms

Dissolve in 5 liters distilled water and adjust pH to 7.2 with 1N NaOH or IN HCl. Distribute in 500ml saline bottles. Autoclave at 10lb for 10 min. Store at 4°C.

Sodium bicarbonate 3.5%

$NaHCO_3$	3.5g
Phenol Red (0.5%)	0.2ml

Dissolve in 100ml DWts CO_2 till pH is about 7.0. Autoclave at 10 lbs for 10mts. Cool and Store at +4°C.

TPCK trypsin (500ug/ml)

10mg of TPCK trypsin is dissolved in 20ml of MEM(E) & filter with 0.45 um Millipore filter. Distribute 0.2ml per vial and store at –20°C.

TPVG (Trypsin in PBS with Versene and Glucose)

PBS (10x)	50.00ml
DDH_2O	371.5ml
Phenol red (0.5%)	1.00ml
Autoclave Add Sterile	
Trypsin (2%)	25.00ml
EDTA (0.2%)	50.00ml
Glucose (10%)	2.5ml

Adjust pH 7.5 with 0.1 N NaOH. Test sterility. Store at 4°C. Add 1ml P & S before use. Use within 2-3 weeks.

Trypan Blue (0.05%)

Dissolve 50mg Trypan Blue in 100ml of phosphate buffered saline
Filter through whatman filter paper No.4 and store at +4°C.

Trypsin (2%)

Weigh 2g trypsin accurately and dissolve in 100ml sterile millipore distilled water with magnetic stirrer for 1/2hrs. Filter through membrane filter. Store at –20°C. Check sterility.

Viral transport medium

Hank's A (20X)	25ml
Hank's B (20X)	25ml
GDW	394ml
Phenol red (0.5%)	1ml
Autoclave 10lbs, 10 min 110°C. Add sterile	
Bovine serum albumin (10%)	50ml
$NaHCO_3$ (3.5%)	5ml
Adjust pH to 7.2% with 1N NaOH	
Test sterility, store at 4°C	

Precaution

The volume of Millipore water required for dissolving the MEM salt and ingredients will depend on the brand of MEM used. Hence care should be taken to record the brand of MEM used. Check the label before proceeding. Before adding the ingredients check the label for proper name and date of manufacture.

Reagents used for surveillance

Sr. No.	Reagent	Manufacturer	Catalogue No.
1	Amido black	Sigma	N3005
2	Amphotericin B	Sigma	A9528
3	BSA	Himedia	RM113
4	$CaCl_2$	Sigma	C1016
5	Chloroform	Qualigen	12305
6	Citric acid	Qualigen	41095
7	Crystal violet	Qualigen	38583
8	Dextrose	Qualigen	15405
9	EDTA	Qualigen	12634
10	EMEM	Himedia	ATO-17
11	FCS	Gibco BRL	10082-147
12	$Fe(NO_3)_3 . 9H_2O$		
13	Gentamycin (Magenta)	Wockkardt Ltd.	
14	Glucose	Qualigens	15405
15	Glutamine	Himedia	RM049
16	Glutaraldehyde	Merck	4239
17	Hypochlorite	Local Purchase	
18	KCl	Qualigen	13305
19	KH_2PO_4	Qualigen	19465

20	Lysol	Local purchase	
21	$MgSO_{4}.7H_{2}O$	Qualigen	13005
22	$Na_{2}HPO_{4}.2H_{2}O$	Merck	17550
23	NaCl	Merck	6404
24	$NaH_{2}PO_{4}.H_{2}O$	Merck	1/17874
25	Nystatin	Sigma	N3503
26	Penicillin	Alembic	
27	Phenol red	Qualigen	39852
28	Sodium citrate $Na_{3}C_{6}H_{5}O_{7}.2H_{2}O$	Qualigens/BDH	14005
29	Spirit	Local purchase	
30	Streptomycin	Sarabhai Pirmal	
31	TPCK trypsin	Sigma	T1426
32	Trypan blue	Sigma	T0776
33	Trypsin	Sigma	T8003

Equivalents chemicals from other sources can also be used.

Disposable material used

Sr. No.	Material	Manufacturer	Catalogue No.
1	1.5ml vials	Laxbro	SV-1.5D
2	10ml vials	Laxbro	SV-10
3	15ml vials	Laxbro	SV-15DS
4	5.0ml vials	Laxbro	SV-5S
5	50ml droppers	Top Syringe Mfg.	
6	Cotton swabs	Local purchase	-
7	Gloves	Surgicare	-
8	Mask	Local purchase	-
9	Needles	B-D	
10	Syringes	B-D Glass	
11	T25 flask	TPP	90025
12	Tips	Axygen	
13	U bottom HA/HI plates	Laxbro	MT 96 U and MT 96 V

Equivalent material from other sources can be used.

Source

WHO Reference Laboratory for influenza A/H5 infection (Hong Kong);
http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf;
National Institute of Virology, Indian Council of Medical Research

Annex 5

GUIDELINES FOR MOLECULAR DIAGNOSIS OF INFLUENZA A (H5N1) VIRUS

Polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful technique for the identification of influenza virus genomes. The influenza virus genome is single-stranded RNA, and a DNA copy (cDNA) must be synthesised first using a reverse transcriptase (RT) polymerase. The procedure for amplifying the RNA genome (RT–PCR) requires a pair of oligonucleotide primers. These primer pairs are designed on the basis of the known HA sequence of influenza A subtypes and of N1 and will specifically amplify RNA of only one subtype. DNAs generated by using subtype-specific primers can be further analysed by molecular genetic techniques such as sequencing. The primers listed below are recommended by the WHO H5 Reference Laboratory Network ii. A WHO technical working group is being established for timely primer update and development.

Materials required

- QIAamp Viral RNA Mini Kit or equivalent extraction kit
- QIAGEN OneStep RT-PCR kit
- RNase inhibitor (ABI) 20U/µl
- Sterile microcentrifuge tubes, 0.2, 0.5 and 1.5ml
- Primer sets
    - HA gene primers for H5 amplification (modified from Yuen et al. 1998)
    - H5-1: GCC ATT CCA CAA CAT ACA CCC
    - H5-3: CTC CCC TGC TCA TTG CTA TG
    - Expected product size: 219 bp
    - HA gene primers for H9 amplification:
    - H9-426: GAA TCC AGA TCT TTC CAG AC
    - H9-808R: CCA TAC CAT GGG GCA ATT AG
    - Expected product size: 383 bp
    - NA gene primers for N1 amplification (Wright et al. 1995):
    - N1-1: TTG CTT GGT CGG CAA GTG C
    - N1-2: CCA GTC CAC CCA TTT GGA TCC
    - Expected product size: 616bp
- Positive control (Obtained upon request from a WHO H5 Reference Laboratory)
- Adjustable pipettes, 10, 20 and 100µl
    - Disposable filter tips
    - Microcentrifuge, adjustable to 13 000 rpm
    - Vortex mixer
    - Thermocycler
    - Agarose gel casting tray, electrophoresis chamber and power supply
    - UV-light box or hand-held UV light (302 nm)

Procedure

1. Extract viral RNA from clinical specimen with QIAamp viral RNA mini kit or equivalent extraction kit according to manufacturer's instructions.
2. One step RT-PCR

H5 or N1

Prepare master mixture for RT-PCR as below:
5x QIAGEN RT-PCR buffer 10µl
dNTP mix 2µl
5x Q-solution 10µl
Forward primer (5 µM) 6µl
Reverse primer (5 µM) 6µl
Enzyme mix 2µl
RNase inhibitor (20U/µl) 0.5µl
Water (Molecular grade) 9µl

Total 45µl

Add 5µl viral RNA

H9

Prepare master mixture for RT-PCR as below:
5x QIAGEN RT-PCR buffer 10µl
dNTP mix 2µl
Forward primer (5 uM) 6µl
Reverse primer (5 uM) 6µl
Enzyme mix 2µl
RNase inhibitor (20U/ul) 0.5µl
Water (Molecular grade) 19µl

Total 45µl

Add 5 µl viral RNA

3. RT-PCR reaction for H5, N1, H9
   Set the follow PCR conditions:
   Reverse transcription 30 min 50°C
   Initial PCR activation 15 min 95°C
   3-step cycling
   Denaturation 30 sec 94°C
   Annealing 30 sec 55°C
   Extension 30 sec 72°C
   Number of cycles 40
   Final extension 2 min 72°C
4. Agarose gel electrophoresis of PCR product.
5. Prepare agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of maker and PCR product bands under UV light.

Interpretation of results

The expected size of PCR products for influenza A/H5 is 219 bp, for A/H9 is 383 bp, and for N1 is 616 bp. If the test is run without a positive control, products should be confirmed by sequencing and comparison with sequences in deposited databases. The absence of the correct PCR products (i.e. a negative result) does not rule out the presence of influenza virus. 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 or H9 should be tested by other methods (IFA, virus culture or serology) to rule out influenza A (A/H5 or H9) infection.

Source

- WHO Reference Laboratory for influenza A/H5 infection (Hong Kong);
- http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf

Annex 6

GUIDELINES FOR ANIMAL INFLUENZA A (H5N1) SURVEILLANCE AND DIAGNOSIS

• Virus/Viral Antigen Detection by Immunoflourescence Test

Immunoflourescence test, either direct or indirect, can be applied for detection of avian influenza virus. Indirect test is the preferred option.

The test can be applied to clinical specimens or in cell cultures infected with the virus.

Procedure

- The test samples (clinical specimen or infected cells) are fixed in chilled acetone for 30 minutes and stored at cold temperature until test.
- Following washing of the slide or cell cultures in PBS (pH 7.2) three times, the antiserum is applied and incubated for 30-45 minutes. The antiserum could be monoclonal or polyclonal.
- Following three washings in PBS, the slides/cell culture are stained with antiglobulin IgG-FITC conjugates for 30 minutes. If the antiserum is monoclonal, anti-mouse IgG-FITC conjugate is to be used. In case of polyclonal antiserum, anti-species FITC conjugate is used in case the polyclonal antibody is derived from the species.
- After three washings in PBS, the specimen is mounted with a coverslip. The mountant used is 50% buffered glycerine.
- The mounted specimen is examined under fluorescence microscope.
- Suitable control should be used for comparison.
- Specific apple-green fluorescence of the virus/ viral antigen can be seen in the cytoplasm, nucleus or both

• Virus Isolation

Processing of samples for virus isolation

- Prepare a 10% suspension in antibiotic diluent
- Centrifuge at 150g for 20 minutes and collect the supernatant in a sterile vial
- Incubate at room temperature for 1 hour.

Chicken embryo inoculation for virus isolation:

- 9–11 day's old embryos from specific pathogen-free (SPF) flocks should be used. Alternatively, eggs from AIV negative flocks can be used.
- Inoculate 0.2ml of the suspension through allantoic route (9–11 days) or through chorio-allantoic sac (10–11 days)
- Label the eggs and seal the inoculation points with glue.
- Candle the eggs and mark the sites for allantoic or chorio-allantoic sac route inoculation and drill holes.

- Candle the eggs daily for 4–7 days. Embryos dead within 24 hours should be discarded. However, in case of HPAI, the embryos may die within 24 hours.
- The dead and live embryos are kept at refrigerated temperature for overnight.
- Harvesting of amnio-allantoic fluid (AAF) is done from dead and live chilled embryos inoculated with a particular specimen can be pooled and tested by hemagglutination (HA) and hemagglutination inhibition (HI) tests.

Procedure for hemagglutination test

- Label V-bottom microtitre plates
- Add 50µl PBS to all the wells.
- Make two-fold serial dilutions of AAF by putting 50µl to the first well and then transferring the same amount serially.
- The last well should be reserved for RBC control
- To each well add 50µl of washed RBCs (0.5–1%) and mix well by tapping
- Read the plate after 30 minutes when the RBCs of control well settle properly. HA is determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBCs. Titre is calculated by reading the highest dilution giving complete HA (no streaming).

Procedure for hemagglutination Inhibition test

- Into each well of V-bottom micro titre plate dispense 25µl of PBS.
- Add 25µl of subtype specific serum to the first well and make twofold dilutions by transferring 25µl volume of serum across the plate.
- Determine the HA titre of the virus as indicated above.
- Calculate the dilution to achieve 4 or 8 HAU/25µl (e.g. an end point of 1:64=64 HAU/50µl. To get 4HA /25µl dilute the virus in PBS 1:8, to get 8HAU/25µl dilute the virus in PBS 1:4)
- To each well add 4 or 8 HAU/25µl (e.g. an end point of 1:64 = 64 HAU/50µl. To get 4HA/25µl dilute the virus in PBS 1:4.
- To each well add 4 or 8 HAU of the virus and incubate for 30 minutes at room temperature.
- To each well add 25µl of chicken RBC (1%) and mix gently.
- Allow the RBCs to settle for 30–40 minutes at room temperature (20°C) or for 60 minutes at 4°C
- Suitable virus control, serum control and RBC control should be included in this test.
- Read the HI titre. The wells in which the RBCs stream at the same rate as the control is considered as inhibition.
- Any inhibition with the subtype specific serum is considered positive for the particular subtype of the virus.

• Detection of Antibodies

Agar gel immunodiffusion (AGID) test for detection of antibodies of type A influenza virus

By AGID test antibodies to Type A influenza group specific antigens (matrix and nucleoprotein) are detected. In this test the antigen and antibody migrate concurrently through an agar gel matrix. The migrated antigen and antibody combine to form a precipitation line which can be visualized.

Procedure

- Agar gel is prepared by boiling 9.0g of agarose, 80g NaCl and one liter of PBS (0.01M, pH 7.2).
- Allow the agar to cool at room temperature (25°C) before pouring into petri plates.

- Pipette 15–17ml of melted agar on to a 100X15 mm petri plates so that the thickness the agar is approximately 2.8 mm.
- Allow in agar to solidify for 15-30 minutes and punch holes using a template cutter—7 well pattern. The wells are 5.3 mm in diameter and 2.4 mm apart. Up to 7 templates pattern can be cut.
- The agar plugs are removed by aspiration.
- Dispense about 50–55µl of known positive serum into alternate peripheral wells and test serum in rest of the wells. Dispense 50–55µl known antigen into the central well.
- Allow the plates to set for few minutes and incubate at 37°C for 24 hrs in a humid chamber.
- Examine the plates for precipitation lines. In negative reaction the control lines continue into the test sample well without bending or with a slight bend away from the antigen well and towards the positive control serum well.
- In positive reaction control lines joins with and form a continuous line with the line between the antigen and test serum. Weekly positive samples may not have complete line but may only cause the tip or end of the control line to bend inward to the test well.
- Non-specific line does not form continuous line with the positive control lines.
- Hemagglutination inhibition test for AI antibody.
- The test is similar to that described for virus detection. Subtype specific antigen to be used for the test.

Hemagglutination inhibition test for detection of antibodies of type A influenza virus

The test is similar to that described for virus detection. Subtype specific antiserum is to be used for this test.

Micro-serum neutralization test

Reference Virus

Highly pathogenic avian influenza virus (HPAIV) A/Ck/Navapur/Maharashtra/7972/2006 has been adapted to grow in MDCK cell line. The MDCK adapted virus at passages 6 and having an infectivity titre ($TCID_{50}$) of 10^{-7}/ml is used in micro-serum neutralization test. This virus is available in the repository of the High Security Animal Disease Laboratory.

Reference H5 N1 serum

Hyper-immune chicken serum raised against the reference virus and available at the High Security Animal Disease Laboratory is used as reference positive serum in micro-serum neutralization test.

Procedure

- Micro-serum neutralization test employing varying virus-constant (1:10) serum method has been standardized at HSADL (Bhopal) and is conducted to determine the level of H5 N1 virus neutralizing antibody level in the human serum samples. The test envisages estimation of $TCID_{50}$ (tissue culture infective dose 50) of the reference virus in the absence and in the presence of test/reference serum, and the difference between the two is determined as the (log_{10}) neutralization-index (NI) of the serum tested.
- Reference H5N1 chicken serum is used as (H5N1) antibody positive control in the test.
- All serum samples are diluted 1:10 in serum-free culture medium (Glasgow's MEM with L-glutamine; Sigma), inactivated at 56°C for 30 min in a water bath, and filtered through 0.45 syringe filter before use in the test.
- Serial log_{10} dilutions (10^{-1} to 10^{-8}) of the reference H5N1 virus are prepared in serum- free cell culture medium as mentioned above.

- Suspension of MDCK cells at a concentration of $4x10^5$ cells/ml in Glasgow's MEM with L-glutamine and 5% fetal calf serum (Hyclone is used as the indicator systems in the tests).
- The test is conducted in 96-well cell culture plates (Nunc), and all reagents (virus, serum and cell suspension) are used in 50µl volumes.
- Tissue culture (MDCK) infectivity ($TCID_{50}$) of the reference virus in the presence of each test serum is determined wherein three wells are used per dilution of the virus.
- Virus-serum mixtures (50µl each) in the plates are incubated at 37°C for 30 minutes in an incubator with 5% CO_2 tension to effect neutralization of the virus.
- Virus control wells as well as serum control wells receive 50µl of serum-free cell culture medium in lieu of test serum, where as cell control wells receive 100µl of serum-free cell culture medium in lieu of both virus and test serum.
- Following incubation of the virus-serum mixtures, freshly prepared MDCK cell suspension, as mentioned above, is added to all test and control well in 50µl volumes.
- The plates are gently tapped from all sides to ensure proper distribution of cells in all the wells and incubated in CO_2 incubator.
- On completion of 48 hr incubation period, individual wells are checked under inverted microscope for presence or absence of virus mediated cytopathic effect as indicator of no virus neutralization and virus neutralization, respectively.
- $TCID_{50}$ of virus in the absence of test/reference serum is calculated as per Reed and Muench (1938), and the level of virus neutralizing activity in the reference/test serum samples is express as log_{10} N1.

• Molecular Diagnosis of Animal Influenza A (H5N1) Virus

This has to be done by one-step Reverse-Transcription PCR and one-step Real-Time PCR using total RNA extracted from fecal material/cloacal swab chorioallantoic fluid from infected embryonated chicken egg/infected ell culture fluid/tissue and organ suspension, etc. Suspension of clinical materials must be clarified by centrifugation at 8000xg for 10 min in a refrigerated centrifuge. Additionally, faecal material suspensions must be filtered through 0.8µ cut-off syringe filter (Millipore/Pall).

1. Extraction of total RNA

- For extraction of total RNA from materials other than tissue/organ suspensions, 'QIAamp Viral RNA Mini Kit' (Qiagen, product number 52904/52906/52908) may be used with 1µg carrier RNA per ml of AVL buffer provided in the kit. Step-wise procedure outlined by the manufacturer must be followed. Similar RNA extraction kits/systems of other manufacturers can also be used.
- For extraction of total RNA from tissue/organ suspensions (10% w/v in PBS, pH 7.2), 'RNeasy Mini Kit' (Qiagen, Product Number 74103/74104/74106) along with 'QIAshredder' spin columns (Qiagen, Product Number 79654/79656) may be used. Use of QIAshredder spin columns will help in fast and efficient homogenization of tissue/cell lysates without cross-contamination of the samples during extraction procedure. Step-wise procedure outlined by the manufacturer must be followed. Similar RNA extraction kits/systems of other manufacturers can also be used.
- Starting volume of virus/clinical material for extraction of RNA using either of the above mentioned kits be 280µl, and final elution (of RNA) be done in 50µl AVE buffer for RNase-free water available in the kit(s).
- Total RNA extracted be treated with 25–30 Kunitz units of RNase-free-DNase (Qiagen, Product Number 79254) for 15 min at room temperature (20–30°C) followed by enzyme inactivation at –70°C for 15 min in a water bath.

- Each RNA sample be stored in 2 aliquots, one at –70°C and the second one at –20°C for immediate use.
- Two nuclease-free water samples (280µl volume each) should be included in each batch of RNA extraction to monitor cross-contamination during extraction procedure.

2. Reverse Transcription – PCR (RT-PCR)

- One-step RT-PCR employing H15 specific primer pairs recommended by WHO and OIE should be used for screening each total RNA preparation.
- The nucleotide sequences of these primers are mentioned below.

WHO primer

(+) 5′ GCCATTCCACAACATACACCC 3′

(-) 5′ CTCCCCTGCTCATTGCTATG 3′

This pair flanks nucleotide positions 915-1133 of the HA gene of H5. Expected size of the amplicon is 219bp.

OIE primer

(+) 5′ ACACATGCYCARGACATACT 3′

(-) 5′ CTYTGRTTYAGTGTTGATGT 3′

This pair flanks nucleotide positions 155-699 of the HA gene of H5. Expected size of the amplicon is 545bp.

- Access RT-PCR System (Promega, Product number A1250/A1260/A1280) or Qiagen One Step RT-PCR Kit (Qiagen, Product Number, 210210/210212) may be used for one-step amplification of the above target sequences in the H5 gene. Any equivalent one-step kit of other manufacturers can also be used.
- One-step RT-PCR reaction mixes should be prepared as per manufacturer's instructions. In each 25 µl reaction, 25pmol of the forward primer, 20pmol of the reverse primer and 4µl of total (test) RNA must be included, in addition to the other ingredients of PCR recommended by the manufacturer.
- Temperature and duration for Reverse transcription (by MMLV or AMV RTase) recommended by the manufacturer (of the one-step RT-PCR Kit used) must be followed.
- In PCR amplification step, both sets of primers work well at the annealing temperature of 50°C for 30 sec. The primer extension (polymerization by Taq or Tfil DNA polymerase) temperature as recommended by the manufacturer be followed, and it has to be for a duration of 40 sec. After 35 cycles of amplification, a final extension step (1 cycle) of 10 min may be incorporated, followed by hold at 4°C.
- Positive and negative controls.
    1. H5 positive control in the form of a recombinant plasmid can be provided by the High Security Animal Disease Laboratory, Bhopal.
    2. Nuclease free water samples subjected to RNA extraction procedure along with clinical/virus materials should be included as negative controls.
    3. In addition, during PCR set up, two nuclease free water samples must be included (in lieu of RNA template) to monitor carry over contaminations, if any.
- At the end of RT-PCR, all amplicons in 10µl volumes must be electrophoresed in 1% agarose gel (in TAE/TBE) containing 0.5µg EtBr/ml.

- Result.
  1. Both primer sets must yield singular amplicon of expected size (219bp in case of WHO primer and 545bp in case of OIE primer) in the positive control.
  2. All negative controls must be negative; otherwise the test has to be repeated.
  3. Test samples showing desired amplification with both the primer sets are positive.
  4. Test samples showing amplification with only one of the primer pairs should be carefully interpreted and sent to HSADL, Bhopal for confirmation.

3. Real-Time PCR (Taq man Assay)

- Taq Man Assay targeting 'M' (matrix) gene and H5 gene must be conducted on each RNA sample.
- Sybr Green Assay targeting HA cleavage site must be done to assess pathogenicity of the virus at molecular level.

- **Taq Man assay for AIV type A diagnosis.**
  This assay targets 'M' gene and sequences of the primers and probe are mentioned below (designed by HSADL, Bhopal).
  1. Forward primer:
     5′ AGGCTCTCATGGAATGGCTAAAG 3′
  2. Probe:
     5′ FAM AGTCCTCGCTCACTGGGCACGGT-BHQL 3′
  3. Reverse primer:
     5′ GGCATTTTGGACAAATCGTCTACG 3′

- **Taq Man assay for H5 sub typing.**
  This assay targets HA gene of H5 subtype and the following two sets of primer-probe as recommended by CDC, USA and commercially supplied by Operon (Product Number SPH5N1) may be used. These primer and probes can be
  I. First set of primer-probe:
     Forward primer H5-1F: 5′ TGCCGGAATGGTCTTACATAGTG 3′
     Probe H5-1P: 5′ FAM- AGAAGGCCAATCCAGTCAATG-TAMRA
     Reverse primer H5-1R: 5′ TCTTCATAGTCATTGAAATCCCCTG 3′

  II. Second set of primer-probe:
     Forward primer H5-2F: 5′ GTGGCGAGCTCCCTAGCA 3′
     Probe H5-2P: 5′ FAM- TGGCAATCATGGTAGCTGGTC-TAMRA 3′
     Reverse primer H5-2R: 5′TCTGCATTGTAACGACCCATTG 3′

- **Taq Man assay set up for the three sets of primer-probe**
  Each RNA sample has to be tested using the type A primer-probe as well as both the sets two H5 sub typing primer-probe.

1. Brilliant QRT/PCR Master Mix (Stratagene, Product Number 600551) or any equivalent product can be used.
2. All reaction in 25µl volumes must contain ROX dye (final concentration of 30nm), 9 pmol forward primer, 6 pmol reverse primer, 5 pmol probe and 1.5µl test RNA, in addition to QPCR master mix and RTase as recommended by the manufacturer. It is better to use 0.2ml PCR tubes with cap instead of 96-well PCR plate.

3. Positive control in the form of viral RNA or HA-H5 recombinant plasmid has to be included in each test batch.
4. Negative controls in the form of no template control, no probe control and nuclease free water (in place of RNA template) control are also to be included in each test batch.
5. The real-time PCR machine has to be programmed as follows:
   - 50°C for 30 min (reverse transcription): 1 cycle
   - 95°C for 10 min (Hot start Taq activation): 1 cycle
   - 35 cycles of amplification encompassing denaturation at 95°C for 30 sec, annealing at 50°C for 1 min and primer extension at 72°C for 30 sec.
6. Result and interpretation:
   - All positive controls must show amplification signal at Ct values of 9–18.
   - All negative controls must not show any amplification signal up to the last cycle.
   - Test RNA samples showing amplification signal only with type A primer-probe (not with either of the H5 primer-probe) are to be interpreted as type A influenza virus only (certainly not of subtype H5).
   - Test RNA samples showing amplification signals (Ct values of 9-25) with Type A primer-probe as well as both the H5 primer-probe are to be diagnosed as H5 influenza virus.
   - Test RNA samples showing amplification signals with type A primer-probe and one of the H5 primer-probe sets should be interpreted with caution and passed on to HSADL, Bhopal for confirmation.

Real time PCR (Sybr Green I assay)

- Sybr Green I assay targeting HA-H5 region 1001-1124 (a subset sequence with in the WHO H5 primer flanking region 915-1133 which includes the HA cleavage region) maybe conducted on all RNA samples to supplement the Taq Man assay results and to interpret the level of pathogenicity from the Tm value.
- The primer (Payungporn, etal, 2006) to be used are as below:
  1. Forward primer, 5′ AACAGATTAGTCCTTGCGACTG 3′
  2. Reverse primer: 5′ CATCTACCATTCCCTGCCATCC 3′

Sybr Green assay set up

Each RNA sample has to tested using the primer pair mentioned above.

1. Brilliant Sybr Green QRT-PCR Master mix (Stratagene, Product Number 600552) or any equivalent product can be used
2. All reactions in 25µl volumes must contain ROX dye (final concentration of 30nm), 9 pmol forward primer, 6pmol reverse primer and 1.5µl test RNA, in addition to QPCR master mix and RTase as recommended by the manufacturer. It is better to use 0.2ml PCr tubes with cap instead of 96 well PCR plate.
3. Positive control in the form of viral RNA or HA-H5 recombinant plasmid has to be included in each batch test.
4. Negative controls in the form of no template control, no primer control and nuclease free water (in place of RNA template) control are also to be included in each test batch.
5. The real time PCR machine has to programmed as follows:
   - 50°C for 30 min(reverse transcription):1 cycle
   - 95°C for 10 min hot start Taq activation) :1 cycle
   - 40 cycles of amplification encompassing denaturation at 95°C for 30 sec, annealing at 50°C for 1 min and primer extension at 72°C for 30 sec

- This would follow dissociation curve analysis(Tm) of the accumulated product at the end of the 40th cycle where in the product has to heated from 50°C through 95°C at the rate of 1–1.5°C/ sec and fluorescence data collected for calculation for Tm value of the accumulated product.

6. Result and interpretation:
   - All positive controls must show amplification signal at Ct values of 9-18 with Tm of 77-77.6°C indicating the presence of HPAI virus and Tm, of higher than this (up to approximately 79.5°C) would indicate presence of LPAI virus
   - All negative controls must not show any amplification signal up to the last cycle. Tm of primer-dimer would be around 72–74°C
   - Test RNA samples showing amplification signals (Ct values of 9-35) with specific dissociation temperature(Tm) of
   - All RNA samples positive by H5 Taq Man assay must be positive in this assay with specific tm value to qualify them as highly pathogenic avian influenza (HPAI) virus

Source

- High Security Animal Disease Laboratory (Bhopal), Indian Council of Agricultural Research

Annex 7

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