Diagnostic Microbiology

Identification of Microbes

Lecture: 12
Electron Microscopy

- 106 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

- **Skin scrapings**  papillomavirus

Electronmicrographs
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
Measles on human lung carcinoma

vaccinia on monkey kidney

Low multiplicity of infection

High
Assay of viruses

– Biological
  • Plaque assay
  • Transformation (Any differences between transformed and non-transformed cells can be used to assay the transforming ability of viruses. E.g. morphology, increased differentiation.
  • Endpoint dilution Method (Quantal Assay) (dilutions of virus are prepared- constant volume of each dilution is injected into test animals- at each dilution the proportion of infected individuals is scored (death or disease, cytopathic effect) - titration is carried out to find the end point, the highest dilution that still show the virus activity. This point is often defined as the highest dilution showing 50% infectivity of units used often the end point is difficult to define)

– Physical, biochemical
  • Hemagglutination
  • Direct particle count
  • Immunological tests for proteins
  • Assay for nucleic acid (PCR)
  • Enzymatic (reverse transcriptase for retroviruses)
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.
Haemadsorption

Hemadsorption of erythrocytes to cells infected with influenza viruses. This virus express a hemagglutinin, which bind erythrocytes of selected animal species.
Hemagglutination assay: influenza virus

The hemagglutination assay (HA) is a most common indirect method to quantify amount of virus particles. This assay depend on the fact that many viruses contain proteins (for example influenza virus hemagglutinin (HA) protein) that can bind and agglutinate red blood cells (RBC).

During the test, RBC will fall to the bottom of a culture well, forming a sharp dot; however presence of HA protein on viral surface would agglutinate the RBC, thus preventing them from settling out of suspension. Therefore by serially diluting a virus and adding a consistent amount of RBC, an estimation of virus titer can be made. in contrast to nonagglutinated cells, which form a distinct button at the bottom of the well. (Results: Tight button: Negative - Spread out RBC: Positive)
Plaque assay

unknown virus

serial dilution

plate 1 ml

1:100

1:10

1:10

1:10

1:10

1:10

10⁻²

10⁻³

10⁻⁴

10⁻⁵

10⁻⁶

10⁻⁷

plaques

(100,000)

(10,000)

(1000)

100

10

1

Titer = 1 x 10⁷ pfu/ml

Titer = 1.2 x 10⁸ pfu/ml
directly count virus particles, requires:
* means to visualize virus (ELECTRON MICROSCOPE)
* requires a standard to count against (known concentration of reference material)

known concentration of polystyrene beads, is added to the virus preparation. Dilutions, then made and a drop placed on the grid of an EM to dry. The grids are placed in the microscope and the number of virus particles and beads are counted.
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

• **Serological test**
  • Neutralization
  • Hemagglutination inhibition
  • Western blot
  • ELISA
  • radioimmune assay (RIA)
Neutralization and hemagglutination. 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.
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.
ELISA for HIV antibody

Microplate ELISA for HIV antibody: colored wells indicate reactivity
Immunofluorescence

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.
Molecular Methods

Methods based on the detection of viral genome are also commonly known as molecular methods. 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 in vitro amplification of specific target DNA sequences

- **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 - A positive result may be difficult to interpret, especially with latent viruses, where any sero positive 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 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>
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<tr>
<td>Influenza virus</td>
<td>ELISA, antigen, culture</td>
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<tr>
<td>Parainfluenza virus</td>
<td>Antigen, PCR, culture</td>
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<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>
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</tbody>
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ELISA: enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.
Summary

• Main clinical diagnostic techniques
  – Culture, serology, nucleic acid detection

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

• Virus quantitation
  – Biological
  – Physical

• Basic serological techniques

• Molecular techniques
Limitations of the present methods for virus identification and quantification.

<table>
<thead>
<tr>
<th>TECHNIQUE</th>
<th>DETECTION PRINCIPLE</th>
<th>REPRODUCIBILITY</th>
<th>Time</th>
<th>LABOUR</th>
<th>cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral Plaque assay</td>
<td>Infectivity assay</td>
<td>poor</td>
<td>Days</td>
<td>High</td>
<td>inexpensive</td>
</tr>
<tr>
<td>Immune Precipitation</td>
<td>Viral protein</td>
<td>good</td>
<td>Days</td>
<td>moderate</td>
<td>inexpensive</td>
</tr>
<tr>
<td>ELISA</td>
<td>Viral protein</td>
<td>Good</td>
<td>hours</td>
<td>moderate</td>
<td>inexpensive</td>
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<tr>
<td>Hemagglutination assay</td>
<td>Viral protein</td>
<td>good</td>
<td>hours</td>
<td>moderate</td>
<td>inexpensive</td>
</tr>
<tr>
<td>Transmission Electron microscopy</td>
<td>Viral particle</td>
<td>Excellent</td>
<td>weeks</td>
<td>high</td>
<td>Expensive</td>
</tr>
</tbody>
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