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WHO Manual on Animal Influenza Diagnosis and Surveillance

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The World Health Organization (WHO) Animal Influenza Training Manual was based on the Human Influenza Training Manual provided by Dr Nancy Cox and developed by staff from the Centers for Disease Control and Prevention in Atlanta, GA, USA. The volume was edited by Professor Robert G Webster and Mr Scott Krauss from the Virology Division, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis TN, USA, in conjunction with the members of the WHO Animal Influenza Network including; Drs Nancy Cox, Centers for Disease Control and Prevention, Atlanta GA, USA; Yi Guan, Hong Kong University, SAR China; Alan Hay, National Institute of Medical Research, London, UK; Kangzhen Yu, Harbin Veterinary Research Institute, Harbin, China; Kennedy Shortridge, University of Hong Kong, SAR, China; Malik Peiris, University of Hong Kong, SAR, China; Hiroshi Kida, Hokkaido University, Japan; Ian Brown, Central Veterinary Laboratory Agency – UK; Klaus Stöhr, World Health Organization, Geneva, Switzerland. Each member was assigned one or more chapters for revision. The introduction and background information on surveillance for animal influenza orients the trainee in these areas and sets out the overall goals of the proposed WHO Animal Influenza Network. The laboratory procedures for the isolation and antigenic characterization of influenza viruses from humans and lower animals are essentially the same and the present manual reflects these similarities. However, there are a significant number of differences in sampling and field strategies. The chapters on the neuraminidase assay and neuraminidase inhibition assay that were not previously included in the Human Influenza Manual were developed specifically for this Animal Influenza Training Manual. The chapters on the identification of influenza viruses by Reverse Transcriptase – Polymerase Chain Reaction, the intravenous pathogenicity tests for influenza viruses and Newcastle Disease Virus were developed for this manual as was the chapter on the agar gel precipitation tests for the detection of antibodies for avian influenza.

This is the first edition of the WHO Animal Influenza Training Manual and it must be emphasized that the work was done on a voluntary basis and the manual should be considered a work in progress. We fully acknowledge that there is room for improvement and that errors may be present. We request that any omissions and errors be brought to the attention of members of the Animal Influenza Network.

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I. Surveillance for influenza

A. Introduction

Influenza is caused by a zoonotic virus that occurs in lower animals and birds as well as in humans. Influenza viruses belong to the Orthomyxovirideae family of RNA viruses and is constituted of four gencera; Influenza virus A, Influenza virus B, Influenza virus C and Thogotovirus. These viruses have segmented negative-strand RNA genomes. In lower animals and birds influenza A viruses are of primary concern while influenza B virus has been reported in seals and influenza C virus in swine.

Influenza A viruses infect a variety of animals, including humans, pigs, horses, sea mammals, and various bird species. Phylogenetic studies of influenza A viruses have revealed species-specific lineages of viral genes. There is convincing evidence that all 15 hemagglutinin (HA) subtypes of influenza A viruses are perpetuated in the aquatic bird populations of the world, especially in ducks, shorebirds and gulls. There is no evidence that influenza viruses persist for extended periods in individual birds. In aquatic birds, influenza viruses replicate preferentially in the cells lining the intestinal tract and are excreted in high concentrations in the feces; waterfowl transmit influenza viruses by the fecal-oral route through contaminated water. Studies on the ecology of influenza viruses have led to the hypothesis that all mammalian influenza viruses derive from the avian influenza reservoir. Phylogenetic analyses of the nucleoprotein gene show that avian influenza viruses have evolved into five host-specific lineages: a classical equine lineage, which has not been isolated in over 15 years; a recent equine lineage; a lineage in gulls; one in swine; and one in humans.

Studies of nucleoprotein and other gene lineages in avian species reveal separate sublineages of influenza in Eurasia and the Americas, indicating that migratory birds which move between these continents (latitudinal migration) have a lesser role in the transmission of influenza. In contrast, birds that migrate longitudinally appear to play a key role in the continuing process of virus evolution.

A surprising discovery from phylogenetic analyses was that avian influenza viruses, unlike mammalian strains, show low evolutionary rates. In fact, influenza viruses in wild aquatic birds appear to be in evolutionary stasis, with no evidence of net evolution over the past 60 years. Nucleotide changes have continued to occur at a similar rate in avian and mammalian influenza viruses, but these changes no longer result in amino acid changes in the avian viruses, while all eight mammalian influenza virus gene segments continue to accumulate changes in amino acids. The high level of genetic conservation suggests that avian viruses are approaching or have reached an adaptive optimum, wherein nucleotide changes provide no selective advantage. However, after transmission to other avian species of wild or domestic aquatic birds these influenza A viruses then show marked evolutionary changes.

Antigenic drift and antigenic shift are the mechanisms which contribute to the characteristic epidemic pattern of influenza viruses in humans. Frequently

occurring point mutations in the genes coding for the two surface proteins, the HA and the neuraminidase (NA), allow these viruses to escape existing immunity to previously circulating influenza viruses in an individual and in the population. Through this process of antigenic drift, new variants evolve, in humans throughout the world, and cause epidemics almost every year. Antigenic drift is less pronounced in avian, swine and equine influenza viruses. It does occur, but at a reduced rate, and insufficient knowledge is available to know if immune mechanisms play a role in selection of variants. The available evidence indicates that transfer between species results in an increase in antigenic drift. During antigenic shift, on the other hand, viruses emerge which contain an HA and/or a NA not present in the previously circulating human influenza viruses. Such viruses emerge after direct transmission of viruses from other hosts or after reassortment in a host which is simultaneously infected with two distinct subtypes of influenza virus type A. These reassortant viruses cause worldwide pandemics at irregular intervals. Such pandemics of influenza are often associated with high levels of morbidity and mortality worldwide. The importance of antigenic shift for the evolution of influenza viruses relevant for animal health is less well resolved and one of the areas where more information is needed.

The World Health Organization has established a worldwide network for the surveillance of human influenza. The primary goal of this international network is to detect and identify newly emerging epidemic variants in a timely manner and to contribute to the selection of appropriate vaccine strains. The goal of surveillance in lower animals and birds is to complement the human surveillance network, to understand the ecology of influenza viruses that are relevant to human and animal health and to determine the molecular basis of host range transmission and spread in new hosts. The long-term goals are to identify molecular markers of viruses that can transmit between species especially to mammals including humans.

A well-organized network of diagnostic laboratories forms the basis for the successful surveillance of respiratory viruses and other infectious diseases. The clinical specimens taken from animals are an important source of data for surveillance. Every laboratory receiving clinical specimens for the diagnosis of virus infections should maintain well-established laboratory methods which allow for the accurate identification of viruses expected to be in these specimens. The methods should be based on well-characterized and standardized reagents, and they should allow for analysis of a large number of specimens. Isolation of influenza viruses in cell cultures or in embryonated chicken's eggs is a highly sensitive method, but often requires several days to produce a conclusive result. However, for the surveillance of respiratory virus disease, in particular for influenza, successful isolation of the virus is crucial for determining its type and subtype, and for further characterization.

B. Goals of the proposed WHO Animal Influenza Network

1. There is a need to complement the existing WHO Influenza Surveillance Programme with a collaborative laboratory network which pursues the following medium and long-term objectives:
   - Promote the collection and characterization (antigenic and genetic) of animal influenza viruses of potential significance to human health from various parts of the world, especially Asia (focusing initially on China and adjacent areas), and facilitate the exchange of reference virus strains, antisera, and other biological materials
   - Facilitate scientific exchange and ensure rapid communication between the network members, the WHO secretariat and participants in the WHO Influenza Surveillance Programme
   - Coordinate standardization of diagnostic techniques and biologicals and develop reagents for rapid identification of animal influenza viruses to assist surveillance, including:
     - Antibody based tests (Polyclonal and monoclonal antibodies)
     - PCR based diagnosis (primers for sequence analysis and future development of DNA chip technology)
   - Develop and coordinate research projects on the ecology and molecular biology of animal influenza viruses
   - Facilitate training and academic exchange on diagnosis and surveillance of animal influenza viruses
   - Expand the genomic database for animal influenza viruses
   - Assist production of non-pathogenic high-growth reassortant influenza viruses of all HA subtypes suitable for future vaccines (to combat initial threat of a pandemic)
   - Increase understanding of gene constellations responsible for interspecies transmissibility and human pathogenicity of animal influenza viruses
2. The proposed WHO Animal Influenza Network [AIN] would be an integral component of the WHO Influenza Surveillance Programme, focusing on aspects of ecology and molecular biology of animal influenza viruses in the context of human health, and would be coordinated by the WHO Secretariat.
3. During the establishment and development of the AIN, due attention must be paid to potential conflicts of interest between public health and the agricultural/veterinary services in relation to surveillance and reporting of animal influenza. Collaboration with, or endorsement by all interested parties (international organizations such as Food and Agricultural Organization (FAO) and Office International des Epizooties (OIE), national public health and veterinary authorities) is therefore critical.
4. To sustain longterm the collaborative work of the AIN at the interface between interest of human and animal health authorities, it must generate information of mutual benefit which includes a discernible contribution to agricultural/veterinary services and is of significance for public health action and policies.
5. To promote the long-term development of a sustainable network, the following issues are also considered particularly important:
   - The work of the network should have a strong academic basis.

- Network activities should address practical issues and benefit all participants
- Trust and confidence between network members should be developed and maintained
- Sufficient flexibility in collaboration must be present to accommodate the diverse interest of the various partners
- Informal information exchange should be facilitated by the AIN WHO Secretariat
- Strategic development and expansion of the network should come about through consultation within the AIN network, coordinated by the AIN WHO Secretariat
- Momentum within the network by standardization of reagents, external quality assurance programmes, training courses and annual meetings
- Separate laboratory facilities be used for the isolation of influenza viruses from lower animals and from humans. The isolation of novel influenza viruses from animals including humans should be confirmed by resolution and where possible by direct immuno fluorescence, diagnostic PCR and/or serology

C. Background information on surveillance for animal influenza

Representatives of each of the 15HA and 9NA subtypes of Influenza A viruses have been isolated from aquatic birds. These viruses have been isolated worldwide from both domestic and wild species. The largest numbers of viruses have been isolated from feral water birds including ducks, geese, terns, shearwaters, and gulls as well as from a wide range of domestic avian species such as turkeys, chickens, quail, pheasants, geese, and ducks. In ducks, the majority of avian strains of influenza virus replicate predominantly in the cells lining the intestinal tract and also in the lungs and upper respiratory tract. The viruses gain access by passage through the digestive tract of the duck, despite the low pH of the gizzard and are shed in high concentration in the feces. The disease signs associated with influenza A virus infections in avian species vary considerably with the strain of virus. Infection of ducks (or birds) with most strains of influenza virus are completely asymptomatic. However, a few strains produce systemic infection accompanied by central nervous system (CNS) involvement, and death. The viruses that fall into this category are members of the H5 and H7 subtypes such as A/FPV/Dutch/27(H7N7) and A/Tern/South Africa/1/61(H5N3). The HAs of these H5 and H7 viruses is readily cleaved in tissue culture cells and does not require an exogenous protease for infectivity plaque formation. The virulent H5 and H7 viruses differ from the HAs of other influenza A subtypes in that their HAs possess multiple basic amino acids at the carboxyl terminus of HA1. This permits cellular proteases that recognize multiple basic amino acids to cleave the HA and render the virus infectious and capable of systemic spread. Influenza viruses of the other subtypes (excluding H5 and H7) can also cause disease and economic loss in domestic poultry. An example is H9 which has been associated with severe economic loss in turkeys in USA and in chickens and quail in Asia and Europe. This economic loss is often associated with co-infection with bacteria or other microbial agents.

Pigs are naturally infected with H1N1, H3N2, and H1N2 viruses. These include the H1N1 viruses of classic swine influenza, the H1N1 viruses similar to viruses isolated from avian sources, as well as the H1N2 viruses with H1 similar to H1N1 strains from humans. Influenza viruses antigenically similar to human H3N2 strains infect swine and can cause clinical signs of disease. The available evidence suggests that different variants of human H3N2 viruses since 1968 have been transmitted to pigs. There is evidence that H3N2 variants can persist in pigs after they have disappeared from the human population; thus, the A/Port Chalmers/1/73 variant of H3N2 has continued to circulate and cause disease in pigs in Europe through 2001. Influenza in swine was first observed in the United States during the catastrophic 1918 human influenza pandemic. The disease signs in pigs, as in humans, are characterized by nasal discharge, coughing, fever, laboured breathing, and conjunctivitis. Pigs are susceptible to experimental infection with all subtypes of avian influenza A viruses and H9N2 influenza viruses have been isolated from pigs in Hong Kong (SAR).

Two different subtypes of influenza A viruses have infected and caused disease in horses, H7N7 (eg A/Equine/Prague/1/56) commonly known as equine 1 and H3N8 (eg A/Equine/Miami/1/63), known as equine 2. Both viruses produce similar disease signs in horses, but equine 2 infections are usually more severe.

The signs of disease include a dry hacking cough, fever, loss of appetite, muscular soreness, and tracheobronchitis. Secondary bacterial pneumonia almost always accompanies equine influenza. Equine 1 influenza viruses have not been isolated anywhere in the world for over fifteen years, raising the possibility that this virus has disappeared. However, antibodies to equine 1 influenza virus have been detected sporadically in horses, either from vaccination or from subclinical infections. H3N8 viruses of avian origin caused severe influenza in horses in China in 1989-90 with significant mortality, but these avian H3N8 viruses do not appear to have established a permanent lineage in horses.

Influenza viruses of multiple subtypes including H3N3, H4N5, H4N6 and H7N7 have been isolated from seals, H1N3 and H13N2 from whales and H10N7 from mink. These infections can cause mortality in the infected animals notably H7N7 in seals, and have also caused conjunctivitis in humans in close contact with the seals. The available evidence indicates that these influenza viruses originate from aquatic birds, cause localized outbreaks of disease but have not established permanent lineages in seals, whales or mink.

D. Transmission of influenza viruses from lower animals to humans

There have been a number of avian influenza viruses isolated from humans and serological reports indicate that animal influenza viruses may transmit to humans especially in southern China. Convincing evidence has come from studies on swine influenza and the H5N1 and H9N2 bird flu incidents in Hong Kong (SAR). In 1976, swine influenza virus (H1N1) was isolated from military recruits at Fort Dix USA. Antigenically and genetically indistinguishable isolates were subsequently obtained from a man and a pig on the same farm in Wisconsin. These studies confirmed the earlier serological and virus isolation studies that implicated swine viruses in human disease. Sequence analysis of the 1918 influenza virus genome from archival material stored in paraffin blocks or frozen in the permafrost indicates that these viruses were genetically most closely related to influenza viruses of classical swine H1N1 influenza viruses. H1N1 and H3N1 influenza viruses swine have been isolated from children in Holland and Hong Kong (SAR). Thus influenza viruses in swine have been recognized as a potential source of viruses for humans.

The direct transmission of avian H5N1 and H9N2 influenza viruses to humans in Hong Kong (SAR) in 1997 and 1999 resulted in renewed interest in the role of avian influenza viruses as zoonotic disease agents. In May, November, and December 1997, 18 cultured or serologically confirmed cases of influenza A (H5N1) illness occurred among persons living in Hong Kong (SAR). All case-patients were hospitalized; 8 required mechanical ventilation and 6 died. Influenza A (H5N1) infections in humans coincided with outbreaks of highly pathogenic avian influenza A (H5N1) among poultry on Hong Kong (SAR) farms during March through May and among poultry in wholesale and retail markets during November and December. Before 1997, only influenza A viruses with H1, H2, or H3 HA subtypes had been known to cause outbreaks in humans. Documented human influenza A (H5N1) infections raised concerns about the pandemic potential of this subtype, which had previously only been isolated from birds. Although the exact mode of transmission of H5N1 viruses to humans is not known, exposure to poultry was a risk factor for developing disease. Human-to-human transmission, though rare, likely did occur in situations where close contact between patient and health care workers took place. The H9N2 influenza virus that transmitted to 2 children in Hong Kong caused mild influenza symptoms and was antigenically and molecularly similar to a virus circulating in quail (A/quail/HongKong/G1/97;H9N2). Other cases of H9N2 infection were reported in other parts of China.

These two incidents raise the possibility of the emergence of a pandemic influenza virus by direct transmission of avian influenza viruses to humans. Neither of these incidents resulted in viruses that transmitted successfully from human to human, but they might have brought avian influenza viruses into direct contact with human influenza viruses with the possibility of reassortment and acquisition of gene segments that are commensurate with human transmission.

E. Surveillance systems for detection of influenza viruses in lower animals and birds

Surveillance of lower animals for influenza viruses provides information that is relevant for both human and veterinary public health. Surveillance can be in response to clinical outbreaks of disease in animals or by systematic surveillance of lower animals and birds. The procedures used include detection of virus by direct virus isolation, by detection of specific genes or gene products by amplification procedures of polymerase chain reaction (PCR), rapid diagnostic kits or by detection of serological responses. Each of these procedures will be dealt with in detail in later sections.

Veterinary diagnostic laboratories
Respond to clinical outbreaks of disease in domestic poultry including pigs, horses as well as pets animals, exotic animals, and wild animals in game parks or zoological gardens.

Wildlife disease groups
Respond to disease outbreaks in wild life and conduct surveillance in response to disease in domestic animals and wild species. Also included under this heading are organizations like the Audabon Society/and Sea Mammal standing monitors.

Research based programmes
Systematic surveillance in lower animals and birds is carried out in veterinary and in human public health institutes and can be based at university or government agencies that are independent or affiliated with the OIE and/or FAO and/or World Health Organization.

The information on influenza obtained by the above groups is not necessarily coordinated and one of the goals of the present programme is to establish an informal WHO Animal Influenza Network to bring together information from these groups as well as from FAO and OIE.

II. Laboratory procedures

A. Collection of specimens

Introduction

The success of virus diagnosis largely depends on the quality of the specimen and the conditions for transport and storage of the specimen 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 for influenza. In mammals including humans, pigs and horses influenza is primarily a respiratory tract infection while in avian species influenza can be an infection of both the respiratory and large intestinal tract.

Specimens for diagnosis

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

- nasal swab
- throat swab
- tracheal swab

From **slaughter house samples** or **post mortem samples** of mammals the following samples are suitable for the diagnosis of virus infection of the lower respiratory tract:

- tracheal swab/trachea
- bronchoalveolar lavage
- lung biopsy sample

Sampling of avian species for influenza infection should include sampling of both the respiratory and large intestinal tract; samples include:

- cloacal swab
- fecal sample

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

If dead birds are detected, highly pathogenic avian influenza virus should be suspected when representative internal organs including brain, spleen, heart, lung, pancreas, liver and kidney should be sampled together with sampling of the respiratory and intestinal tract.

Transport and storage of specimens

Specimens for direct detection of viral antigen by immunofluorescence staining of infected cells should be kept on ice and processed within 1-2 hours. Specimens for virus isolation should be chilled in an ice pack immediately after collection and inoculated into susceptible cell cultures and eggs as soon as possible. If the specimen cannot be processed within 48 - 72 hours, the specimen should be kept frozen at or below -70°C. Samples for influenza should not be stored or shipped in dry ice (CO_2) unless the samples are sealed in glass or are sealed, taped and double plastic bagged. CO_2 can rapidly inactivate influenza viruses if it gains access to the sample during shrinkage of tubes during freezing.

Specimens should be collected and transported in a suitable transport medium in ice or in liquid nitrogen. Some transport systems which have proven to be satisfactory for the recovery of a wide variety of viruses are commercially available. Hanks balanced salt solution, cell culture medium, phosphate buffered saline, tryptose-phosphate broth, veal infusion broth, and sucrose-phosphate buffer are commonly used transport media. They should be supplemented with protein, such as bovine serum albumin (BSA) or gelatin, to a concentration of 0.5% to 1%, to stablilize the viruses. The addition of antibiotics and antimycotics helps prevent microbial growth.

Materials required

- 1.0 - 3.0 ml plastic screw capped nunc tubes
- Polyester or cotton fiber-tipped swabs Viral transport medium*
- Instruments for post mortem examination

**Virus Transport Medium for Clinical specimens*

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

(A) Medium 199 for transport

1. Tissue culture medium 199 containing 0.5% BSA
2. Penicillin G (2 X 106 U/liter), Streptomycin 200 mg/liter, polymyxin B (2 x 106 U/liter), gentamicin (250 mg/liter), nystatin (0.5 X 106 U/liter). Ofloxacin HCl (60 mg/liter), and sulfamethoxazole (0.2 g/liter).
3. Sterilize by filtration and distribute in 1.0ml - 2.0ml volumes in screw capped tubes.

(B) Glycerol medium

1. PBS

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
dH_2O	QS to 1 liter

2. Autoclave the PBS and mix 1:1 with sterile glycerol to make 1 liter.
3. To 1 liter PBS/Glycerol add:
   - Penicillin G (2 X 10^6 U/liter), streptomycin 200 mg/liter, polymyxin B (2 X 10^6 U/liter), gentamicin (250 mg/liter), nystatin (0.5 X 10^6 U/liter). Ofloxacin HCl (60 mg/liter), and sulfamethoxazole (0.2 g/liter).

Prepare sample vials

To sterile vials (glass or plastic screw cap) dispense 1.0 - 2.0 ml of transport media. It is preferable to store these vials at -20°C until used, but they can be stored at 4°C (or room temperature for short periods of 1-2 days only)

Preparing to take samples

Number the vials to correspond with those on the field data sheets (see attached sheet). When possible the following information should be recorded: type of animal sampled, species, type of sample (i.e., fecal), date, time, and location of sample.

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

Use of trade names and commercial sources is for identification only and does not imply endorsement by WHO or by U.S. Department of Health and Human Services.

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

Nasal swab

A dry cotton or polyester swab is inserted into the nostril parallel to the palate and left in place for a few seconds. Then it is 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 2-3 ml of transport medium and the applicator stick is broken off.

Nasopharyngeal swab for horses.

Custom swabs consisting of cotton gauze attached to the end of a 60-cm length of twisted 18-guage soft stainless steel wire encased in rubber tubing are used. This swab is sterilized by autoclaving and is reused after cleaning and replacement of the cotton gauze. For swabbing, the gauze end is inserted as far as possible into the horse's nasopharynx, about 30 cm for an adult horse, 25 cm for a pony, and 20 cm for a foal. Twitching or tranquilization of the horse may be necessary to safely perform satisfactory nasopharyngeal swabbing. Care must be taken that the guaze does not come loose from the swab and remain in the nasopharynx. For ponies or foals the gauze may require trimming to be accomodated by the nasal passage. Upon withdrawal the gauze is removed and immersed in a vial containing 5 ml of transport medium.

The diagnostic laboratory processes the swab by stirring the transport vial and swirling the gauze to free trapped mucosal material; then the gauze is transferred to the barrel of a 10 ml syringe and the fluid squeezed into a test tube together with the remaining contents of the vial. Swab fluid thus obtained is used directly for testing, and is not filtered or otherwise treated.

Throat swab

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

Tracheal swab

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

The trachea of dead animals including pigs at slaughter houses can be swabbed after the lungs and trachea have been removed from the animal. The trachea is held in a gloved hand and the swab inserted to its maximal length with vigorous swabbing of the wall. The swabs are placed in transport medium as above.

Cloacal swab

The cloaca of live birds is swabbed by inserting a swab deeply into the vent and vigorously swabbing the wall. The swab should be deeply stained with fecal material and is then placed in transport medium as above.

Fecal samples

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

Tissue samples

Tissue samples are best frozen immediately without transport medium and later ground in transport medium prior to inoculation of eggs or tissue culture. Small samples of tissue are suspended in transport medium.

Sera collection for influenza diagnosis and surveillance

Optimally an acute phase serum specimen (3 - 5 ml of whole blood) should be taken soon after onset of clinical symptoms but not later than 7 days, and a convalescent phase serum specimen should be collected 2 to 4 weeks later. In serological surveillance studies at slaughter houses or from free flying wild birds that are bled and released only a single sample of serum is available. The serum is allowed to clot then the sera should be centrifuged at 2500 rpm/15 mins to separate the RBCs and serum. The serum should be pipetted off, and the RBCs may be discarded. Single serum specimens cannot be used for individual diagnosis. Serum samples are stored at -20°C.

Transporting specimens to the laboratory

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

B. Isolation of influenza viruses

Introduction

Virus isolation is a highly sensitive and very useful technique for the diagnosis of viral infection when used with clinical specimens of good quality. In fact, isolation of a virus in eggs or cell culture along with subsequent identification by immunologic or genetic techniques (or by electron microscopy) are standard methods for virus diagnosis. One important advantage of virus isolation is that the virus is available for further antigenic and genetic characterization, and also for vaccine preparation or drug-susceptibility testing if required.

Several factors limit the use of virus isolation as a diagnostic technique. Each of the currently available mammalian cell lines supports the replication of only a limited number of clinically important viruses; therefore, the laboratory must maintain several cell lines. On the basis of the clinical information and epidemiologic situation, appropriate cell lines should be selected. MDCK cells are the preferred host for influenza but other cells can be used providing they will support the replication of influenza viruses.

Most avian viruses grow readily in embryonated eggs and this is the option of choice for investigation of avian hosts. However, some human and porcine viruses grow poorly in eggs, especially if inoculated by the allantoic route alone. In such instances, MDCK cells are an useful additional approach to isolating virus. In general, embryonated eggs alone would suffice for investigation of avian hosts, but for other animals a combination of embryonated eggs and cell culture is desirable.

The following protocols provide instructions for the isolation of influenza viruses in cell culture and eggs. This procedure allows virus identification by immunofluorescence and provides isolates for further analysis.

Processing clinical material for virus isolation

(A) Nasal tracheal, cloacal and fecal swabs

1. Thaw if necessary and add 1/10th volume of 10^{x}-X concentrated antibiotics.
2. Vigorously agitate 1-2ml collection vial with swab on vortex mixer.
3. Leave at room temperature for 30 minutes to settle.
4. For cloacal and fecal samples the swab can be used as a filter enabling filling of the sample into the syringe.
5. Inoculate the specimen and store the remainder at -70^{o}C.

(B) Tissue samples

1. Grind tissue in a sterile glass on glass tissue grinder or in a sterile mortar and pestle with crushed glass from a pasteur pipette, making a 10% suspension with transport medium.

2. Add 1/10 volume of 10x antibiotic mixture. Transfer the specimen to a centrifuge tube and centrifuge at 400xg for 10 min to remove extraneous materials.
3. Remove supernatant, inoculate, and store an aliquot at -70°C.

C. Isolation in cell culture

Materials required

- Madin-Darby Canine Kidney cells (MDCK)
  American Type Culture Collection
  ATCC CCL 34
- T-75 tissue culture flasks, canted neck
  Corning Cat. #430720
- Dulbecco's Modified Eagle Medium
  (D-MEM)*
  GIBCO BRL Cat. # 11965-092
- RPMI Medium 1640 (RPMI 1640)*
  GIBCO BRL Cat. # 11875-036
- Penicillin-Streptomycin, stock solution
  (10,000 U/ml penicillin G; 10,000 µg/ml streptomycin sulfate)
  GIBCO BRL Cat. # 15140-023
- HEPES Buffer, 1 M stock solution
  GIBCO BRL Cat. # 15630-023
- Bovine serum albumin fraction V, 7.5% solution
  GIBCO BRL Cat. # 15260-011
- Fetal bovine serum, 40 nm filtered
  HyClone Laboratories, Inc. Cat. # A-1111-L
- Trypsin-EDTA
  (0.05% trypsin; 0.53 mM EDTA . 4Na)
  GIBCO BRL Cat. # 25300-054
- Trypsin, TPCK treated
  (type XIII from bovine pancreas)
  Sigma Cat. # T-8642
  (See below for preparation of stock solution)
- Gentamicin reagent solution (50mg gentamicin sulfate/ml)
  GIBCO BRL Cat. # 15750-011

**Suggestion: Always check label for expiration date.*

Preparation of media and reagent formulas

(A) Preparation of complete D-MEM with L-glutamine (MDCK cells):

to 500 ml D-MEM add:

- Penicillin/Streptomycin
  5 ml (Final concentration: 100 U/ml Penicillin and 100 µg/ml Streptomycin)
- Bovine serum albumin
  12.5 ml (Final concentration: 0.2 %)
- HEPES buffer
  12.5 ml (Final concentration: 25 mM)
- Reagent (for growth media only)
  Fetal bovine serum 10 ml added to 90 ml of complete D-MEM to make approx 10% FBS.
- Media for virus growth:
  Add 0.5 ml of TPCK-trypsin stock solution containing 2mg/ml per ml of complete D-MEM; (without serum) this results in a final concentration of 2 µg/ml.

(B) Preparation of TPCK-trypsin stock solution

a. Dissolve 20 mg TPCK-trypsin* in 10 ml.
b. Filter through 0.2 µm membrane.
c. Store in aliquots at -20°C.

**Suggestion: Always check label for expiration date.*

Preparation of MDCK cells in tissue culture flasks

Procedure

The procedure for preparing an MDCK cell suspension is described for confluent T-75 flasks. If cell culture flasks of other sizes are used, the volumes have to be adjusted accordingly. One T-75 flask with a confluent monolayer of MDCK cells contains approximately 107 cells.

1. Decant medium and add 5 ml of trypsin-EDTA pre-warmed to 37°C.
2. Distribute trypsin-EDTA over entire cell sheet by gently rocking the flask for 1 min. Remove trypsin-EDTA with pipette.
3. Add another 5 ml of trypsin-EDTA solution and rock flask as described above for 1 min. Remove trypsin-EDTA with pipette.
4. Add 1 ml of trypsin-EDTA solution. Distribute trypsin-EDTA over entire cell sheet and incubate flask at 37°C until all cells detach from plastic surface (5 - 10 min). The flasks may need shaking or tapping to detach cells.
5. Add 1 ml of FBS to inactivate remaining trypsin.
6. Add 8 ml of complete D-MEM. Pipette up and down gently to break up cell clumps.
7. Transfer the 10 ml mixture to 90 ml of complete D-MEM containing 10% FBS for a final concentration of 10% FBS. (This cell suspension contains approximately 105 cells per ml.)
8. Add 6 ml (600,000 cells) of this cell suspension to an appropriate number of T-25 flasks. The remaining cell suspension can be used to seed T-75 flasks for cell passage. Usually 5 ml cell suspension added to 20 ml of media provides adequate cells for obtaining a confluent monolayer in several days.
9. Incubate flasks at 37°C.

Quality control

Over a number of passages, MDCK cells might lose their susceptibility to respiratory viruses. For this reason, the laboratory should keep a stock of cells at a low passage level frozen in liquid nitrogen with 7.5% Dimethyl Sulfoxide (DMSO) and 15% fetal bovine serum. After 15-20 passages of MDCK cells, a new vial of cells must be thawed out, if sensitivity of the isolation system is to be maintained. At intervals, the sensitivity of the MDCK cell line must be assessed using positive control virus of known titre. Cell lines should be free of mycoplasma contamination.

Inoculation of cell culture

NOTE: All working steps during this procedure must be performed in a class 2 biosafety cabinet.

Materials required

- Confluent monolayer of MDCK cells in flasks or tubes
- Trypsin, TPCK treated (type XIII from bovine pancreas) Sigma Cat. # T-8642
- HEPES Buffer, 1 M stock solution GIBCO BRL Cat. # 15630-023
- Dulbecco's Modified Eagle Medium (D-MEM)* GIBCO BRL Cat. # 11965-092
- Penicillin-Streptomycin, stock solution (10,000 U/ml penicillin G; 10,000 µg/ml streptomycin sulfate) GIBCO BRL Cat. # 15140-023
- Bovine serum albumin fraction V, 7.5% solution GIBCO BRL Cat. # 15260-011
- 3 clinical specimens in 2ml wheaton vials (For teaching purposes the clinical specimen has been prepared for inoculation.)
- 4 T-25 flasks seeded with MDCK cells (NB: cells grown in tissue culture tubes may be used, in place of flasks, when large numbers of specimens are being processed. Cell numbers would be adjusted accordingly).
- 1ml pipettes
- 10ml pipettes

Procedure

I. Preparation of flasks

1. Check the cells with microscope at 40X magnification.
2. Replace growth medium with medium for virus growth. Be sure to use proper media as indicated.
3. Decant growth medium into a beaker and wash three times with 6 ml of D-MEM containing 2 µg/ml of TPCK-trypsin.

II. Inoculation of flasks

1. Remove D-MEM from flask with sterile pipette.
2. Inoculate 200 µl of each specimen into a T-25 flask using sterile pipettes.
3. Allow inoculum to adsorb for 30 minutes at 370C.
4. Add 6ml of complete media (D-MEM) containing 2 µg/ml of TPCK-trypsin without calf serum to T-25 flasks.
5. Observe daily for cytopathogenic effect (CPE).

III. Harvesting of flasks

1. Harvest the cell culture if 3+ or 4+ CPE is observed by collecting supernatant fluid and adding stabilizer such as glycerol gelatin or bovine serum albumin to a final concentration of 0.5%. Harvest by day 6 or 7, even if no CPE is observed.
2. Perform a hemagglutination test and store at 40C. If no HA is present, passage up to 2 more times before reporting inability to recover virus from the specimen.
3. If necessary, centrifuge the tubes at 3000 rpm/5 minutes to remove excess cells. Identify the isolate by hemagglutination inhibition testing and store the isolate at -70°C within 1 days of harvest.

D. Inoculation of embryonated chicken eggs

NOTE: All working steps during this procedure must be performed in a class 2 biosafety cabinet.

Materials required

- embryonated chicken eggs, 10 days old
- egg candler
- 70% ethanol
- needle, 22 gauge, 1½ inch
- syringe, 1ml
- egg hole punch
- glue or varnish
- 15ml tubes & rack
- 10ml pipettes
- forceps (sterile)

Materials provided

3 clinical specimens in 2ml wheaton vials (For teaching purposes the clinical specimens have been prepared for inoculation.)

Procedure

I. Candling of eggs

1. Examine eggs with an egg candler and place with blunt end up into egg trays.
2. Discard any eggs that are infertile, have cracks, are underdeveloped, or that appear to have a porous shell.

II. Inoculation of eggs

1. Place eggs with blunt end up into egg trays and label each egg with a specific identification number (3 eggs per specimen).
2. Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac. Three eggs per specimen are usually inoculated.
3. Aspirate 1ml of processed clinical specimen into a tuberculin syringe with a 22 gauge, 1½ inch needle.
4. Holding the egg up to the candler, locate the embryo. Insert the needle into the hole of the egg. Using a short stabbing motion, pierce the amniotic membrane and inoculate 100 µl into the amniotic cavity. Withdraw the needle about ½ inch and inoculate 100 µl of the specimen into the allantoic cavity. Remove the needle.
5. Inoculate the two other eggs in the same manner with the same syringe and needle for a total of three eggs inoculated per specimen.
6. Discard syringe into a proper safety container.

7. Seal the holes punched in the eggs with a drop of glue.
8. Incubate the eggs at 33-34°C for 2-3 days.

Note: Avian influenza 35ºC or 37ºC; mammalian influenza 35ºC (avian viruses also grow well at 35ºC)

III. Harvesting of inoculated chicken eggs

1. Eggs are chilled at +4°C overnight or for 4 hours before harvesting.
2. Label one plastic tube (15ml) for each egg with the specimen number. Clean off the top of each egg with 70% ethanol.
3. With sterile forceps, break the shell over the air sac and push aside the allantoic membrane with the forceps. Using a 10ml pipette, aspirate the allantoic fluid and place in a labeled plastic tube. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Place harvest in a separate tube, but because of the low volume of amniotic fluid obtained from each egg, it is usually necessary to combine the amniotic fluid from the three eggs inoculated per specimen.
4. Centrifuge harvested fluids at 3000rpm/5minutes to remove blood and cells and perform a hemagglutination test and incubate at 4ºC/30 mins. If no HA is present, passage the specimen 2 more times before reporting inability to recover virus from the specimen.
5. If necessary, centrifuge the tubes at 3000rpm/5minutes to remove excess blood and tissues. Identify the isolate by hemagglutination inhibition testing and store the isolate at -70°C within 1 days of harvest.

Precautions

I. Never use -20°C for storage of isolates! Influenza viruses are very unstable at this temperature.

II. Be aware of contamination of clinical specimens with laboratory strains:

Proper safety procedures always must be observed when handling influenza viruses, however, special precautions should be observed when working with clinical specimens and laboratory-adapted influenza virus strains in the same work area.

Some laboratories prepare their own laboratory-adapted influenza viruses for positive controls. In addition, laboratories frequently use commercially available influenza reference viruses for their quality assurance program. These viruses are selected because of their optimal growth properties. Consequently, laboratory-adapted and reference influenza virus strains are a frequent source of cross contamination with clinical specimens. It is extremely important that all laboratory-adapted controls be prepared, tested, and stored well in advance of the influenza season. If laboratory-adapted control viruses must be replenished during the influenza season, this should be done on days when clinical material is not being inoculated. Likewise, quality assurance tests with commercial reference viruses should be performed outside the influenza season or on days when clinical material is not being inoculated. Acceptable laboratory practice always requires that known viruses and unknown materials be worked

with at different times and in separate biosafety cabinets or rooms. Likewise, it is critical that clinical material obtained from swine or birds be processed in separate laboratories by different laboratory staff.

Identification of contamination

Because laboratory-adapted viruses and commercial reference viruses are prepared using well adapted strains they usually grow to high titers. If in doubt, complete antigenic analysis by HAI using selected reference antisera and sequencing can be performed to determine if an isolate has been contaminated inadvertently.

GOLDEN RULE 1: NEVER PROCESS CLINICAL SPECIMENS FOR VIRUS ISOLATION AND LABORATORY-ADAPTED INFLUENZA STRAINS AT THE SAME TIME.

GOLDEN RULE 2: NEVER PROCESS CLINICAL SPECIMENS FROM HUMANS AND FROM SWINE OR BIRDS IN THE SAME LABORATORY.

E. Identification of influenza isolates by hemagglutination inhibition

Introduction

The hemagglutinin (HA) protein agglutinates erythrocytes; hence, the derivation of its name. The traditional method for identifying influenza field isolates takes advantage of this property. Specific attachment of antibody to the antigenic sites on the HA molecule interferes with the binding between the viral HA and receptors on the erythrocytes. This effect inhibits hemagglutination and is the basis for the hemagglutination inhibition (HAI) test. The HAI test, originally described by Hirst (1942) and later modified by Salk (1944), is currently performed in microtiter plates. In general, a standardized quantity of HA antigen is mixed with serially diluted antisera, and red blood cells are added to determine specific binding of antibody to the HA molecule.

The HAI test is extremely reliable, provided reference antisera are available to all subtypes. Disadvantages of the HAI test include the need to remove non-specific inhibitors which naturally occur in sera, to standardize antigen each time a test is performed, and the need for specialized expertise in reading the results of the test. However, the HAI assay remains the test of choice for WHO global influenza surveillance.

Hemagglutination and Hemagglutination Inhibition Test

Materials required

I. Reference antisera to HA subtypes.

Reference antisera to many but not all of the 15 HA subtypes have been prepared in goats or sheep. These reference antisera were prepared with isolated HA and are considered monospecific. In the cases where monospecific antisera is not yet available polyclonal antisera to the reference virus in those subtypes has been prepared. In some subtypes that contain human, swine, and equine viruses, multiple antisera are included to more accurately reflect the antigenic diversity within a subtype.

These reference sera are designed to distinguish between subtypes but are broadly cross reactive to detect as many different variants as possible within a subtype. Reference antisera for field isolate identification are prepared in sheep by multiple intramuscular injections with purified HA or in chickens by intravenous inoculation with virus grown in embryonated eggs. Control antigens consist of infected allantoic fluid inactivated by beta-propiolactone. The preparations are derived from either the wild type vaccine strain or a high growth reassortant made using the wild type strain or an antigenically equivalent strain.

The categories of required reagents to identify all subtypes are listed in Table1.

Table 1: Reference antisera and antigens to the HA of influenza A and B viruses

Influenza A	
H1a	A/PR/8/34 (H1N1)
H1b	A/FM/1/47 (H1N1)
HIc	A/swine/IA/30 (H1N1)
H2a	A/Japan/305/57 (H2N2)
H3a	A/Hong Kong/1/68 (H3N2)
H3b	A/equine/Miami/63 (H3N8)
H3c	A/duck/Ukraine/63 (H3N8)
H4	A/duck/Czech/56 (H4N6)
H5	A/tern/So.Af./61 (H5N3)
H6a	A/turkey/MA/65 (H6N2)
H7a	A/equine/Prague/56 (H7N7)
H7b	A/FPV/Rostock/34 (H7N1)
H8	A/turkey/Ont./6118/68 (H8N4)
H9	A/turkey/WI/66 (H9N2)
H10	A/chicken/N/Germany/49 (H10N8)
H11a	A/duck/England/56 (H11N6)
H11b	A/duck/Memphis/546/74 (H11N9)
H12	A/duck/Alberta/60/76 (H12N5)
H13	A/gull/MD/707/77 (H13N6)
H14	A/mallard/Ast./263/82 (H14N5)
H15	A/shearwater/W.Aus./79 (H15N8)
Influenza B	
B/Lee/40	

Monospecific antisera to H12 - H15 are in preparation and may be provided as polyclonal sera

II. Buffers and reagents

- Red blood cells in Alsever's solution (may be chicken, turkey, human type "O", or guinea pig RBCs)
- Sterile distilled or deionized H_20
- Phosphate-buffered saline (0.01M), pH 7.2 (PBS)
- Physiological saline, 0.85% NaCl

III. Equipment

- 37°C waterbath
- 56°C waterbath
- Centrifuge

IV. Supplies

- Pipettes and pipettor
- Multichannel pipettors
- Tubes and racks
- Centrifuge tubes (graduated conical 50 ml and 15 ml)
- Gauze squares
- 96-well microtiter plates
  Costar #3897: V-shaped for use with chicken or turkey RBCs
  Costar #3797: U-shaped for use with human type "O" or guinea pig RBCs
- 50-ml tubes for antigen dilutions

Preparation of reagents and solutions

1. Phosphate-buffered saline (0.01M), pH 7.2 (PBS)

   a. Prepare stock 25 times concentrated (25X) phosphate buffer containing in 100 ml: 2.74 g dibasic sodium phosphate (Na_2HPO_4) and 0.79 g monobasic sodium phosphate monohydrate (NaH_2PO_4 x H_2O).

   b. To prepare PBS, mix and dissolve in deionized, distilled water, and q.s. to 1 liter: 40 ml of 25X phosphate buffer and 8.5 g of sodium chloride (NaCl).

   c. After thorough mixing, check pH = 7.2 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary.

   d. Autoclave or filter to sterilize.

   e. Store opened PBS, pH 7.2 at 4°C for no longer than 3 weeks.

2. Alsever's

   a. Weigh out, dissolve in distilled water, and q.s. to 1 liter:
   20.5 g dextrose
   8.0 g sodium citrate dihydrate ($Na_3C_6H_5O_7$ x $2H_2O$)
   4.2 g sodium chloride (NaCl)
   0.55 g citric acid ($C_6H_8O_7$)

   b. After thorough mixing, check pH = 6.1 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary. Sterilize by filtration through a membrane filter with 0.22 µm pore size. Contamination is likely in this solution if it is not adequately sterilized.

3. Physiological saline, 0.85% NaCl
    a. Prepare a 20x stock solution by dissolving 170 g of NaCl in deionized water q.s. for 1000 ml.
    b. Sterilize by autoclaving at 121°C.
    c. To prepare physiological saline, 0.85% NaCl, add 50 ml 20x stock solution to 950 ml deionized water.
    d. Sterilize by autoclaving at 121°C.
    e. Store opened physiological saline at 4°C for no longer than 3 weeks.
4. Standardized RBCs (See Appendix)

Procedure for HAI identification of field iIsolates

I. Treatment of Reference Antisera for Inactivation of Nonspecific Inhibitors*

Reference antisera to influenza viruses prepared in most animals (ferret, rabbit, goat, sheep) must be treated with receptor destroying enzyme (RDE) to remove nonspecific inhibitors. Poultry antisera (e.g. chicken) can be used without RDE treatment but it is still advisable to treat with RDE to confirm positive results.

1. Reconstitute lyophilized reference antisera with sterile distilled H_2O to volume indicated on label. Store reconstituted antisera at -20°C to -70°C.
2. Reconstitute and store RDE (receptor destroying enzyme). [see below]
    a. Reconstitute the RDE with 25 ml physiological saline, 0.85% NaCl.
    b. Aliquot and store at -20°C to -70°C.
3. Add 3 vol of RDE to 1 vol serum (0.9 ml RDE + 0.3 ml serum). *Note: This volume is sufficient for testing 50-55 field isolates.*
4. Incubate overnight in a 37°C waterbath.
5. Heat in a 56°C waterbath for 30 min to inactivate remaining RDE.
6. Allow antisera to cool to room temperature. Add 6 vol (1-8 ml) of physiological saline, 0.85% NaCl. The final dilution of antisera is 1:10.
    - The reconstitution of RDE is done according to the instructions in the package insert and can vary from batch to batch. Alternative protocols for removing non-specific inhibitors from serum are give in the Appendix.

II. Identification of Nonspecific Agglutinins in Treated Sera

1. Choose the appropriate plate and add 25 µl of PBS (pH 7.2) to B through H (B1-H6) wells of each of six numbered columns.
2. Add 50 µl of PBS (pH 7.2) to the first well (A6) in column number 6 for a RBC control.
3. Add 50 µl of each treated antiserum to the first well (A1 - A5) of row A.
4. Prepare serial twofold dilutions of the antisera by transferring 25 µl from the first well of number columns 1-6 to successive wells in each column. Discard the final 25 µl after row H.
5. Add 25 µl of PBS, pH 7.2 to all wells of each of the six numbered columns.

6. Add 50 µl of standardized RBCs.
   (NOTE: For the course, standardized chicken RBCs will be provided to the student.)
7. Mix by using a mechanical vibrator or by manually agitating the plates thoroughly.
8. Incubate the plates at room temperature (22° C to 25° C) for the appropriate time by checking the cell control for complete settling of RBCs. Thirty minutes is usually required for chicken or turkey RBCs. Sixty minutes is required for guinea pig or human type "O" RBCs. (Table 2)

Interpretation

If the RBCs completely settle, the antiserum is acceptable for use in the HAI test. The presence of nonspecific agglutinins will be evident by hemagglutination of the RBCs by diluted antisera. In this case, the antisera must be adsorbed with RBCs according to Section III below.

III. Adsorption of Antisera to Remove Nonspecific Agglutinins

1. To one volume of packed RBCs, add 20 vol of RDE-treated serum.

Table 2 Influenza Hemagglutination with Different Species of RBCs

	Chicken	Turkey	Guinea Pig	Human Type O
Concentration	0.5%	0.5%	0.75%	0.75%
Microtiter plate	"V"	"V"	"U"	"U"
Incubation time, 25°C	30min	30min	1 hour	1 hour
Appearance of control cells	button*	button*	halo	halo

* = flows when tilted

2. Mix thoroughly and incubate at 4°C for 1 hr, mixing at intervals to resuspend cells.
3. Centrifuge at 1200 rpm for 10 min.
4. Carefully remove the adsorbed serum without disturbing the packed cells.
5. Repeat serum controls as described above.
6. Repeat adsorption with RBCs until the serum controls are negative.

IV. HA Titration of Control Antigens and Field Isolates

1. Choose V-shaped 96-well microtiter plates if using chicken or turkey RBCs. Choose U-shaped microtiter plates if using guinea pig or human type O RBCs.

2. Add 50 µl of PBS (pH 7.2) to #2 through #12 (A2 - H12) wells of each lettered row.
3. Add 100 µl of each control antigen or field isolate to the first well (A1-F1) of the lettered rows except rows G & H.
4. Prepare an RBC control well in row H (H1) by adding 100 µl of PBS.
5. Make serial twofold dilutions by transferring 50 µl from the first well of lettered rows to successive rows. Discard the final 50 µl.
6. Add 50 µl of RBC suspension to each well on the plate.
7. Mix by using a mechanical vibrator or by manually agitating the plates thoroughly.
8. Incubate the plates at room temperature (22° to 25°C). Check cell control for complete settling of RBCs.
9. Record results.

Interpretation

Hemagglutination occurs when the RBCs are in suspension after the RBC control has settled completely. This is recorded using a "+" symbol. When a portion of the RBCs is partially agglutinated or partially settled, a "+/-" symbol is used. In the absence of hemagglutination, chicken or turkey RBCs form a compact button on the bottom of the wells. A " - " symbol is used to record the absence of hemagglutination. Hemagglutination can be determined by tilting the plates and noting the absence of tear-shaped streaming of erythrocytes which flow at the same rate as RBC controls. Guinea pig or human type O RBCs will appear as a "halo" or circle of settled cells on the bottom of the wells. The RBC control should be completely settled either as a compact button or "halo"

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

V. Preparation of Standardized Antigen for the HAI Test and "Back Titration"

A "unit" of hemagglutination is not a measure of an absolute amount of virus, but is an operational unit dependent on the method used for HA titration. An HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension.

1. Determine the volume of standardized antigen needed for the HAI test. For example, 1 ml of antigen will test 5 sera, each of which is diluted in 8 wells, with 25 µl of antigen added to each well (5 sera X 8 wells X 25 µl = 1 ml of standardized antigen). Prepare an additional 1.0 ml additional volume for "back titration" and wastage.
2. The standard for the HAI test is 4 HA units of virus/antigen added to twofold dilutions of antisera. Since we are adding 25 µl of antigen in the test, we need a virus dilution that contains 4 HA units/ 25 µl or 8 HA units/50 µl. Calculate the antigen dilution by dividing the HA titer (which is based on 50 µl) by 8 because you wish to have 8 HA units/50 µl. For example, an HA titer of 160 divided by 8 is 20. Mix 1 part of virus with 19 parts PBS to obtain the desired volume of standardized antigen (Ex:

Add 0.1 ml antigen to 1.9 ml of PBS). Calculate and prepare dilution. Keep a record of the dilution prepared.

3. Perform a "back titration" to verify units by performing a second HA test using the standardized antigen dilution preparation. Store the diluted antigen at 4°C and use within the same day.
4. Record results.

Interpretation

Standardized antigens must have an HA titer of 4 HA units/25µl. This titer will hemagglutinate the first four wells of the back titration plate. If an antigen does not have an HA titer of 8, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units. For example, if complete hemagglutination is present in the fifth well, the virus now has a titer of 16 and the test antigen should be diluted twofold. Conversely, if hemagglutination is only present to the third dilution, the antigen has a titer of 4 and an equal volume of virus must be added to the test antigen as was used when the antigen was initially diluted. This will double the concentration of virus in the test antigen to give a titer of 8. Continue adjusting the concentration of antigen until 4 HA units/25µl (8 units/50µl) is obtained.

VI. HA-HAI Test: Identification of Field Isolates

1. Label appropriate microtiter plates.
   - The actual antisera to be used in the training course will be provided in the class room and will include antisera to distinguish to two different lineages of H9 influenza viruses.
2. Add 25 µl of PBS to wells B through H (B1 - H12) of each numbered column.
3. Using the set of sera provided which are already diluted 1:10, add 50 µl of each serum to the first well of the appropriate numbered column. For example, serum #1 should be added to well A1 and well A8; serum #2 to A2 and A9, etc.
4. Add 50 µl of PBS to the first well of columns 6 and 7 (A6 - A7) for cell control.
5. Prepare serial twofold dilutions of the treated sera by transferring 25 µl from the first well of numbered columns 1-12 to successive wells. Discard the final 25 µl after row H.
6. Add 25 µl of standardized control antigen #1 to all wells of a complete set of diluted treated sera (Ex: A1 - H5). Continue with remaining standardized control antigens and test antigens.

Note: Four HA units are added to the test in 25µl because the HA unit calculations were based on a volume of 50µl and the antigen concentration was adjusted to give an HA titer of 8 units/50µl.

7. Add 25 µl of PBS instead of antigen to serum control plate.
8. Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec or by agitating the plates manually.

10. Cover the plates and incubate at room temperature (22° to 25°C) for 15 min.

11. Add 50 µl of standardized RBCs to all wells. Mix as before.

    - Cover the plates and allow the RBCs to settle at room temperature (22° to 25°C) for the appropriate time according to the RBCs being used.

12. Record the results.

13. Record HAI titers.

Interpretation
If an antigen/antibody reaction occurs, hemagglutination of the RBCs will be inhibited. Symbols of "+" for HA, "+/-" for partial, and "- " for inhibition of HA are used. The HAI titer is the reciprocal of the last dilution of antiserum that completely inhibits hemagglutination.

To identify a field isolate, compare the results of the unknown field isolates to those of the antigen control. An isolate is identified as a particular type or subtype if the field isolate reacts with one reference antiserum to a fourfold or greater HAI titer than to other antisera. The higher titer is assumed to be homologous.

Quality control/quality assurance limitations

Note: To ensure optimal HAI test performance when identifying field isolates or diagnosing infections serologically, it is essential that test procedures be followed exactly.

1. Standardized antigen dilutions must contain 4 HA units/25 µl. The antigen dilutions must be prepared and back titrated each test day.
2. Incubation times must be strictly observed. Plates must be read promptly when the RBC control has completely settled. Elution of the RBCs from the virus can occur with some virus strains. When this happens, the plates may be read earlier or placed at 4°C.
3. RBC suspension must be standardized consistently.
4. Test reagents must be handled and stored in the prescribed manner. To minimize freeze-thaws and to avoid bacterial contamination, dispense reagents in small volumes using sterile techniques.
5. Lyophilized reagents should be reconstituted to the volume described and stored according to instructions.
6. Depending on the choice of RBCs, appropriate microtiter plates ("U" or "V" shaped) must be used.
7. Avoid bacterial contamination. Agglutinins of non-influenza origin in contaminated specimens may react nonspecifically with all antisera.

Note: To monitor HAI test performance when identifying field isolates or diagnosing infections serologically, it is essential to include appropriate controls.

1. RBC controls allow adjustments in incubation times. There should be an RBC control on each plate, if possible.
2. Each field isolate antigen and the control antigens must be tested with a negative serum control. This control will allow identification of anti-

host component antibodies. When this occurs, the sera may be adsorbed with uninfected host cells.

3. Serum controls for all sera are required. Nonspecific agglutinins which must be removed will be detected with this control.
4. Reference antisera must be included as positive control sera in each diagnostic serologic test.
5. Appropriate record sheets should be kept of each test performed to monitor reproducibility.

Limitations

Growth properties of the virus

Evolution of influenza viruses can result in changes in host susceptibility. Influenza viruses from avian hosts usually grow best in chicken embryos. Influenza viruses from mammals especially from pigs may grow poorly in chicken embryos and should also be grown in MDCK cells (See section on preparation of cell cultures)

Differences in the ability of viruses to hemagglutinate RBCs

Avian and mammalian erythrocytes hemagglutinate influenza viruses. Amino acid changes located in and around the receptor binding pocket of the HA molecule sometimes result in a loss of sensitivity to certain RBCs. Chicken RBCs are frequently chosen for HAI testing because the settling time is shorter, inhibition patterns are clearer, and the cells are readily available. It is known that some strains of influenza during initial and early passage may not hemagglutinate chicken RBCs. Guinea pig RBCs have consistently been more sensitive for the detection of human influenza viruses and, therefore, should be used for maximum sensitivity in detecting newly isolated viruses. Currently circulating human influenza viruses react with turkey RBCs or chicken RBCs. Selection of RBCs should take into account the host of origin of influenza virus isolate/reference strain.

Serum nonspecific inhibitors

Non-antibody molecules present in serum are capable of binding to the hemagglutinin, resulting in nonspecific inhibition and leading to false interpretations. This effect is believed to occur because these serum components contain sialic acid residues that mimic the receptors of RBCs, and compete with RBC receptors for the influenza hemagglutinin. To perform a valid HAI test, one must ensure that the serum does not contain nonspecific inhibitors reactive with the virus antigen being tested. Three molecular types of inhibitors, designated alpha, beta, and gamma, have been described in human or animal sera. The inhibitors exhibit different levels of activity against the hemagglutinin of different influenza strains. Several methods exist for inactivating nonspecific inhibitors in sera of different species. When nonspecific inhibitors create a problem with interpretation in HAI tests, different treatment methods may need to be investigated.

F. Serologic diagnosis of influenza virus infections by hemagglutination inhibition

Introduction

Influenza virus diagnosis by virus isolation definitively identifies the infecting strain and is usually more rapid than serologic diagnosis. However, serologic diagnosis is an important approach when clinical specimens are unavailable or when the laboratory does not have the resources for virus isolation. Many laboratories rely on serology for determining recent individual infections. Individual diagnosis must be based on acute- and convalescent-phase sera collected 2 to 3 weeks apart. The acute phase serum sample should be taken as close to symptom onset as possible. Antibody response will be reflected by a rise in titer to one type or subtype of virus. Since most human and many chicken, pig and horse sera contain antibodies to influenza viruses, diagnosis of individual cases using a single convalescent-phase serum is unreliable and should not be attempted.

Although a single serum is unreliable for individual diagnosis, single sera can be used for presumptive diagnosis in an outbreak situation. If a statistically significant number of sera from individuals in the acute phase of illness and an equal number matched according to age and other demographic factors is collected from individuals in the convalescent phase of illness, the sera can be tested simultaneously for antibody. Based on geometric mean titers, a significant statistical difference between the two groups must be shown before the outbreak can be determined to be due to influenza. Another procedure is to collect single sera from cases bled several weeks after onset and pair these samples with matched non-ill controls from the outbreak or from historical bleeds before the outbreak. In this case, the analysis is more difficult since non-ill persons or historical controls may have antibody titers sufficiently high enough to obscure the results. This could happen for various reasons such as asymptomatic infection or, with historical controls, undocumented influenza virus circulating in the population.

Although other diagnostic tests such as complement fixation and enzyme immunoassay can be used for serological diagnosis, the HAI test is preferred.

Sera from poultry particularly from chickens and quail have low or undetectable levels of non-specific inhibitors of hemagglutination. Consequently it is possible to do serological tests without RDE treatment of sera. However we do not know if avian sera are uniformly free of non-specific inhibitors so preliminary testing is needed to varify absense of non-specific inhibitor to any particular subtype of influenza virus. In principal a batch of RDE sera should always be included as control.

Procedure for serologic diagnosis

Materials required

See section on Identification of Influenza Isolates by Hemagglutination Inhibition.

Procedure

I. Treatment of sera

1. See section on Identification of Influenza Isolates by Hemagglutination.
2. Adsorb sera to remove nonspecific agglutinins as described above.

II. Standardization of RBCs

- Standardize RBCs according to the procedure in Appendix.
  Note: For the course, participants will be provided with standardized chicken RBCs.

III. HA Titration of control antigens

- Following the procedure for titration of the control antigens given above.

IV. Preparation of standardized antigens for HAI test and "Back Titration"

- Each control antigen must be standardized to contain 4 HA units/25 µl, 8 HA units/50 µl.
  Note: Four HA units are added to the test in 25 µl because the HA unit calculations were based on a volume of 50 µl.

V. HAI Test for serologic diagnosis

1. Label appropriate microtiter plates.
2. Add 25 µl of PBS to wells B through H (B1 - H12) of each numbered column.
3. Add 50 µl of each treated serum (1:10) to the appropriate first well (A1 - A12) of the numbered column.
4. Prepare serial twofold dilutions of the treated sera by transferring 25 µl from the first well of the numbered columns 1-12 to successive wells. Discard the final 25 µl after row H.
5. Add 25 µl of standardized antigen to all wells (A1 - H12) in a set of treated sera.
6. Add 25 µl of PBS instead of antigen to the set of treated sera for serum controls (A1 - H12).
7. Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec or by agitating the plates manually.
8. Cover the plates and incubate at room temperature (22 to 25°C.) for 15 min.
9. Add 50 µl of standardized RBCs to all wells. Mix as before.
10. Cover the plates and allow the RBCs to settle at room temperature (22 to 25°C) for the appropriate time according to RBCs being used.
11. Record the HAI titers.

Interpretation

Hemagglutination and inhibition of hemagglutination are read as previously described. The positive control antigens and corresponding antisera should give consistent results when compared with previous tests. A fourfold titer increase between the acute and convalescent serum is considered diagnostically positive for that influenza type/subtype.

G. Neuramindase assay and Neuraminidase inhibition assay

Introduction

The neuraminidase (NA) is the second most abundant glycoprotein on the surface of influenza viruses. There are 9 different antigenic subtypes of influenza A NA and antigenic drift occurs in the NA as it does in the HA. Immunity to NA plays a role in protection against influenza virus infection, and anti-NA antibodies prevent virus release from infected cells. NA is an enzyme (sialidase) that cleaves terminal sialic acid residues from cell surface receptors of the influenza virus. This activity enables release of virions from infected cells and removes sialic acid from newly synthesized HA and NA molecules. Another role of NA is projected to be the cleavage of mucus on the surface of the respiratory tract permitting the virus to contact the cell surface and to spread within the respiratory tract. Inhibition of the action of NA with antibodies or specific inhibitors restricts the virus to a single round of replication showing that the NA has no role in attachment, fusion, replication, assembly or budding. The structure of NA is established and is a mushroom shaped molecule with four enzyme active sites on top. The discovery and approval of two potent NA inhibitors (Relenza and Tamiflu) provides a strategy for the prophylaxis and treatment of influenza. While the selection of NA resistant mutants resistant to NA inhibitors is difficult, they can be obtained. Studies of these resistant mutants show that the HA and NA function together in attachment and release of virus. Selection of resistant mutants of the NA with these inhibitors shows selection of mutants first in the HA then in the NA. The mutants combine with cells with lower affinity and require less NA for release.

Materials required

I. Reference antisera to NA subtypes.

Antisera to eight of the nine NA subtypes of reference influenza A antigens have been prepared in goats. These reference antisera were prepared using purified preparations of NA and are considered monospecific. In the cases where monospecific antisera not available, polyclonal antisera to the reference virus has been prepared. Reference antisera are designed to distinguish between different NA subtypes but are broadly cross-reactive to detect as many different variants as possible within a certain NA subtype.

The following categories of reagents are provided:

Reference antisera to the NA subtypes of influenza A viruses

N1	A/New Jersey/8/76
N2	A/Singapore/1/57
N3	A/Tern/South Africa/61 and A/Turkey/England/63
N4	A/Turkey/Ontario/6118/68
N5	A/Shearwater/Australia/1/72
N6	A/Duck/England/56
N7	A/Equine/Prague/1/56
N8	A/Equine/Miami/1/63
N9	In preparation

Reference antigens to the NA subtypes of influenza A viruses

N1	A/New Jersey/8/76	(H1N1)
N2	A/Singapore/1/57	(H2N2)
N3	A/Tern/South Africa/61	(H5N3)
N4	A/Turkey/Ontario/6118/68	(H8N4)
N5	A/Shearwater/Australia/1/72	(H6N5)
N6	A/Duck/England/56	(H11N6)
N7	A/Equine/Prague/1/56	(H7N7)
N8	A/Duck/Khabarovsk/1610/72	(H3N8)
N9	A/Duck/Memphis/546/74	(H11N9)

II. Buffers and reagents

- Phosphate Buffer, pH 5.9
- Fetuin (substrate)*
- Periodate Reagent
- Arsenite Reagent
- Thiobarbituric Acid Reagent
- Phosphate-buffered saline (0.01M), pH 7.3 (PBS)
- Warrenoff Reagent
- Sterile distilled or deionized H2O

** Fetuin may be obtained from Sigma Chemical Co., or from Gibco (Life Sciences), or from JRH Biosciences*

III. Equipment

- 37° C water bath
- Boiling pan
- Tripod stands
- Bunsen burner
- Centrifuge
- Spectrophotometer

IV. Supplies

- Pipettes and pipettor (For volumes 50µl, 100µl, 1µl, 2.5µl)
- Borosilicate glass culture tubes (13x100)
- Polypropylene rack, 72 hole
- Cuvettes-micro for a spectrophotometer (polysterene, 1-cm wide)

V. Preparation of reagents

1. Phosphate buffer, pH 5.9

   A. 0.4M sodium dihydrogen orthophosphate ($NaH_2PO_{4)}$
   Weigh 27.6g of NaH_2PO_4 and dissolve it in 500 ml ddH_2O

   B. 0.4M disodium hydrogen orthophosphate (Na_2HPO_4)
   Weigh 28.4g of Na_2HPO_4 and dissolve it in 500 ml ddH_2O

Mix 81ml solution A with 19ml solution B to give 0.4M buffer, pH 5.9.Adjust pH if necessary with appropriate phosphate component (solution A or B). Store at room temperature.

2. Fetuin (substrate)

• Fetuin	0.5g
• Phosphate Buffer, pH 5.9	20µl
• ddH_2O	20µl
• Store in 5ml aliquots at -20°C.	

3. Periodate reagent

• Sodium meta-Periodate	4.28g
• ddH_2O	38ml

Dissolve by heating. Cool at room temperature and then add 62ml of 85% ortho-phosphoric acid. Mix well and store in dark bottle away from light at room temperature.

4. Arsenite reagent

• Sodium arsenite (meta)	10.0g
• Anhydrous sodium sulfate	7.1g
• ddH_2O	100ml

Dissolve by heating. Cool at room temperature and then add 0.3ml concentrated sulfuric acid. Store at room temperature.

5. Thiobarbituric acid reagent

• Anhydrous sodium sulfate	35.5g
• Thiobarbituric acid	3.0g
• ddH_2O	500ml

Dissolve by heating in a boiling water bath. Store at room temperature. Reagent may precipitate after about 10 days depending on the quality of the thiobarbituric acid, whereupon fresh reagent should be prepared.

6. Warrenoff reagent

• 1-Butanol	475ml
• Concentrated hydrochloric acid	25ml

Store in a dark bottle in a location suitable for flammable reagents.

7. Phosphate-buffered saline (0.01M), pH 7.3 (PBS)

• Sodium Chloride (NaCl)	80.0g
• Potassium Chloride (KCl)	2.0g
• Sodium Phosphate, dibasic, anhydrous (Na_2HPO_4)*	11.5g
• Potassium Phosphate, dibasic, anhydrous (KH_2PO_4)	2.0g
• ddH_2O	10 L

**Sodium Phosphate, monobasic ($Na_2HPO_4.H_2O$) 21.7g may be substituted*

Neuraminidase assay (NA assay)

The first step in the procedure is to estimate the amount of NA activity in your influenza virus sample. In this assay the viral neuraminidase (an enzyme) acts on the substrate (fetuin) and releases sialic acid. Arsenite reagent is added

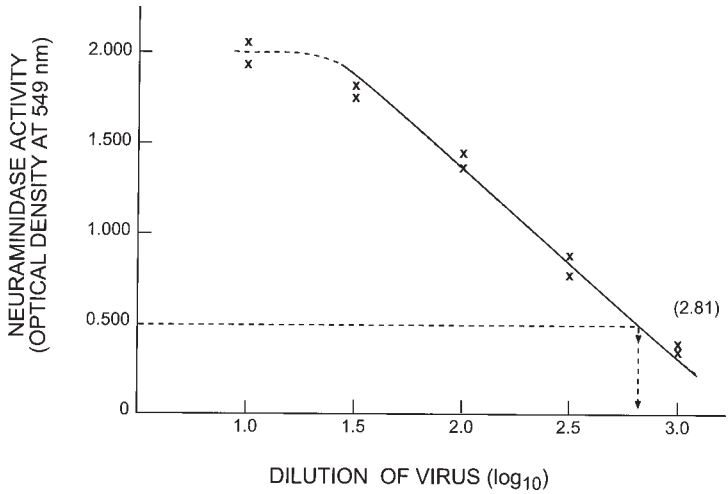
to stop enzyme activity. The amount of sialic acid liberated is determined chemically with the thiobarbituric acid that produces a pink color in proportion to free sialic acid. The amount of color (chromophor) is measured in a spectrophotometer at wavelength 549nm.

1. Prepare serial dilutions of influenza virus (reference antigen or field isolate) in PBS (pH 7.3), usually in 0.5 log10 (1/3.16) steps: undiluted, $10^{-0.5}$,10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3}. For example, $10^{-0.5}$ dilution: 0.1µl of undiluted virus + 0.216 µl PBS; 10^{-1} dilution: transfer 0.1 µl of virus from $10^{-0.5}$ dilution + 0.216 µl PBS etc.
2. Prepare duplicate tubes for each virus dilution to be assayed, i.e. two tubes for undiluted, two tubes for 10-0.5, etc. Set up tubes as follows:
   a) virus sample: 50 µl of each virus dilution + 50 µl of PBS + 100 µl fetuin;
   b) negative control (fetuin control): 100 µl of PBS + 100 µl fetuin
3. Shake the tubes, cover them with plastic wrap and incubate at 37°C water bath for 30 minutes. For influenza viruses with low NA activity (field isolates), the tubes must be incubated for 18 hours (overnight) at 37°C water bath.
4. Cool the tubes for 2 min at room temperature, add 0.1ml periodate reagent to each tube (including negative controls). Shake well, and leave at room temperature for 20 minutes.
5. While the samples are incubating (step 4) prepare a water bath by mounting a boiling pan filled approximately half full on tripod stands. Place Bunsen burners below and ignite so that the water will be boiling when the samples are prepared
6. Add 1.0ml of arsenite reagent to each tube (this stops the reaction). A brown color will appear, and the tubes should be shaken vigorously until the brown color disappears. The contents will appear milky. The test may be stopped at this stage and stored at 4°C, in that case the tubes must be covered with plastic wrap.
7. Add 2.5ml of thiobarbituric acid reagent to each tube. Shake the tubes vigorously and quickly place the rack with the tubes into a boiling water bath for 15 minutes. The thiobarbituric acid develops a pink color in the proportion to the amount of free sialic acid.
8. Cool the tubes in ice bath. (The whole rack with tubes must be put into an ice bath). Remove the rack with the tubes from the ice bath and add 3.0ml of warrenoff reagent. Cover each tube separately with plastic wrap and vortex. Centrifuge at 200rpm for 10 minutes. Carefully transfer upper (butanol) phase to a cuvette and measure the optical density (OD) on spectrophotometer at wavelength 549nm. Before measuring the OD of the virus samples blank the spectrophotometer against fetuin control tubes.
9. Determine the proper virus dilution to be used in the neuraminidase inhibition (NAI) assay by constructing a NA activity curve for each influenza sample tested (reference antigen or field isolate). The curves must be constructed by plotting NA activity (ODvirus sample at 549nm)

versus the virus dilution (Figure 1). One unit of NA activity was defined as that dilution of virus that gave an OD reading at 549nm of 0.500 under standard conditions.

In the example given in Figure 1 a dilution of virus of 1:650 (10-2.81) would give one unit of NA activity per 50 µl (50 µl, the volume of virus sample used in the assay).

Figure 1. Plot of neuraminidase activity versus viral dilution.



Neuraminidase inhibition assay (NAI)

The next step in the procedure is to standardize the reference antisera to each of the nine NA subtypes. This serum can be prepared in animals (goats, sheep, chickens, etc.) to the isolated NA when it is monospecific for that NA. If monospecific sera is not available (e.g. for influenza N9 NA subtype) then antiserum to the whole virus is used. In this case care in interpretation is needed as antibodies to HA can mask the NA and give spurious results (see "Pitfalls and Problem Solving," below).

I. Standardization of reference antiserum

1. For each reference antiserum (N1 - N9) use appropriate reference antigen, which must be diluted in PBS to a value of 0.500 units of OD. This dilution of the reference antigen must be determined in the NA assay (Section VI) before starting the procedure of the reference antiserum standardization.
2. Prepare serial dilutions of each antiserum to be standardized in PBS, usually $0.5\log_{10}$ (1/3.16) steps: undiluted, $10^{-0.5}$, 10^{-1}, $10^{-1.5}$,10^{-2}, $10^{-2.5}$, 10^{-3}, $10^{-3.5}$. For example, $10^{-0.5}$ dilution: 0.1ml of undiluted virus + 0.216 µl PBS; 10^{-1} dilution: transfer 0.1 µl of virus from $10^{-0.5}$ dilution + 0.216 µl PBS; etc.
3. Prepare duplicate tubes for each antiserum dilution to be assayed, i.e. two tubes for undiluted, two tubes for $10^{-0.5}$, etc. Set up tubes as follows:

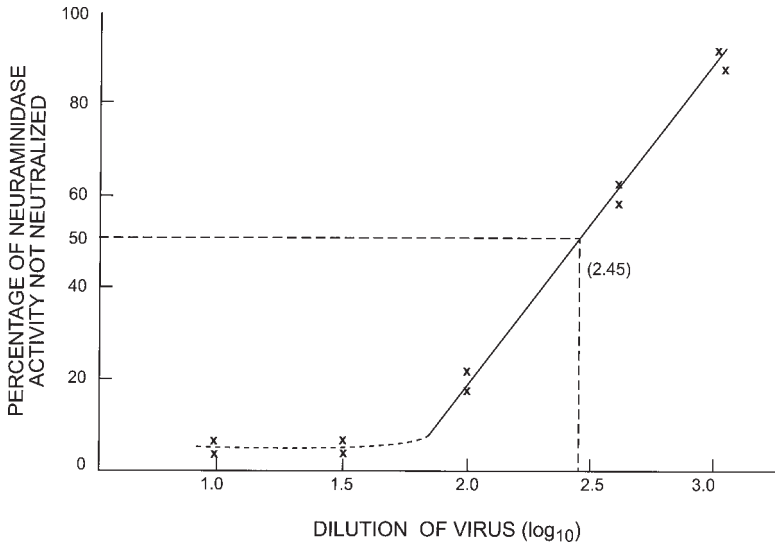
a) antiserum: 50 µl of each serum dilution + 50 µl of reference virus (diluted to 0.500 units of OD);

b) negative control (fetuin control): 100 µl of PBS

4. Incubate all tubes for 30-60 minutes at room temperature.
5. Add 100 µl fetuin to each tube. Proceed to steps 3-8 as described for the NA assay.
6. Determine the proper serum dilution to be used in the NAI assay by constructing a NA activity curve for each antiserum tested. Before measuring the OD of the antiserum tested blank the spectrophotometer against fetuin control tubes. The curves must be constructed by plotting percentage of NA activity not neutralized by antiserum versus the antiserum dilution. Determine the dilution of serum that caused 50% inhibition of NA activity. This volume of antiserum is then increased fivefold for the NAI assay.

In the example given in Figure 2 a dilution of antiserum of 1:280 ($10^{-2.45}$) would give 50% inhibition of NA activity 50 µl (50 µl, the volume of antiserum used in the assay).

Figure 2. Plot of the percentage of NA activity not neutralized by antiserum versus antiserum dilution.

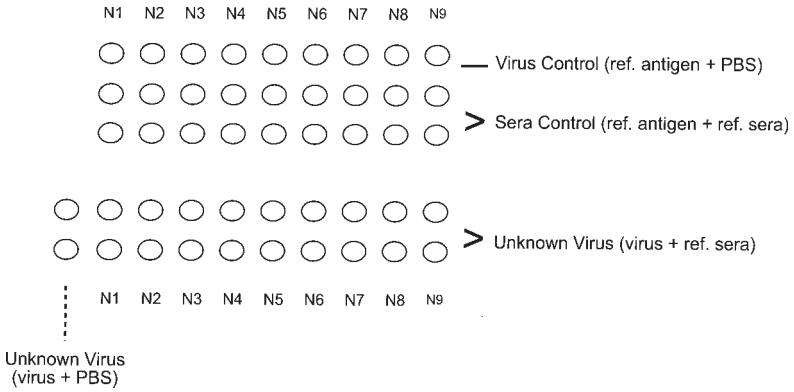


II. Identification of the NA subtype in NAI assay

The next step in the procedure is the identification of the NA subtype of an unknown influenza virus using the specific standardized antisera. In the NAI test the antibodies in the serum discriminate between the different NA subtypes and inhibit the action of the viral enzyme on the fetuin substrate and therefore no pink color is developed in the test.

1. Dilute assayed influenza viruses (or field isolates) in PBS to a value of 0.500 units of OD and place on ice. For each influenza virus (or field isolate) volume of 1.5ml will be required.

2. Prepare and label duplicate tubes representing each of the nine NA subtypes of reference antisera (N1, N2, N3 ….N9). Label two tubes with the number of virus sample (or field isolate) to be tested (#1, 2, 3, etc.). Dilute each reference antiserum according to the protocol for the standardization of reference antiserum (Section VII, A) and then increased the volume fivefold for the NAI assay.



3. Set up the tubes as follows:

   a) N1 (virus sample + reference antisera N1): 50 µl virus sample tested + 50 µl reference antisera;

   b) # 1 (virus sample): 50 µl virus sample tested + 50 µl PBS.

   Note: The same sets of tubes must be prepared for each virus sample to be tested.

4. Dilute reference antigens representing all nine NA subtypes (N1-N9) in PBS to a value of 0.500 units of OD as determined by NA assay. The tubes with the reference antigens must be placed on ice. For each reference antigen the volume of 200ml will be required.

5. Prepare and label three sets of tubes for each of the nine NA subtypes (whole amount 27 tubes). Label one set of tubes for virus control (VC1, VC2…… VC9). Prepare and label duplicate tubes for serum control (SC1, SC2…… SC9).

6. Set up the tubes as follows:

   a) VC1 (reference antigen N1): 50 µl reference antigen N1 + 50 µl PBS;

   b) SC1 (reference antiserum N1): 50 µl reference antigen N1 + 50 µl reference antiserum N1.

   Note: The same sets of tubes must be prepared for all nine NA subtypes.

7. Incubate the test and control tubes for 30-60 minutes at room temperature.

8. Add 100 µl fetuin to each tube. Shake the tubes, cover them with plastic wrap and continue through the rest of the assay following steps 3-8 as described for the NA assay.

III. Interpretation of results

Each virus control tube containing reference antigen (N1-N9) plus PBS must be compared with the duplicate serum control tubes containing the reference antigen plus reference antiserum. All the virus control tubes should appear pink, indicating the presence of the NA activity in the reference antigen. The serum control tubes should appear clear or have only slightly developed color, indicating that the NA activity has been inhibited by the specific reference antiserum. The above results showed that the assay was set up correctly.

Each tube with influenza virus sample assayed, containing the virus sample plus reference antiserum must be compared to the tube containing virus sample plus PBS (no antiserum). Absence or significant reduction of color in the sets of tubes "virus sample + reference antiserum" indicates that the NA activity was inhibited by the reference antiserum of a certain NA subtype. Each virus sample assayed must be inhibited by a reference antiserum of one NA subtype, according to that the identification of the NA subtype must be done. However, there could be cross-reactions between some NA subtypes and in these cases some additional assays may be required (see "Pitfalls and Problem Solving," below).

IV. Pitfalls and problem solving

It is always necessary to include a negative control serum of the animal species from which the antiserum was prepared. If low dilutions of antiserum are used (e.g. 1/10, 1/20) there can be free sialic acid that can mask antibody. These nonspecific reactions can be solved by dialyzing the antiserum or more dilute antiserum can be used.

Some subtypes of NA do show cross reactivity (for example N1 and N4, N4 and N8). This can be resolved by using more dilute antiserum in the inhibition test, using alternate sera, or by reporting that it is N1/N4 and further resolving the question by sequencing the NA gene.

Antibodies to the HA present in polyclonal antiserum can non-specifically block NA activity. To solve this problem it is necessary to use antiserum against two different viruses. For example, for N9 identification with polyclonal sera we could use an a-H1N9 and an a-H6N9: if only one serum inhibits then we would suspect that it was due to the antibodies to HA; if both inhibit then it is likely to be antibodies to NA, and we could conclude that this virus sample tested possessed an NA of the N9 subtype.

Screening of sera for evidence of NA antibodies

Serological studies to detect evidence of infection of animals with influenza virus are usually based on two tests: HAI and neutralization of infectivity. A useful and confirmatory test can be an NAI assay with known antigens. This test can be done according to the method for standardization of reference antisera.

Estimation of the titer of antibodies in serum samples is done as given in section VII. There are some advantages to serological tests using NAI assay in that few sera contain nonspecific inhibitors to NA whereas many sera contain inhibitors to HA. A good policy is to use both HAI and NAI assays.

H. Neutralization assay for antibody detection

Introduction

Serology methods such as the hemagglutinin inhibition test (HI), the virus neutralization test, and enzyme-linked immunosorbent assay (ELISA) are useful in epidemiologic and immunologic studies as well as in the evaluation of vaccine immunogenicity.

The virus neutralization test is a sensitive and specific assay applicable to the identification of virus-specific antibody in animals and humans. The neutralization test is performed in two stages consisting of (1) a virus-antibody reaction step, in which the virus is mixed and inoculated with the appropriate antibody reagents, and (2) an inoculation step, in which the mixture is inoculated into the appropriate host system (e.g. cell cultures, embryonated eggs, or animals) to detect residual virus infectivity. The absence of infectivity constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in human or animal sera.

The neutralization test has several advantages for detecting antibody to influenza virus. First, like HI tests, the assay primarily detects antibodies to the influenza virus HA, and thus can identify functional, strain-specific antibodies in animal and human serum. Second, since infectious virus is used, the assay can be developed quickly upon recognition of a novel virus and is available before suitable purified viral proteins become available for use in other assays. Because of their sensitivity and specificity, neutralisation tests can be used to confirm the results of HI tests. Neutralising antibody is less cross-reactive between antigenically related viruses than HI antibody and when used together, neutralisation tests provide additional information on the identity of the infecting virus.

In the neutralization assay, it is expected that serum neutralizing antibodies to influenza virus HA will inhibit the infection of MDCK cells with virus. Serially diluted sera are pre-incubated with a standardized amount of virus prior to the addition of MDCK cells. There are different options for the detection of virus neutralisation. In conventional neutralisation tests, as described here, the neutralisation of virus activity is based on directly visualising the suppression of CPE under an inverted microscope. These are more easily set up with a new virus, but take 3-4 days for a result to be obtained, and are more labor intensive and are less sensitive. Neutralisation tests with influenza differ from that done with other viruses in that the culture medium needs to be serum free and contain trypsin to allow influenza viruses to undergo productive replication.

More recently described methods, combine culture and antigen detection by ELISA and estimate 50% reduction of viral antigen as the end point. The newer methods combining culture and antigen detection methods can yield results within 2 days and are more sensitive (e.g. Rowe T et al. J Clin Microbiol. 1999; 37: 937-943). They are particularly useful in situations where antibody responses are poor e.g. avian influenza virus infections in humans.

Not all avian viruses grow well or produce CPE in cell culture and an alternative detection system (e.g. inoculation of embryonated eggs) may have to be used in such instances.

Procedure for influenza virus neutralization assay

Materials required

A. Cell cultures and reagents

1. MDCK cell culture monolayer (Madin-Darby Canine Kidney cells) Low passage (< 25-30 passages) at low crowding (70-95% confluent)
2. Complete D-MEM for MDCK cells.
   - 500 ml Dulbecco's Modified Eagles Medium (DMEM) Gibco, Cat. # 11965-092
   - 5.5 ml 100X Antibiotics (10,000U/ml Pencillin- 10,000µg/ml streptomycin sulphate) Gibco, Cat. # 15140-023
   - 13.5 ml 1 M HEPES buffer Gibco Cat # 15630-023
3. D-MEM for cell growth (GM). To complete D-MEM (above), add heat inactivated (56oC, 30 min) Fetal Bovine Serum (FBS) to final concentration of 10%. Fetal Bovine serum from Hyclone, Cat. # A-1115-L
4. Trypsin/EDTA: Gibco, Cat. # 25300-054
5. Virus growth Medium (VGM): Complete D-MEM (above) with 2µg/ml TPCK-trypsin solution. (e.g. 0.5 ml of 2mg/ml TPCK–trypsin stock solution added to 500 ml of Complete D-MEM).
6. Falcon 96-well tissue culture plates, Flat-bottom, Becton Dickinson, Cat # 3072.
7. TPCK-trypsin: Sigma, Cat # T 8642. (see section on Cell culture for preparation of stock solution at 20mg TPCK trypsin in 10 ml).

B. Supplies

1. Sterile capped tubes.
2. Assorted sterile pipettes and pipetting device including multi-channel pipette.
3. Autoclavable containers for discarding cultures.

C. Equipment

1. Class II biological safety cabinet.
2. Water baths, 37°C and 56°C.
3. Incubator, 37°C, 5% CO_2.
4. Inverted microscope or standard microscope for the observation of cells.
5. Freezer, - 70°C (for long term virus storage) or - 20°C (for serum storage).
6. Low speed, bench top centrifuge preferably with refrigeration.
7. Liquid nitrogen for cell storage.

Quality Control

A. Serum controls:

Make multiple aliquots of all control sera and store at - 20°C. Sera should not be repeatedly frozen and thawed.

Include both negative and positive serum controls for each virus used in assay.

1. Negative (normal) serum control:
   Include a normal serum to determine whether the virus is non-specifically inactivated by serum components. Wherever possible, use non-immune serum from same animal species that is being tested. For human sera, use age-matched normal serum from a population not exposed to the particular virus subtype in question. Use the normal serum at the same dilution as the matching viral antiserum.
2. Positive (infected or immunised) serum controls:
   a. For animal sera, use sera raised in infected ferrets or other (chicken, goat, rabbit or mouse)immunized animals. Ideally, use positive control sera from the same species that is being tested. The best results will be obtained if all (negative and positive) control animal sera are treated with receptor destroying enzyme (RDE) prior to use in the neutralization assay.
   b. For human sera, an optimal positive control would be acute and convalescent serum samples.

B. Virus and cell controls

Include a virus back-titration and positive and negative cell controls with each assay.

1. Negative and positive cell controls:
   Set up four wells as positive cell controls which receive 100ul of the virus-medium mixture and four wells as negative cell controls 100 µl medium alone in parallel with the neutralization test.
2. Virus titration check:
   In each assay, include a back-titration of the test dilution of virus in quadruplicate. The virus is diluted in ½ log10 steps as described later (see section on virus titration).

Procedure of the Neutralisation Assay

Part A: Virus titration

(A) Generation of stock virus: grow up virus to high titer in MDCK cells.

1. Prepare 3 T-75 flasks seeded with MDCK cells (as described previously). Check the cells with microscope at 40X magnification. Decant growth medium into a beaker and wash three times with 6 ml of VGM (D-MEM containing 2 µg/ml of TPCK-trypsin).

(B) Inoculation of flasks

1. Dilute virus sample (usually 1:10 to 1:1000) in VGM (see previously).

2. Remove medium from flask with sterile pipette.
3. Inoculate 2ml of each virus dilution into a T-75 flask using sterile pipettes.
4. Allow inoculm to adsorb for 45 minutes at 37^{0}C.
5. Add 15ml of VGM to each T-75 flask.
6. Observe for cytopathogenic effect (CPE).

(C) Harvesting of flasks

1. Harvest the cell culture when 50-100% of the cells show CPE. Collecting supernatant fluid and add a stabilizer such as glycerol gelatin or bovine serum albumin to a final concentration of 0.5%.
2. Centrifuge the tubes at 3000 rpm/5 minutes to remove excess cells. Aliquot in 1-2ml volumes and store at -70^{0}C.

(D) Preparation of microtitre plates with MDCK cells

1. Check MDCK cell monolayer in the T-75 flask.
2. Gently rinse monolayer with 5 ml trypsin-EDTA and remove.
3. Add 4-5 ml trypsin-EDTA to cover the cell monolayer.
4. Lie flask flat and incubate at 37^{o}C in 5% CO_2 until monolayer detaches (approximately 10-20 min).
5. Add 5-10 ml of MDCK medium to each flask, remove cells and transfer to centrifuge tube.
6. Wash cells 2x with PBS (5 min at 12,000 rpm).
7. Resuspend cells in D-MEM cell growth medium and count cells with a hemacytometer.
8. Adjust cell number to 1.5x10^5 cells/ml with D-MEM growth medium.
9. Add 200 µl Preparation of Standardized Antigen for the HAI Test and "Back Titration" cells to each well of microtiter plate. Typically, a confluent T-75cm^2 flask should yield enough cells to seed up to 3 microtiter plates (~1x 10^7 cells/flask).
10. Incubate cells overnight at 37^{o}C, 5% CO_2 (18-22 hrs). Use plate when cells have just reached confluence. For optimum results, the cells should be in growth phase.

(E) Virus titration

1. Thaw an ampule of virus.
2. Dilute virus 1/10 in VGM (1ml virus + 9 ml VGM) (working stock dilution of virus).
3. Add 100 µl of VGM (±TPCK-trypsin, 2 µg/ml*) to all wells, except column 1 of a 96-well tissue culture plate. (Perform titration of virus in quadruplicate cultures).

**Some avian viruses, including H5 viruses, do not require the addition of trypsin. When determining $TCID_{50}$ of new test viruses, it is best to perform titration with and without trypsin to determine optimal conditions for each virus.*

4. Add 146 µl virus of working stock dilution of virus to column 1. Perform ½ $\log_{10}$ dilutions of virus: Transfer 46 µl serially from column 1,2,3, —to column 11. Discard 46 µl from the last dilution row. Each well will have 100 µl of dilutions from 10^{-1},$10^{-1.5}$,10^{-2} - - -10^{-6} .
5. Add 100 µl of medium to each well and incubate at 37°C for 2 hours.

Prepare 96 well microtitre plate containing confluent MDCK cells for inoculation.

1. Remove medium from plate.
2. Add (fill each well) 350 µl with D-MEM serum free medium containing TPCK trypsin, 2µg/ml. Remove and replace with same medium to wash away fetal bovine serum.
3. After completion of the 2 hour incubation period in the virus titration plate above, transfer 100 µl of each virus dilution to corresponding wells in the MDCK cell plate.
4. Allow adsorption for 2 hours at 37°C in a CO_2 incubator.
5. Remove inoculum using a multi-channel pipette. Wash microtitire wells by adding and removing 250 µl of VGM.
6. Add 200 µl of VGM to each well an incubate as before (37°C in a CO_2 incubator) for 3-4 days. Observe under inverted microscope for viral CPE and record results.

   Note: When setting up the assay with a new virus, it is advisable to observe the plates daily and record the results. Once experience is gained on how long CPE takes to appear, the reading can be confined to this time. Initially, it may be necessary to confirm that the CPE endpoint corresponds to the end point by hemagglutination. After reading the CPE, transfer 50 µl aliquots to a separate U-bottomed plate for hemagglutination. Add chicken or turkey erythrocytes (as described elsewhere) and observe hemagglutination.

7. Calculate the $TCID_{50}$ by the Reed-Muench method (see Table 3 below).

Table 3 Calculation of $TCID_{50}$ by the Reed-Muench method

			Cumulative Value			
Dilution	(1) Positive	(2) Negative	(3) Positive	(4) Negative	(5) Ratio	(6) %Positive
10^{4}	4	0	11	0	11/11	100
$10^{4.5}$	4	0	7	0	7/7	100
10^{5}	3	1	3	1	3/4	75
$10^{5.5}$	0	4	0	5	0/5	0

Part B: Virus neutralization assay

(A) Preparation of Test Sera

1. Sera should be tested in quadruplicate. Three sera can be tested on each microtiter plate.
2. Heat inactivate sera for 30 min at 56°C.
3. Add 60 µl of VGM to each well of the microtiter plates.
4. Add an additional 48 µl of VGM to Row A (wells A1-A11).
5. Add 12 µl of the heat-inactivated sera to Row A in quadruplicate.
6. Perform twofold serial dilutions by transferring 60 µl from row to row (A,B,C,—H). Discard 60 µl from the last row (row H).

(B) Addition of virus

1. Dilute virus to 100 $TCID_{50}$ per 50 µl (200 TCID50 /100 µl) in VGM (approximately 7 ml/plate).
2. Add 60 µ diluted virus to all wells containing antibody dilutions except the cell controls CC.
3. Add 60 µl VGM to the cell control wells.
4. Set up back-titration as follows. Add 438 µl of the test virus dilution (100 $TCID_{50}$ per 50µl) to the first of a series of 8 x 1 ml ampoules. Add 300 µl of VGM to each of the others. Transfer 138 µl from the virus test dilution serially down the row of tubes using a separate pipette tip for each transfer. This results in a serial ½ $\log_{10}$ dilution series of the test virus.
5. Add 60 µl of VGM to each of the virus back titration wells. Transfer 60 µl of respective back titration dilution in quadruplicate, to the microtitre plate.
6. Gently agitate the virus-serum mixtures and incubate the plates for 2 hrs at 37°C, 5% CO_2.

Prepare 96 well microtitre plate containing confluent MDCK cells for inoculation.

1. Remove medium from plate.
2. Add (fill each well) 350 µl with D-MEM serum free medium containing TPCK trypsin, 2 µg/ml. Remove and replace with same medium to wash away fetal bovine serum.

Inoculation of 96 well microtitre plate with virus – antibody mixture.

1. After completion of the 2 hour incubation period of the virus antibody mixtures above, transfer 100 µl of each well of the neutralisation plate to corresponding wells in the MDCK cell plate using a multi-channel pipette. Use a separate pipette tips for each transfer.
2. Incubate plates for 2 hours at 37°C in a CO_2 incubator.
3. Remove the virus antibody mixture from each well. Wash microtitre wells by adding 250 µl of D-MEM and removing.

4. Add 200 µl of D-MEM (+/- 2µg/ml TPCK trypsin) medium.
5. Incubate for 3-4 days at 37°C. Observe under inverted microscope for viral CPE and record results.
6. Read the plates when the virus back titration shows 50% or more CPE in the cell sheet in at least half the wells inoculated with the 1$TCID_{50}$ dose of virus.

(C) Data analysis

The highest dilution of each serum completely protecting the cell sheet from CPE in at least 2 wells of 4 is is taken to be the viral antibody titre. (See Table 3 page 54)

1. Record the number of positive (1) and negative (2) values at each dilution.
2. Calculate the cumulative numbers of positive (3) and negative (4) wells.

   (3) Obtained by adding number in (1), starting at bottom.

   (4) Obtained by adding number in (2), starting at top.
3. Calculate the percentage of positive wells (5) by using value in (3)/ [value in (3) + (4)].
4. Calculate the proportional distance between the dilution showing > 50% positive (6) and the dilution showing < 50% positive (6).

Proportional = % positive value above 50%-50 x Correction distance % positive value above 50%- % positive value below 50% factor

$= 75\text{-}50 \times 0.5 = 0.3 \times 0.5 = 0.15$

75-0

5. Calculate the $TCID_{50}$ by adding the proportional distance factor to the dilution showing > 50% positive. Since each well was inoculated with 0.1 ml of each virus dilution, the $TCID_{50}$ is expressed as $TCID_{50}$/0.1 ml. One $TCID_{50}$ in this case is $10^{-5.15}$ / 0.1 ml, and therefore 100 $TCID_{50}$ equals $10^{-3.15}$ / 0.1 ml, or $10^{-2.8}$ / 50 ml.
6. If other dilution series are used, other factors must be used. For example, in this case, the correction factor for ½ $\log_{10}$ dilution series would be 0.5, that for 10-fold dilution series would be 1, that for a twofold dilution series would be 0.3, and that for a fivefold dilution series would be 0.7.

I. Identification of influenza viruses by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Introduction

The co-circulation of up to 15 different HA subtypes in combination with 9 different NA subtypes of influenza A viruses in avian species, the co-circulation of H1N1, H3N2 and the occasional introduction of avian influenza viruses to pigs, H3N8 in horses, and the zoonotic nature of all avian subtypes pose major challenges for the diagnosis and identification of influenza in animals and birds. Influenza viruses of the H5, H6, and H9 subtypes are considered of particular interest at this time for one lineage of each of these subtypes has an internal gene composition closely related to viruses like A/Hong Kong/156/97 (H5N1) that caused the 'bird flu' incident in Hong Kong. Hemagglutination, hemagglutination inhibition (HI) assays, and immunofluorescence are routine diagnostic methods used for detecting and subtyping influenza A viruses. However, the use of molecular techniques to directly detect virus in samples from animals facilitates the rapid identification and genetic characterization of influenza A viruses.

Polymerase Chain Reaction (PCR) is a powerful technique for the identification of influenza virus genomes even when they are present at very low levels. Since the genome of an influenza virus is single-stranded RNA, a DNA copy (cDNA) which is complementary to viral RNA must be synthesized prior to the PCR reaction. Reverse transcriptase (RT) is a polymerase used to synthesize such cDNA. The RNA template is first denatured by heating at 72°C in the presence of the primer, which is complementary to the termini of all eight viral RNA segments (primer 'Uni12'). The reaction mixture is then cooled to a temperature (42 °C) that allows the primer to anneal to the RNA target sequence. The annealed primer is then extended with RT to synthesize full length cDNA. After cDNA synthesis, an aliquot of the RT-reaction is used for the PCR reaction. The PCR reaction consists of 25-30 cycles of the three steps: denaturation, annealing and extension. TAQ DNA polymerase is a thermostable DNA polymerase that is not inactivated at 94 °C, and does not need to be replaced at every round of the amplification cycle. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product. Thus, this method is very sensitive and negative controls have to be included to detect possible contaminations of the reagents.

For the sensitivities and specificities of RT-PCR-based methods the choice of primer sequences is the most crucial parameter. Primer pairs used in PCR reactions are designed based on known sequences. In this course, primer pairs specific for the hemagglutinin (HA) gene of currently circulating influenza viruses are used. Primers HA-1140 and HA-Reverse are influenza A specific primers which were designed to amplify the HA genes of H5, H6 and H9 subtypes. The subtype of a virus can be determined by sequencing of the PCR products and comparison of the sequences with sequences deposited in databases. Primer pairs HA-1144/H6-1480R, HA-1144/H5-1735R and H9-1/H9-808R are specific for each subtype and allow the separation of the subtypes based on the presence or absence of a specific amplified PCR product.

In this course, viral RNA is extracted by the teaching staff using a commercially available RNA extraction kit (Qiagen). The protocol for viral RNA extraction is included in this course manual. The emphasis of the course will be on the synthesis of cDNA, amplification of the cDNA by PCR and gel electrophoresis.

RNA extraction from viral isolate

Materials required

- Qiagen RNAeasyTM Total RNA Isolation Kit (Catalog #74104):
  - RNeasy mini spin columns
  - Collection tubes (1.5ml)
  - Collection tubes (2 ml)
  - Buffer RLT
  - Buffer RW1
  - Buffer RPE
  - RNase-free water
- ß mercaptoethanol
- 70% ethanol
- sterile 1.5 ml microcentrifuge tubes
- 10, 20, and 100µl adjustable pipettes and tips
- microcentrifuge, adjustable to 13 K
- vortex

Procedure

1. Mix together the following materials:
   - 350 µl Lysis Buffer RLT
   - 3.5 µl ß -mercaptoethanol
   - 200 µl virus suspension
   - 550 µl 70% ethanol
2. Pipette the mixture onto the column (pink) and centrifuge for 15 seconds at 10,000 rpm.
3. Remove the column and empty the liquid from the bottom collection tube. Replace the column in the collection tube.
4. Add 700 µl Wash Buffer RW1 to the column and centrifuge for 15 seconds at 10,000 rpm.
5. Transfer the column to a clean collection tube and add 500 µl Wash Buffer RPE to the column.
6. Centrifuge for 15 seconds at 10,000 rpm. Remove the column and empty the liquid from the collection tube.
7. Add 500 µl Wash Buffer RPE to the column and centrifuge for 2 minutes at 12,000 rpm.
8. Transfer column to a 1.5 ml microcentrifuge tube and pipette 20 µl RNase free H20 directly onto the filter of the column. Wait 1 minute.
9. Centrifuge for 1 minute at 10,000 rpm. Sample is now ready for cDNA synthesis. RNA can be stored at –20ºC.

Synthesis of cDNA

Materials required

- sterile 0.5 ml microcentrifuge tubes
- 10, 20, and 100 µl adjustable pipettes and tips
- microcentrifuge, adjustable to 13K
- vortex
- water bath or thermocycler for 90ºC and 42ºC incubation
- viral RNA (Extracted by teaching staff)
- 10 mM dideoxynucleotide triphosphate (dNTP) mix
- ultrapure water
- primer 'Uni12': AGCAAAAGCAGG (1 µg/µl)
- AMV Reverse Transcriptase (25 units/µl, Life Sciences, Inc, Cat# ARB-45)
    - 5x Reverse Transcriptase buffer
- RNase Inhibitor (40 units/µl)
- ice

Procedure

Note: All reactions should be carried out on ice.

1. Label one 0.5 ml microcentrifuge tube for each RNA used.
2. The negative control is water.
3. To 4 µl of RNA and negative control (waterblank) add 0.5 µl of primer 'Uni-12'
4. Incubate for 5 minutes at 72 ºC
5. Make a cocktail of the following:
    - 1.5 µl of H20.
    - 2.0 µl of Reverse Transcriptase buffer
    - 0.5 µl of 10 mM dNTP mix
    - 0.5 µl of RNasin
    - 1.0 µl of Reverse Transcriptase
6. Add 5.5 µl of the cocktail to each tube
7. Incubate the RNA/Primer mix with the cocktail , total volume 10 µl at 42ºC for 1 hour.
8. Then stop the RT-reaction by incubation for 5 minutes at 95 ºC.

PCR reaction

Materials required

A. Amplification of different subtypes with one primer pair

- Forward and reverse primers at 1 µg/µl
- HA-1144 and HA-Reverse

B. Amplification of different subtypes with subtype specific primer pairs

- Forward and reverse primers at 1 µg/µl
- H5: HA-1144 and H5-1735R
- H6: HA-1144 and H6-1480R
- H9: H9-1 and H9-808R

C. Positive Control (optional)

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

D. Sterile 0.5 ml microcentrifuge tubes

- 10, 20, and 100 µl adjustable pipettes and tips
- microcentrifuge, adjustable to 13K
- vortex
- thermocycler for polymerase chain reaction
- TAQ DNA Polymerase (5 units/µl, Promega Cat #M1901)
- 10x PCR Buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton, pH 9.0)
- 25 mM $MgCl_2$
- 10mM d NTP mix
- H_20
- sterile mineral oil
- ice

Procedure

1. From the cDNA synthesized take only 1.5 µl for each PCR reaction. This is added to 48.5 µl of the master mix.
2. Make a PCR reaction Master Mix as follows: (Run blank for each primer pair)
   a. 5 µl PCR buffer
   b. 38.65 µl H_20
   c. 1 µl 10 mM d NTP mix
   d. 3 µl 25 mM $MgCl_2$
   e. 0.25 µl Taq DNA polymerase
   f. 0.3 µl Forward primer (1µg/µl)
   g. 0.3 µl Reverse Primer (1µg/µl)

h. Spin briefly to collect.
i. Add a drop of mineral oil to the top of the tube.

3. Place tube in thermocycler. Program for amplification:
   a. 94 ºC for 2 minutes
   b. 94 ºC for 1 minute (denature)
   c. 50 ºC for 1 minutes (anneal)
   d. 72 ºC for 3 minutes (extend)
   e. Repeat from step 2, 30 times
   f. 72 ºC for 8 minutes
   g. 4 ºC until usage

Agarose Gel Electrophoresis of the PCR products

Materials required

- agarose gel casting tray and electrophoresis chamber
- power supply and electrode leads
- hand-held UV light (302 nm) or a UV-light box
- camera and film
- 10µl adjustable pipette and tips
- 1% agarose gel in 1x TBE (prepared by teaching staff)
- 1x TBE Buffer
- ethidium bromide (10 µg/µl)
- gel loading buffer (GLB, 30%Glycerol, 0.25% BPB and 0.25% XC)
- molecular weight marker (Low DNA Mass Ladder, Gibco, Cat # 10068-013)
- microtubes containing the PCR product from the overnight reactions

Procedure

1. Remove tape from gel frame and place the gel into the electrophoresis chamber; cover the gel with 1x TBE.
2. Label the 0.5 ml microcentrifuge tubes separately.
3. Remove 4 µl of the PCR product from each reaction tube to a corresponding 0.5 ml microcentrifuge tube (remove PCR product from underneath oil); mix with 3 µl gel loading buffer.
4. Load 4 µl molecular weight marker to the first well of the 1% agarose gel.
5. Pipette 7 µl of PCR reaction, positive control and negative control to wells of the gel separately.
6. Close lid on chamber and attach the electrodes. Run the gel at 120V for 30-40 minutes.

7. Visualize presence of marker and PCR product bands with a hand-held UV light. It is desirable to have an ultraviolet light source emitting light at 302 nm wavelength (254 nm or 366 nm give results, but not as good).
8. Document gel with a photograph
9. Compare the size of the PCR-fragments with the marker.

Interpretation of results

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

Chemicals and enzymes used in the molecular assays

1% Agarose: 1g Agarose in 100 ml 1X TBE. Heat in hot water bath or microwave until dissolved. Agarose is the most common medium for nucleic acid electrophoresis. When an electric field is applied across a gel, DNA, which is negatively charged at neutral pH, migrates toward the anode.

AMV Reverse Transcriptase (AMV RT): AMV RT is an enzyme that can be used for the synthesis of cDNA corresponding to a particular RNA.

RNase Inhibitor (RNasin): RNasin is a noncompetitive inhibitor of pancreatic RNase. It is used in the synthesis of cDNA to prevent RNA degradation. This inhibitor should be used at a final concentration of 0.5 units/µl. Avoid freeze-thaw cycles as they denature the protein.

TAQ DNA Polymerase (TAQ): TAQ is a thermostable DNA-dependent DNA polymerase. Because TAQ is heat-stable, it will not be inactivated during the denaturation steps of the PCR reaction. TAQ requires Mg++, but the melting and annealing temperatures are affected by the concentration of cations.

Ethidium Bromide (EtBr): EtBr is a planar, multi-ring compound. The molecule intercalates between stacked bases of the DNA. The DNA fragments can then be visualized by exposing the gel containing the EtBr to ultraviolet light (250-310 nm). Using a 10 µg/µl stock solution, add to the agarose gel to a final concentration of 0.5 µg/ml.

Bromophenol Blue (BPB): BPB is a component of the gel loading buffer (GLB). BPB is widely used as a tracking dye for nucleic acid and protein gel electrophoresis and is usually added to samples at a final concentration of 0.01%.

Xylene Cyanole FF (XC): XC is a component of the gel loading buffer (GLB). It is a useful dye marker for electrophoresis of low molecular weight (less than 1

KB) nucleic acids. It migrates slower than the BPB so that there are two reference points for dye migration, allowing for more precise control of sample migration.

10 X TBE Buffer:		
	Tris	107.8 g
	Boric Acid	55.0 g
	EDTA(Na2)	8.2 g

Bring up to 1 liter with deionized water. Check the pH. If the pH is out of the range of 8.3 ±0.3, prepare the solution again. DO NOT adjust the pH; a change in ion concentration will affect the migration of the DNA through the gel.

Low DNA Mass Ladder: (Gibco Life Technologies, Cat# 10068-013) consists of an equimolar mixture of six DNA-fragments from 100 to 2000 bp.

Fragment #	Number of base pairs
1	2000 bp
2	1200 bp
3	800 bp
4	400 bp
5	200 bp
6	100 bp

Primers used for RT-PCR:

Uni12:	AGCAAAAGCAGG
HA-1144:	GGAATGATAGATGGNTGGTAYGG
HA-Reverse:	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT
H5-1735R :	GTGTTTTTAAYTAMAATCTGRACTMA
H6-1480R:	AATTCAAAGCAACCATTCCC
H9-1:	AGCAAAAGCAGGGGAAYWWC
H9-808R:	CCATACCATGGGGCAATTAG
M-WSN-8:	GAAGGTAGATATTGAAAGATG
M-1023R:	GAAACAAGGTAGTTTTTTACTC
	W = (AT); Y = (CT); N = (AGTC); M = (AC)

J. The Intravenous Pathogenicity Test (IVPI) for influenza viruses and Newcastle Disease Virus (NDV)

Introduction

As the terms highly pathogenic avian influenza (HPAI) and 'fowl plague' refer to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Whereas all virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 8 days.This procedure is a means of testing the level of pathogenicity of an isolate by observing clinical signs in infected birds over a ten day period.

Method

The IVPI test is carried out as follows.

1. Working in a High Security Laboratory, harvest infective allantoic fluid from Specific Pathogen Free (SPF) eggs previously inoculated with virus. The hemagglutinating titre must be >1/16 (>24 or >$\log_2$ 4 when expressed as the reciprocal).
2. Dilute the harvested material 1:10 in sterile isotonic saline. (Antibiotics must not be used so as to avoid the i/v inoculation of birds with relatively high concentrations of antibiotics.) Any suspicion of bacterial contamination in allantoic fluid is checked by bacterial culture on nutrient agar plates prior to IVPI test.
   All the following in-vivo procedures should ideally be done in a category 3 high security animal facility.
3. Inoculate 0.1ml of the diluted/undiluted virus intravenously into each of 10 six-week-old chickens. These birds should be hatched from eggs obtained from an SPF flock. Inoculate two chicks with 0.1ml of the diluent to act as controls.
4. Examine the birds for clinical signs at intervals of 24 hours over a ten day period.
5. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death.)

6. Kill any bird unable to feed or drink and record as dead at the following day's observation time.

7. The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period. Calculate the pathogenicity index as shown in the example below:
(The numbers recorded are the numbers of birds showing clinical signs on the specified day.)

Clinical signs	D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10	Total	Score
Normal	10	10	0	0	0	0	0	0	0	0	20 X 0	= 0
Sick	0	0	3	0	0	0	0	0	0	0	3 X 1	= 3
Paralyzed	0	0	4	5	1	0	0	0	0	0	10 X 2	= 20
Dead	0	0	3	5	9	10	10	10	10	10	67 X 3	= 201
											Total	**= 224**

The index is calculated as the mean score per bird per observation. In the above example this would be: -224/100 = 2.24

Definitions

The OIE criteria for classifying an avian influenza virus as highly pathogenic using the IVPI test is:

> *Any influenza virus that is lethal for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid.*

In the European Union, a similar definition has been adopted although in this case the intravenous pathogenicity index (IVPI) test was used as a method of assessing virulence. For the purposes of confirming disease and implementing the control measures in the EU Directive, the following definition applies:

> *'an infection of poultry caused by an influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens >1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin.'*

Examples of IVPI indices

Highly pathogenic	2.0-3.0 (range 1.74-3.0)
Non pathogenic	0 (range 0-1.0)
Intermediate	1.2-1.4*

** Exceptionally viruses of H10 subtype have given IVPI's marginally in excess of 1.2 and would, according to the European Union definition, be classified as highly pathogenic irrespective of the amino acid motif at the cleavage site.*

K. Agar Gel Precipitation Test (AGP) for the detection of antibodies to avian influenza using type specific Ribonucleoprotein (RNP) antigen

Introduction

The AGP test is based on the principle of concurrent migration of antigen and antibody toward each other through an agar medium. The medium contains a high salt concentration, which enhances the formation of the antigen-antibody immune complex precipitate. As all influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens, AGP tests are used to detect antibodies to these antigens. Concentrated virus preparations containing either or both type antigens are used in such tests.

Safety

Hazards.

Wear gloves and mask when working with phenol, working in the fume hood whenever possible. Wash all apparatus and electrode in cold running water.

First aid.

Wash hands etc. immediately in cold running water if there has been contact with phenol.

Materials

Gel diffusion Agar

Sodium chloride	80g
Phenol	5g
Distilled water	1 litre
Oxoid Agar No.	112.5g

Dissolve phenol and sodium chloride in distilled water. Adjust the pH to 7.5 using 1N sodium hydroxide. Add Oxoid Agar No.1 or equivalent agar and boil to dissolve. Dispense into 20ml amounts in glass bottles, allow to cool and solidify at room temperature. Once cool, store at +4°C. Re-boil to liquefy, pour into petri-dish and allow to solidify.

Test method

4.1 Using a template and cutter, wells of approximately 5 mm in diameter, and 2-5 mm apart, are cut in the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. This will make a continuous line of identity between the known positive, the suspect serum and the RNP antigen.

4.2 Add the reagents at approximately 50ml per well.

4.3 Incubate the agar plates at room temperature in a sealed container. Place a piece of wetted cotton or paper in the container to prevent the plates from drying out.

4.4 Precipitin lines can be detected after approximately 24-48 hours, but this may be dependent on the concentrations of the antibody and the antigen. These lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be due to the test serum lacking identity with the antibodies in the positive control well.

III. Appendix

A. Forms

INFLUENZA SURVEILLANCE FIELD DATA

Page _____

Individual	Reference Number	Field Number	Animal Type	Species	Type Sample	Age	Sex	Health H or S or D	Wild Domestic	Migrating Nonmigrating	Date	Results +/–

B. Definitions

Antigenic drift. The gradual alteration by point mutations of the hemagglutinin and neuraminidase within a type or subtype which results in the inability of antibody to previous strains to neutralize the mutant virus. Antigenic drift occurs in both influenza A and B viruses and causes periodic epidemics.

Antigenic shift. The appearance in the human population of a new subtype of influenza A virus containing a novel hemagglutinin or a novel hemagglutinin and neuraminidase that are immunologically different from those of isolates circulating previously. Antigenic shift is responsible for worldwide pandemics.

Epidemic. An epidemic is the occurrence of more cases of disease than expected in a community or given area over a particular period of time. The terms epidemic and outbreak have similar meanings.

Epidemiology. The study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to the control of health problems.

Hemagglutinin titer. The HA assay provides a measure of the concentration of influenza virus in a given sample. The HA titer is the reciprocal of the highest dilution of virus causing agglutination of an equal volume of standardized RBCs.

Hemagglutinin unit. One unit of hemagglutinin is contained in the end point dilution of the HA titration. The "unit" of hemagglutinin is not a measure of an absolute amount of virus but is an operational definition dependent on the method used for the HA titration. Details of the titration volume must be included in the definition of the "unit" to understand how much virus is represented by the term. For example, HA titration in a volume of 0.5 ml and microtitration in a volume of 0.05 ml will give identical titers. A unit should therefore be defined as the amount of virus in 0.5 ml or 0.05 ml giving agglutination, i.e. 1 unit/0.5 ml or 1 unit/0.05 ml depending on the method.

Influenza pandemic. By convention, worldwide outbreaks caused by influenza A strains which exhibit antigenic shift are called "pandemics." Outbreaks caused by influenza A or B strains which exhibit antigenic drift are termed "epidemics," although they may also occur in many parts of the world during the same season.

Public Health Surveillance. The systematic, ongoing assessment of the health of a community, based on the collection, interpretation, and use of health data. Surveillance provides information necessary for public health decision making.

C. Direct detection of influenza virus type A & B by Enzyme immunoassay

Introduction

Commercial enzyme immunoassays which allow rapid detection of influenza virus type A in clinical specimens have been on the market for several years. These tests can be performed in any laboratory, and all required equipment and reagents are included in the test kit. A result can be obtained within 15 to 20 minutes.

The greatest use in animal influenza studies is in initial detection of viruses from infected hosts. Tracheal or, nasal swabs, or throat swabs are suitable specimens for these assays but fecal samples are not suitable. This test is useful to confirm the presence of influenza A in egg or cell culture grown material.

In the first step of the Directigen Flu A Kit, the specimen is treated with an extraction buffer containing detergent to lyse cells and a mucolytic reagent. The diluted specimen is homogenized and then applied to a filter membrane. Viral antigens are bound to the membrane. In a following step, unbound material is washed off and nonspecific binding of subsequent reagents is prevented by blocking the membrane with rabbit IgG. Enzyme-labeled monoclonal antibody specific to influenza virus is added and after repeated washing, activity of bound enzyme is detected by subsequent incubation with two substrate-chromogen solutions. In the presence of viral antigens, a purple triangle becomes visible on the membrane. Virus antigen on a small round area in the center of the membrane serves as a test control. Positive and negative control material is included in the kit.

The FLU OIA test involves the extraction and detection of an influenza A or B nucleoprotein. The Optical ImmunoAssay technology enables the direct visual detection of a physical change in the optical thickness of molecular thin films. The change is a result of antigen-antibody binding on an optical surface which is a silicon wafer. When extracted specimen is placed directly on the optical surface, the immobilized specific antibodies capture the antigen. After washing, the substrate is added, increasing the thickness of the molecular thin film. This change in thickness alters the reflected light path and is visually perceived as a color change. Slight changes in optical thickness produce a distinct visible color change. A positive result appears as a purple spot on the gold background. When antigen is not present in the specimen, no binding takes place. Therefore, the optical thickness remains unchanged and the surface retains the original gold color indicating a negative result.

In this course, the detection of influenza virus type A antigens will be demonstrated using the Directigen Flu A Kit, and detection of influenza virus type A & B antigens will be demonstrated using the FLU OIA Kit, according to the instructions included in the kits.

Directigen Flu A kit

Materials required

- Directigen Flu A Kit
  Becton Dickinson, Cat. # 8560-20
- "Clinical" specimens
  Note: Influenza A reference virus has been added to some of these specimens in order to obtain "positive" and "negative" specimens.

Procedure

The test will be performed following the instructions included in the Directigen Flu A Kit, Becton Dickinson, Cat. # 8560-20.

1. Remove Tri-Pak device from package and place focuser tightly.
2. Add 8 drops of Reagent A to plastic dispense tube.
3. Add 125 µl of clinical specimen to dispense tube and mix thoroughly.
4. Insert dispense tip onto dispense tube.
5. Dispense entire content of dispense tube to Tri-Pak membrane.
6. Allow to absorb completely.
7. Fill well with Reagent 1 (washing step).
8. Allow to absorb completely.
9. Remove focuser.
10. Dispense 4 drops of Reagent 2 into well (washing and blocking).
11. Allow to absorb completely.
12. Dispense 4 drops of Reagent 3 into well (enzyme-labeled monoclonal antibodies).
13. Allow to absorb completely and let stand for 2 - 5 min.
14. Fill well with Reagent 4 (washing step).
15. Allow to absorb completely.
16. Dispense 4 drops of Reagent 5 into well (washing step).
17. Allow to absorb completely.
18. Dispense 4 drops of Reagent 6 into well (first substrate-chromogen solution).
19. Allow to absorb completely

    NOTE: during this step the membrane will turn yellow
20. Dispense 4 drops of Reagent 7 into well (second substrate-chromogen solution).
21. Allow to absorb completely.
22. Let reaction develop for 5 min.
23. Stop enzyme-substrate reaction by adding 4 drops of Reagent 8.
24. Read test result.

Reading and interpretation

- A purple triangle of any intensity is indicative of a positive test result.
- With less intense staining of the purple triangle, a purple dot in the center of the triangle may be visible; appearance of this purple dot indicates appropriate function of the membrane and the test reagents.
- A purple dot in the center of the filter with no evidence of a stained triangle is indicative of a negative test result. Appearance of this purple dot indicates appropriate function of the membrane and the test reagents.
- Any other appearance of the membrane after completing the test indicates that the test has not worked properly and should be repeated. With high concentrations of influenza virus (e.g. egg grown virus), the whole members may form purple and the test should be repeated after detection.

Quality control

The virus antigen spotted on the center of the filter serves as internal test control. If the spot does not show up after the test has been completed, make sure that neither the filter cartridge nor any of the reagents have passed the expiration date. Positive and negative control material is included in the kit. Virus-positive and negative specimens can be stored frozen at -20°C to be used as additional controls in future tests.

Limitations

The monoclonal antibodies used in this test have been shown to react with subtypes H1N1, H2N2, and H3N2 of human influenza viruses. Avian, swine, and equine influenza viruses can also be detected with this assay.

The sensitivity of the assay is greatly influenced by the quality of the specimen. When laboratory-grown viruses are used, the detection level is between 1,000 and 2,000 plaque forming units of cell free virus or approximately 20 infected cells. It is therefore important to obtain a significant number of epithelial cells by any means of sampling which yields a specimen compatible with the assay format. A negative result does not exclude the possibility that the patient has an influenza A infection and should be interpreted as: No influenza A virus detected.

If mucus is not efficiently dissolved or removed from the specimen, it can prevent viral antigens from attaching to the membrane. This may cause a false-negative result. Such specimens are identified by very slow absorption to the membrane, and they should be retested after a further dilution of 1:4.

The reagents and the filter device included in the kit have variable shelf-lives. Always make sure that neither the reagents nor the filter device have passed the expiration date.

FLU OIA KIT

Materials required

- FLU OIA Kit
  Biostar, Cat. # FLU30
- "Clinical" specimens

NOTE: Influenza A or B reference virus has been added to some of these specimens in order to obtain "positive" and "negative" specimens.

Procedure

The test will be performed following the instructions included in the FLU OIA Kit, Biostar, Cat. # FLU30

1. Remove reagents from refrigerated storage and allow to warm to room temperature (18º to 30ºC.). Store Wash at room temperature after opening kit. If stored refrigerated, Wash will take up to 2 hours to warm to room temperature.
2. Add 3 drops of Sample Diluent and 2 drops of Reagent 2 into the Extraction Tube.
3. Immediately add the patient's specimen swab to the Extraction Tube. Thoroughly mix the solution with the swab so that the liquid migrates into the fiber tip. Wait at least 3 but no more than 5 minutes.
4. Hold the swab shaft to the side of the Extraction Tube, while adding 1 drop of Reagent 2. Use swab to thoroughly mix reagents. Squeeze sides of the flexible tube and twist the shaft as the swab is withdrawn, expressing as much liquid as possible into the Extraction Tube. Discard the swab.
5. Use a clean Transfer Pipette to place 1 drop of the extracted sample directly onto the center of the test surface. Wait at least 6 but no more than 7 minutes.
6. Wash the test surface vigorously with Wash Solution, taking care not to exceed the capacity of the absorbent material surrounding the test surface.
7. Confirm that the blotter in the Test Device lid is in position I. Close the Test Device at the corners. Leave closed for 10 seconds to remove residual moisture from the test surface.
8. Open the lid, change blotter to position II, and apply 1 drop of Substrate centrally on the test surface. Wait at least 6 but no more than 7 minutes.
9. 10 seconds. Open and examine for color change.

Reading and interpretation

- A positive test result will show a solid blue/purple reaction circle of any intensity surrounding the internal control dot.
- A negative result will show no reaction circle of any intensity, only the internal control dot in the center will be displayed.

- If the internal control dot is missing, the entire test is invalid and will need to be repeated.

Quality control

Each test device has a built-in antigen control which appears as a small blue/purple dot in the center of each test surface following the completion of a test. The antigen control, inactivated influenza B virus, serves as a reagent check for the conjugate and capture antibody. The absence of the antigen control indicates an invalid test and test results should not be reported.

Additionally, positive and negative controls may be used to monitor the antigen extraction process, performance of the test reagents, and procedure technique. The positive antigen control contains inactivated influenza A virus. The negative control contains a protein solution.

Limitations

As with other diagnostic procedures, the results obtained with this test should be used as an adjunct to clinical observations and other information available to the physician. Reliable results are dependent on adequate specimen collection and good sampling technique. Because antigen detection methods do not require virus viability, the FLU OIA test may produce a positive result in the absence of living virus. Negative results are not intended to rule out other non-influenza viral infections. A negative result does not exclude the possibility that the patient has an influenza infection and should be interpreted as: No influenza A or B virus detected.

D. Laboratory safety

Safety is the responsibility of everybody working in the laboratory and safe procedures must be adhered to at all times. The following statements provide some basic rules for safety in the laboratory.

- Blood and body fluid precautions are to be consistently used with all clinical specimens of blood or other potentially infectious material (Universal Precautions).
- Use barrier protection at all times (laboratory coats, gloves, or other appropriate barriers).
- Good laboratory practices should be followed. Eating, drinking, or smoking is not permitted in the laboratory.
- Mechanical pipetting devices are to be used for all liquids in the laboratory. Mouth pipetting is dangerous.
- Biosafety Level 2 practices should be followed when handling most specimens, for highly pathogenic influenza viruses like H5 and H7 Biosafety Level 3 is required. Class I or II biological safety cabinets or other physical containment devices should be used for all manipulations of agents that cause splashes or aerosols of infectious materials.
- Adequate and conveniently located biohazard containers for disposal of contaminated materials should be available.
- Countertops and surfaces of biological safety cabinets should be wiped with a disinfectant (0.5% sodium hypochlorite is preferred) routinely after working with infectious agents or clinical specimens.
- Wash hands often especially before leaving the laboratory and before eating. Protective clothing should be removed before leaving the laboratory.

E. Hazardous chemicals

Acetone is used for fixation of cell cultures in the rapid culture assay.
Caution: Acetone is extremely flammable as liquid and vapor, and harmful if inhaled.
Eye and skin contact: Immediately flush eyes with plenty of water; get medical attention.
Ingestion: Get medical attention.
Inhalation: Remove to fresh air.

Hydrogen peroxide is used as the substrate for the HRPO-conjugate in the neutralization assay.
Caution: Hydrogen peroxide may cause severe irritation of skin, eyes, and mucous membranes and respiratory irritation.
Eye and skin contact: Flush with plenty of water; get medical attention.
Ingestion: Give large volumes of water or milk if conscious; DO NOT INDUCE VOMITING; get medical attention.
Inhalation: Remove to fresh air.

Sodium azide is added as a preservative in a concentration of 0.1% to some of the reagents included in the WHO Influenza Reagent Kit and the monoclonal antibodies.
Caution: Sodium azide may be fatal if swallowed, inhaled, or absorbed through skin.
Eye and skin contact: Flush with plenty of water, remove contaminated clothing.
Ingestion: Give large quantities of water if conscious and not convulsive.Induce vomiting; get medical attention.
Inhalation: Remove to fresh air, give artificial respiration or oxygen if required.

Sulfuric acid is used to stop the substrate reaction in the EIA procedure of the microneutralization test.
Caution: Can cause severe burns.
Eye contact: Flush with plenty of water for at least 15 min.
Skin contact: Flush with plenty of water for at least 15 min, while removing contaminated clothing.
Ingestion: DO NOT INDUCE VOMITING! Give large amounts of water and call a physician.
Inhalation: Remove to fresh air, and if necessary, give oxygen.

Chloroform is used in the alternate methods for RNA extraction and the dsDNA purification.
Caution: Chloroform is a possible human carcinogen and may be fatal if swallowed or inhaled. Inhalation or ingestion may cause nausea, vomiting, headache, or dizziness. Eye contact may cause corneal damage. Skin contact may cause irritation or dermatitis.
Eye and skin contact: Flush with water for at least 15 min.
Ingestion: If swallowed, give large amounts of water, if conscious. Induce vomiting. Call a physician.
Inhalation: Remove to fresh air. Give oxygen if breathing becomes difficult. Call a physician.
Ethidium Bromide is an intercalating dye that is used to visualize DNA in agarose gels.
Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves

should be worn when working with solutions containing this dye and a mask should be used when weighing it out.
Eye and skin contact: Flush with water for at least 15 min.
Ingestion: Call physician immediately. Do not induce vomiting unless instructed by medical personnel.
Inhalation: Remove to fresh air. Give oxygen if breathing becomes difficult. Call a physician.

Phenol is used in the alternate methods for RNA extraction and dsDNA purification.
Caution: Phenol is highly corrosive and can cause severe burns. This agent is rapidly absorbed through the skin. Wear gloves, lab coat, and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood.
Eye and skin contact: Rinse with large volumes of water and wash with soap and water.DO NOT USE ETHANOL!
Ingestion: If conscious, give activated charcoal in water, olive oil, or margarine. Induce vomiting. Call a physician.
Inhalation: Remove to fresh air. Give oxygen if breathing becomes difficult. Call a physician.

F. Alternate protocols

Influenza HAI serum treatment

Sensitivity to nonspecific inhibitors present in sera differs between influenza strains. The procedures recommended here to remove serum non-specific inhibitors include RDE (receptor destroying enzyme) treatment and trypsin-heat-periodate treatment.

I. RDE Treatment

Because batches of RDE may differ, the treatment procedures may also change. The procedure written in this manual is specifically for the RDE supplied in the 1998-1999 WHO Influenza Reagent Kit. The following procedure is recommended for use with RDE prepared by Sigma.

1. Reconstitute the RDE with 5 ml sterile distilled water.
2. Dilute to 100 ml with calcium saline, pH 7.2.
3. Add 4 vol of RDE to 1 vol of serum (0.4 ml RDE + 0.1 ml serum).
4. Incubate overnight at 37°C.
5. Add 5 vol of 1.5% sodium citrate (0.5 ml).
6. Heat 56°C for 30 min to inactivate remaining RDE.

Reagents:

Calcium saline solution, pH 7.2

a. Weigh out and dissolve in 1 liter of distilled water:
   - 1 g calcium chloride, dihydrate ($CaCl_2 . 2 H_2O$)
   - 9 g sodium chloride, anhydrous (NaCl)
   - 1.2 g boric acid, anhydrous (H_3BO_3)
   - 0.052 g sodium borate, decahydrate ($Na_2B4O7 . 10H_2O$)

b. Autoclave or filter to sterilize; pH should be 7.2.
c. Store opened bottle at 4°C for no longer than 3 weeks.

1.5% sodium citrate solution, pH 7.2

a. 1.6 g of sodium citrate, dihydrate ($Na_3C_6H_5O_7 . 2H_2O$) is dissolved in 100 ml of PBS, pH 7.2
b. Autoclave or filter to sterilize.
c. Dispense into amber bottles to prevent photodegradation.
d. Store opened bottle at 4°C for no longer than 3 weeks.

II. Trypsin-Heat-Periodate treatment

A. Procedure

1. Add ½ vol of trypsin solution to 1 vol of serum (0.15 ml trypsin + 0.3 ml serum).
2. Inactivate at 56°C for 30 minutes.
3. Cool to room temperature.

4. Add 3 vol of 0.011 M metapotassium periodate (KIO4), (0.9 ml).
5. Mix and leave at room temperature for 15 min.
6. Add 3 vol of 1% glycerol saline (0.9 ml).
7. Mix and leave at room temperature for 15 min.
8. Add 2.5 vol of 0.85% saline (0.75 ml) and mix.
9. Final serum dilution is 1:10.

B. Reagents

Trypsin solution

a. Dissolve 200 mg of trypsin (P-300) in 25 ml of 0.1 M phosphate buffer, pH8.2.
b. Sterilize by filtration.
c. Dispense solution in small quantities and store at -20°C or -70°C. Prepare fresh trypsin every six months.

0.1 M Phosphate buffer, pH 8.2

a. Prepare 0 .1 M monobasic sodium phosphate monohydrate by dissolving 13.8 g $NaH_2PO_4HO_2$ in sterile distilled water and q.s. to 1000 ml.
b. Prepare 0.1 M dibasic sodium phosphate by dissolving 14.2 g Na_2HPO_4 in sterile distilled water and q.s. to 1000 ml.
c. Mix 1 vol of monobasic sodium phosphate monohydrate with 31 vol of dibasic sodium phosphate to give a working solution of 0.1 M phosphate buffer, pH 8.2.
d. Check pH. If necessary, adjust pH by adding extra volumes of 0.1 M monobasic sodium phosphate monohydrate to lower pH or 0.1 M dibasic sodium phosphate to raise pH.

0.011 M metapotassium periodate (KIO_4)

a. Dissolve 230 mg metapotassium periodate (KIO_4) in PBS pH 7.2 and q.s. to 100 ml.
b. Sterilize by filtration.
c. KIO4 solution must be prepared fresh and used within one week.

1% Glycerol saline

a. Add 1 ml glycerol to 99 ml PBS pH 7.2.
b. Sterilize by filtration.

Influenza HAI standardization of RBCs

The following procedure is commonly used for standardization of RBCs.

A. Procedure

1. The final concentration of chicken and turkey RBCs is 0.5%. Guinea pig and human “O” RBCs require a final concentration of 0.75%. A higher concentration of guinea pig and human type O RBCs is desirable for visualization of complete settling of RBCs.

2. Filter approximately 5 ml of blood through gauze into a 50-ml conical centrifuge tube.
3. Centrifuge at 1200 RPM for 10 min.
4. Aspirate supernatant and the buffy layer of white cells.
5. Add 50 ml PBS (pH 7.2). Mix gently.
6. Centrifuge at 1200 RPM for 5 min. Aspirate supernatant.
7. Repeat PBS wash two times.
8. Resuspend RBCs to a final volume of 12 ml using a 15-ml graduated conical centrifuge tube.
9. Centrifuge at 1200 RPM for 10 min.
10. Estimate the volume of packed cells and dilute to appropriate concentration.
11. Determine concentration with a hemacytometer and adjust accordingly.

B. Determination and adjustment of concentration by hemacytometer counting

1. Prepare an approximate RBC suspension.
2. Prepare a 1:100 dilution of the suspension by adding 0.5 ml of RBC suspension to 49.5 ml PBS pH 7.2.
3. Transfer 10 µl onto the hemacytometer channel and allow the cells to spread throughout the unit.
4. Count the RBCs in each of the 4 smaller corner squares of the unit.
5. Calculate the final volume of RBC suspension.
   a. Final volume for 0.5% suspension of avian cells
   $$= \frac{\text{\# of cells counted}}{160} \times \text{volume of initial RBC suspension}$$
   b. Final volume for 0.75% suspension of mammalian cells
   $$= \frac{\text{\# of cells counted}}{240} \times \text{volume of initial RBC suspension}$$

Note: The above formulas were determined by the following:

$$\text{Volume}_{\text{final}} = \frac{\text{\# cells}}{160 \text{ (avian)}\ \text{or}\ 240 \text{ (mammalian)}} \times \text{Volume}_{\text{initial}}$$

Alternate method for isolation of RNA

I. Equipment

- benchtop centrifuge capable of 1,000 x g
- ultracentrifuge capable of 100,000 x g with 700 µl ultracentrifuge tubes and appropriate rotor
- 1.5 ml microcentrifuge tubes
- vortex

- 56°C water bath or hot block

II. Reagents

- 10X RSB
- 0.1 M Tris
- 0.1 M KCl
- 0.015 M $MgCl_2$ X 6 H_2O
- Sterilize by autoclaving or filtration.
- 10% SDS
  Dissolve 100 g of SDS in 900 ml H_2O. Heat to 68°C. Adjust pH to 7.2 by adding concentrated HCl. Adjust volume to 1000 ml with H_2O.
- Proteinase K (5 mg/ml)
- Dissolve 100 mg Proteinase K in 20 ml of 1X RSB. Incubate for 10 minutes at 56°C. Store at -20°C.
- 0.4 M Acetate buffer
- 62.5 ml H_2O, adjust pH to 4.9 with 1N NaOH. Add 2.85 ml of Acetic Acid. Add H_2O to 125 ml.
- 5.6 M LiCl
- 29.67 g LiCl, bring up to 125 ml with H_2O.
- 10X LiCl Bu
- Chloroform:isoamyl alcohol (24:1) Combine 96 ml chloroform with 4 ml isoamyl alcohol. Store at room temperature in an amber bottle.
- Phenol saturated with RSB buffer (pH 4.91)
- Wash phenol with equal volumes of RSB 3-5 times.
- TSE Buffer (pH 7.8)
- 2.42 g Tris
- 8.77 g NaCl
- 0.74 g EDTA
- 900 ml H_2O
- Adjust pH to 7.8 with 1N HCl, add H_2O to 1000 ml.
- Sterilize by autoclaving or filtration.
- 100% ethanol (ice-cold)
- 70% ethanol (ice-cold)

III. Protocol

A. Growth and concentration of virus

1. Grow virus in embryonated hens' eggs or in MDCK cell cultures.
2. Harvest allantoic fluid or cell culture supernatant.
3. Clarify cell culture supernatant by low speed centrifugation (1,000 x g, 15 minutes).
4. Concentrate virus by pelleting in an ultracentrifuge (100,000 x g, 1 hour).
   a. Start from 500 µl and 700 µl of allantoic fluid or cell culture supernatant.
   b. Resuspend virus pellet in 350 µl of TSE and transfer to a 1.5 ml microcentrifuge tube.

B. Extraction and precipitation of RNA

1. Add 40 µl 10X RSB to virus suspension in microcentrifuge tube.
2. Add 12 µl 10% SDS.

3. Add 40 µl of Proteinase K.
4. Vortex.
5. Incubate at 56^{0}C for 15 minutes.
6. Add 8 µl 10X LiCl buffer.
7. Add 400 µl RSB-saturated phenol.
8. Vortex.
9. Incubate at 56^{0}C for 15 minutes, vortexing every 3 minutes.
10. Centrifuge at 10,000 rpm for 1 minute.
11. Transfer aqueous, cloudy upper layer to a new 1.5 ml microcentrifuge tube.
12. Add 400 µl RSB-saturated phenol.
13. Vortex.
14. Centrifuge at 10,000 rpm for 1 minute.
15. Transfer aqueous, clear upper layer to a new 1.5 ml microcentrifuge tube.
16. Add 200 µl RSB-saturated phenol.
17. Add 200 µl chloroform:isoamyl alcohol (24:1).
18. Vortex.
19. Centrifuge at 10,000 rpm for 1 minute.
20. Transfer aqueous, clear upper layer to a new 1.5 ml microcentrifuge tube.
21. Add 400 µl chloroform:isoamyl alcohol (24:1).
22. Vortex.
23. Centrifuge at 10,000 rpm for 1 minute.
24. Transfer aqueous, clear upper layer to a new 1.5 ml microcentrifuge tube.
25. Add 1 ml 100% ethanol.
26. Place tube at -70^{0}C for 1 hr, or -20^{O}C overnight, or on a dry-ice/ethanol bath for 5 minutes to precipitate the RNA.
27. Centrifuge at 10,000 rpm for 15 minutes at 4^{O}C.
28. Pour off ethanol carefully.
29. Add 1 ml 70% ethanol.
30. Centrifuge at 10,000 rpm for 5 minutes at 4^{0}C.
31. Pour off ethanol carefully.
32. Add 1 ml 70% ethanol.
33. Centrifuge at 10,000 rpm for 5 minutes at 4^{0}C.

34. Dry pellet under vacuum or inverted on the benchtop.
35. Resuspend the dry pellet in 10 µl ultrapure water.

Alternative method: Micro-Neutralisation Assay

Introduction

Serology methods rarely yield an early diagnosis of acute influenza virus infections; however, the demonstration of a significant increase in antibody titers (greater than or equal to fourfold) between acute- and convalescent-phase sera may establish the diagnosis of a recent infection even when attempts to detect the virus are negative. Apart from their diagnostic value, serologic techniques such as the hemagglutinin inhibition test (HI), the virus neutralization test, and enzyme-linked immunosorbent assay (ELISA) are the fundamental tools in epidemiologic and immunologic studies as well as in the evaluation of vaccine immunogenicity.

The virus neutralization test is a highly sensitive and specific assay applicable to the identification of virus-specific antibody in animals and humans. The neutralization test is performed in two stages consisting of (1) a virus-antibody reaction step, in which the virus is mixed and inoculated with the appropriate antibody reagents, and (2) an inoculation step, in which the mixture is inoculated into the appropriate host system (e.g. cell cultures, embryonated eggs, or animals). The absence of infectivity constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in human or animal sera.

The virus neutralization test gives the most precise answer to the question of whether or not an animal has antibodies that can neutralize infectivity of a given virus strain. The neutralization test has several additional advantages for detecting antibody to influenza virus. First, the assay primarily detects antibodies to the influenza virus HA, and thus can identify functional, strain-specific antibodies in animal and human serum. Second, since infectious virus is used, the assay can be developed quickly upon recognition of a novel virus and is available before suitable purified viral proteins become available for use in other assays.

The microneutralization test is a sensitive and specific assay for detecting virus-specific antibody to avian influenza A (H5N1) virus in human serum and potentially, for detecting antibody to other avian subtypes. The microneutralization test could detect H5-specific antibody in human serum at the titers that could not be detected by the HI assay, the traditional test used for the detection of antibodies to human influenza A and B viruses. Because antibody to avian influenza subtypes is presumably low or absent in most human populations, single serum samples may be used in screening for the prevalence of antibody to avian viruses. However, if infection of humans with avian viruses is suspected, the testing of paired acute and convalescent sera in the microneutralization test would provide a more definitive answer as to whether infection has occurred.

Conventional neutralization tests for influenza viruses based on the inhibition of cytopathogenic effect (CPE)-formation in Madin-Darby Canine Kidney (MDCK) cell cultures are laborious and rather slow, but in combination with rapid culture assay principles, the neutralization test can yield results within 2 days. In our influenza virus microneutralization assay, it is expected that serum neutralizing antibodies to influenza virus HA will inhibit the infection of MDCK cells with

virus. Serially diluted sera should be pre-incubated with a standardized amount of virus prior to the addition of MDCK cells. After an overnight incubation, the cells are fixed and the presence of influenza A virus NP protein in infected cells is detected by ELISA. This protocol is divided into 3 parts: 1)Virus Titration, 2)Virus Neutralization Assay and, 3) ELISA.

I. Materials required

A. Cell cultures and reagents

1. MDCK cell culture monolayer (Madin-Darby Canine Kidney cells)
   Low passage (< 25-30 passages) at low crowding (70-95% confluent)

2. MDCK sterile cell culture maintenance medium: DMEM, 5% FBS, Antibiotics
   - 500 ml Dulbecco's Modified Eagles Medium (DMEM)
     Gibco, Cat. # 11960-051
   - 5.5 ml 100X Antibiotics (100 U/ml penicillin, 100X ìg/ml streptomycin)
     Gibco, Cat. # 15140-122
   - 5.5 ml 2 mM L-Glutamine
     Gibco, Cat. # 25030-081
   - 25.5 ml heat inactivated (56°C, 30 min) Fetal Bovine Serum (FBS)
     Hyclone, Cat. # A-1115-L

3. Trypsin/EDTA: Gibco, Cat. # 25300-054

4. Diluent: DMEM, 1% BSA, Antibiotic- freshly made
   - 429 ml DMEM
   - 66 ml 7.5% Bovine serum albumin (BSA), Gibco Cat. # 15260-037.
   - 5 ml 100X Antibiotics, Gibco, Cat. # 15140-122.

5. Fixative: 80% Acetone in PBS- freshly made
   - 400 ml Acetone
   - 100 ml 0.01 M Phosphate Buffered Saline, pH 7.2 (PBS)
     Gibco Cat. # 20012-019, Store at 4x°C

6. Wash Buffer: PBS, 0.05% Tween-20, Sigma Cat. # P-1379
   - 4 L PBS
   - 2 ml Tween-20

7. Blocking Buffer: PBS, 1% BSA, 0.1% Tween-20
   - 867 ml PBS
   - 132 ml BSA
   - 1.0 ml Tween-20

8. 96-well microtiter plates: -Flat-bottom; Dynatech, Immulon-2 plates
   - Dynatech Cat. # 011-010-3455

9. 1X-antibody: Anti-Influenza A pool
   - Anti-NP mouse monoclonal antibody, CDC Lot.# 90-0026
   - Dilute 1:4000 in blocking buffer or at optimal concentration

10. 2X-antibody: Goat anti-mouse IgG conjugated to horseradish peroxidase (HRP)
    - Kirkegaard and Perry Cat. # 474-1802
    - Dilute 1:2000 in blocking buffer or at optimal concentration

11. Substrate: HRP-Substrate
    - For each 20 ml of Citrate buffer (2-plates/20 ml), add 10 mg
    - o-phenylenediamine dihydrochloride (OPD) tablet just before use.
    - Sigma, Cat. # P8287
    - For citrate buffer, prepare as follows:
      a) Make with Sigma Citrate Buffer capsules Cat. # P4922 (1 capsule to 100 ml dH_2O)

      OR

      b) 58.8 g trisodium citric acid
      2 L dH_2O
      pH to 5.0 with HCl
    - Add 10X ml 30% hydrogen peroxide (0.015% H_2O_2) to each 20 ml of substrate just prior to use.

12. Stop Solution: 1N Sulfuric acid (28 ml stock sulfuric acid to 1L dH_2O)

13. Serum specimens: If samples are to be tested repeatedly, it is better to make several aliquots of sera. Sera should not be repeatedly freeze-thawed. Sera can be stored at -20 to -70 °C.

14. Virus strains: Allantoic fluid (determine 100 $TCID_{50}$/100 ml before use).

B. Supplies

1. Sterile capped tubes.
2. Assorted sterile pipettes and pipetting device.
3. Autoclavable containers for discarding cultures.

C. Equipment

1. Class II biological safety cabinet.
2. Water baths, 37°C and 56°C.
3. Incubator, 37°C, 5% CO_2.
4. Inverted microscope or standard microscope for the observation of cells.

5. Freezer, - 70°C (for long term virus storage) or - 20°C (for serum storage).
6. Automatic ELISA reader with 490 nm filter.
7. Automatic plate washer (not essential but would be optimal).
8. 4 °C refrigerator.
9. Low speed, bench top centrifuge preferably with refrigeration.
10. Liquid nitrogen for cell storage.

II. Quality control

A. Serum controls

Make multiple aliquots of all control sera and store at - 20°C. Include both animal and human normal serum controls and positive serum controls for each virus used in assay.

1. Negative (normal) serum control:
   Include a normal serum to determine whether the virus is nonspecifically inactivated by serum components.
   a. For animal sera, use nonimmune serum from same animal species used for positive control.
   b. For human sera, use age-matched normal serum from a population not exposed to the particular virus subtype in question.
   c. Use the normal serum at the same dilution as the matching viral antiserum.
2. Positive (infected or vaccinated) serum controls:
   Include anti-sera to test viruses as positive control.
   a. For animal sera, use sera raised in infected ferrets or other (goat, rabbit or mouse) immunized animals. The best results will be obtained if all (negative and positive) control animal sera are treated with receptor destroying enzyme (RDE) prior to use in the neutralization assay.
   b. For human sera, an optimal positive control would be acute and convalescent serum samples.

B. Virus and cell controls

Include a virus back-titration and positive and negative cell controls with each assay.

1. Negative and positive cell controls:
   Set up four wells as positive cell controls-VC (50 µl medium + 50 µl test dilution of virus + 100 µl of MDCK cells) and four wells as negative cell controls-CC (100 µl medium + 100 µl of MDCK cells) and assay in parallel with the neutralization test.
2. Virus titration check:
   In each assay, include a back-titration of the test dilution of virus. Add 50 µl of medium to each well (B to H). Add 100 µl of the test dilution of virus (100 $TCID_{50}$) to the first well (A well). Titrate in 2-fold serial

dilutions down the plate (7 wells, B to H). Add an additional 50 µl of virus diluent to virus titration wells. Add 100 µl of MDCK cells (1.5x104/ well) and incubate overnight with rest of assay.

III. Procedure

Part I: Virustitration

Generation of stock virus

1. Grow up virus to high titer in the allantoic cavity of 10-day old embryonated hens' eggs.
2. Dilute virus sample (usually 1:100 to 1:1000) in PBS + antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mg/ml gentamycin).
3. Pierce hole in the tops of eggs.
4. Inoculate eggs with virus inoculum (100 µl/egg) using a syringe fitted with a 22 gauge/1" needle.
5. Seal hole in eggs with glue or paraffin wax.
6. Incubate eggs for 20-72 hrs at 37°C or 35°C (H5N1 viruses for 24 hrs at 37°C, H3N2 and H1N1 viruses for 2 days, and influenza B viruses for 3 days at 35°C).
7. Chill eggs (overnight at 4°C or 30 min at -20°C).
8. Remove tops of eggs and harvest allantoic fluid with pipette.
9. Clarify allantoic fluid by low-speed centrifugation (600 x g, 10 min).
10. Aliquot virus in 1 ml ampules and freeze at -70°C.
11. Thaw virus and determine HA activity and $TCID_{50}$.

Preparation of MDCK cells

1. Check MDCK cell monolayer (should be 70-95% confluent). Do not allow to overgrow. Typically, a confluent 162 cm^2 flask should yield enough cells to seed up to 7-10 microtiter plates (~$2X10^7$ cells/flask). Split confluent monolayer 1:10 two days before use for optimum yield and growth. (CELLS MUST BE IN LOG PHASE GROWTH FOR MAXIMUM VIRUS SENSITIVITY).
2. Gently rinse monolayer with 5 ml trypsin-EDTA and remove.
3. Add 4-5 ml trypsin-EDTA to cover the cell monolayer.
4. Lie flask flat and incubate at 37°C in 5% CO_2 until monolayer detaches (approximately 10-20 min).
5. Add 5-10 ml of MDCK medium to each flask, remove cells and transfer to centrifuge tube.
6. Wash cells 2x with PBS (5 min at 12,000 rpm).
7. Resuspend cells in diluent and count cells with a hemacytometer.
8. Adjust cell number to $1.5x10^5$ cells/ml with diluent.
9. Add 100 µl cells to each well of microtiter plate.

10. Incubate cells overnight at 37°C, 5% CO_2 (18-22 hrs).

Virus titration

1. Thaw an ampule of virus.
2. Dilute virus 1/100 in diluent (100 µl virus + 9.9 ml diluent).
3. Add 100 µl of diluent (±TPCK-trypsin, 2 mg/ml*) to all wells, except column 1, of a 96-well tissue culture plate. (Perform titration of virus in quadruplicate cultures).

   **Some avian viruses, including H5 viruses, do not require the addition of trypsin. When determining $TCID_{50}$ of new test viruses, it is best to perform titration with and without trypsin to determine optimal conditions for each virus.*
4. Add 146 µl virus of 1/100 working stock to column 1. Perform ½ $\log_{10}$ dilutions of virus: Transfer 46 µl serially from column 1,2,3,11. Dilutions will be 10^{-2},$10^{-2.5}$,10^{-3},10^{-7} .

Assay and determination of 50% Tissue Culture Infectious Doses (TCID50)

1. Remove medium from plate.
2. Wash each well with 250 µl PBS.
3. Remove PBS (Do not allow wells to dry out) and add 100 µl / well of cold fixative.
4. Cover with lid and incubate at room temperature for 10 min.
5. Remove fixative and let plate air-dry.
6. Perform ELISA (see Part III).
7. Calculate the mean absorbance (OD) of the cell controls.
8. Any test well with an OD > 2 times OD of cell control wells (CC) is scored positive for virus growth.
9. Once all test wells have been scored positive (+) or negative (-) for virus growth, the titer of the virus suspension can be calculated by the method of Reed and Muench. This will determine $TCID_{50}$ per 100 µl volume.
10. Dilute the virus suspension so that 50 µl contains 100 $TCID_{50}$. (Initial virus titration will determine if addition of TPCK-trypsin to virus diluent is optimal for virus infection of MDCK cells).

Part II: Virus neutralisation assay

Preparation of MDCK cells

1. Check MDCK cell monolayer (should be 70-95% confluent). Do not allow to overgrow. Typically, a confluent 162 cm^2 flask should yield enough cells to seed up to 8-10 microtiter plates.
2. Gently rinse monolayer with 5 ml trypsin-EDTA and remove.
3. Add 4-5 ml trypsin-EDTA to cover the cell monolayer.

4. Lie flask flat and incubate at 37°C in 5% CO_2 until monolayer detaches (approximately 10-20 min).
5. Add 5-10 ml of MDCK medium to each flask and remove cells.
6. Wash cells 2X with PBS to remove FBS.
7. Resuspend cells in diluent and count cells with a hemacytometer.
8. Adjust cell number to 1.5x105 cells/ml with diluent.
9. Add 100 µl cells to each well of microtiter plate (1.5x104 cells/well).

Preparation of test sera

1. 10 µl of sera are needed for each virus to be tested once. Sera should be tested in duplicate (requires 20 µl when possible). Eleven sera can be tested on each microtiter plate.
2. Heat inactivate sera for 30 min at 56°C.
3. Plate setup.
4. Add 50 µl of diluent to each well of the microtiter plates.
5. Add an additional 40 µl of diluent to Row A (wells A1-A11).
6. Add 10 µl of heat-inactivated sera to Row A (1 serum/well except A12).
7. Perform twofold serial dilutions by transferring 50 µl from row to row (A,B,C,H).

Note: If assaying for 2 virus strains, dilute 2 separate plates.

Addition of virus

1. Dilute virus to 100 $TCID_{50}$ per 50 µl ($2x10^2$ $TCID_{50}$ /100 µl) in virus diluent ±TPCK-trypsin (approximately 5 ml/plate).
2. Add 50 µl diluted virus to all wells except CC (wells E12, F12, G12, and H12).
3. Add 50 µl diluent to CC wells.
4. Set up back-titration, start with the virus test dilution (100 $TCID_{50}$), prepare additional serial 2-fold dilution with diluent.
5. Gently agitate the virus-serum mixtures, and incubate them and the virus back-titration for 2 hrs at 37°C, 5% CO_2.
6. Incubate plates overnight at 37°C, 5% CO_2 (18-22 hrs). To ensure even distribution of heat and CO_2, stack plates only 4 to 5 high in incubator.

Fixation of the plate(s)

1. Remove medium from plate.
2. Wash each well with 250 µl PBS.
3. Remove PBS (Do not let wells dry out) and add 100 µl / well of cold fixative.

4. Cover with lid and incubate at room temperature for 10 min.
5. Remove fixative and let plate air-dry.

PART III: Elisa

Antibody

1. Wash plate(s) 3X with wash buffer. Fill wells completely with wash buffer for each wash.
2. Dilute 1-antibody (anti-Influenza A pool; NP monoclonal) 1:4000 in blocking buffer.
3. Add diluted 1-antibody to each well (100 µl /well).
4. Cover plate(s) and incubate for 1 hr at room temperature.

Antibody

1. Wash plate(s) 4X with wash buffer.
2. Dilute 2-antibody (goat anti-mouse IgG; HRP conjugated) 1:2000 in blocking buffer.
3. Add diluted 2-antibody to each well (100µl /well).
4. Cover plate(s) and incubate for 1 hr at room temperature.

Substrate

1. Wash plate(s) 6X with wash buffer.
2. Add freshly prepared substrate (10 mg OPD to each 20 ml citrate buffer + H_2O_2) to each well (100 µl/well).
3. Incubate for 5-10 min (or until color change in VC is intense and before background CC begins to change color) at room temperature.
4. Add stop solution (100 µl/well) to all wells.
5. Read absorbance (OD) of wells at 490 nm.

Data analysis

1. Calculations are determined for each plate individually.
2. Determine virus neutralization antibody end point titer of each serum utilizing the equation below:

   (Average of VC wells) - (Average of CC wells) + (Average of CC wells) = X

   X = 50% of specific signal. All values below this value are positive for neutralization activity.
3. The value of OD in the normal serum control should be similar to that observed in the virus control (VC).
4. The virus test dose (100 $TCID_{50}$) is confirmed by virus back-titration. In most cases, the test dose of virus is acceptable if the back-titration is positive in 3-5 wells containing the lowest dilutions of test virus.

5. The negative cell control (CC) should show OD < 0.2 and the monolayer quality should be acceptable.
6. The serum positive controls should give titers within twofold of expected.

   Occasionally, the neutralization test may be difficult to interpret. In such cases, consider the factors presented in Table 4.

$TCID_{50}$ (50% tissue culture infectious dose) is the dilution of virus at which half of cell cultures are infected.

1. Record the number of positive (1) and negative (2) values at each dilution.
2. Calculate the cumulative numbers of positive (3) and negative (4) wells.

   (3) Obtained by adding number in (1), starting at bottom.

   (4) Obtained by adding number in (2), starting at top.
3. Calculate the percentage of positive wells (5) by using value in (3)/[value in (3) + (4)].

4. Calculate the proportional distance between the dilution showing > 50% positive (6) and the dilution showing < 50% positive (6).

   Proportional = % positive value above 50%-50 X Correction

   distance % positive value above 50%- % positive value below 50% factor

   = 75-50 X 0.5 = 0.3 X 0.5 = 0.15

   75-0
5. Calculate the $TCID_{50}$ by adding the proportional distance factor to the dilution showing > 50% positive. Since each well was inoculated with 0.1 ml of each virus dilution, the $TCID_{50}$ is expressed as $TCID_{50}$/0.1 ml. One $TCID_{50}$ in this case is $10^{-5.15}$ /0.1 ml, and therefore 100 $TCID_{50}$ equals $10^{-3.15}$ /0.1 ml, or $10^{-2.8}$ /50 µl.
6. If other dilution series are used, other factors must be used. For example, in this case, the correction factor for ½ $\log_{10}$ dilution series would be 0.5, that for 10-fold dilution series would be 1, that for a twofold dilution series would be 0.3, and that for a five-fold dilution series would be 0.7.

Table 4 Table 4. Problems associated with interpretation of the neutralization test

Probler	Problem	Possible cause(s)	Solution
Weak c color in cell cor wells	Weak or no color in positive cell control (CC) wells	i) Problem with ELISA:	
		-Using wrong antibodies or substrate	Check the antibodies and substrate
		-Buffer solutions incorrect	Prepare fresh buffers
		ii) Test dose of virus too weak or forgot to add virus to VC wells	Redetermine endpoint titers
		iii)Virus inactivated during incubation step	Check incubator and cabinet temperature
Weak c neutrali positive sera	Weak or no neutralization by positive control sera	Test dose of virus too strong	Redetermine endpoint titers
		Serum deteriorated	Obtain new antisera
			Check storage conditions
Neutral by nega control	Neutralization by negative control sera	Non-specific reaction or cross-reactivity	Heat inactivate serum (56°, 30 min)
			Check samples for cross-reactive properties
		Test dose of virus too weak	Redetermine endpoint titers
Nonspe virus inactiva	Nonspecific virus inactivation	Serum not heat inactivated or contains non-specific viral inhibitors	Heat inactivate serum (56°C, 30 min)
			For animal serum, treat with RDE
Monola toxicity	Monolayer toxicity	Serum not heat inactivated or is toxic to cells	Heat inactivate serum
			Run toxicity check on normal MDCK cells and check microscopically for toxicity

Table 5 Calculation of $TCID_{50}$ by the Reed-Muench method

	Observed Value (OD)		Cumulative Value			
Dilution	(1) Positive	2) Negative	(3) Positive	(4) Negative	(5) Ratio	(6) %Positive
10^{4}	4	0	11	0	11/11	100
$10^{4.5}$	4	0	7	0	7/7	100
10^{5}	3	1	3	1	3/4	75
$10^{5.5}$	0	4	0	5	0/5	0

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