Laboratory Diagnosis of Viral Diseases

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Koch’s Postulates

1. Organism present only in diseased individuals
2. Organism cultivated in pure culture from diseased individual
Koch’s Postulates

3. Organism causes disease when injected into healthy individuals
4. Organism re-isolated from infected individual from point 3.

<table>
<thead>
<tr>
<th>The Postulates:</th>
<th>Tools:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Cells from a pure culture of the suspected organism should cause disease in a healthy animal.</td>
<td>Experimental animal</td>
</tr>
<tr>
<td>4. The organism should be reisolated and shown to be the same as the original.</td>
<td>Laboratory reisolation</td>
</tr>
</tbody>
</table>

KOCH'S POSTULATES

Remove blood or tissue sample and observe by microscopy

Pure culture (must be same organism as before)
River’s Postulates

- T.M. River, 1937
- Modified from Koch’s Postulates (proof of bacterial diseases)

1. Isolate virus from diseased hosts.
2. Cultivation of virus in host cells.
4. Production of a comparable disease when the cultivated virus is used to infect experimental animals.
5. Re-isolation of the same virus from the infected experimental animal.
6. Detection of a specific immune response to the virus.
Procedures for laboratory viral diagnosis

Virus infected patients

Collected specimens

Check the infected cells under light microscope

Observe virus particles under electronic microscope

Virus culture and isolation

Detection of viral antigen or genome

Serology test antibody test

Identify viral propagation

Antigen

Genome

Neutralization test

Hemagglutination inhibition test

EIA/ELISA

Western Blot

CPE

Hemadsorption

Hemagglutination

Virus interference

Neutralization test

Plaque assay

TCID50 assay

Immunofluorescence

EIA/ELISA

Western Blot

Immunoelectrophoresis

Radio immune assay

PCR

Southern blot

Northern blot

Dot blot

In situ hybridization
Viral Diagnostics in the Clinical Laboratory

- Over 70% of all infectious disease cases seen by a physician are due to viral infections.

- Quality of patient specimens and their transport to the laboratory is important.
Relation of stage of illness to presence of virus in test material & to appearance of specific antibody

<table>
<thead>
<tr>
<th>Stage or period of illness</th>
<th>Virus detectable</th>
<th>Specific antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>Rarely</td>
<td>No</td>
</tr>
<tr>
<td>Prodrome</td>
<td>Occasionally</td>
<td>No</td>
</tr>
<tr>
<td>Onset</td>
<td>Frequently</td>
<td>Occasionally</td>
</tr>
<tr>
<td>Acute phase</td>
<td>Frequently</td>
<td>Frequently</td>
</tr>
<tr>
<td>Recovery</td>
<td>Rarely</td>
<td>Usually</td>
</tr>
<tr>
<td>Convalescence</td>
<td>Very rarely</td>
<td>Usually</td>
</tr>
</tbody>
</table>
Storage and Collection of Biological Specimens for Viral Testing

What types of specimens are collected to diagnose?

- *Respiratory tract infections*: Nasal and bronchial washings, throat and nasal swabs, sputum
- *Eye infections*: throat and conjunctival swab/scraping
- *Gastrointestinal tract infections*: stool and rectal swabs
- *Vesicular rash*: vesicle fluid, skin scrapings
- *Maculopapular rash*: throat, stool, and rectal swabs
- *CNS (encephalitis and meningitis cases)*: stool, tissue, saliva, brain biopsy, cerebrospinal fluid
- *Genital infections*: vesicle fluid or swab
- *Urinary tract infections*: urine
- *Bloodborne infections*: blood
Collection of vesicular fluid from palmar lesions for virological diagnosis of HFMD
Three General Approaches for Laboratory Diagnosis of Viral Infections

- **Direct detection**
  - Microscopy or staining
  - Detection of nucleic acid, antigens

- **Virus Isolation (Indirect Examination)**
  - CPE and other characters
  - Nucleic acid, antigens for detection

- **Serology**
  - Antibodies
Indirect Examination

1. Cell Culture  
   - cytopathic effect (CPE)  
   - haemabsorption  
   - immunofluorescence

2. Eggs  
   - pocks on CAM  
   - haemagglutination  
   - inclusion bodies

3. Animals  
   - disease or death
Virus Isolation-animal inoculation

Choice of animal and inoculation pathways
Mouse, rat, rabbit, monkey, and so on.
Virus Isolation-embryos inoculation

Chorioallantoic membrane inoculation
(chorionic epithelium)

Amniotic cavity

Shell

Albumin

Air sac

Yolk sac

Allantoic cavity

Chorioallantoic membrane

Amniotic inoculation
(amniotic epithelium)

Yolk sac inoculation
(yolk sac lining)

Allantoic inoculation
(allantoic epithelium)

Davis, Duylbecco, Eisen, Ginsberg “Microbiology” 4th ed, J.B. Lippincott 1990, Fig. 48-1
9-11 days embryos taken out after virus infection

Virus propagation and vaccine production in enterprise industry
Virus Isolation-cell cultures

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, Vero, Hep2, LLC-MK2, MDCK

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.
Cultured cells

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

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

- **Continuous cell line**
  - Immortal
  - Most homogeneous
  - Genetically weird – furthest from animal
  - Hassle free
  - Suspension or monolayer
Laminar Flow Hoods

- Virologist’s facility
- Laminar vertical flow hoods
  - Contains HEPA filter
  - Removes 99.97% of particles of 0.3 μM or higher

Figure 5-3: Vertical laminar flow hood.
Primary cell culture
Subculture

enzymes

time
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 accumulate if all were retained in culture. (From Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001 Fig. 2-2.)
Cytopathic Effects

- Visible results of viral infection
- Cell death by
  - Multiplying viruses
  - Inhibition of DNA, RNA or protein synthesis
  - Effects on permeability of membrane
- Cytopathic effects (CPEs) of infected cells can be observed with inverted light microscopes
  - Rounding/detachment from plastic flask
  - Syncytia/fusion
    - Fusion of cells
  - Shrinkage
  - Increased refractiability
  - Aggregation
  - Loss of adherence
  - Cell lysis/death
- Common observations of CPEs
  - Inclusion body formation
    - Intracellular virus parts (replication or assembly)
  - Hemadsorption assays
Cultured cell morphologies

Epithelial-like
(human lung carcinoma, A549)

Fibroblast like
(baby hamster kidney, BHK)

Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Fig. 2-3
CPE: Measles on human lung carcinoma (A549)
CPE: vaccinia on monkey kidney (BSC40)

Low multiplicity of infection (moi)
single plaque

High moi, 48 hr

Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Fig. 2-4
Herpesvirus CPE

Poliovirus CPE
Cytopathic Effect - inclusion bodies
Cytopathic Effect- Syncytium formation

Syncytium formation in cell culture caused by RSV (top), and measles virus (bottom).

(courtesy of Linda Stannard, University of Cape Town, S.A.)
measles virus

SARS virus syncytia
Hemadsorption
Hemagglutination:

Hemagglutination Inhibition test:
Hemadsorption Test

- Some viruses agglutinate RBCs
  - Mumps, measles, influenza
  - Hemagglutination
    - Clumps RBCs
Virus infected cells - interference

Without significant CPE but interference other virus infection

rubella virus + cell $\Rightarrow$ CPE -
enterovirus + cell $\Rightarrow$ CPE +

rubella virus
\[ \downarrow \]
cell + enterovirus
\[
\quad \downarrow \quad \downarrow \\
\quad CPE - \\
\quad CPE - \\
\]

Interference - 
+
Transformation

- Immortalization
- Loss of contact inhibition
- Anchorage independence
  - Growth in soft agar
  - Growth in suspension
- Tumor formation in athymic (nude) mice
Common Methods

- Four methods:
  - Quantitative assays
    - Plaque assays
    - TCID$_{50}$
  - Hemmagglutination assays
  - Transformation assays
Quantitative Assays

- Plaque assays
  - Lytic viruses only
  - Steps
    - Serial dilution of virion-containing solution
    - Create tissue culture plates
    - Spread diluted virus
    - Overlay with agar—prevents diffusion
  - Count number of plaques
  - Each plaque represents 1 PFU (Plaque Forming Unit)

Figure 5.8: Plaque assays used to quantitate a viral stock.

Courtesy of Teri Shors
Plaque assay: method

virus

1:100

1:10

1:10

1:10

1:10

1:10

serial dilution

10^{-2}

10^{-3}

10^{-4}

10^{-5}

10^{-6}

10^{-7}

plate 1 ml

plaques

Titer = 1 \times 10^7 \text{ pfu/ml}
Quantitative Assays- TCID$_{50}$

- Tissue Culture Infectious Dose: TCID$_{50}$
  - Measure cytopathic effects other than lysis
  - Concentration of virus it takes to produce cytopathic effect (CPE) in 50% of the dishes of cells infected with virus

<table>
<thead>
<tr>
<th>Virus Dilution</th>
<th>CPE Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-2}$</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>+ + + + + - + + + + +</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>- + - + + + - - - + -</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>- - - - - + - - - - -</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>- - - - - - - - - - -</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>- - - - - - - - - - -</td>
</tr>
</tbody>
</table>
Five replicate wells of cells are infected with one ml of each of four different virus dilutions, incubated, and scored for infection by looking for CPE. In this example, the final titer is $10^{6.3}$ TCID$_{50}$ per ml. (TCID = tissue culture infective dose)
Hemagglutination test: method

Titer = 32 HA units/ml
Hemagglutination assay. Seven different samples of influenza virus, numbered 1 through 7 at the left, were serially diluted as indicated at the top, mixed with chicken red blood cells (RBC), and incubated on ice for 1 to 2 hours. Wells in the bottom row contain no virus. Agglutinated RBCs coat wells evenly, in contrast to nonagglutinated cells, which form a distinct button at the bottom of the well. The HA titer, shown at the right, is the last dilution that shows complete hemagglutination activity. (From Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Fig. 2-8)
## Comparison of quantitative methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Amount (per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct electron microscope count</td>
<td>$10^{10}$ EM particles</td>
</tr>
<tr>
<td>Quantal infectivity assay in eggs</td>
<td>$10^9$ egg ID$_{50}$</td>
</tr>
<tr>
<td>Quantal infectivity assay by plaque formation</td>
<td>$10^8$ pfu</td>
</tr>
<tr>
<td>Hemagglutination assay</td>
<td>$10^3$ HA units</td>
</tr>
</tbody>
</table>

*Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Table 2-4*
Three General Approaches for Laboratory Diagnosis of Viral Infections

- **Direct detection**
  - Microscopy or staining
  - Detection of nucleic acid, antigens

- **Virus Isolation**
  - CPE and other characters
  - Nucleic acid, antigens for detection

- **Serology**
  - Antibodies
# Direct Examination

1. **Electron Microscopy**
   - morphology of virus particles
   - immune electron microscopy

2. **Light Microscopy**
   - histological appearance
   - inclusion bodies

3. **Antigen Detection**
   - immunofluorescence, ELISA etc.

4. **Viral Genome Detection**
   - hybridization with specific nucleic acid probes
   - polymerase chain reaction (PCR)
Direct Detection

- Electron Microscopy
  - Examine specimen for viruses
- Immuno-electron microscopy
  - Labeled antibody
- Immunofluorescence
  - Fluorescent tag bound to Fc region of Ab
Electronmicrographs

Adenovirus

Rotavirus

(courtesy of Linda Stannard, University of Cape Town, S.A.)
Direct electron microscopic particle count. An electron micrograph of a spray droplet containing 15 latex beads (spheres) and 14 vaccinia virus particles (slightly smaller, brick-shaped particles). (From Fields Virology, 4th ed, Knipe & Howley, eds, Lippincott Williams & Wilkins, 2001, Fig. 2-7.)
# Rapid Diagnosis Based on the Detection of Viral Antigens

<table>
<thead>
<tr>
<th>Nasopharyngeal Aspirate</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influenza A and B</td>
</tr>
<tr>
<td></td>
<td>Parainfluenza</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Faeces</th>
<th>Rotaviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenoviruses</td>
</tr>
<tr>
<td></td>
<td>Astrovirus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Skin</th>
<th>HSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VZV</td>
</tr>
</tbody>
</table>

| Blood                   | CMV (pp65 antigenaemia test) |
Immunofluorescence

**Direct method**
- Fluorescein tagged antibody
- Antigen
- Attached fluorescein tagged antibody visualized by UV microscopy

**Indirect method**
- First step: Untagged antibody
- Antibody attached to antigenic determinant
- Second step: Fluorescein tagged anti-immunoglobulin
- Attached fluorescein tagged anti-immunoglobulin visualized by UV microscopy
Immunofluorescence

Positive immunofluorescence test for rabies virus antigen. (Source: CDC)

(Virology Laboratory, Yale-New Haven Hospital)
CMV pp65 antigenaemia test

Figure 4 CMV pp65 antigens detected in nuclei of peripheral blood neutrophils

(Virology Laboratory, Yale-New Haven Hospital)
Advantages and Disadvantages

Advantages

- Result available quickly, usually within a few hours.

Potential Problems

- Often very much reduced sensitivity compared to cell culture, can be as low as 20%. Specificity often poor as well.
- Requires good specimens.
- The procedures involved are often tedious and time-consuming and thus expensive in terms of laboratory time.
Methods for Rapid Diagnosis of Viral genome

1. Polymerase Chain Reaction (PCR);

2. Molecular hybridization (Southern or Northern Blot);
Each cycle doubles the copy number of the target.
PCR machine

Real-time PCR machine

PCR machine
Polymerase Chain Reaction

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 such as CMV, 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 such as the Roche Cobas Amplicor 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.
Simple and rapid detection of human EV 71 by RT-LAMP
Nucleic acid molecular hybridization

Specimen DNA
  └── Digested by endonuclease
      └── Running agarose gel
          └── Hybrid membrane
              └── Denatured into ssDNA
                  └── Hybridized with ssDNA* (probe)
                      └── Radioactivity or labeled enzyme to develop color
DNA applied to gel

Electrophoresis

Salt solution 'blots' DNA onto filter by capillary action

Migration

Paper towels

Sponge

Nitrocellulose filter

Gel

Probe hybridizes with complementary DNA sequence

Filter in "Seal-a-Meal" bag

DNA transferred to filter

Expose X-ray film to filter

Hybridize with unique nucleic acid probe

remove unbound probe

autoradiogram

Southern Blot analysis of DNA
(after Griffiths et al. 1996)
DNA Microarray
(Chip assay)

A Comparative Hybridization Experiment
in situ hybridization
Dot Blot Hybridization
## Comparison of nucleic acid hybridization methods

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Southern/Northern blot</th>
<th>Dot blot</th>
<th>In situ Hybr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen</td>
<td>DNA/RNA</td>
<td>serum</td>
<td>Tissue slide</td>
</tr>
<tr>
<td>Agarose gel</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Denature</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Probe</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Develop color</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Meanings</td>
<td>Diagnosis</td>
<td>Diagnosis</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>MEW assay</td>
<td></td>
<td></td>
<td>DNA/RNA Location</td>
</tr>
</tbody>
</table>
Three General Approaches for Laboratory Diagnosis of Viral Infections

- **Direct detection**
  - Microscopy or staining
  - Detection of antigen and viral genome

- **Virus Isolation**
  - Nucleic acid, antigens

- **Serology**
  - Antibodies
  - Antigens
Serology

Detection of rising titres 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. Enzyme linked immunosorbent assay (EIA)</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>
Viral Serology

- Indirect
  - Primary and secondary responses to viral infections
    - IgM (1st exposure)
    - IgG (2nd exposure)

Figure 5.18: Primary (1 degree) and secondary (2 degree) antibody responses toward a viral pathogen.
Viral Serology

- Enzyme-Linked Immunosorbent Assays (ELISAs)
  - Enzyme reacts with substrate to produce colored product
  - Very sensitive
    - HIV test
      - If positive twice, Western Blotting is performed next
  - Could detect viral antigens or antibodies
ELISA Procedures

1. Antibodies in patient’s serum
2. Enzyme-labeled antihuman IgG
3. Substrate
4. Viral antigen in patient’s sample
5. Antibodies coated

Figure 5.20: HIV ELISA test.
ELISA Competition assay

- Patient serum
- Marked Antibody
- Antigen
ELISA Competition assay
Viral Serology

Western Blotting

- Viral proteins are separated in SDS-PAGE gel
- Transferred to a nitrocellulose filter
- Detected by labeled antibodies
Figure 5.21a: The basic principles behind the Western blotting procedure.
Antibody detection: western blot

From Medical Microbiology, 5th ed., Murray, Rosenthal & Pfaller, Mosby Inc., 2005, Fig. 51-7.
Figure 5.21b: The structure of HIV-1.

Figure 5.21c: The typical results of a Western blot testing patient serum for HIV-1 antibodies.
Antigen, antibody detected by neutralization and hemagglutination or inhibition assay
Assays for viral proteins and nucleic acids

**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.
Summary

- 4 main clinical diagnostic techniques
  - Culture, serology, antigen detection, nucleic acid detection
  - Virus culture
    - Cultured cell types
    - Cytopathic effect
    - Not all viruses can be cultured
  - Virus quantitation
    - Biological
    - Physical
  - Basic serological techniques
Procedures for laboratory viral diagnosis

Virus infected patients

Collected specimens

Check the infected cells under light microscope

Observe virus particles under electronic microscope

Virus culture and isolation

Detection of viral antigen or genome

Serology test antibody test

Identify viral propagation

Antigen

Genome

Neutralization test

Hemagglutination inhibition test

EIA/ELISA

Western Blot

Immunofluoresence

EIA/ELISA

Western Blot

Immunoelectrophoresis

Radio immune assay

PCR

Southern blot

Northern blot

Dot blot

In situ hybridization

CPE

Hemadsorption

Hemagglutination

Virus interference

Neutralization test

Plaque assay

TCID50 assay
Terms & Questions

1. Primary cell, continuous cell, cell strain.
2. Molecular hybridization, viral molecular diagnosis.
3. Neutralization test, hemagglutination inhibition test, $\text{TCID}_5\text{O}_0$, $\text{ID}_5\text{O}_0$, $\text{LD}_5\text{O}_0$, PFU, MOI.
4. CPE, inclusion body, syncytia, virus interference.
5. How to determine a patient with HIV infection?