Diagnostic Microbiology
320 MIC

Lecture: 10
Diagnosis of Virus Infections
### Specimens for viral diagnosis

<table>
<thead>
<tr>
<th>Common Pathogenic Viruses</th>
<th>Specimens for Culture</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiratory Tract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus; influenza virus; enterovirus</td>
<td>Nasal washing, throat swab,</td>
<td>Enterovirus is also shed in stool</td>
</tr>
<tr>
<td>(picornavirus); rhinovirus; paramyxovirus;</td>
<td>nasal swab, sputum</td>
<td></td>
</tr>
<tr>
<td>rubella virus; HSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gastrointestinal Tract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reovirus; rotavirus; adenovirus; Norwalk</td>
<td>Stool, rectal swab</td>
<td>Samples are analyzed by electron microscopy and antigen detection (ELISA); viruses are not cultured</td>
</tr>
<tr>
<td>virus, calicivirus</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maculopapular Rash</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus; enterovirus (picornavirus)</td>
<td>Throat swab, rectal swab</td>
<td></td>
</tr>
<tr>
<td>Rubella virus; measles virus</td>
<td>Urine</td>
<td></td>
</tr>
<tr>
<td><strong>Vesicular Rash</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus; echovirus; HSV; VZV</td>
<td>Vesicle fluid, scraping, or swab, enterovirus in stool</td>
<td>Initial diagnosis of HSV and VZV can be obtained from vesicle scraping (Tzanck smear)</td>
</tr>
<tr>
<td><strong>Central Nervous System (Aseptic Meningitis, Encephalitis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterovirus (picornavirus)</td>
<td>Stool</td>
<td>PCR</td>
</tr>
<tr>
<td>Arboviruses (e.g., togaviruses, bunyavirus)</td>
<td>Rarely cultured</td>
<td>Diagnosis is by serologic tests</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Tissue, saliva, brain biopsy</td>
<td>Diagnosis is by immunofluorescence analysis for antigen</td>
</tr>
<tr>
<td>HSV; CMV; mumps virus; measles virus</td>
<td>Cerebrospinal fluid</td>
<td>PCR, virus isolation, and antigen are assayed</td>
</tr>
<tr>
<td><strong>Urinary Tract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus; CMV</td>
<td>Urine</td>
<td>CMV may be shed without apparent disease</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV; human T-cell leukemia virus; hepatitis B, C, and D viruses</td>
<td>Blood</td>
<td>Serologic antigen or antibody detection (ELISA), PCR, and RT-PCR are performed</td>
</tr>
</tbody>
</table>
B. **Direct Examination:** Direct demonstration of the virus, its antigens or nucleic acid.
   - widely used
   - fast method of virus diagnosis

Virus or virus antigen is detected in lesions, fluids, tissues or excretions from the patient and a result can be obtained within an 1 or 2 hour of receipt specimen.

B. **Indirect Examination:**
   Virus Propagation Isolation
   - Chick embryo
   - Cell culture (cell line)

C. **Animal inoculation**

D. **Serology**
A- Direct Examination

1- Serology (Antigen Detection) immunofluorescence, ELISA etc.

2- Electron Microscopy morphology of virus particles number of virus particles immune electron microscopy

3- Light Microscopy histological appearance changes in cell morphology inclusion bodies

4- Viral Genome Detection hybridization with specific nucleic acid probes polymerase chain reaction (PCR)
B- Indirect Examination

1- Cell Culture
   cytopathic effect (CPE)
   haemabsorption
   immunofluorescence

2- Eggs
   pocks on chorioallantoic membrane (CAM)
   haemagglutination
   inclusion bodies

3- Animals
   disease or death
Electron Microscopy (EM)

- $10^6$ virus particles per ml required for visualization. 50,000 - 60,000 magnification normally used.

**Viruses may be detected in the following specimens:**

- Faeces → Rotavirus, Adenovirus
- Vesicle Fluid → HSV
- Skin scrapings → papillomavirus
Immune Electron Microscopy

The sensitivity and specificity of EM may be enhanced by immune electron microscopy.

There are two variants:

• **Classical Immune electron microscopy (IEM)** - the sample is treated with specific anti-sera before being put up for EM. Viral particles present will be agglutinated and thus congregate together by the antibody.

• **Solid phase immune electron microscopy (SPIEM)** - the grid is coated with specific anti-sera. Virus particles present in the sample will be absorbed onto the grid by the antibody.

Problems with Electron Microscopy

• Expensive equipment
• Expensive maintenance
• Require experienced observer
• Sensitivity often low
Epithelial-like (human lung carcinoma, A549)

Measles on human lung carcinoma

Fibroblast like (baby hamster kidney, BHK)

www.freelivedoctor.com
Vaccinia on monkey kidney

Low multiplicity of infection

High
Cell Cultures are most widely used for virus isolation.

There are 3 types of cell cultures:

1. Primary cells - Monkey Kidney
2. Semi-continuous cells - Human embryonic kidney and skin fibroblasts
3. Continuous cells (Hela)

Primary cell culture are widely acknowledged as the best cell culture systems available since they support the widest range of viruses. However, they are very expensive and it is often difficult to obtain a reliable supply.

Continuous cells are the most easy to handle but the range of viruses supported is often limited.
Virus Isolation

- In the laboratory, solid samples are minced, homogenized, centrifuged at low speed to remove cellular debris that may be toxic to the cultured cells, and sterilized by 0.2 µm filter.

- A backup aliquot is stored at 4 or -70 C.

- Next step is the inoculation of a system supporting virus replication: cell cultures, embryonating eggs, organ cultures, laboratory animals or host animals.
Virus Isolation (chick embryo)
Cell Cultures

Growing virus may produce:

1. **Cytopathic Effect (CPE):**
such as the ballooning of cells or syncytia formation, may be specific or non-specific.

2. **Haemadsorption:**
cells acquire the ability to stick to mammalian red blood cells.
Types of Cytopathic Effects (CPE):

**Cytopathic Effect (1)**
Cytopathic effect of enterovirus and HSV in cell culture: note the ballooning of cells.
(Virology Laboratory, Yale-New Haven Hospital, Linda Stannard, University of Cape Town).

**Fig. 1, Cytopathic effects of enterovirus 71 in rhesus monkey kidney cells**

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**Cytopathic Effect (2)**
Syncytium formation in cell culture caused by RSV and measles virus. (courtesy of Linda Stannard, University of Cape Town, S.A.)

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Primary cell culture
Growth of cells in culture. A primary culture is defined as the original plating of cells from a tissue, grown to a confluent monolayer, without subculturing. A cell strain (solid line) is defined as a euploid population of cells subcultivated more than once in vitro, lacking the property of indefinite serial passage. Cell strains ultimately undergo degeneration and death, also called crisis or senescence. A cell line (dashed line) is an aneuploid population of cells that can be grown in culture indefinitely. Spontaneous transformation or alteration of a cell strain to an immortal cell line can occur at any time during cultivation of the cell strain. The time in culture and corresponding number of subcultivations or passages are shown on the abscissas. The ordinate shows the total number of cells that would
Cultured cells

a) **Primary**
- Heterogeneous – many cell types
- Closest to animal
- Technical hassle

b) **Diploid cell strain**
- Relatively homogeneous – fewer cell types
- Further from animal
- Technically less hassle

c) **Continuous cell line**
- Immortal • Most homogeneous • Genetically weird – furthest from animal
- Hassle free • Suspension or monolayer
Problems with cell culture

• Long period (up to 4 weeks) required for result.
• Often very poor sensitivity, sensitivity depends on a large extent on the condition of the specimen.
• Susceptible to bacterial contamination.
• Susceptible to toxic substances which may be present in the specimen.
• Many viruses will not grow in cell culture e.g. Hepatitis B, Diarrheal viruses.
Haemadsorption:

Syncytial formation caused by mumps virus and haemadsorption of erythrocytes onto the surface of the cell sheet.

(courtesy of Linda Stannard, University of Cape Town, S.A.)
Haemadsorption:

Ebola virus infection, inclusion bodies (arrows)
Haemadsorption

1. Add red blood cells.
Hemadsorption of erythrocytes to cells infected with influenza viruses. This virus express a hemagglutinin, which bind erythrocytes of selected animal species.
Haemagglutination: to Detect Viral Antigen
Haemagglutination: to Detect Viral Antigen

<table>
<thead>
<tr>
<th>Dilution</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1024</th>
<th>No virus</th>
</tr>
</thead>
</table>

![Image of haemagglutination test result](image)

Titre
Serology

• Detection of rising titers of antibody between acute and convalescent stages of infection, or the detection of IgM in primary infection.

<table>
<thead>
<tr>
<th>Classical Techniques</th>
<th>Newer Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complement fixation tests (CFT)</td>
<td>1. Radioimmunoassay (RIA)</td>
</tr>
<tr>
<td>2. Haemagglutination inhibition tests</td>
<td>2. (ELISA)</td>
</tr>
<tr>
<td>3. Immunofluorescence techniques (IF)</td>
<td>3. Particle agglutination</td>
</tr>
<tr>
<td>4. Neutralization tests</td>
<td>4. Western Blot (WB)</td>
</tr>
<tr>
<td>5. Counter-immunoelectrophoresis</td>
<td>5. RIBA, Line immunoassay</td>
</tr>
</tbody>
</table>
Serology

Criteria for diagnosing Primary Infection

• 4 fold or more increase in titre of IgG or total antibody between acute and convalescent sera
• Presence of IgM

Criteria for diagnosing Reinfection

• fold or more increase in titre of IgG or total antibody between acute and convalescent sera
• Absence or slight increase in IgM
Serology

- Neutralization
- Hemagglutination inhibition
- Western blot
- ELISA, radioimmune assay (RIA)
Antibody Detection: Neutralization & Haemagglutination

In the assay shown, 10 fold dilutions of serum were incubated with virus. Aliquots of the mixture were then added to cell cultures or erythrocytes.

In the absence of antibody, the virus infected the monolayer (indicated by CPE) and caused hemagglutination.

In the presence of the antibody, infection was blocked (neutralization), and hemagglutination was inhibited, allowing the erythrocytes to pellet.
Antibody Detection: Western Blot

Western blot analysis of HIV antigens and antibody.

HIV protein antigens are separated by electrophoresis and blotted onto nitrocellulose paper strips. The strip is incubated with patient antibody, washed to remove the unbound antibody, and then reacted with enzyme-conjugated antihuman antibody. Serum from an HIV-infected person binds and identifies the major antigenic proteins of HIV.
HIV Antigen Detection: ELIZA

Sample to be tested

virus

Enzyme -> colour

Detecting antibody

Capturing antibody
Microplate ELISA for HIV: coloured wells indicate reactivity
Virus Antigen Detection: Immunofluorescence

Antibody binds to antigen in fixed cells;
- fluorescein-labeled anti-IgG binds;
- fluorescence by UV microscopy
Virus Antigen Detection: Immunofluorescence

BHV-1 antigens in neuron in trigeminal ganglion

Positive immunofluorescence test for rabies virus antigen. (Source: CDC)
Usefulness of Serological Results

• How useful a serological result is depends on the individual virus.

• For example, for viruses such as rubella and hepatitis A, the onset of clinical symptoms coincide with the development of antibodies. The detection of IgM or rising titres of IgG in the serum of the patient would indicate active disease.

• However, many viruses often produce clinical disease before the appearance of antibodies such as respiratory and diarrheal viruses. So in this case, any serological diagnosis would be retrospective and therefore will not be that useful.

• There are also viruses which produce clinical disease months or years. e.g. HIV. In this case, the presence of antibody is sufficient to make a definitive diagnosis.
Problems with Serology

• Long period of time required for diagnosis for paired acute and convalescent sera.

• Mild local infections may not produce a detectable humoral immune response.

• Extensive antigenic cross-reactivity between related viruses e.g. HSV may lead to false positive results.

• Immunocompromised patients often give a reduced or absent humoral immune response.

• Patients given blood or blood products may give a false positive result due to the transfer of antibody.
Assay of viruses

- **Biological**
  - Plaque assay
  - Transformation
  - Endpoint Method

- **Physical and biochemical**
  - Hemagglutination.
  - Direct particle count.
  - Immunological tests for proteins.
  - Assay for nucleic acid (PCR).
  - Enzymatic (reverse transcriptase for retroviruses).
Plaque Assay: Method

Titer = 1 x 10^7 pfu/ml

Titer = 1.2 x 10^8 pfu/ml
Plaque Assay: Result
Direct Particle Count

Beads \((10^4/\text{ml})\)

1.5 \times 10^4 \text{ virus/ml}

10 beads => 1 ul

15 virus => 1.5 \times 10^4 \text{ virus/ml}
## Assay for Viral Protein & Nucleic Acid

### Proteins
- Protein patterns (electrophoresis)
- Enzyme activities (e.g., reverse transcriptase)
- Hemagglutination and hemadsorption
- Antigen detection (e.g., direct and indirect immunofluorescence, enzyme-linked immunosorbent assay, Western blot)

### Nucleic Acids
- Restriction endonuclease cleavage patterns
- Size of RNA for segmented RNA viruses (electrophoresis)
- DNA genome hybridization in situ (cytochemistry)
- Southern, Northern, and dot blots
- PCR (DNA)
- Reverse transcriptase polymerase chain reaction (RNA)
- Real-time PCR
- Branched-chain DNA and related tests (DNA, RNA)

PCR, Polymerase chain reaction.
Molecular Methods

- Methods based on the detection of viral genome are also commonly known as molecular methods. It is often said that molecular methods is the future direction of viral diagnosis.

- However in practice, although the use of these methods is indeed increasing, the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods.

Classical Molecular Techniques

- hybridization are examples of classical techniques. They depend on the use of specific DNA/RNA probes for hybridization.

- The specificity of the reaction depends on the conditions used for hybridization. However, the sensitivity of these techniques is not better than conventional viral diagnostic methods.

- However, since they are usually expensive than conventional techniques, they never found widespread acceptance.
Polymerase Chain Reaction

PCR allows the in vitro amplification of specific target DNA sequences in an extremely sensitive technique.

• it is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest.

• These oligonucleotides act as primers for the thermostable Taq polymerase. Repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (50°C), and primer extension (72°C) result in the exponential production of the specific target fragment.

• Detection and identification of the PCR product is usually carried out by agarose gel electrophoresis, restriction enzyme analysis, or DNA sequencing.
Advantages of PCR:
Extremely high sensitivity, may detect down to one viral genome per sample volume - Easy to set up - Fast turnaround time

Disadvantages of PCR:
Extremely liable to contamination - High degree of operator skill required - Not easy to set up a quantitative assay- A positive result may be difficult to interpret, especially with latent viruses, where any seropositive person will have virus present in their blood irrespective whether they have disease or not.- These problems are being addressed by the arrival of commercial closed systems which requires minimum handling. The use of synthetic internal competitive targets in these commercial assays has facilitated the accurate quantification of results. However, these assays are very expensive.
## Diagnostic Methods for most Common Human Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus (gastrointestinal)</td>
<td>ELISA</td>
</tr>
<tr>
<td>Adenovirus (respiratory)</td>
<td>Antigen, PCR, culture</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Antigen, PCR, culture</td>
</tr>
<tr>
<td>Enteroviruses (echo, coxsackie, polio)</td>
<td>PCR, culture</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>PCR, serology</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Serology</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Antigen, PCR, serology</td>
</tr>
<tr>
<td>Hepatitis C virus</td>
<td>PCR, serology</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>Antigen, PCR, culture</td>
</tr>
<tr>
<td>HIV</td>
<td>Antigen, PCR, culture, serology</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>ELISA, antigen, culture</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>Antigen, PCR, culture</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>ELISA, antigen, PCR, culture</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>ELISA</td>
</tr>
<tr>
<td>Varicella-zoster virus</td>
<td>Antigen, PCR</td>
</tr>
</tbody>
</table>

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.
Summary

• **Four main clinical diagnostic techniques:**

  – Culture, serology, antigen detection, nucleic acid detection.

• **Virus culture**
  – Not all viruses can be cultured – Cultured cell types – Cytopathic effect

• **Virus quantitation**
  – Biological
  – Physical

• **Basic serological techniques**