بسم الله الرحمن الرحيم
Laboratory Diagnosis of Viral Infections affect Respiratory Tract

M Parsania, Ph.D.
Tehran Medical Branch, Islamic Azad University
Over 200 viruses

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andenoviruses</td>
<td>+ 41</td>
</tr>
<tr>
<td>Coronaviruses</td>
<td>2</td>
</tr>
<tr>
<td>Influenza viruses</td>
<td>3</td>
</tr>
<tr>
<td>Parainfluenza viruses</td>
<td>4</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>1</td>
</tr>
<tr>
<td>Rhinoviruses</td>
<td>+100</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>+60</td>
</tr>
</tbody>
</table>
# Viruses Associated with Respiratory Infections

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Commonly Associated Viruses</th>
<th>Less Commonly Associated Viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corza</td>
<td>Rhinoviruses, Coronaviruses</td>
<td>Influenza and parainfluenza viruses, enteroviruses, adenoviruses</td>
</tr>
<tr>
<td>Influenza</td>
<td>Influenza viruses</td>
<td>Parainfluenza viruses, adenoviruses</td>
</tr>
<tr>
<td>Croup</td>
<td>Parainfluenza viruses</td>
<td>Influenza virus, RSV, adenoviruses</td>
</tr>
<tr>
<td>Bronchiolitis</td>
<td>RSV</td>
<td>Influenza and parainfluenza viruses, adenoviruses</td>
</tr>
<tr>
<td>Bronchopneumonia</td>
<td>Influenza virus, RSV, Adenoviruses</td>
<td>Parainfluenza viruses, measles, VZV, CMV</td>
</tr>
</tbody>
</table>
Diagnostic Methods for Viral Respiratory Infections

• **Antigen detection tests**
  1- **Rapid Immunoassay**: Flue and RSV
  2- **Direct fluorescent antibody tests (DFA)**: influenza A and B viruses, RSV, parainfluenza viruses 1–3, and adenovirus.
• **Virus isolation**: influenza, parainfluenza viruses and RSV
• **Serology**: influenza
• **electron microscopy (EM)**
• **Nucleic acid amplification tests (RT-PCR &NASBA)**: commonly used for detection of influenza virus and other respiratory viruses.
• **Real-Time PCR assays**: are currently available for the detection of influenza A (including subtypes) and influenza B viruses, RSV, parainfluenza viruses 1, 2, and 3, and human metapneumovirus.
Common Cold Viruses

• Common colds account for one-third to one-half of all acute respiratory infections in humans.

• Rhinoviruses are responsible for 30-50% of common colds, coronaviruses 10-30%.

• The rest are due to adenoviruses, enteroviruses, RSV, influenza, and parainfluenza viruses, which may cause symptoms indistinguishable to those of rhinoviruses and coronaviruses.
Rhinovirus

- **Family:** Picornaviridae
- **Genus:** Rhinoviruses
- **Species:** Human rhinovirus A, Human rhinovirus B
- **Types:** Rhinovirus (at least 100 serotypes are known)
- ss RNA virus
- acid-labile
Rhinoviruses

- Rhinoviruses are associated with upper respiratory tract infections or common colds which may be complicated by otitis media in children and sinusitis in adults.

- Rhinovirus is also able to cause infections in the lower respiratory tract such as pneumonia, wheezing in children and aggravates conditions such as asthma and chronic obstructive pulmonary disease in adults.
Cell culture

• **Human Rhinovirus** has a relatively low optimum growth temperature of 33°C thought to be an evolutionary adaptation to the nasopharyngeal environment.

• **Rhinoviruses** are spread by aerosols and fomites. These viruses produce CPE in NCI-H292, RMK, RD, trypsin-treated MA-104 and human diploid fibroblast cells, preferably in roller cultures at 33°C -34°C.
Normal Cells

Infected Cells
Coronavirus

- ssRNA Virus
- Enveloped, pleomorphic morphology
- 2 serogroups: OC43 and 229E
Coronaviridae

• **Diagnosis**
  – 4-fold increase in IgG
  – Ag Detection
  – Electron Microscopy
  – RT-PCR

• **Epidemiology**
  – Mode of transmission: p-p, direct, respiratory droplets (sneezing)
  – Infections mainly in infants & children

• **Prevention**
  – Prevent respiratory droplet transmission
  – No vaccine
SARS Severe Acute Respiratory Syndrome

February 2003 Guangdong, China

• Viral pneumonia, fever, cough, dyspnea, headache, and hypoxemia
• High case fatality
Laboratory Diagnosis of SARS

- **SARS CoV testing**
  - RNA detection by RT-PCR or real time PCR
  - Serology
Adenovirus

- **Family:** Adenoviridae
- **Genus:** Mastadenovirus
- **Type:** Adenovirus
- ds DNA virus
- non-enveloped
- 51 serotypes are known
- classified into 6 subgenera: A to F
Clinical Syndromes

1. Pharyngitis 1, 2, 3, 5, 7
2. Pharyngoconjunctival fever 3, 7
3. Acute respiratory disease 4, 7, 14, 21
4. Pneumonia 1, 2, 3, 7
5. Follicular conjunctivitis 3, 4, 11
6. Epidemic keratoconjunctivitis 8, 19, 37
7. Pertussis-like syndrome 5
8. Acute haemorrhagic cystitis 11, 21
9. Acute infantile gastroenteritis 40, 41
10. Severe disease in immunocompromized patients 5, 34, 35
11. Meningitis 3, 7
Laboratory Diagnosis

- Detection of Antigen - a rapid diagnosis can be made by the detection of adenovirus antigen from nasopharyngeal aspirates and throat washings.
- Virus Isolation - virus may be readily isolated from nasopharyngeal aspirates, throat swabs.
- Serology - a retrospective diagnosis may be made by serology. CFT most widely used.
- PCR
Cell culture

- Most serotypes other than 40 and 41 replicate readily in any of the following human cell lines: HEK, HEp2, HeLa, A549, HEL, and NC1-H292 cells with or without rolling.

- The adenoviruses are among the easiest viruses to identify because they have a distinctive CPE and are unique in producing prodigious quantities of soluble antigens as they grow in cell culture, and these antigens possess many type and group specific properties that aid diagnosis.
Overview of viral infections affect the lower respiratory tract

- Influenza A, B
- Parainfluenza virus 1-4
- Respiratory Syncytial Virus
- Human Metapneumovirus
- More recently described respiratory virus:
  - Human bocavirus
Influenza virus
ORTHOMYXOVIRUSES

HA - hemagglutinin
NA - neuraminidase
helical nucleocapsid (RNA plus NP protein)
lipid bilayer membrane
polymerase complex
M1 protein

type A, B, C: NP, M1 protein
sub-types: HA or NA protein
Three viral types are distinguished by their matrix and nucleoproteins

<table>
<thead>
<tr>
<th>Type</th>
<th>Host</th>
<th>Clinical Importance</th>
<th>Pattern of Occurrence</th>
<th>Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Humans, birds, horses, other mammals</td>
<td>Moderate to severe disease</td>
<td>Sporadic, epidemics, pandemics</td>
<td>Yes H1-H16† N1-N9‡</td>
</tr>
<tr>
<td>B</td>
<td>Humans</td>
<td>Moderate to severe disease</td>
<td>Sporadic, epidemics</td>
<td>No 2 lineages co-circulate</td>
</tr>
<tr>
<td>C</td>
<td>Humans and swine</td>
<td>Mild disease</td>
<td>Sporadic, localized outbreaks</td>
<td>No</td>
</tr>
</tbody>
</table>

- Influenza A is further classified according to its H and N subtypes, e.g. A/H3N2, A/H1N1

†H = hemagglutinin; ‡N = neuraminidase.
How Influenza Changes Its Surface Proteins

Antigenic Drift

Point Mutation of Hemagglutinin and Neuraminidase gene
How Influenza Changes Its Surface Proteins

**Antigenic Shift**

Human H2N2  
Generation of new Human Virus (H3N2) Possessing Hemagglutinin from Avian Virus (H3N8)

Avian H3N8  
Genetic Reassortment  
Antigenic Shift

Human H3N2
Genetic origins of (H1N1) 2009

N. American H1N1 (swine/avian/human)

Eurasian swine H1N1

Pandemic (H1N1) 2009, combining swine, avian and human viral components

avian, human, and swine components
Embr
y
Egg

Donated

Egg Inoculation

Candle 11-Day Embryonated Egg

Inject Proper Dilution of Virus in Allantoic Sac
Alantoic Fluid Harvesting

- Harvest the Fluid
- Centrifuge to Remove the Egg Stuff
- HA Assay to titer the virus
Haemagglutination (HA)
Virus Propagation in Cell Culture

- MDCK Cell line Adaptation
- Confluent Monolayer Cell
- Inoculation of Serial Dilution of Virus to the Monolayer
- CPE Observation
- HA Titration of Culture Media
- Hemadsorption
- Plaque Assay
- TCID50
MDCK Cell Culture

A. non Infected MDCK
B. Influenza Infected MDCK
Plaque Assay
Molecular Diagnosis

- Nasal & Pharynx Swab Sampling in Transient Media
- RNA Extraction
- Multiplex RT-PCR Using Type- & Subtype-Specific Primers
Paramyxoviridae structure

Hemagglutinin (binding protein)
F (fusion) protein
Lipid envelope
M (matrix) protein
Viral RNA with nucleocapsid, phosphoprotein, and polymerase proteins

Paramyxovirus electron micrograph
Paramyxovirus infections affect the lower respiratory tract.
PARAINFLUENZA VIRUSES

**Croup** (Acute Laryngotracheobronchitis) and pneumonia in **children**

**Common cold** – like disease in **adults**.

5 subtypes: 1, 2, 3, 4a and 4b

Surface spikes consist of **H, N and fusion proteins**. H and N on the same spike while fusion protein is on a different spike.
EPIDEMIOLOGY

Transmission: *respiratory droplets, winter months.*

**Croup** is the commonest clinical manifestation of parainfluenza virus infection, caused by *subtypes 1 and 2.* It occurs in children (below 3 years). **Parainfluenza 3** is prone to produce *bronchiolitis and pneumonia.* The majority of infections with parainfluenza viruses are subclinical.
Laboratory Diagnosis

Croup is a well-defined, easily recognized clinical entity.

- **Detection of Antigen** - a rapid diagnosis can be made by the detection of parainfluenza antigen from nasopharyngeal aspirates and throat washings.

- **Virus Isolation** - virus may be readily isolated from nasopharyngeal aspirates and throat swabs.

- **Serology** - a retrospective diagnosis may be made by serology. **CFT most widely used.**

- **RT-PCR**
Respiratory Syncytial Virus (RSV)

- ssRNA enveloped virus.
- belong to the genus Pneumovirus of the Paramyxovirus family.
- Considerable strain variation exists, may be classified into subgroups A and B by monoclonal sera.
- Both subgroups circulate in the community at any one time.
- Causes a sizable epidemic each year.
RSV causes outbreaks of respiratory infections every winter.

RSV is a major nosocomial pathogen in pediatric wards.

The pathogen may be introduced by infected infants who are admitted from the outside and adults, especially members of staff with mild infections.
Infants at Risk of Severe Infection

1. **Infants with congenital heart disease** - infants who were hospitalized within the first few days of life with congenital disease are particularly at risk.

2. **Infants with underlying pulmonary disease** - infants with underlying pulmonary disease, especially bronchopulmonary dysplasia, are at risk of developing prolonged infection with RSV.

3. **Immunocompromized infants** - children who are immunosuppressed or have a congenital immunodeficiency disease may develop lower respiratory tract disease at any age.
LABORATORY DIAGNOSIS

• Detection of Antigen - a rapid diagnosis can be made by the detection of RSV antigen from nasopharyngeal aspirates. A rapid diagnosis is important because of the availability of therapy.

• Virus Isolation - virus may be isolated from nasopharyngeal aspirates. However, this will take several days.

• Serology - a retrospective diagnosis may be made by serology. CFT most widely used.

• Detection of RNA – RT-PCR
LABORATORY DIAGNOSIS

**Immunofluorescence** on smears of respiratory secretions

ELISA for detection of RSV antigens

*Isolation in cell culture (multinucleated giant cells or syncytia)*

Rise of antibody titre.
Human Metapneumovirus

- Paramyxovirus first recognized in 2001
- hMPV and RSV in Pneumovirinae subfamily of the Paramyxoviridae family.
- 2 major antigenic subgroups (A and B).
- Four major genotypes (A1,A2, B1,B2)
- It is about over 10% of all children with respiratory infection in winter.
- May occur together with other viruses
- It is mainly cause bronchopneumonia and bronchiolitis.
Human Metapneumovirus

• Transmission likely by droplet spread.
  – Healthcare associated infections documented
• Annual epidemics late winter, early spring.
  – Coincides/overlaps with RSV season.
  – Sporadic infection year round.
• Incubation period 3-5 days.
• Viral shedding 1 to 2 weeks.
  – Immunocompromised may shed for months.
Human metapneumovirus (hPMV)  
DIAGNOSIS

• antigen detection  
  – commercially available

• RT-PCR

• culture
Human Bocavirus

- DNA virus, family *parvoviridae*; first identified in 2005 in children with acute RTI.
- Name derives from similarity to bovine parvovirus 1 and canine minute virus.
- **2 distinct genotypes**; no data regarding antigenic variation or distinct serotypes.
## Examples of Parvoviruses

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parvovirinae</td>
<td>Dependovirus</td>
<td>Adeno-associated virus 2</td>
</tr>
<tr>
<td></td>
<td>Parvovirus</td>
<td>Minute virus of mice Feline panleukopenia virus</td>
</tr>
<tr>
<td></td>
<td>Erythrovirus</td>
<td>B 19 virus</td>
</tr>
<tr>
<td></td>
<td>Bocavirus</td>
<td>Human bocavirus</td>
</tr>
<tr>
<td>Densovirinae</td>
<td>Iteravirus</td>
<td>Bombyx mori densovirus</td>
</tr>
</tbody>
</table>
**Human Bocavirus**

- Detection *only described in humans*.
- Transmission presumed *respiratory secretions*.
- Duration of shedding not known.
- Circulates *worldwide and throughout the year*.
- Usually an issue *in fall and winter*.
- May cause *bronchiolitis and pertussis-like illness*.
Human Bocavirus

• Laboratory Diagnosis

• **HBoV PCR** and **serology** mostly used by research labs.
  
  – Now included in **commercial multiplex** assays.
Diagnostic Methods for Viral Respiratory Infections

- Antigen detection tests
  1- Rapid Immunoassay: Flue and RSV
  2- Direct fluorescent antibody tests (DFA): influenza A and B viruses, RSV, parainfluenza viruses 1–3, and adenovirus.
- Virus isolation: influenza, parainfluenza viruses, and RSV
- Serology: influenza
- Electron microscopy (EM)
- Nucleic acid amplification tests (RT-PCR & NASBA): commonly used for detection of influenza virus and other respiratory viruses.
- Real-Time PCR assays: are currently available for the detection of influenza A (including subtypes) and influenza B viruses, RSV, parainfluenza viruses 1, 2, and 3, and human metapneumovirus.
Safety Considerations

- **Specimen collection**
  
  Appropriate hazard labelling according to local policy.

- **Specimen transport and storage**
  
  Compliance with current postal and courier transportation regulations is essential. A suitable virus transport system must be used and the specimen should be placed in a sealed plastic bag, separately from the request form.

- **Specimen processing**
  
  Laboratory procedures that may give rise to infectious aerosols must be conducted in a class 2 microbiological safety cabinet. It is strongly recommended that disposable gloves are worn when inoculating samples or manipulating cell cultures.
Specimen Collection

• Optimal timing of specimen collection
  Respiratory specimens should be taken within three days of illness and no later than five days. All specimens should be taken before anti-viral chemotherapy is commenced.

• Correct specimen type and method of collection
  Successful recovery of viruses from clinical specimens depends on the quality of material received for inoculation. Many viruses are susceptible to drying, adverse pH and varying osmotic potential. For this reason samples should be placed in virus transport medium (VTM) immediately after they have been taken.

• Adequate quantity and appropriate number of specimen
  Duplicate specimens may be required for the exclusion of other microbial pathogens
Specimen Transport and Storage

- **Time between specimen collection and processing**
  - Specimens should be transported to the laboratory and processed as soon as possible. Specimens that may be delayed should be refrigerated prior to transportation to the laboratory.

- **Special consideration to minimize deterioration**
  - Samples should be refrigerated at 4°C if there is likely to be a delay in processing. If the delay is likely to exceed 24 hr, the sample should be stored at -70°C and thawed prior to processing. Repeated freezing and thawing should be avoided.
Specimen Processing

• Test collection

Under circumstances where a more rapid result is required, electron microscopy (EM) or immunofluorescence (IF) or a commercial antigen detection assay may be used to provide an interim report but this result should be confirmed by culture where possible. In certain cases a validated PCR method may also be used.

IF is recommended as a rapid diagnostic test and also enables the quality of the sample taken to be determined. If there is a lack of epithelial cells on the slide it is unlikely that any virus will be detected. The chances of successful culture are also going to be reduced. In this case it is recommended that the doctors are asked to provide a repeat sample.
Culture and Investigation

Specimen processing

- **Nasopharyngeal aspirate**
  - A fine **tip plastic pipette** should be used **to wash any mucoid material from the plastic tube** (disposable mucus extractor) into 2 mL of PBS in a plastic centrifuge tube. The sample should then be **homogenised**
  - Approximately **500 μL** of the mixture should then be transferred into **virus transport medium (VTM)** for virus isolation
  - It is recommended that a further **10 mL of PBS** is added to the plastic centrifuge tube and that the tube is then **centrifuged at 380× g for 10 minutes** in the centrifuge
  - The supernatant should then be removed and **re-suspended in 200 μL of PBS**
  - **Repeat above steps until all mucoid material has been removed.** This aids **clarification of the cells**
Culture and Investigation

Swabs

- Swabs should **arrive broken off into virus transport medium (VTM)**.
- Dry swabs or those in bacteriological transport medium are **unacceptable**, and repeat specimens in the correct format should be requested.
  - Ensure the **container top is tight**.
  - **Vortex mixture** for 15 seconds to dislodge material on the swab into the transport medium
  - Store the specimen at **-80°C**
Culture and Investigation

2-Microscopy technique

- Slides prepared for IF microscopy should preferably be prepared from material taken directly from the aspirate. When this is not possible a slide for examination may be prepared from the specimen if received in virus transport medium (VTM).
- Where the sample is inadequate, a repeat should be requested.
- A slide should be labelled and 10 – 25 μL of cell suspension, depending upon the density of the cell suspension, poured into 8 wells of the slide.
- The slide should then be dried on a Hot Plate. The slides should be fixed in acetone at room temperature for 10 minutes and then air dried. This slide is now ready for immunofluorescent staining which should be carried out according to the manufacturer’s instructions.
Culture and Investigation

3-Isolation

- The purpose of virus isolation is to demonstrate the presence and viability of viruses in clinical specimens.
- A variety of cell cultures are available.
- Traditionally all the viruses discussed grow best in human tissue lines such as human embryonic lung tissues (HEL).
- Some laboratories include dog kidney cells (MDCK) in their cell line profile as parainfluenza and influenza viruses fail to grow in HEL cells.
Culture and Investigation

3-Isolation

- The clarified sample is inoculated into the selected cell culture monolayer(s) in a class I safety cabinet.

- The tubes should be labeled.

- The inoculate are adsorbed to the monolayer at ambient temperature.

- The length of time for adsorption of these organisms the cell layer is crucial to sensitivity.
Culture and Investigation

3-Isolation

• The cultures are then re-fed with maintenance medium and incubated at 35°C - 36°C for seven to fourteen days.

• HEL cell lines should be rolled in a roller drum or agitated on rocker platforms.

• The tubes should be read every 24 or 48 hours to check for the development of CPE and contamination under light microscopy.
Culture and Investigation

4-Identification

Identification of the isolates can be done in a number of ways:

- **Cytopathic effect:** Interpretation of CPEs can be very subjective and experience in reading cell cultures is very important.

  CPEs should be confirmed using immunofluorescence, PCR or via a reference laboratory.

  Immunofluorescence antigen detection is normally carried out to confirm the results of the CPE; this method does not detect all virus types and new virus strains or types will be missed.

- **Haemadsorption:** Haemadsorption of viruses

- **PCR:** either single or multiplex has proved to be very useful.
Limitations

• **Successful isolation** of organisms depends on **correct** specimen collection, transport, storage and processing; the quality and range of cell lines used and the use of **correct** conditions for culture and the provision of adequate/suitable clinical information.

• **Only cell lines proven to be susceptible** to respiratory viruses should be used and susceptibility should be checked on acquisition and at regular intervals. Cells retrieved from liquid nitrogen storage should be checked for sensitivity before use.

• **Other procedures may be required** and professional interpretation by qualified staff is essential.
Impact of Molecular Methods on Respiratory Viral Diagnostics

• **Much greater sensitivity** vs culture and DFA.
  – Better understanding of epidemiology of respiratory viruses.
  – Fewer infections where don’t identify a virus.
  – Potential impacts on clinical care: less antibacterial therapy, shorter hospital stay, reduced mortality if earlier use of antivirals for influenza.

• **Faster turnaround time** – greater opportunity to guide therapy.

• **Discovery of new viruses** in respiratory tract in last decade
  – Metapneumovirus
  – Multiple coronaviruses: SARS, 229E, NL63, OC43, HKU1.
  – Human bocavirus

• **Viral coinfections** recognised as a relatively common entity.
Multiplex PCR

• Multiple viruses can cause same clinical syndrome
  – Respiratory infections
• Can perform multiplex PCR assays to detect multiple viruses in one reaction.
• Multiplex–PCR System for the detection of 13 Respiratory Viruses (Influenza A/B virus, RSV A/B, Rhinovirus, Coronavirus OC43/HKU1, coronavirus 229E/NL63, adenovirus, parainfluenza virus 1-3m bocavirus, enterovirus
Rapid diagnosis by PCR impacts clinical management:

- Earlier hospital discharge
- Fewer additional diagnostic tests
- Decreased antibiotic usage
- Decreased overall health care costs
Thank you for your attention