Detection of avian influenza

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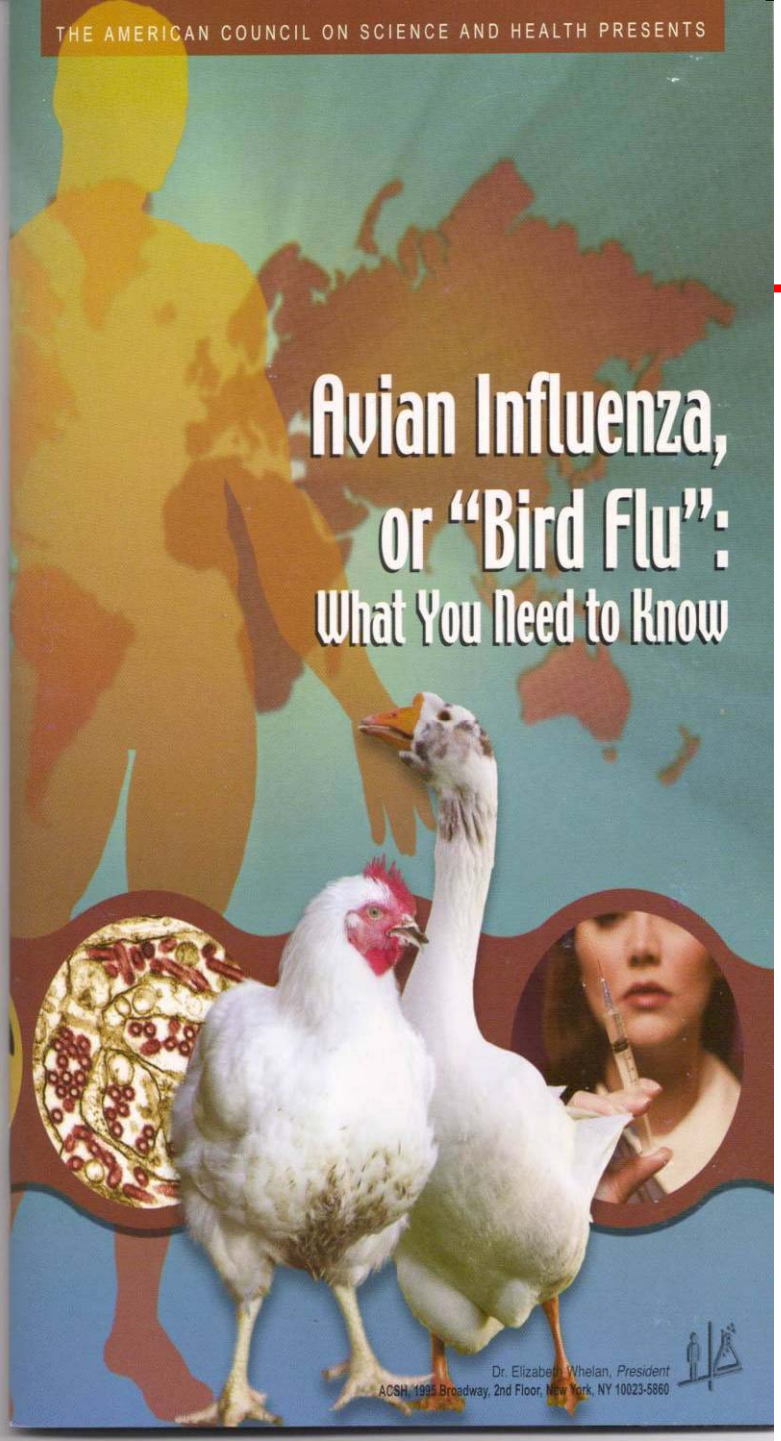


Avian influenza

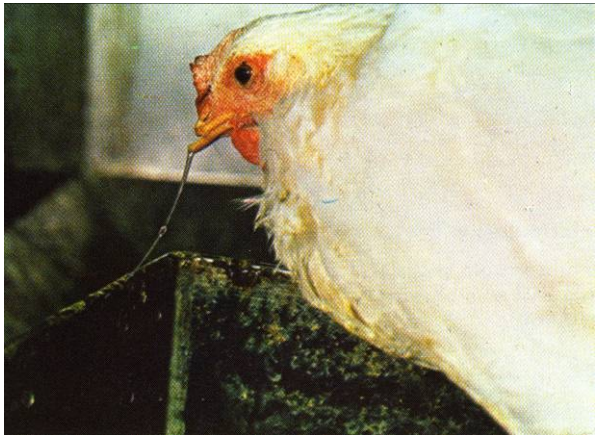
- A contagious disease
- Domestic poultry flocks are especially vulnerable
- The disease in birds has two forms: mild or highly pathogenecity (HPAI)

Common domestic hosts

Clinical Manifestations

- Vary greatly depending on age, species, virus virulence, other infections, and production management

- Low Pathogenic AI
  - -Depression
  - -Respiratory signs
  - -Lower productivity
    egg drop in layers, such as H9N2 infection
  - -"Sleepy" chicks



感染减蛋综合征鸡群所产蛋蛋壳质量变差，软皮蛋、无壳蛋

History of avian influenza

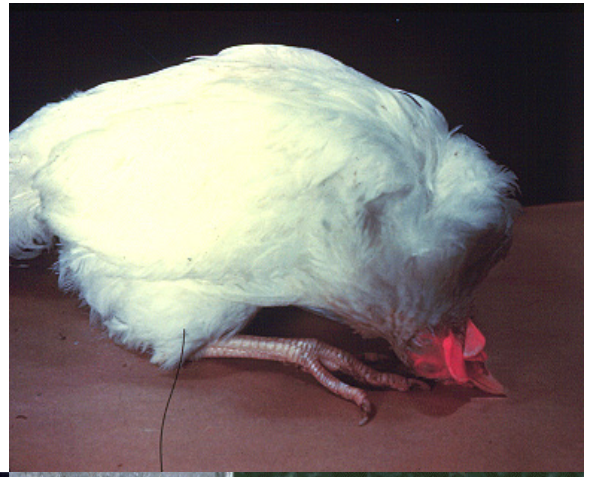
- First report in Italy in 1878 as Fowl Plague
- Frequent outbreaks up to about 1950
- Confirmed to be caused by influenza A virus in 1955
- First duck isolate (H4N6) in Czechoslovakia in 1956
- First chicken isolate (H5N1) in Scotland in 1959
- First turkey isolate (H6N8) in Canada and H7N3 in England in 1963

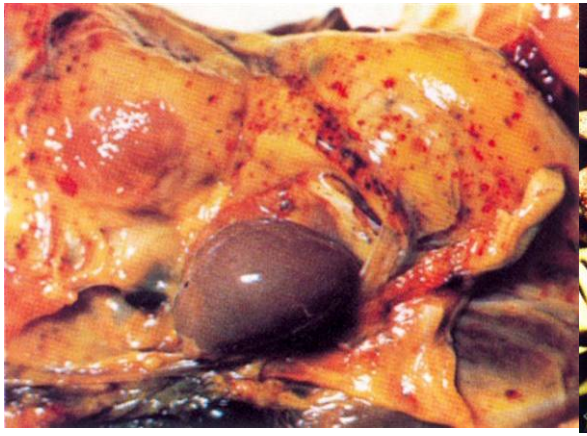
History of avian influenza

- Only 21 outbreaks from 1959 to 1997
- Most outbreaks very small and quickly controlled
- A few large widespread epidemics (market dissemination)
- First instance of human infection (conjunctivitis) caused by AIV (H7N7) in England in 1996
- First instance of human's death caused by AIV (H5N1) in Hong Kong in 1997
- Multiple H5 and H7 outbreaks since 1997

Highly Pathogenic Avian Influenza—Fowl Plague

- Sudden onset high mortality
- Depression, +/- Nervous signs
- Face edema & hemorrhagic lesions





High Pathogenecity Avian influenza

How about HPAI in recent years?

- Since mid-2003, the H5N1virus has caused the largest and most severe outbreaks in poultry on record.
- In 2005, H5N1 HPAI outbreak in migratory birds in Qinghai lake in western China, It was the first case in the history of avian influenza.
- Now, the H5N1 HPAIVs have spread all over the world through the migration of wild birds
- Many human cases

History of HPAI in domestic poultry

1959-Scotland, H5N1

1961-S. Africa, H5N3

1963-England, H7N3

1966-Canada, H5N9

1975-Australia, H7N7

1979-England, H7N7

1983-84 - USA, H5N2

1983-Ireland, H5N8

1985-Australia, H7N7

1991-England, H5N1

1992-Australia, H7N3

1994-Australia, H7N3

1994-95-Mexico, H5N2

Red represents shift from LPAI to HPAI

1995, 2001 & 2004 –Pakistan, H7N3

1997-Australia, H7N4

1997-Italy, H5N2

1996-2007 - Asia, Euro, Africa ,H5N1

Largest epizootic in 50 yrs

1999-2000 - Italy, H7N1

2002 - Chile, H7N3

2003 – Netherlands, H7N7

2004 – USA, H5N2

2004 – Canada, H7N3

2004 – S. Africa, H5N2

2005 – N. Korea, H7N?

The Hong Kong 1997 outbreak

- Substantial outbreak of H5N1 in domestic poultry
- First began to show mammalian virulence
- 18 human cases, 6 deaths
- Pigs have remained insignificant so far in the epidemiology of H5N1 virus
- The HK97 virus was eradicated by eliminating domestic poultry, has never been detected subsequently

Evolution 1997-2003

- H5N1 viruses were isolated on only a few occasions
- Hong Kong had intermittent outbreaks in poultry and a cluster of human cases
- Isolated from two geese in a market in Vietnam in 2001
- Sudden explosive epidemic in Asia and caused much more human cases from 2003

HPAI diagnosis

- Clinical
- Post Mortem Lesions
- Virology (Antigen) : usually by virus isolation

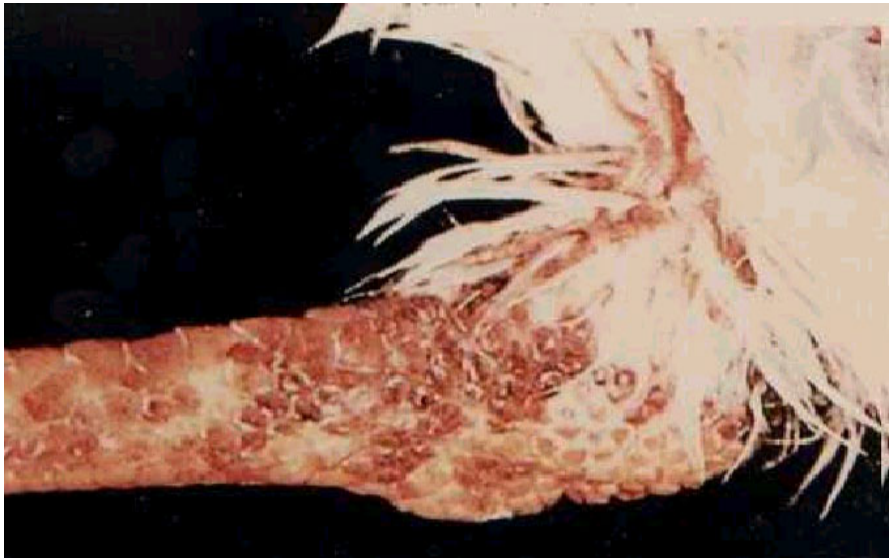
  Presence of virus confirmed by
  AGID
  ELISA
  RT-PCR
- Serology (Antibody) may be helpful

Clinical Signs

- Incubation period: 3-14 days
- Depression, anorexia, ruffled feathers
- Neurological signs
- Conjunctivitis and respiratory signs
- Drop in egg production
- Birds found dead
- Mortality almost 100%

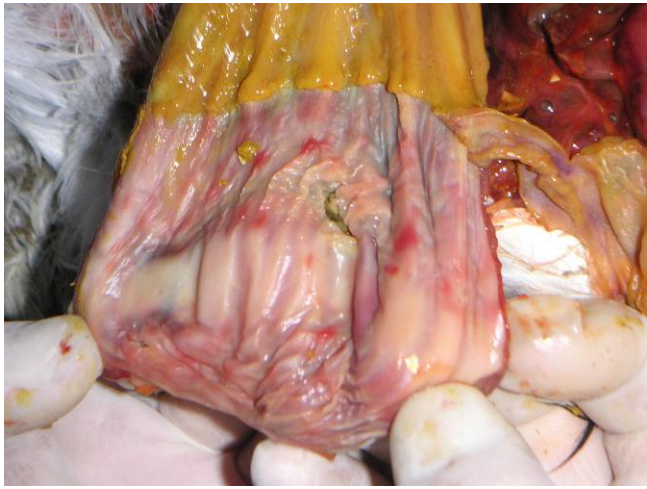
Clinical Signs

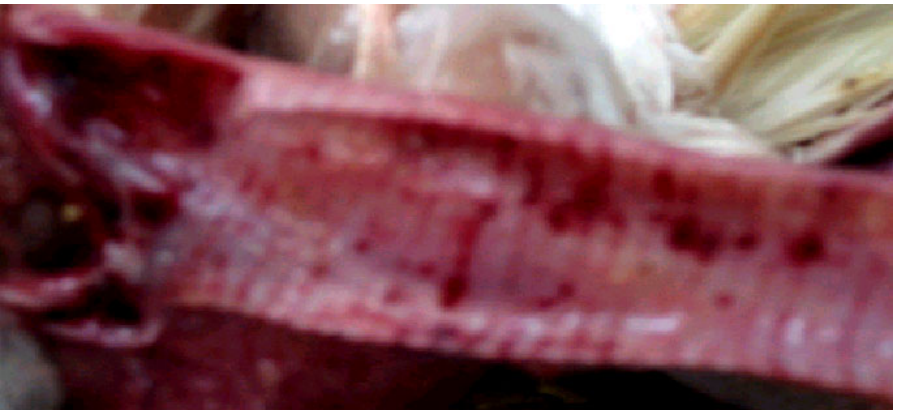
- Facial edema
- cyanotic combs and wattles
- Petechial hemorrhages on the leg

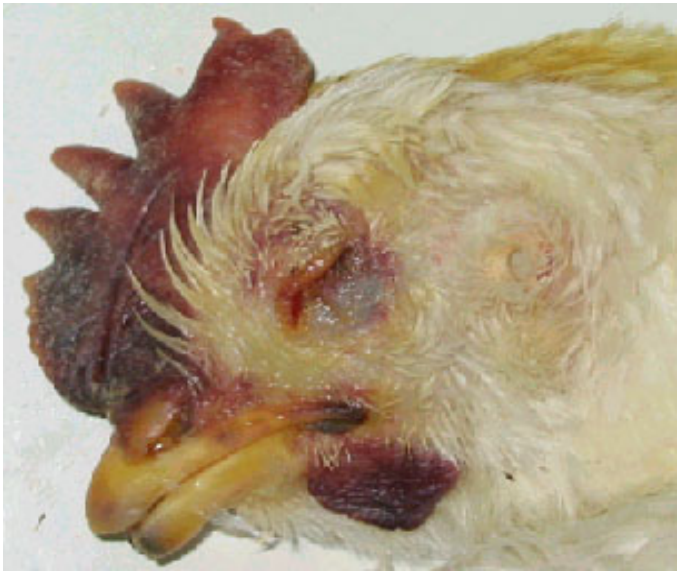


Post Mortem Lesions

- Lesions may be absent with sudden death
- Severe hemorrhage on tissues
- Subcutaneous edema of head and neck area
- Dehydration

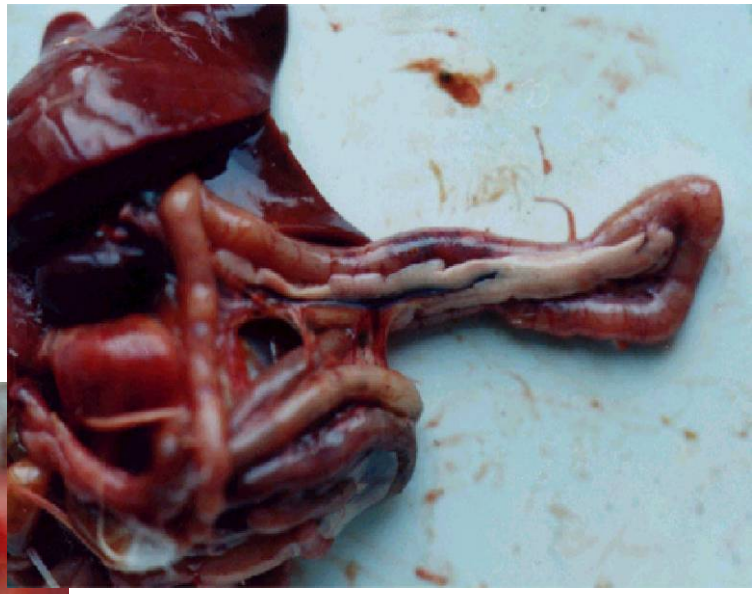


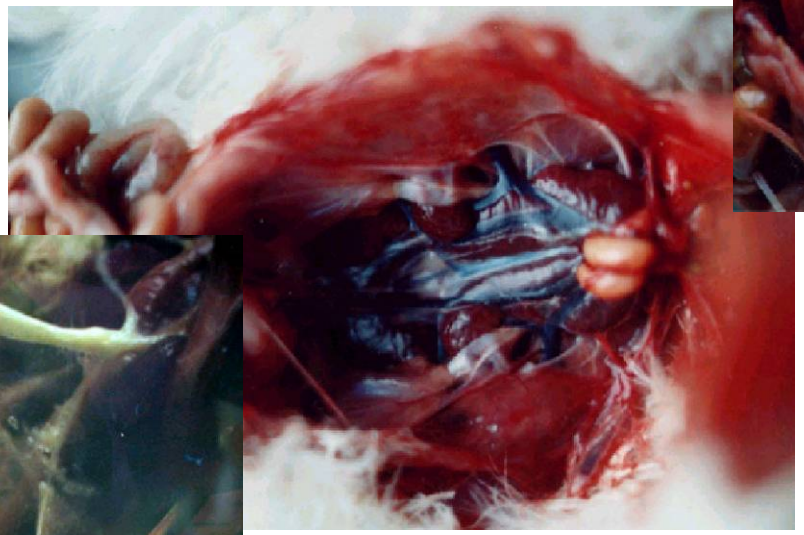


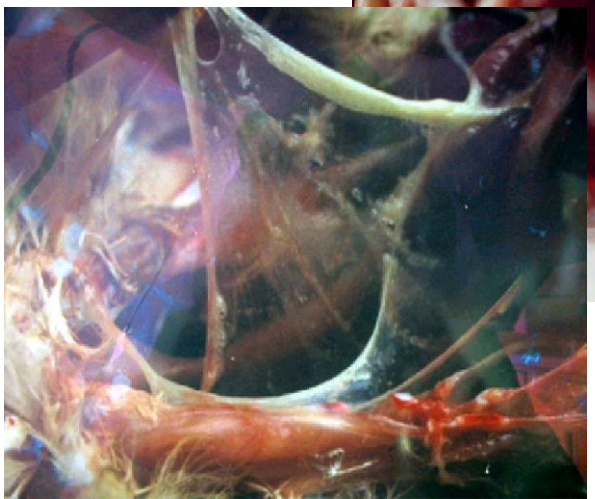


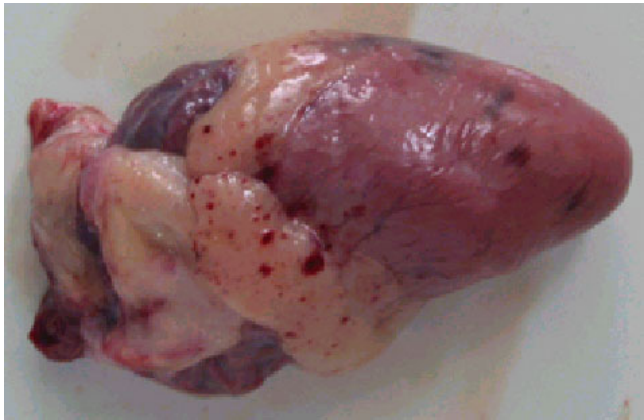
Post Mortem Lesions

- Nasal and oral cavity discharge
- Petechiae on serosal surfaces
- Kidneys severely congested
- Intestine hemorrhage





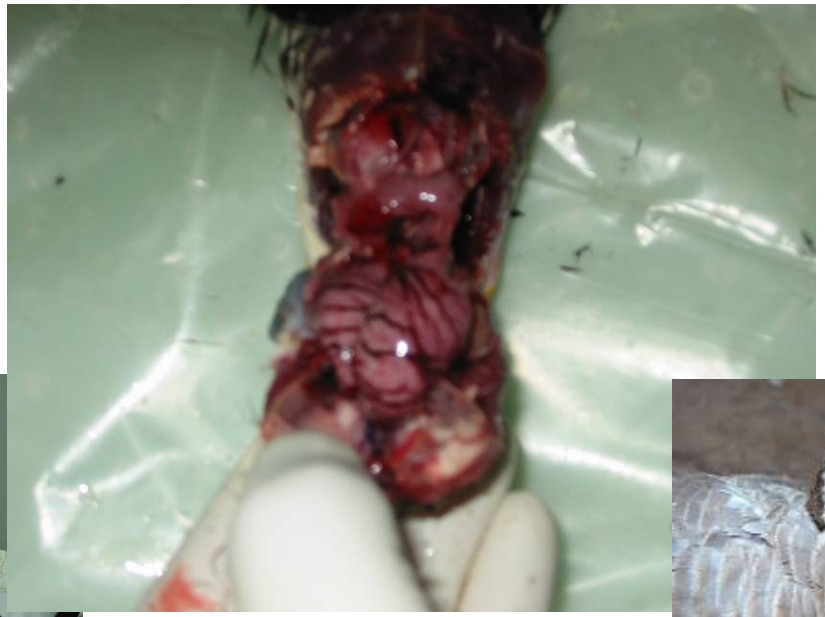




Post Mortem Lesions

HPAIVs always can cross the barrier of Blood and Brain, infect brain, then lead to death

Brain is the first and foremost tissue for isolate the HPAIV



Laboratory Techniques

- Collection of specimens
- Isolation of influenza virus

Collection of specimens

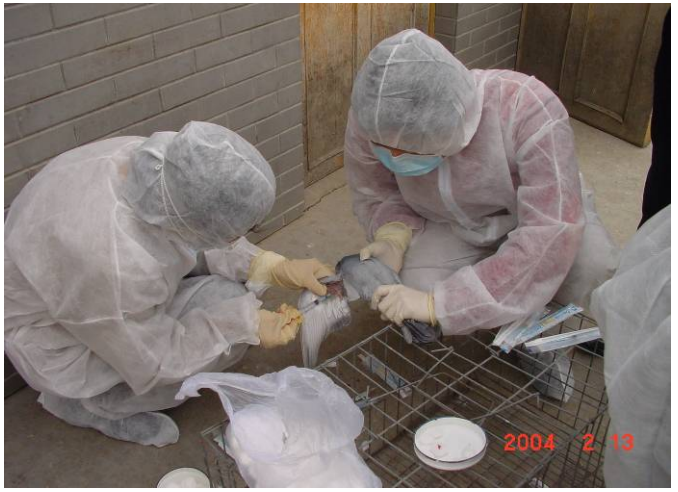
Specimen is a significant factor for virus diagnosis

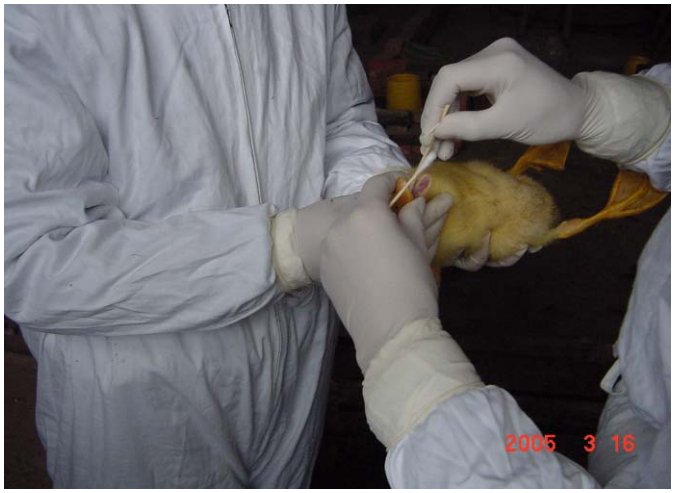
- the quality of the specimen
- the conditions for transport and storage

Representative Requirements of the Samples

30Samples/poultry farm

- 30Samples/live live market
- 20 Samples/habitat
- 20 Samples/swine farm





The quality of the specimen

- Should be taken during the first 3 days
- Respiratory & intestinal tract:Avian
- Respiratory tract:
  Mammals:human,pig,horse
- Upper respiratory tract:nasal swab
  throat swab
  tracheal swab
- Intestinal tract: cloaca swab

Sampling

Samples: a critical element for diagnosis

- Collected in day 3 should be best
- Swabs: cloacal and laryngeal
- Tissues: Brain, spleen, kidney, pancreas, lung, trachean
- Keep them respectively

Kept in special box with ice

Sampling

Samples: a critical element for diagnosis

- Before collecting or sending any samples, the proper authorities should be contacted
- Samples should only be sent under secure conditions and to authorized laboratories to prevent the spread of the disease
- HPAI samples may be zoonotic
- Protect yourself when contect HPAI samples at any time

The quality of the specimen

- **Avian species**

  live: cloacal, tracheal swab, fecal sample,

  dead: together with internal organs

- **Mammals**

  live: tracheal swab/trachea

  dead: together with bronchoalveolar lavage, lung biopsy sample

Transport and Storage

- **Sample should kept on ice** and processed within 1-2h for immunofluorescence staining
- **Chilled** in an ice pack immediately

  for virus isolation ASAP
- **Kept frozen** at or below -70℃

  If cannot be processed withing48-72h
- **Should not** be stored or shipped in dry ice(CO_2), unless be sealed or doubled plastic bagged
- **Repeated** freezing and thawing must be avoided

Transport and Storage

- **Transport medium**: shoule be supplemented with antibiotics, antimycotics, protein
- **Sterilize, distribution, in ice or in liquid nitrogen**
- **Commonly**: Glycerol medium
  Medium 199
- **Commercially**: Hanks balanced salt solution
  phosphate buffered saline
  cell culture medium, etc.

Take samples

- **Nasal swab**: inserted into, parallel to, left for,slowly withdrawn, rotating motion
- **Throat swab**: posterior pharynx, swab vigorously
- **Tracheal swab**: live bird, gently
  dead, vigorously
- **Cloacal swab**: insert deeply, swab vigorously

Take samples

- **Fecal sample**: freshly deposited wet feces
- **Tissue sample**: frozen immediately without transport medium, then ground
- **Water sample**
- **Sera**: acute phase , within 1 week;

  convalescent phase,2-4 weeks.

  Blood clot, then centrifuged at 2500rpm,

  15 mins, discard RBC

  Stored at -20 ℃

Take samples

- **Essential to record**
  - Type of animal sampled
  - Type of sample
  - Location of sampled
  - Date of sampled
- Samples collected form cages are called enviromental since the source animal is usually uncertain

Isolation of influenza virus

Processing clinical material

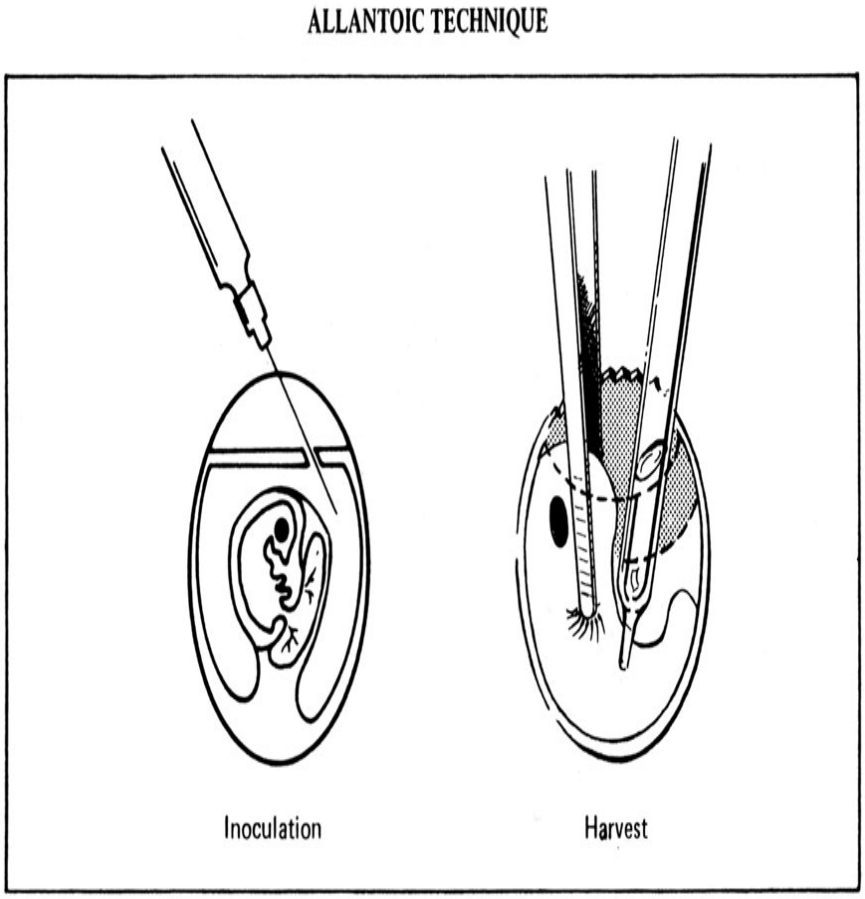
- swab samples: thaw, add antibiotics, agitate on vortex, leave for 30 mins at room temp
- Tissue samples: grind, make a 10% (g/v) suspension, add antibiotics, centrifuge at 400xg for 10 mins, inoculate supernatant

Isolation of influenza virus

Two culture systems

- **Embryonated Egg**: The option of choice for avian influenza viruses, but some of the human or swine influenza virus may grow poorly.
- **Cells**: Used for growing both human and animal influenza viruses. Not suitable for isolation human vaccine candidate
- **Two culture systems combinate**

Isolation from Embryonated Egg



- Canding
- Sterilization
- Inoculation
- Incubation
- Harvesting

Isolation from Embryonated Egg

- **Canding**

  discard those are infertile, underdevelpled, have cracks, have a porous shell

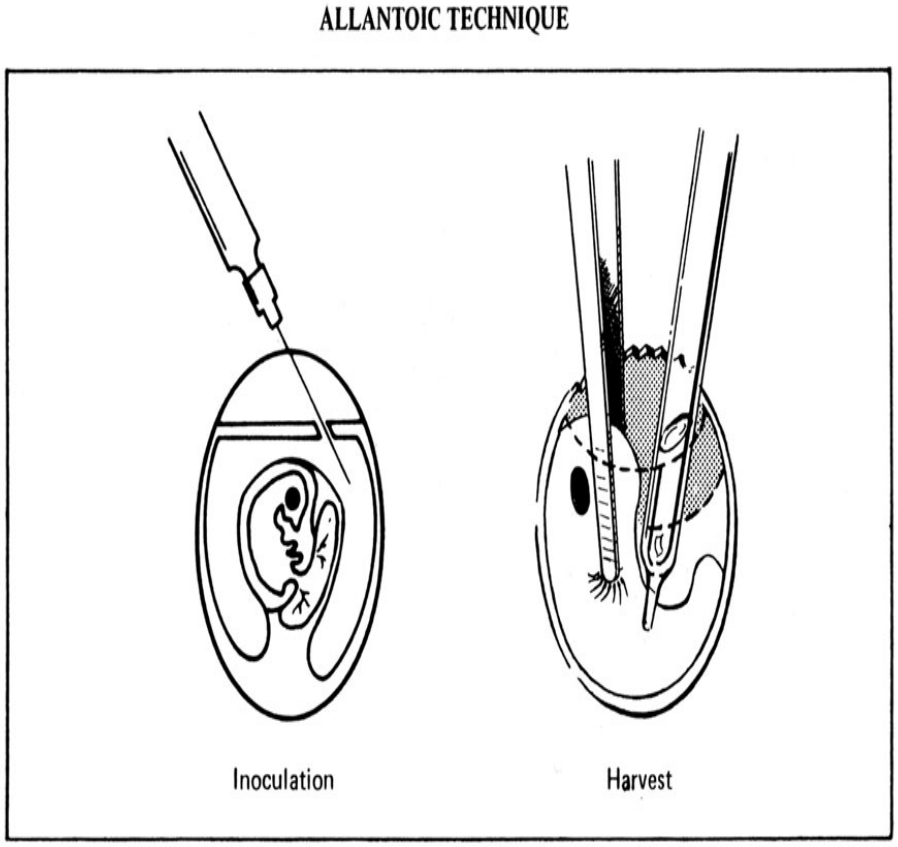
- **Sterilization**

  Blunt end up, wipe with 70% ethanol

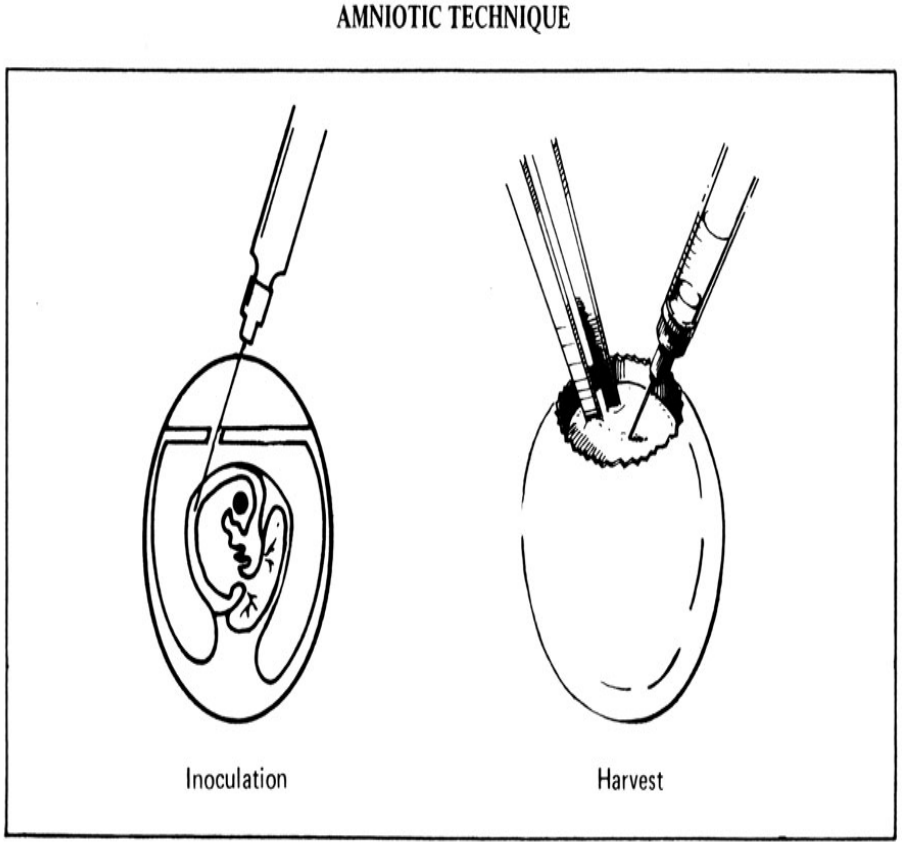
Isolation from Embryonated Egg

- **Inoculation**: two routes

Allantoic cavity



Amniotic



Isolation from Embryonated Egg

- **Incubation**

  Avian influenza virus 35-37 ℃

  Mammalian influenza virus 35 ℃

  HPAIV:often dead in 1-2 days

  LPAIV: last for 2-4 days

Isolation of influenza virus

- **Harvest**

Chill　4 ℃ overnight or 4h before harvest

Sterilize

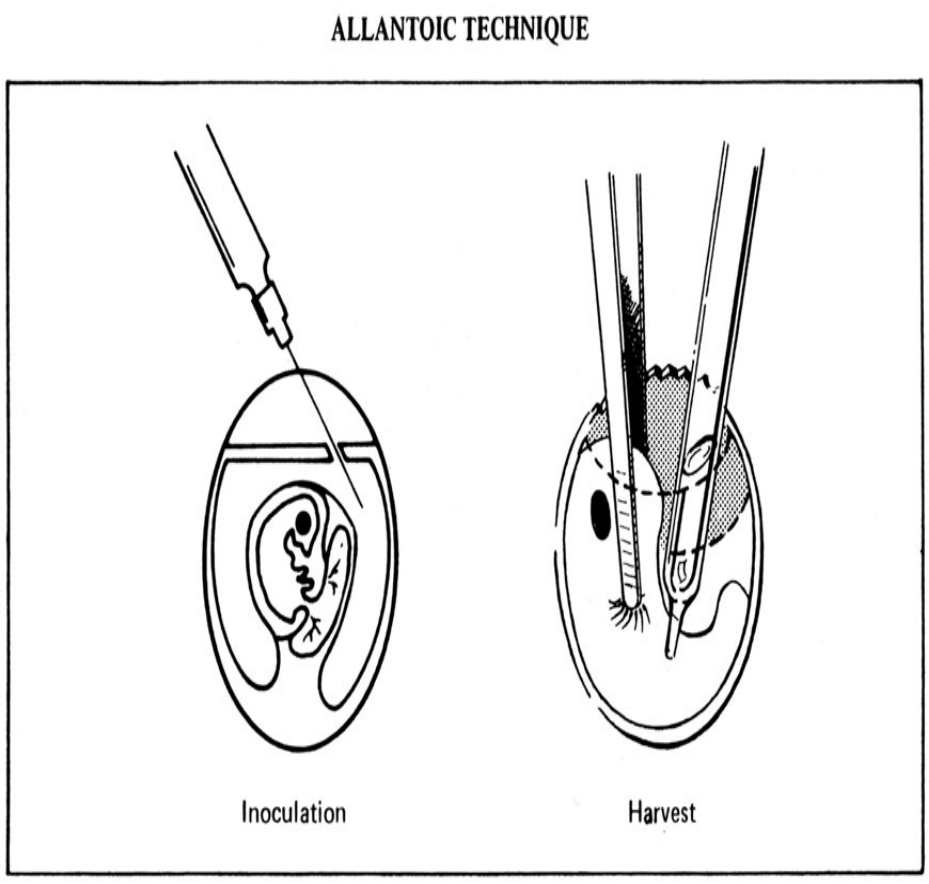
Combine

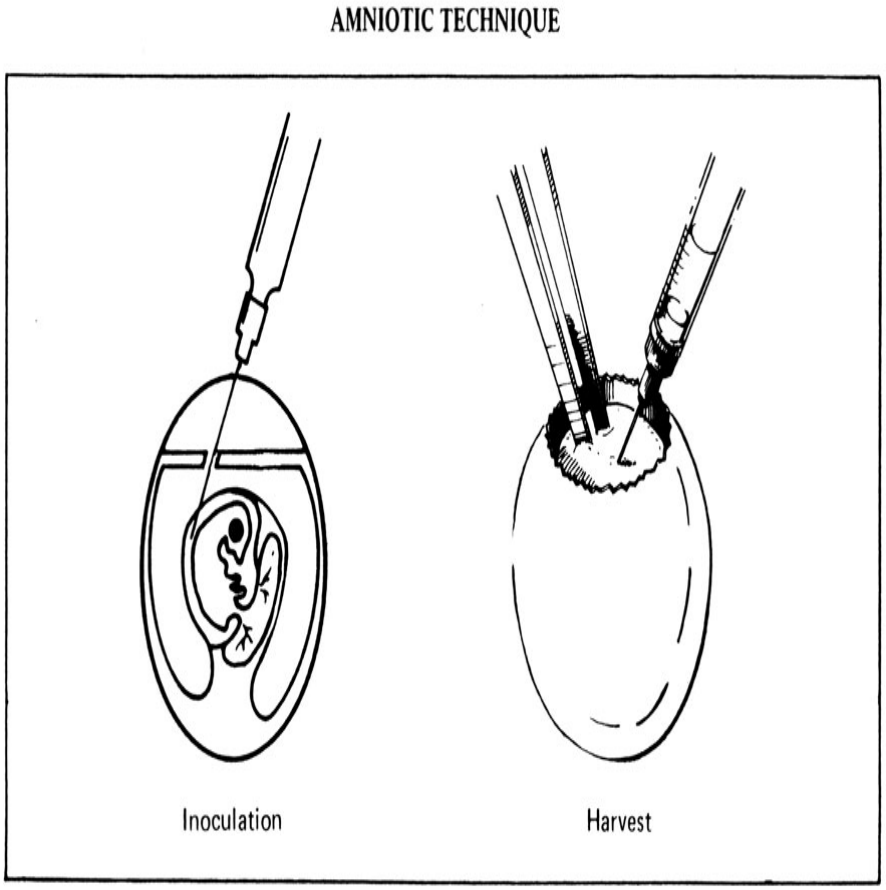
Centrifuge　remove blood cell

HA test

Isolation from Embryonated Egg

- Harvest





Cell systems

- MDCK:Madin-Darby Canine Kidney: most common used cell line
- CEF: Chick Embryo Fibroblast;most sensitive for isolation avian isolates

Cell systems

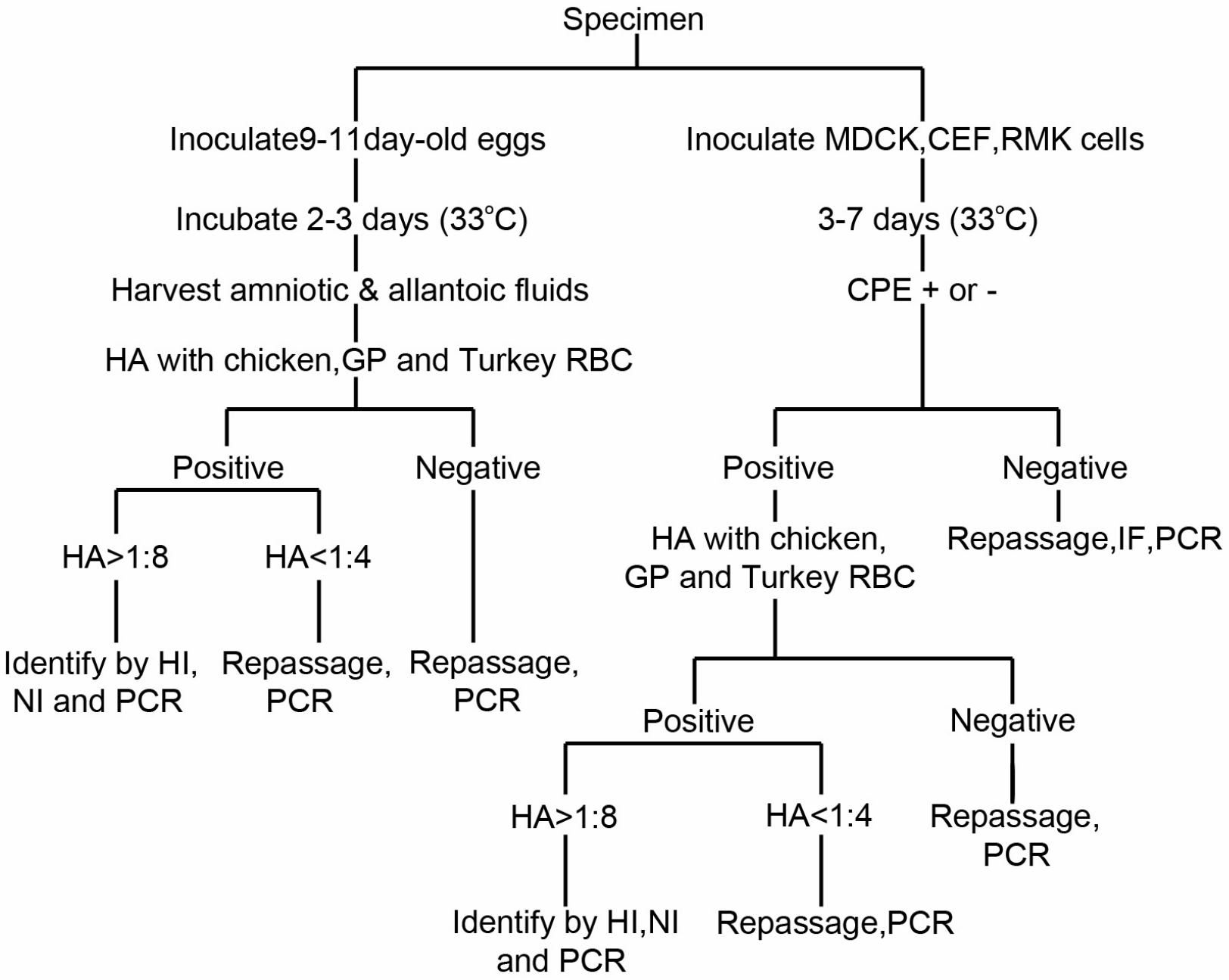
- MDCK:Madin-Darby Canine Kidney: most common used cell line
- CEF: Chick Embryo Fibroblast;most sensitive for isolation avian isolates
- GMK:Green Monkey Kidney;most sensitive for human and swine isolates
- Vero:Special licensed acceptable for human vaccine production

MDCK

- After certain passages , the sensitivity of the isolation become low
- Different source of MDCK cells differ on the sensitivity of the isolation
- Virus isolated from MDCK cells more-like the original clinical samples than egg-grown viruses
- **No serum in the virus culture medium and don't forget add trypsin to virus culture medium especially for isolation attenuated avian or mammlian virus**

Isolation from MDCK

- **Wash** the cells with no serum medium
- **Inoculation** of the sample
- **Absorption**: RT/37 ℃, 30mins
- **Take off** the inoculum and add VGM
- **Incubation**:37 ℃, 3-7 days
- **Observation**: CPE cytopathic effect
- **Harvesting**
- **Detection**:HA,Haemadsorption,IF,PCR
- **Repassage**


Specimen
Inoculate9-11day-old eggs
Inoculate MDCK,CEF,RMK cells
Incubate 2-3 days (33°C)
3-7 days (33°C)
Harvest amniotic & allantoic fluids
CPE + or -
HA with chicken,GP and Turkey RBC
Positive
Negative
Positive
Negative
HA>1:8
HA<1:4
HA with chicken,
GP and Turkey RBC
Repassage,IF,PCR
Identify by HI,
NI and PCR
Repassage,
PCR
Repassage,
PCR
Positive
Negative
HA>1:8
HA<1:4
Repassage,
PCR
Identify by HI,NI
and PCR
Repassage,PCR

Precautions

- **Golden rule1**: Never process clinical specimens for virus isolation and laboratory-adapted influenza strains at the same time
- Golden rule2: Never process clinical specimens from humans and from swine or birds in the same laboratory
- No HA does not mean no virus,before report negative blind passage 2-3 times

Storage of influenza virus

- **4** ℃ : 1-2 days
- **-80** ℃ : years
- **Lyophilised**: most stable

Laboratory Record Keeping

- **Strain designations**

  The antigenic type of nucleoprotein
  A,B,C

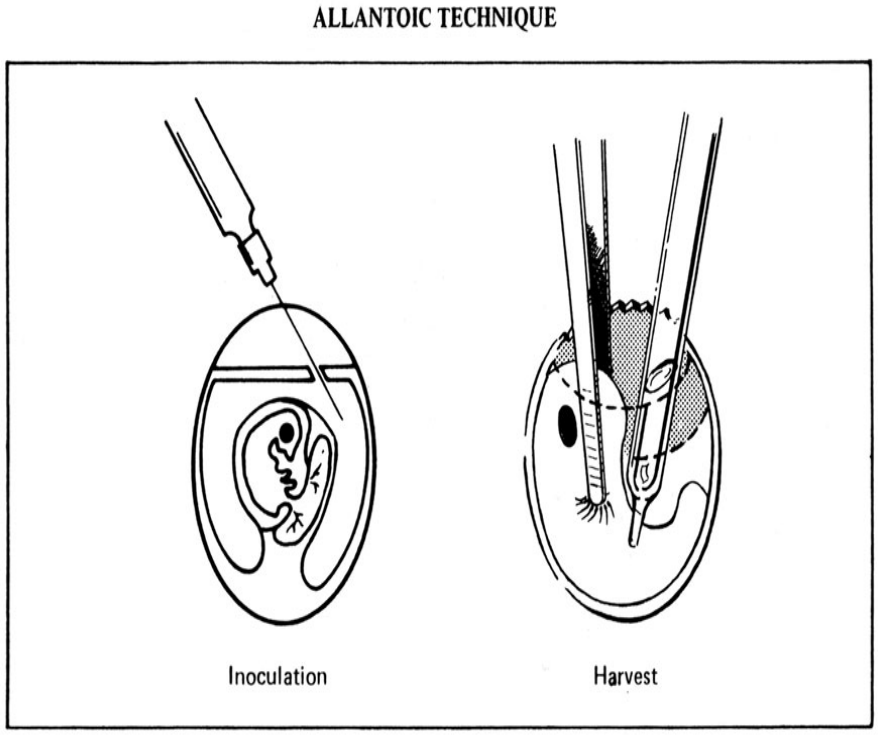
  host origin,locus,year subtype

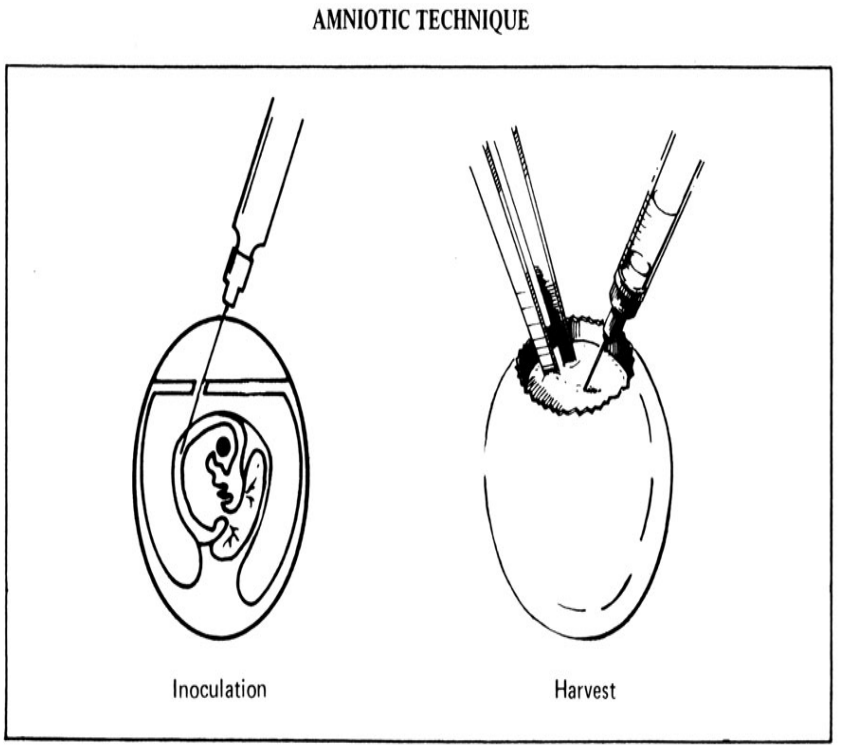
  A/Goose/Guangdong/1996(H5N1)

Diagnosis-Virus isolation

Virus isolation: necessary, useful

- BSL-3 conditions
- Egg or cell culture

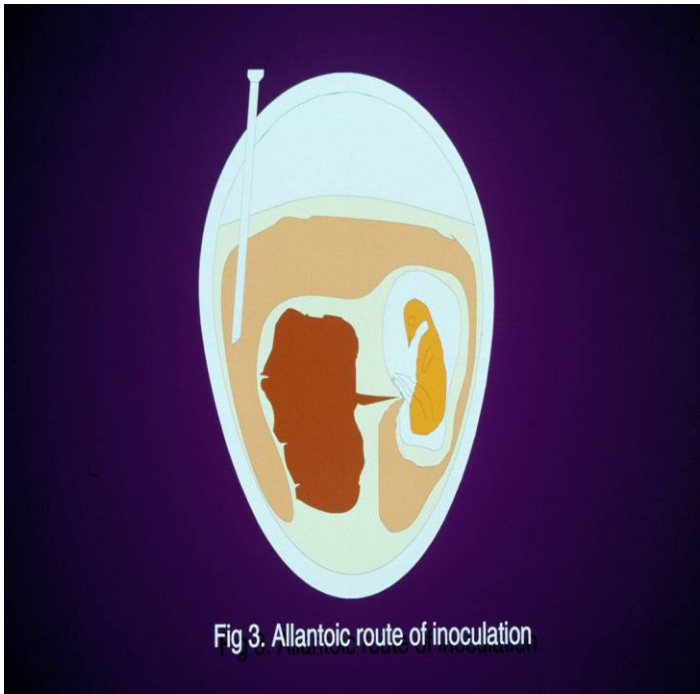




Diagnosis-Virus isolation

Incubation

- Avian influenza virus： 35 ℃ -37 ℃
- Mammalian virus： 35 ℃
- HPAIV： dead in 1~2 days
- LPAIV： last 2~4 days



Diagnosis-Virus isolation

Advantages:

- Gold rule: the recommended method by WHO,OIE
- Accurate: with the highest accuracy
- Useful: for the following researches

Disadvantages:

- Time waste: more than one day usually
- Laboratory security: BLS-3
- Higher quality of sample
- The other pathogen should be exclude
- Intercross contamination

So serology and some rapid diagnosis technology have been developed

Diagnosis-serology

Serology methods:

AGP

ELISA

VNT

IFA

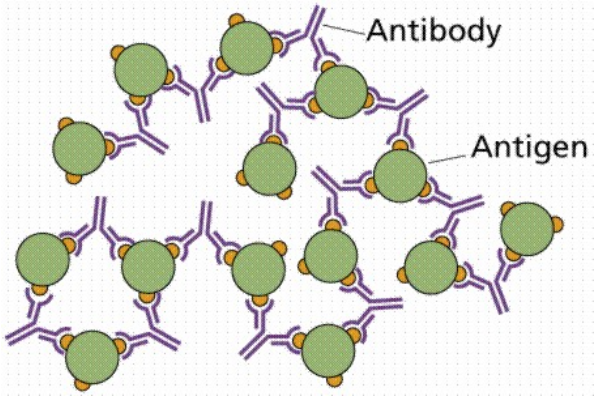
HA-HI

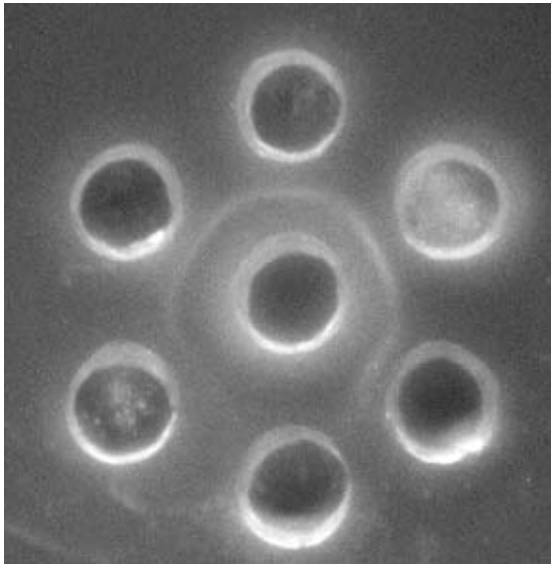
NA-NI

Etc.

Diagnosis-serology

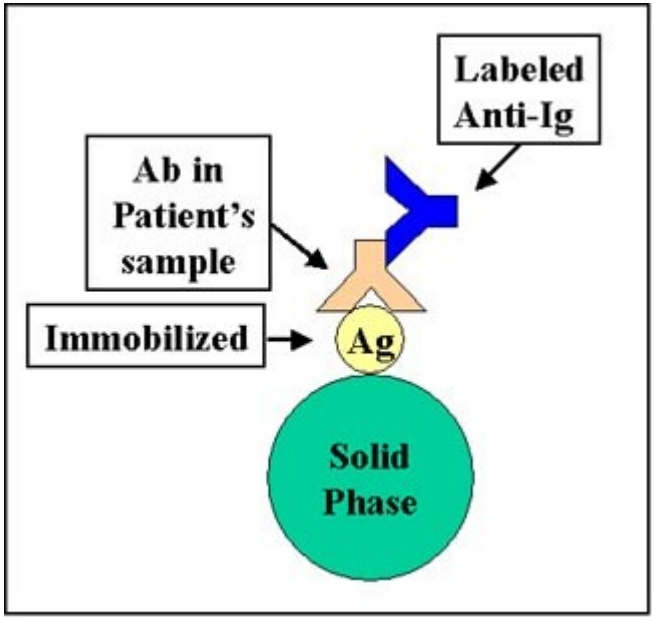
- AGP: Agar Gel Precipitation Test
- Antigen-antibody immune complex precipitate
- migrate through an agar medium
- Type detection





Diagnosis-serology

- ELISA: Enzyme-linked immune assay
- Ag+Ab+Enzyme
- AC-EIA: Antigen-capture enzyme immunoassay
- DAS-ELISA: A double-antibody sandwich Elisa
- Dot-Elisa: Dot-enzyme-linked immunosorbent assay
- Type (NP antigen) or subtype (subty antigen) detection



Diagnosis-serology

VNT: Virus Neutralization Test

a virus-antibody reaction step: the virus is mixed and inoculated with the appropriate antibody

an inoculation step: the mixture of Ag+Ab is inoculated into the host system (e.g. cell cultures, embryonated eggs, or animals)

Detect residual virus infectivity

Ag is known to detect Ab

Ab is known to detect Ag

Detect the subtype

Diagnosis-serology

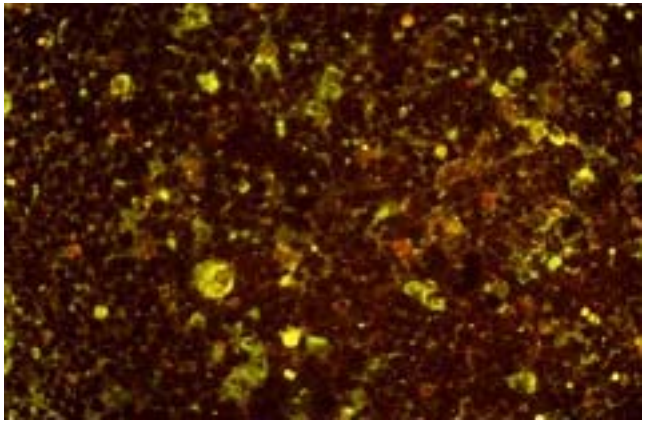
- Conventional serological diagnosis is possible by means of the complement fixation and hemagglutination inhibition tests and allows the detection of type and subtype-specific antibodies, respectively.
- As part of an automated serology, immunofluorescence test and enzyme-linked immunosorbent assay are the methods.

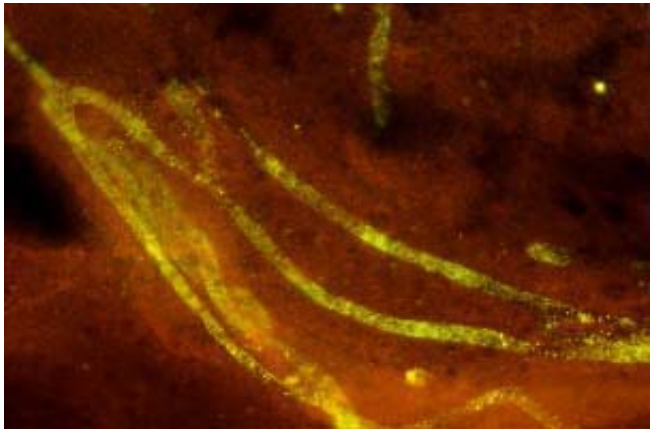
Diagnosis-serology

IFA: Immunofluorescence Assay

Ag or Ab labled by flurescence, then detect the Ag and Ag mixture with flurescence microscope

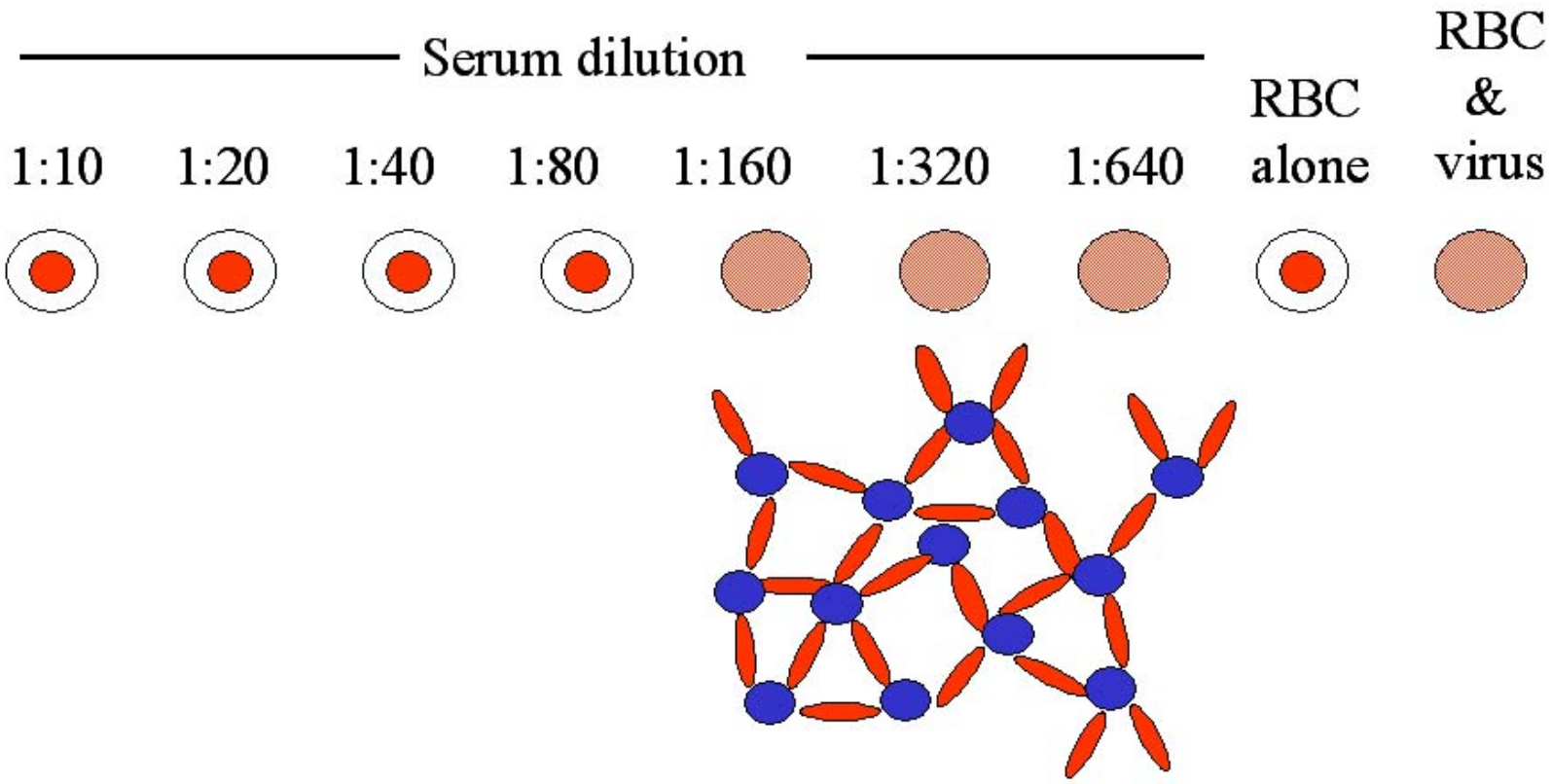
Type and subtype detection





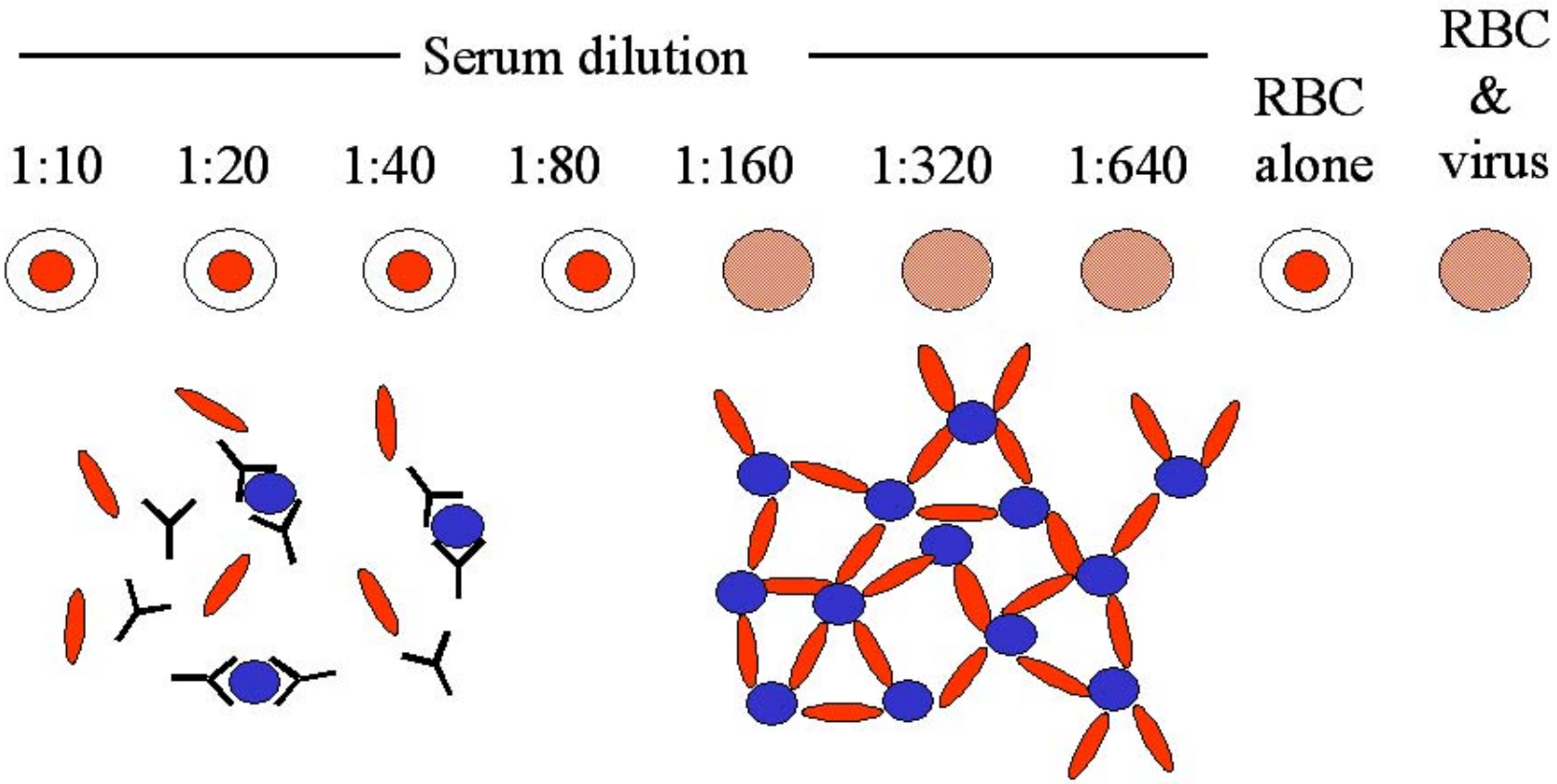
Diagnosis-serology

Hemagglutination test-HA



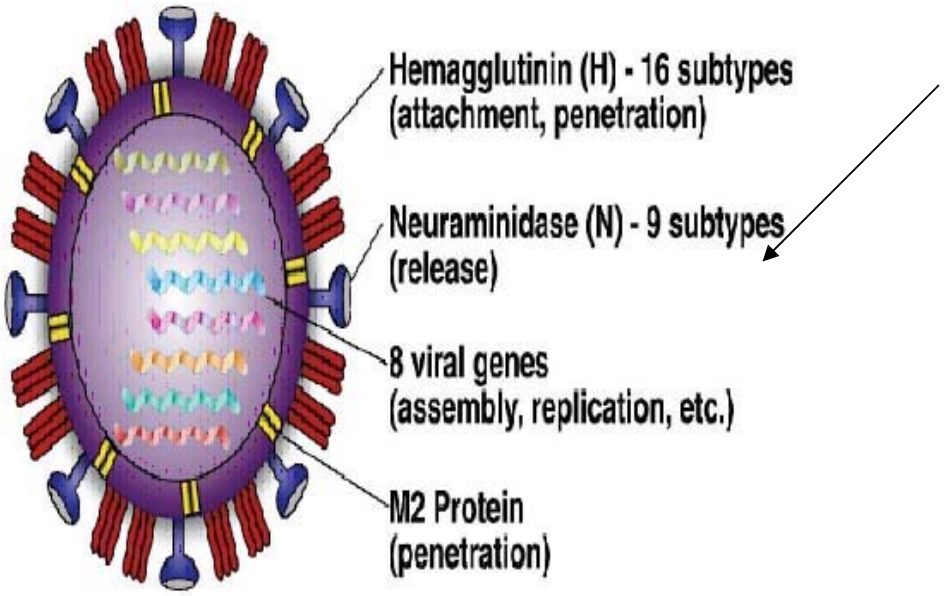
Diagnosis-serology

Hemagglutination Inhibition test-HI



Diagnosis-serology

NA-NI: Neuramindase Assay and
Neuraminidase Inhibition Assay-NA-NI

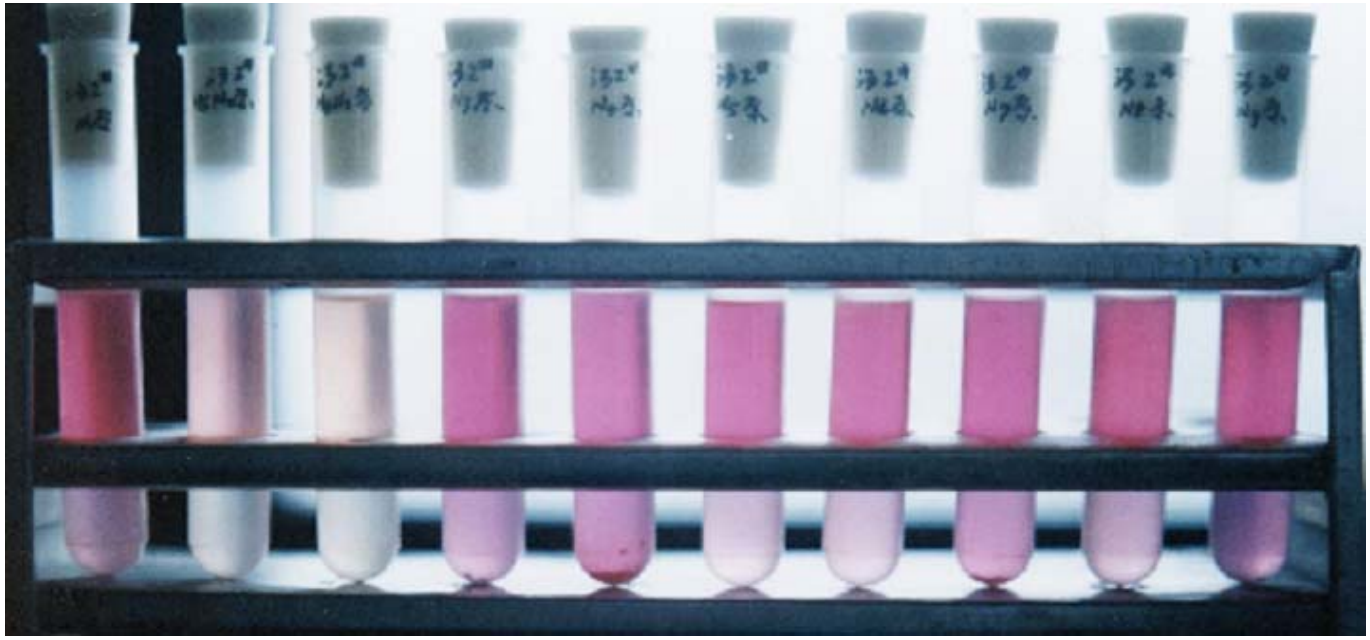


NA is an enzyme (sialidase) that cleaves terminal sialic acid residues from cell surface receptors of the influenza virus.

Specific attachment of antibody inhibits the activity

Diagnosis-serology

NA-NI:



N2(+)

N2 N2

"Classic" methods for influenza diagnosis

- Antigen detection using kit-based test on specimens – 20 mins
- DFA – 2 to 4 hours
- Virus isolation - 1 to 5 days
- Conventional PCR to sub-type e.g. H5N1 – 1 to 2 days
- Strain determination by sequence e.g GS/GD/1/96 – 2 to 7 days
- Need for rapid molecular method

Differential Diagnosis

- Virulent Newcastle disease
- Avian pneumovirus
- Infectious laryngotracheitis
- Infectious bronchitis
- Chlamydia
- Mycoplasma
- Acute bacterial diseases
  - Fowl cholera, *E. coli* infection


Thanks for your attention !