Lab 4:
Orthomyxoviruses

450 MIC

PRACTICAL PART

SECTION (30397)

Learning Outcomes

- 3rd Example of Human Specific Disease:
  - Orthomyxoviruses.
- Classification.
- **Influenza Virus** morphology and structure.
- Pathogenicity and immunity.
- The laboratory diagnosis:
- Specimen.
- Rapid Tests.
- Molecular based tests.

3rd: Orthomyxoviruses

Classification:

Family: Orthomyxoviridae

Genera:

- *Influenzavirus A* (humans and other animals).
  - Species: *Influenza* A virus.
- *Influenzavirus B* (humans only).
- *Influenzavirus C* (rarely cause serious disease).
- *Thogotovirus* (tick-borne arbovirus to humans and livestock).
- *Isavirus* (Infectious salmon anaemia virus).

The Classification System of Influenza viruses:

The classification system for influenza viruses whereby isolates are placed into genera (and species) A, B, or C, then into 18 hemagglutinin and 11 neuraminidase types, for example, H3N2. Viruses are further categorized into subtypes by host (swine, horses, birds, etc.), geographical origin, strain number, and year of isolation.

The emergence of variant viruses depends on:

1- Antigenic drift, genetic point mutations (nucleotide substitutions, insertions, deletions).

2- Antigenic shift, that is genome segment reassortment.

The genetic drift and shift of two genes:

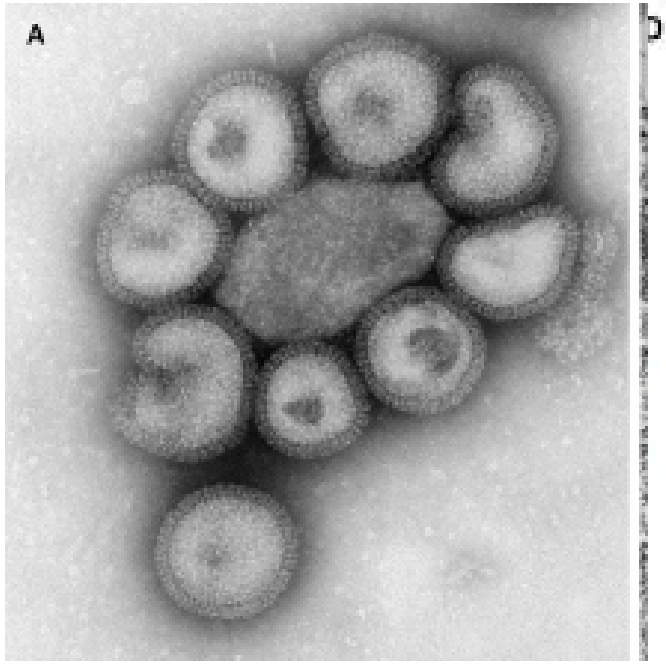
A- Viral hemagglutinin gene (HA).

B- Neuraminidase gene (NA).

Influenza Virus morphology and structure.

80 to 120 nm virions. consist of an envelope with helically symmetrical nucleocapsid segments which consist of a single-stranded RNA.

Influenza viruses are sensitive to heat (56°C, 30 minutes), acid (pH 3), and lipid solvents, and are thus very labile under ambient environmental conditions.



(A) Negative contrast electron microscopy (A/Hong Kong/1/1968 [H3N2] virus), as would be seen in diagnostic settings.

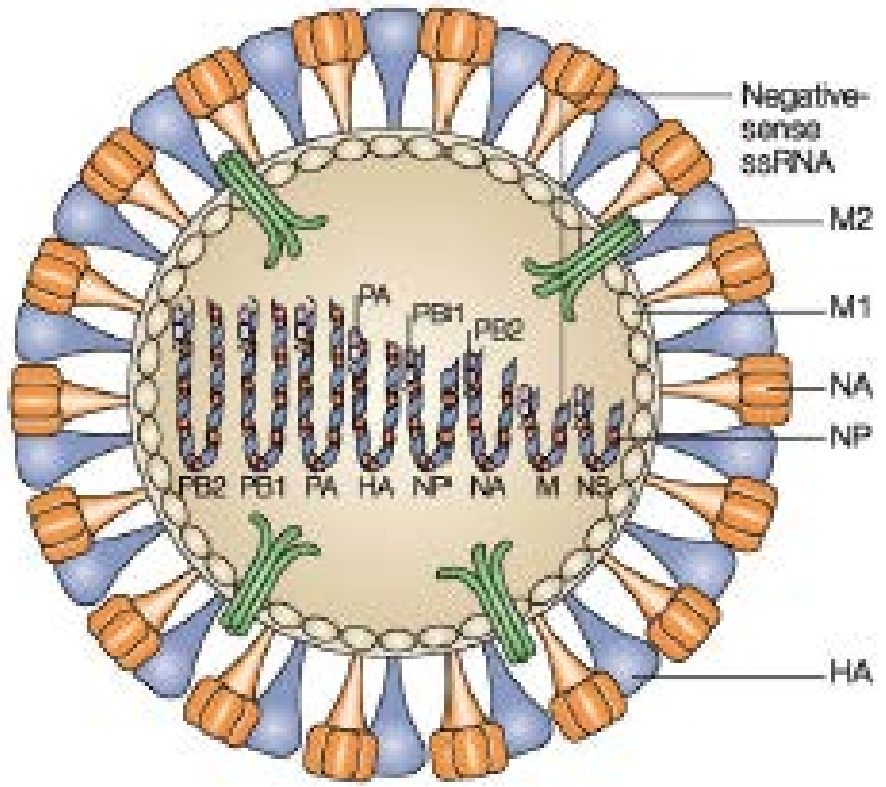


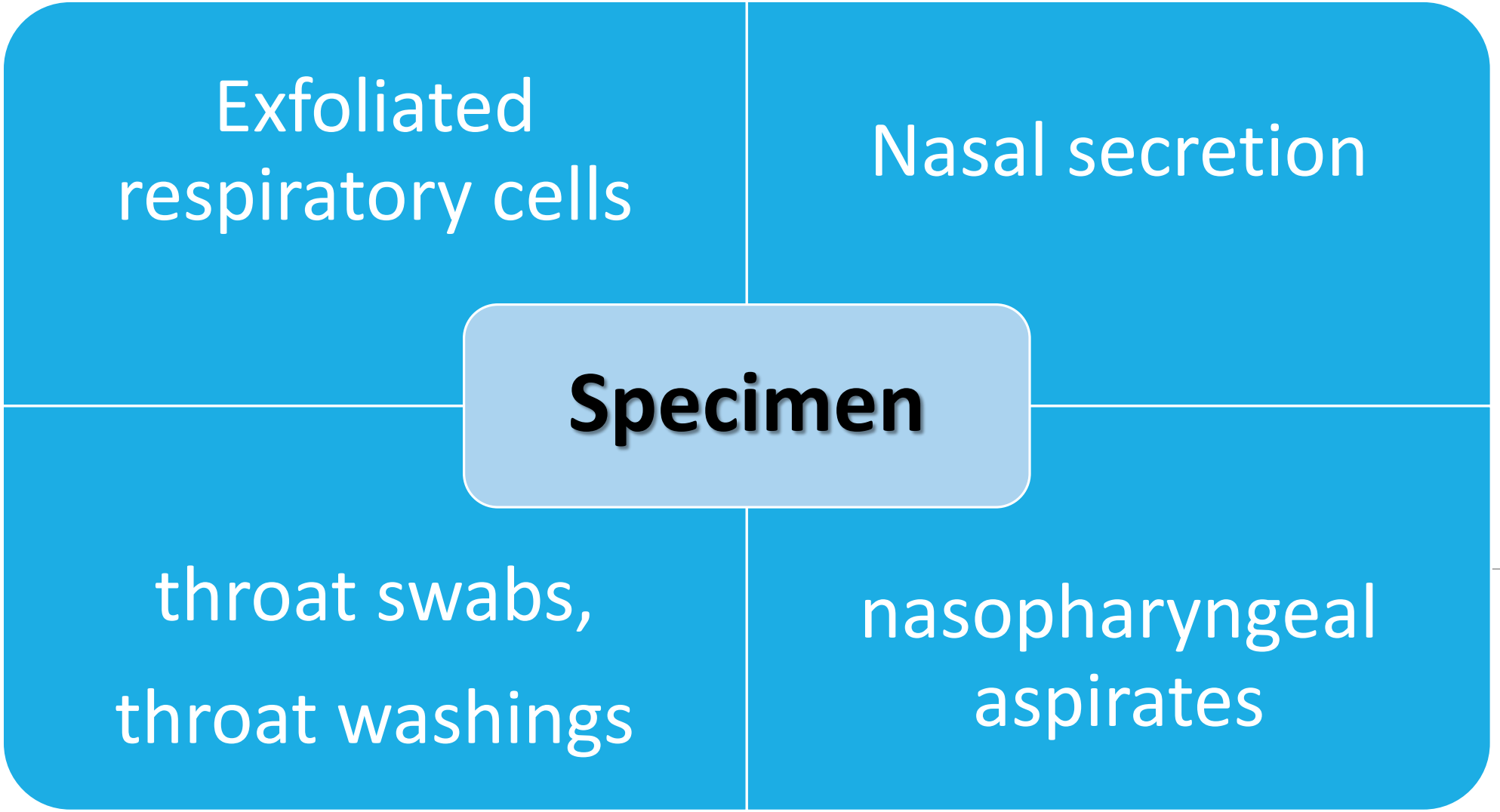
Diagram of influenza virus showing envelope and hemagglutinin (HA), neuraminidase (NA) and matrix (M2) projections, as well as the eight nucleocapsid segments (RNA and associated proteins) of influenza A viruses.

Pathogenicity & Immunity

- Influenza viruses replicate in epithelial cells of the upper and lower respiratory tract.
- Infection causes inflammation, and this leads to a serous nasal discharge.
- The most important changes occur in the lower respiratory tract and include:

laryngitis, tracheitis, bronchitis, bronchiolitis, and interstitial pneumonia accompanied by congestion and alveolar edema.

Laboratory Diagnosis of Influenza Virus


Exfoliated respiratory cells
Nasal secretion
Specimen
throat swabs, throat washings
nasopharyngeal aspirates

1- Virus Culture

Virus isolation by inoculation of chick embryos has now been largely replaced by virus isolation in the Madin-Darby canine kidney (MDCK) cell line at 33 to 34°C. Cytopathology is not usually conspicuous but growth of virus can be recognized after 1to 10 days by hemadsorption. The isolate identified by immunofluorescence on the fixed monolayer.

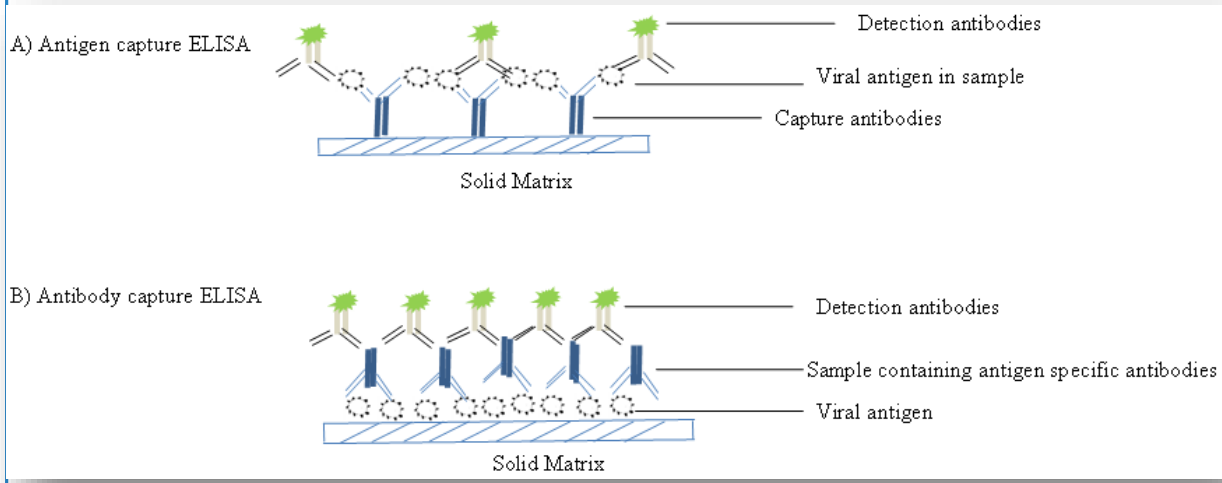
For infections 4.5 ml cells were seeded in 50 ml filter tubes at a cell density of 0.8–1.2 × 10^6 cells/ml. Cells in CDM medium were diluted at a 30/70% ratio into MDCK 33016 PFM medium supplemented with 0.5% of a penicillin/streptomycin solution and 900 IU/ml trypsin. Inoculated cultures were then incubated at 33 °C for 3 days in a 5% CO_2 atmosphere in a ISF-1-W shaker incubator.

For virus harvests the cells were separated by centrifugation (800–1000 × *g* for 10 min) and the supernatant was recovered. Aliquots of the supernatant were frozen at ≤−60 °C.

For Confirmation: Immunofluorescence or enzyme immunoassay (EIA):

For Example, Influenza Virus Antibody EIA 5x96 well plate. It is an ELISA test to detect antibodies against Influenza typa A virus (AIV NP) antigen in serum and plasma samples of animal

- The principle is based on the reaction of (AIV NP) NP proteins with avian antibodies.
- NP expression proteins have been coated to 96 well microtiter strip plate.
- The serum sample is added (dilutted 1:100) to the wells of the coated plate.
- After washing, the bound avain antibodies are detected by an anti-species conjugate.
- Bound anti-species conjugate is made visible by adding substrate/chromagen mix.
- The intensity of the color reaction in the wells is propotrional to the concentration of anti-AIV antibodies in the serum sample.



2-Rapid Direct Antigen Detection:

Rapid immunochromatographic lateral flow test:

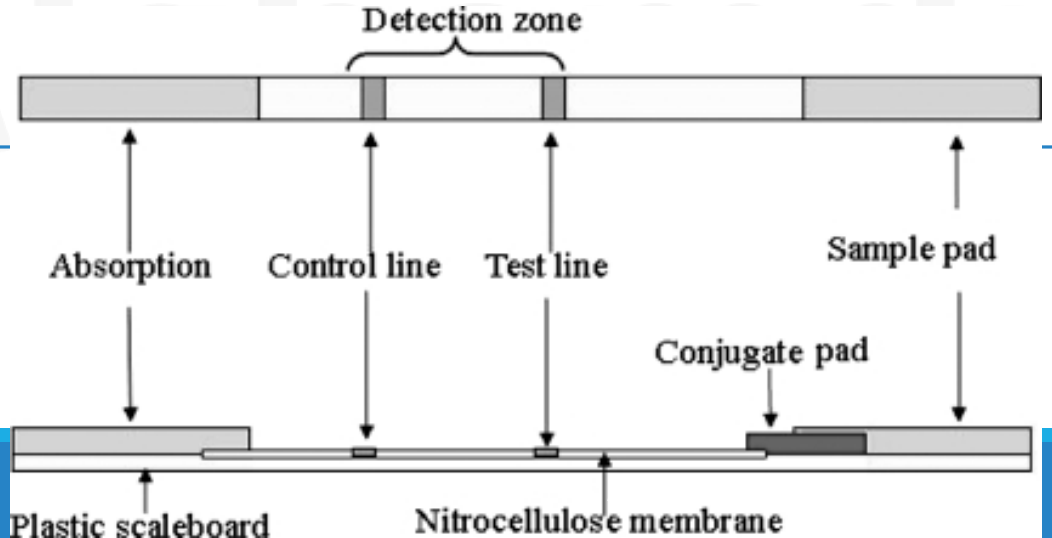
For instance, the detection of the H9 subtype of avian influenza viruses (H9AIVs) in poultry, using two monoclonal antibodies (MAb), 4C4 (labeled with colloidal gold as the detection reagent) for H9AIV hemagglutinin (HA) and 4D4 (blotted on the test line) for nucleoprotein, while a goat anti-mouse antibody was used on the control line of the nitrocellulose membrane.

During the assay process, the liquid sample is applied to the sample pad, and it rapidly diffuses into the conjugate pad.

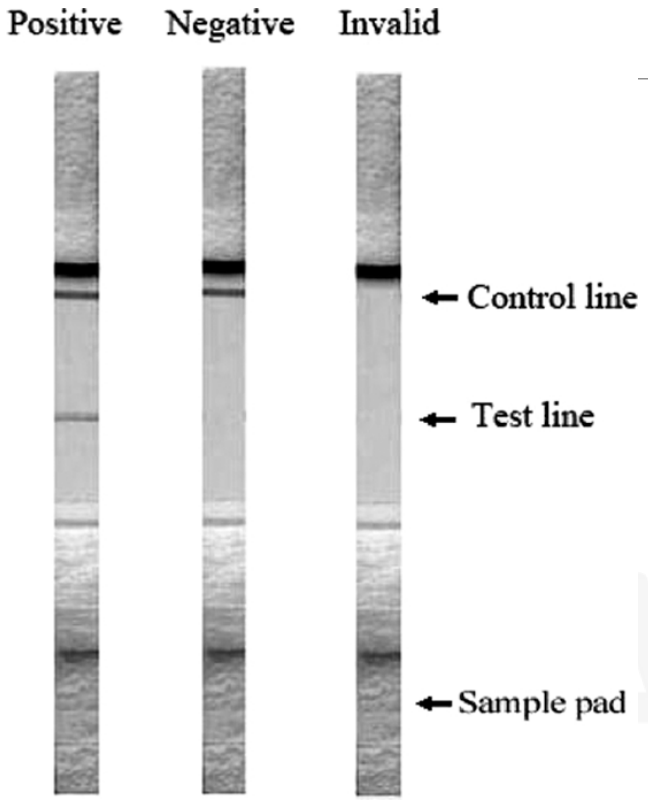
The composition of the immunochromatographic strip test

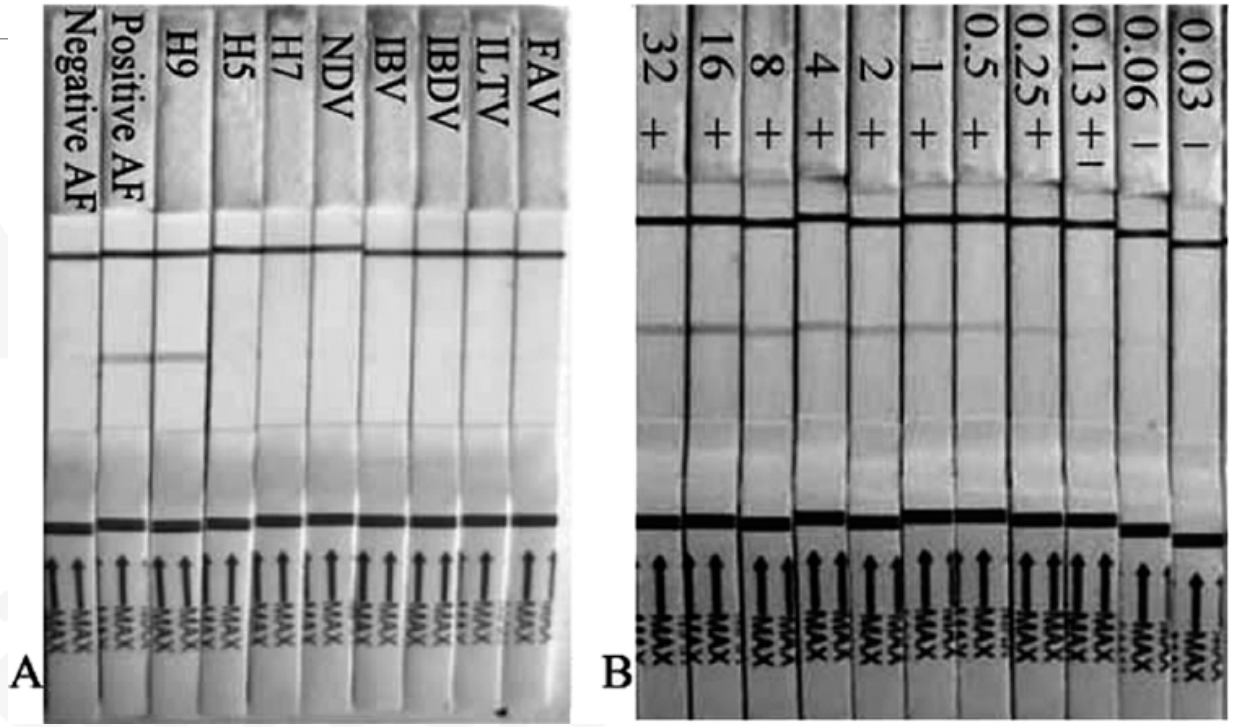
If the sample contains H9AIV antigen, the sample will react with the colloidal gold-4C4 conjugate to form an antigen-colloidal gold-4C4 complex. The complex will move along on the nitrocellulose membrane chromatographically, due to capillary action. Eventually, the complex will react with immobilized anti-NP MAb 4D4 on the test line to form a colored band.

The excess conjugate, or free conjugate if the sample does not contain H9AIV antigen, will migrate along the membrane to the control line, where it will interact with immobilized goat anti-mouse antibody to form a colored band.



The result Interpretation (Sample results).





3- Reverse transcriptase polymerase chain reaction (RT-PCR)

Specimens were subjected to RT-PCR using RNA isolated from 100 µl of each specimen and positive and negative controls.

PCR was performed with 50-µl reaction mixtures coamplifying the 212-bp segment of the influenza virus matrix gene (forward primer, reverse primer,) and a 300-bp segment of the control β2-microglobulin gene (forward primer, reverse primer).

Reagent	Volume of Reagent Added per Reaction
Nuclease-free Water	N x 5.5 µl
Forward Primer	N x 0.5 µl
Reverse Primer	N x 0.5 µl
Probe	N x 0.5 µl
SuperScript™ III RT/Platinum® *Taq* Mix	N x 0.5 µl
2X PCR Master Mix	N x 12.5 µl
Total Volume	**N x 20.0 µl**

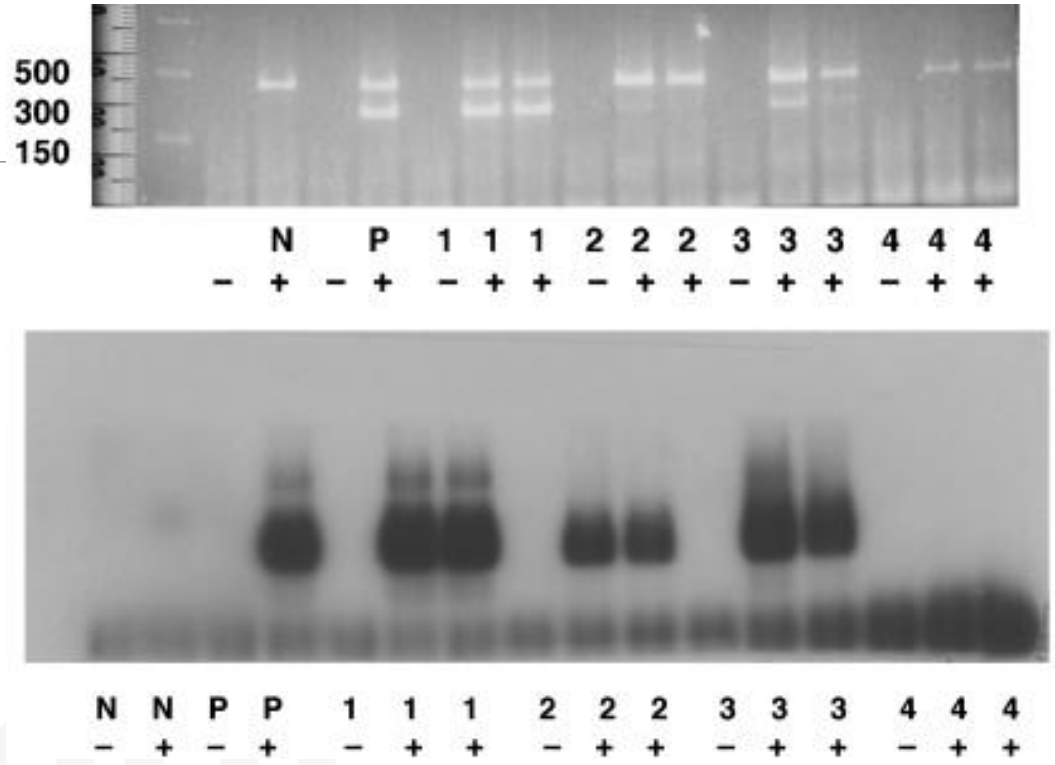
Each reaction mixture contained 10 µl of either the cDNA or mock cDNA Cycling parameters were 50°C for 5 min; 94°C for 10 min; and 36 cycles of 94°C for 10 s, 54°C for 30 s, and 72°C for 30 s.

3- Reverse transcriptase polymerase chain reaction (RT-PCR)

To detect PCR products, 20 µl of each RT-PCR product was incubated at 37°C for 15 min with 20 µl of an internal matrix gene probe end labeled with [γ-32P]ATP.

PCR products and size markers were separated on 0.8% agarose gels, which were subsequently stained with ethidium bromide for the direct detection of PCR products on a transilluminator.

The gels were then exposed to X-ray film for 24 to 48 h, and influenza virus PCR products were detected by autoradiography.



RT-PCR detection of influenza A virus

Treatment

Several antivirals are available for treating influenza virus infections.\

- Depending on the mode of action:

1- Compounds that interfere with the function of the M2 ion channel through the viral envelope (amantadine,

rimantadine), and

2- Inhibitors of neuraminidase function (oseltamivir(Tamiflu) and zanamivir (Relenza)).

Immunization

Existing vaccines are continually being rendered obsolete by antigenic shift and drift, and the composition of the vaccine needs to be modified almost every year to match the current strains of circulating virus.

In the usual process, the current strains ofinfluenza A(H1N1), A(H3N2) and B are grown separately in the

allantois of chick embryos, inactivated with an appropriate chemical such as β-propiolactone, then purified by zonal ultracentrifugation, disrupted with detergent, and pooled.

The resulting polyvalent inactivated vaccine is administered each autumn. Recently, vaccines consisting of virus grown in cell cultures (MDCK cells) have also been approved in the United States and Europe; this approach is expected to avoid problems in recipients who are allergic to eggs, and also to speed up vaccine production.

Prevention & Control

Routine virus isolates are regularly submitted by local laboratories to a network of WHO Influenza Collaborating Centres for characterization and comparison with previous strains. When a new outbreak or epidemic is detected in a local area, its pandemic potential needs to be assessed, based on evidence about its ease of human-to-human spread, case fatality, and the molecular and antigenic relationship of the virus to previous strains.

Control measures that may need to be instituted include quarantining of cases and contacts

coupled with contact tracing; chemoprophylaxis for high-risk individuals using Tamiflu or Relenza; and sometimes rapid initiation of the production of a new vaccine against the new

strain.

For example, the first reports of the new H1N1 swine-related strain in 2009 in Mexico suggested that it had high virulence, leading to national stockpiling of antivirals and fast-tracking of large supplies of new vaccine.

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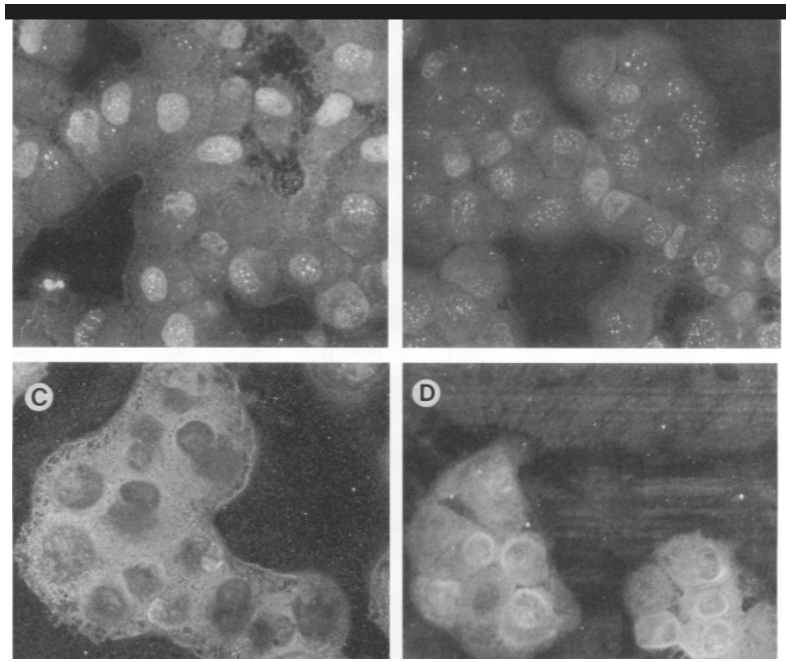

Indirect IFA staining of MDCK (A and C) and MDCKbcl-2 (B and D) cells infected with Ty/Ont and stained with monoclonal antibody to NS (A and B) or to NP (C and D), as described in Materials and Methods. Magnification, _93.

Hinshaw V. S., **Olsen C. W.**, **Dybdahl-Sissoko N.**, **Evans D.** (1994) Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. **68**:3667–3673.