Primary Isolation and Cultivation of Viruses -2

Practical Medical Virology 450 MBIO
2017-18

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Learning Outcome

- Virus Isolation techniques including: virus isolation in cell cultures, rapid culture methods.
- Types of cell culture, genetically modified cell lines.
- Obtaining and processing specimens, conventional cell cultures, inoculation and incubation.
- Detection of virus-induced effects including; Cytopathic effect (CPE), Hemadsorption (HAd), blind immunostaining.
- Describe the CPE.
- Identification of virus isolates.
- Virus common isolated in conventional cell cultures: HSV, CMV, Influenza, RSV.

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Cell Culture Systems of Viruses

Cell Cultures are most widely used for virus isolation in-vitro using monolayer cells cultures. However, some viruses cannot grow in-vitro e.g. Hepatitis C!

1- Primary cells

- e.g. Primary Rhesus Monkey Kidney (PMK) Cells

2- Semi-continuous cells

- e.g. Human embryonic kidney and skin fibroblasts

3- Continuous cells

- e.g. Human diploid fibroblast(HDF), Human Cervix Adenocarcinoma (HeLa), African Green Monkey Kidney(Vero), Human Epithelial Cells (Larynx carcinoma) (Hep2), Rhesus Monkey Kidney (LLC-MK2), Madin-Darby Canine Kidney (MDCK).

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1- Primary Cell Cultures

- The cells in culture divide only a limited number of times, before their growth rate declines and they eventually die.
- Prepared from cells obtained directly from the tissues or organs.
- Viable cell suspensions may be obtained by dissociating tissues or organs, by enzymatic digestion or mechanical dispersion.
  - e.g. human amnion, with trypsin, collagenase or other enzymes.

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1- Primary Cell Cultures

- Primary cell lines 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.

Advantages:

- usually retain many different characteristics of the cell in-vivo.

Disadvantages:

- Initially heterogeneous but later become dominated by fibroblasts.
- The preparation of primary cultures is labor intensive.
- It can be maintained in-vitro only for a limited period of time.

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2- Semi-continuous Cell Cultures (Diploid cell lines or strains)

- Those cell cultures are established with the successful subculture of primary cell monolayers.
- These cultures consist mostly of spindle shaped fibroblast cells.
  - E.g. Established from human embryonic tissue, or neonatal foreskin.

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3- Continuous Cultures

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

- A few cells in culture may acquire a different morphology and get altered. Such cells are capable of growing faster resulting in an independent culture. The progeny derived from these altered cells has unlimited life (unlike the cell strains from which they originated). They are designated as continuous cell lines.

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3- Continuous Cultures

- The transformed cells for continuous cell lines may be obtained from normal primary cell cultures (or cells strains).
- It is derived from a subculture of primary culture.
- The continuous cell lines are *transformed* (spontaneous or engineered), *immortal* and *tumorigenic* by treating them with chemical carcinogens or by infecting with oncogenic viruses.
  - e.g. Cells taken from tumours:
    - **Hela (human cervical carcinoma) cell line.**
    - **RD (human rhabdomyosarcoma) cell line.**

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3- Continuous Cultures

- The following continuous cell lines are **commonly** used:
  - Hela and HEp2 are used for cultivation of HSV, adenovirus, poliovirus and some coxsackie viruses.
  - Vero cells will also support growth of these viruses and are used with BHK21 cells for growth of arboviruses.
  - RK13 cells and BHK21 cells for isolation and propagation of rubella virus .
  - RD cells for the isolation of coxsackie A virus

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3- Continuous Cultures

Characteristics of continuous cell lines:

- Smaller, More Rounded
- Less adherent with a higher nucleus /Cytoplasm ratio
- Fast growth
- Reduced serum and anchorage dependence and grow more in suspension conditions

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What is Cell Cytopathic Effect (CPE)?

- It is the visible result of viral replication within cells and morphological changes occurring in cells due to viral infection is called cytopathic effects.
- The virus responsible are called **cytopathogenic virus**. The development of characteristic CPE in cell culture is often useful for making a presumptive identification of the viral isolate.
- The identification would also be based on the specimen source and the cell line on which the virus has grown.
- Some viruses cause very little or no CPE in cells of their natural host but can be detected via
  - **Inhibition of replication of another virus**
  - **Or presence of viral genome or antigens in cultured cells**
- It can be observed by **Inverted Light Microscope**.

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Several forms of CPE:

- Rounding (detaching from the plastic flask)
- Syncytia (Fusion of the cells)
- Cell lysis (death)
- shrinkage
- Aggregation
- lose of adherence
- Increased refractivity.

Common observation of CPE:

- Inclusion bodies formation (Intracellular virus parts resulting from replication or assembly).
- Hemadsorption assays when cells acquire the ability to stick to mammalian red blood cells.

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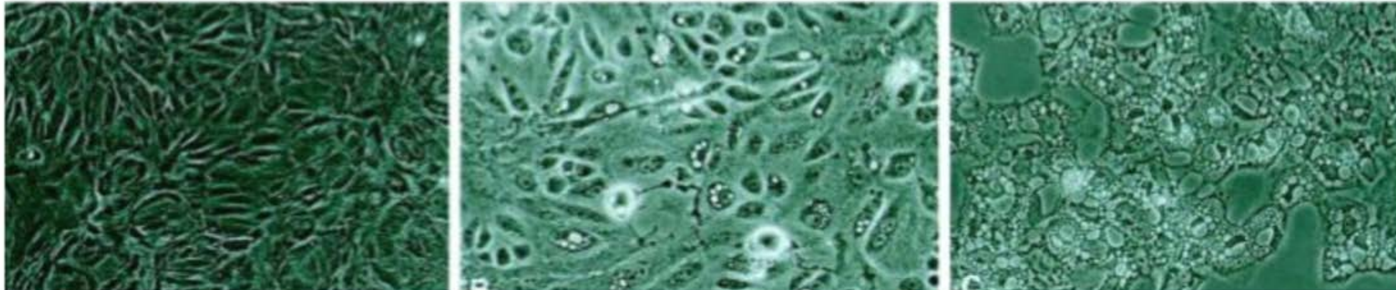
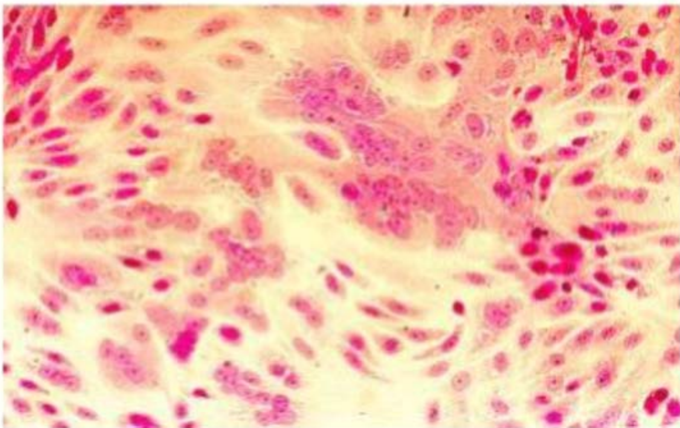
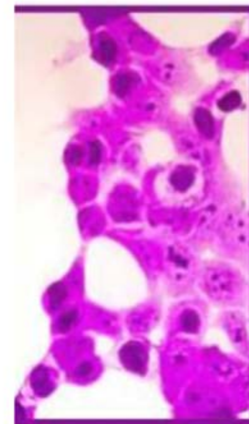
Several forms of CPE:

Cytoplasmic vacuoles characteristic of polyoma virus

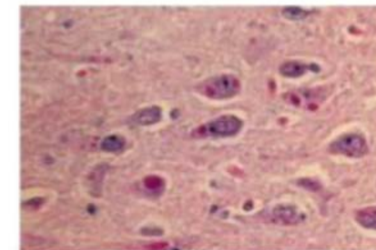


RSV infection induces fusion with adjacent cells to form a syncytia (polykaryocytes)

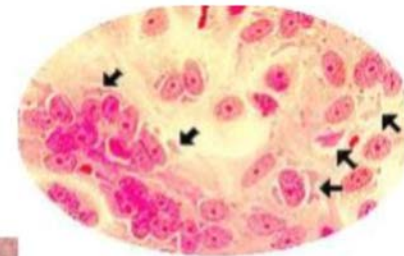




Adenovirus
nuclear inclusions

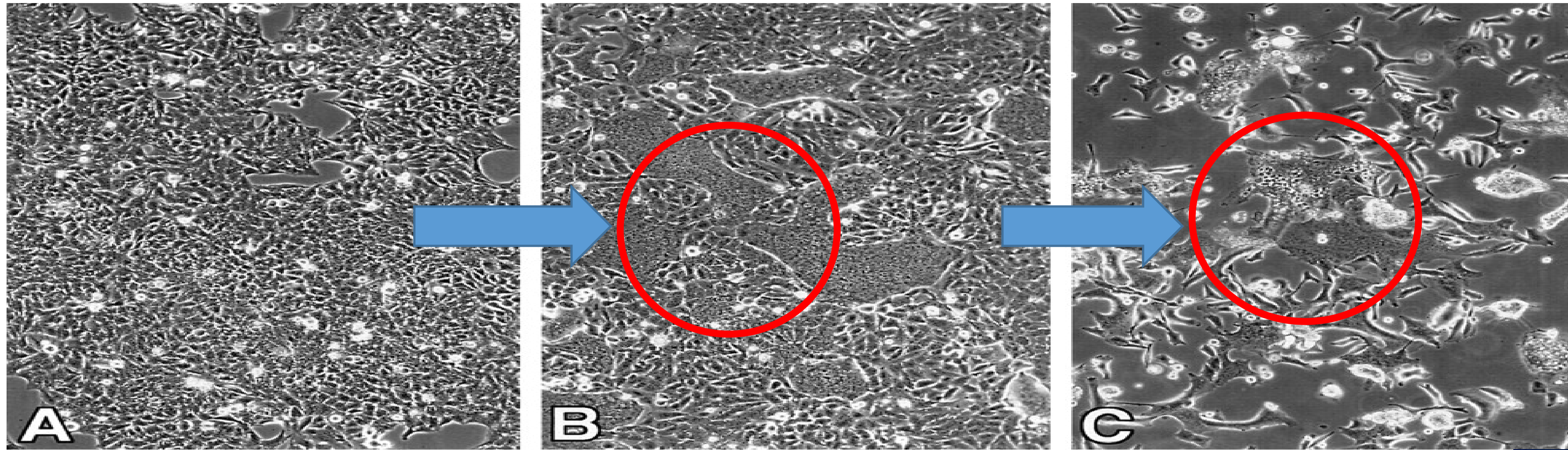


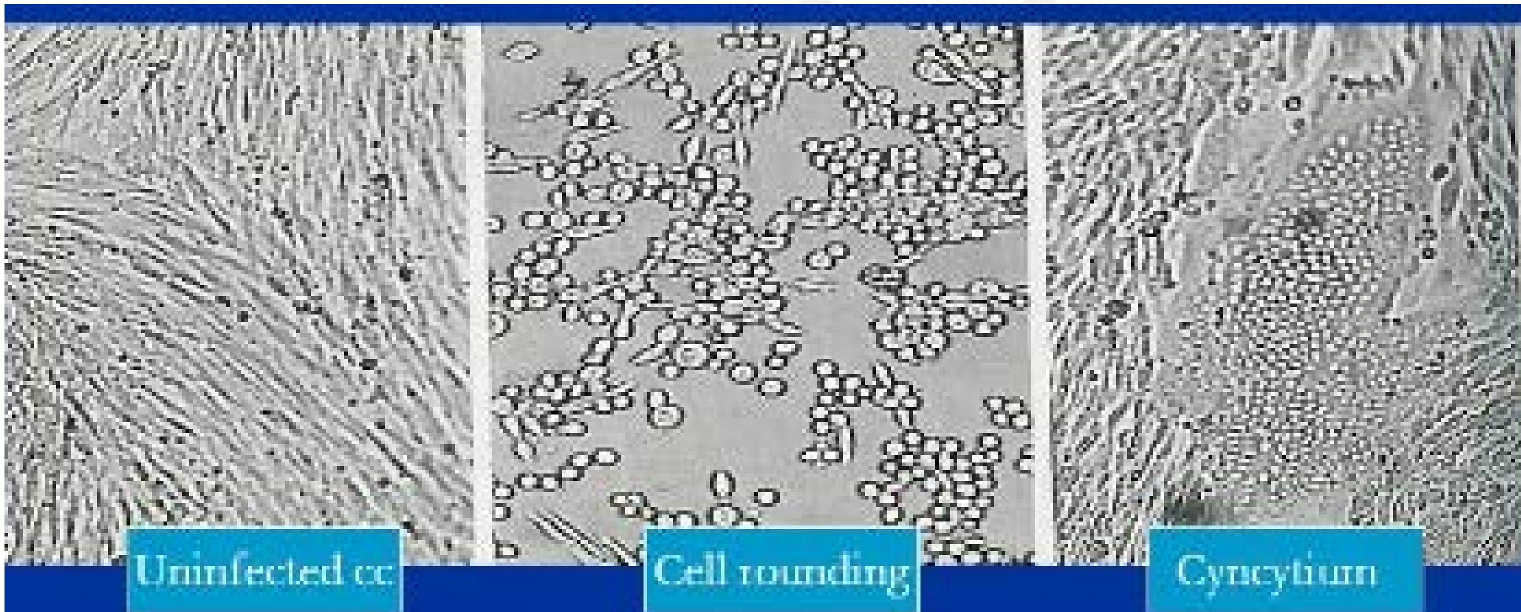
Rabies virus
cytoplasmic inclusions
Negri bodies

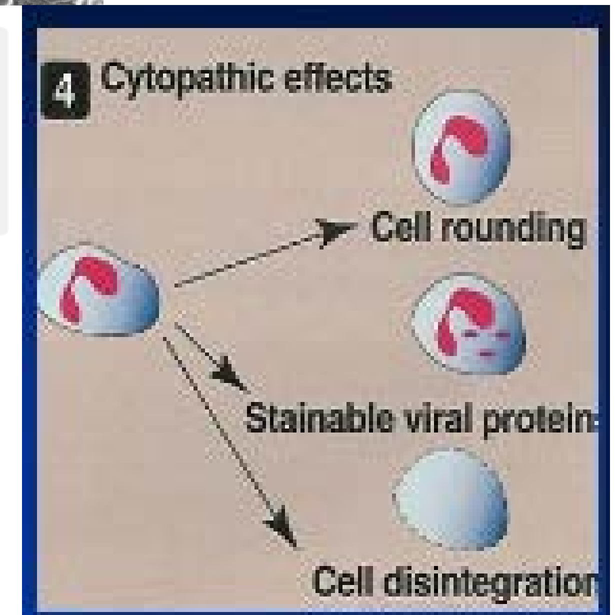


RSV
cytoplasmic inclusions

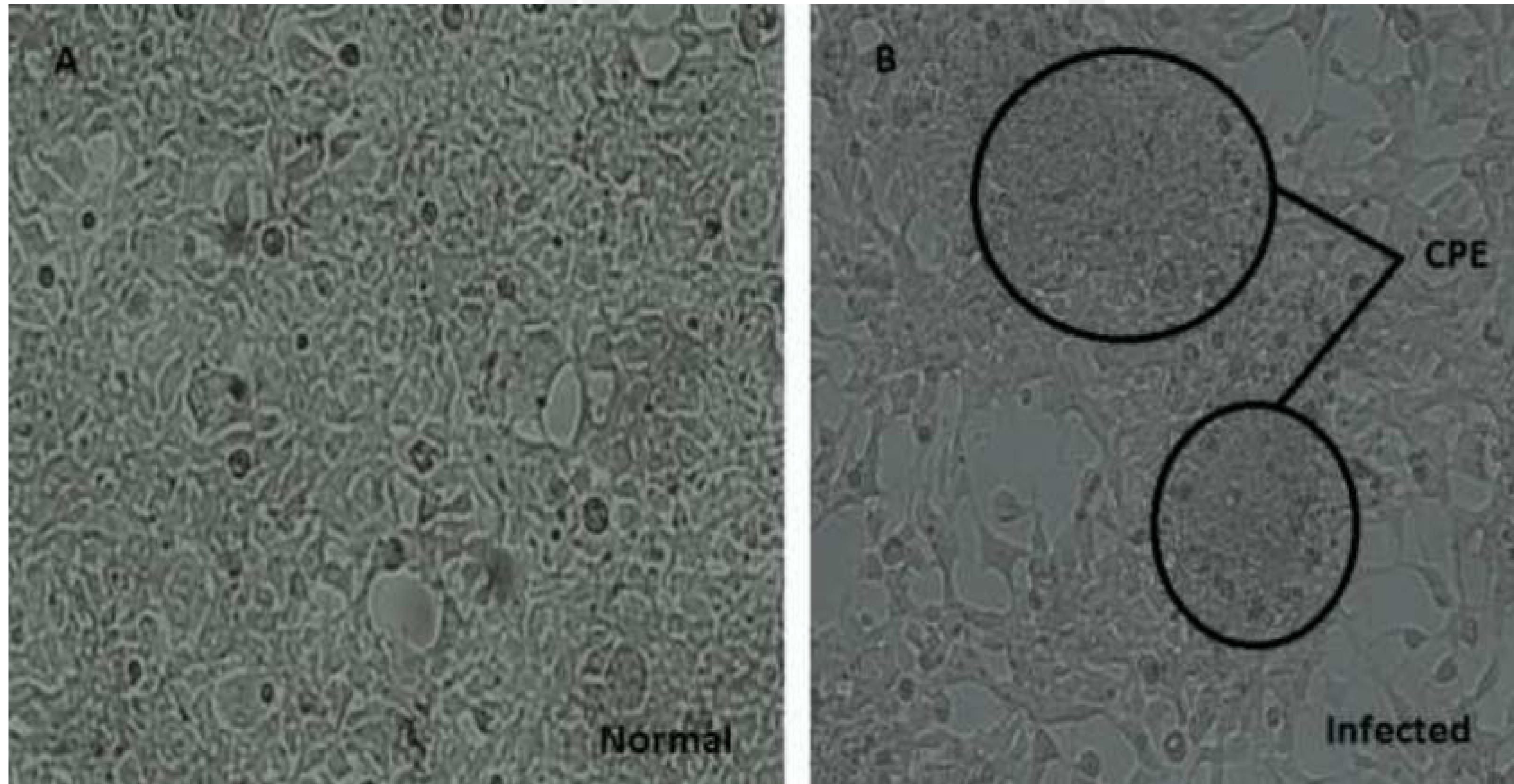
Several forms of CPE:







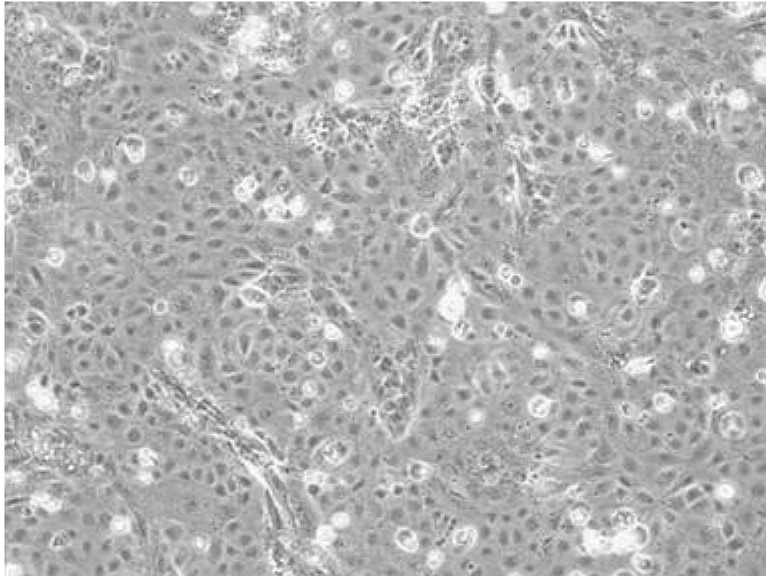
Cytopathic effect (CPE) in HEK-293 cell line inoculated with Nasal/throat swab specimens. (A) Normal morphology of HEK-293 cell line. (B) Cell lysis and aggregation on HEK-293 cells on day 2 of third passage (magnification 200x).



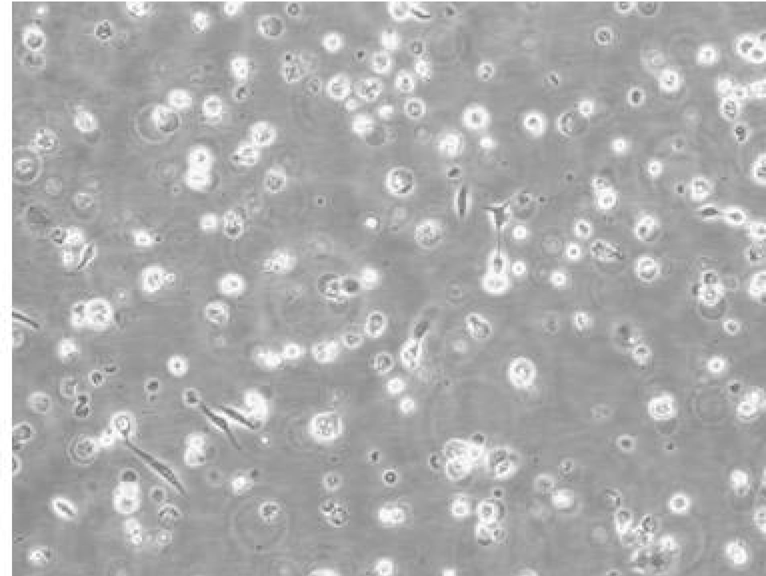
Various Forms of CPE

1- Influenza: INF B Virus (CPE, rounding and cell death or degeneration):

ATCC® Number: **VR-1735™**
Agent: **Influenza B virus**
Strain: **B/Taiwan/2/62**



Uninfected host (ATCC® CCL-34™)

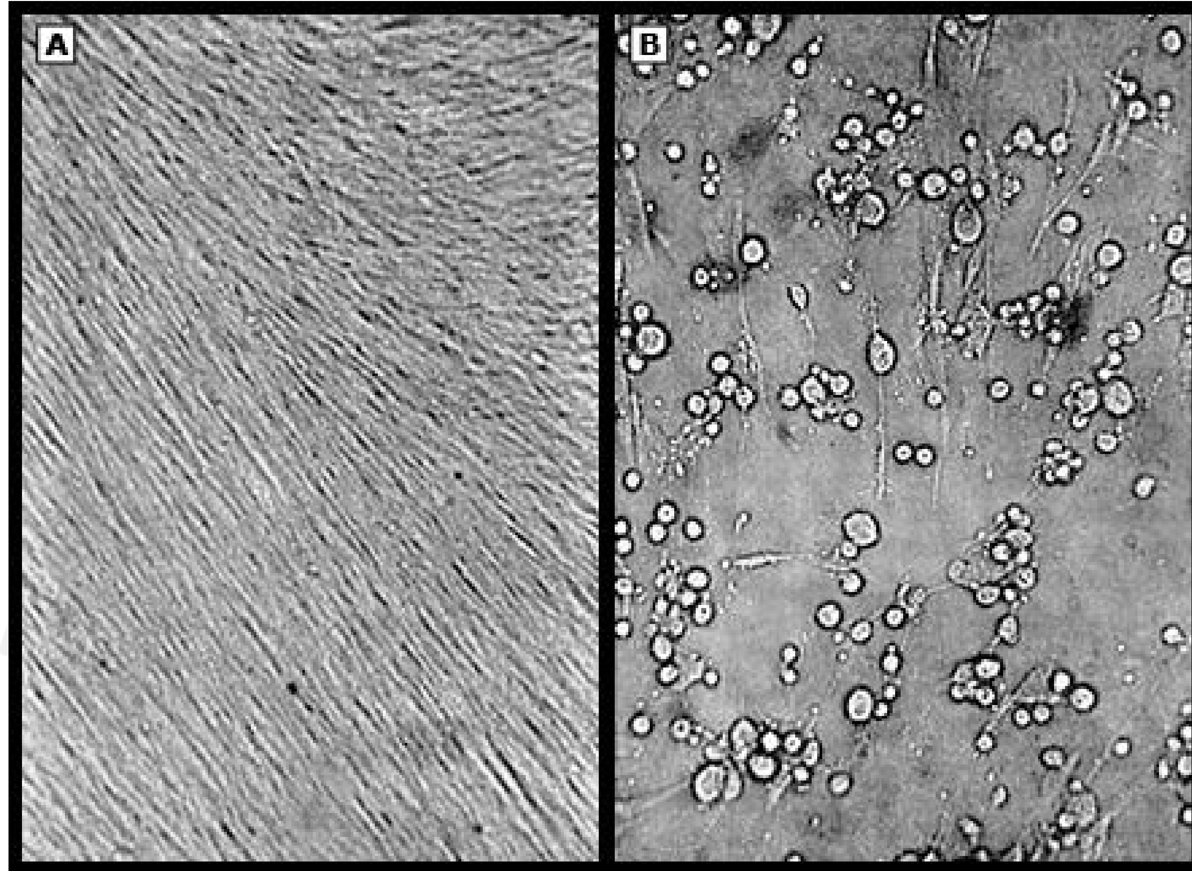


Infected host (ATCC® CCL-34™) showing CPE

Derived by adaptation from allantoic product ATCC® VR-295™ to MDCK cells (ATCC® CCL-34™). ATCC® VR-295™ and ATCC®VR-1735™ have not been compared for sequence or infectivity in chicken embryo culture and tissue culture.

Various Forms of CPE

2- Herpes simplex virus: (CPE, Cell Lysis):

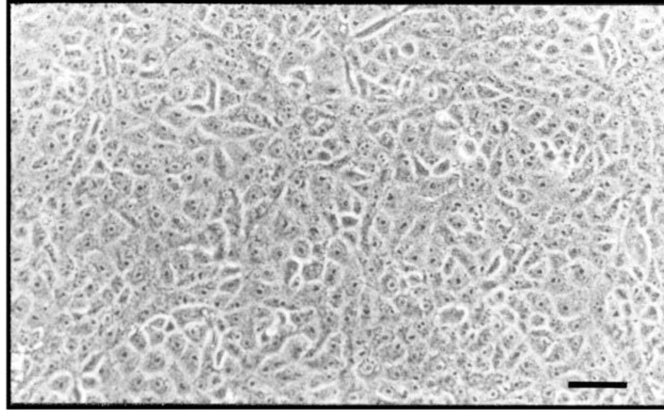


The microscopic appearance of a monolayer of uninfected human fibroblasts grown in cell culture (A) and the same cells after infection with herpes simplex virus (B), demonstrating the cytopathic effect caused by viral replication and concomitant cell lysis.

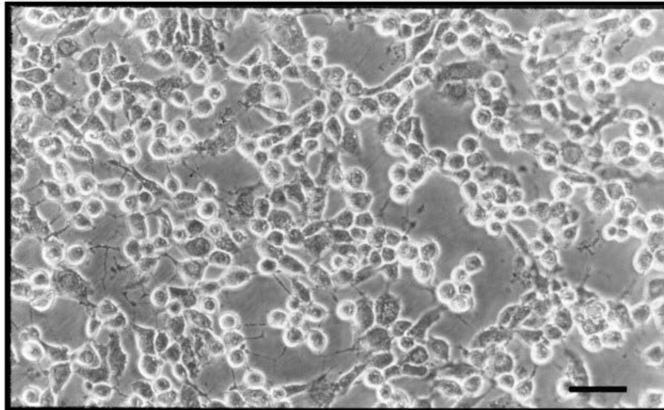
Various Forms of CPE

2- Herpes simplex virus: (CPE, rounding and degeneration, cell lysis):

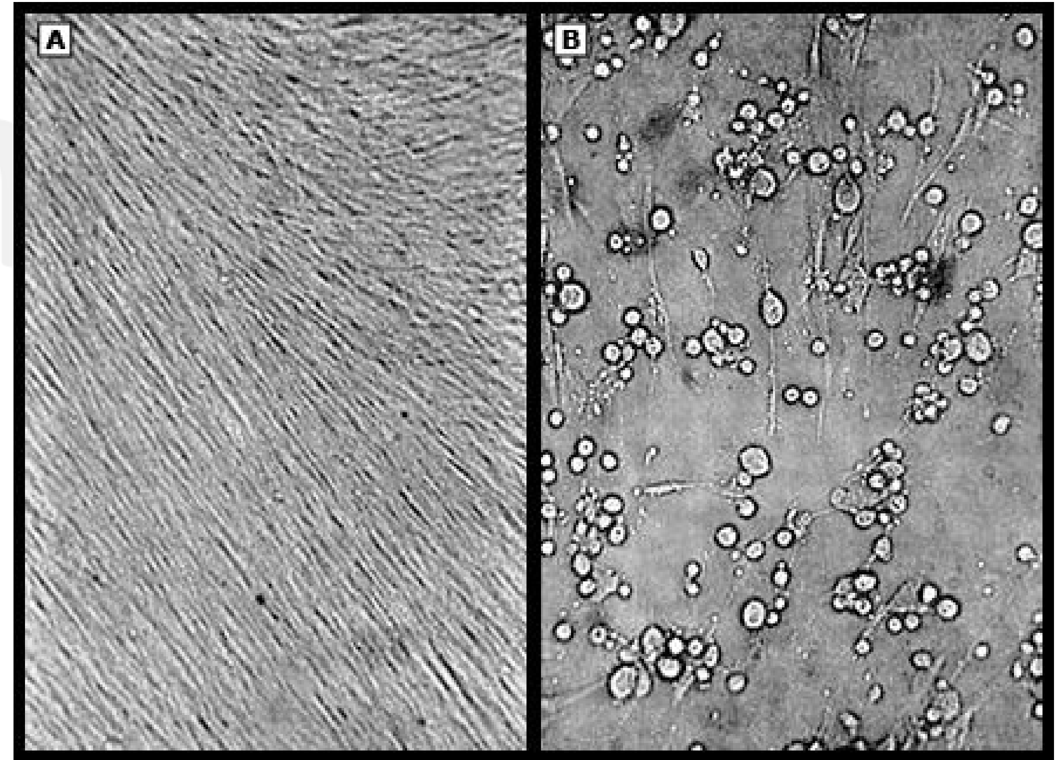
CELLS



CELLS +HSV-1



Cytopathic effect of HSV-1 on **Vero cells**

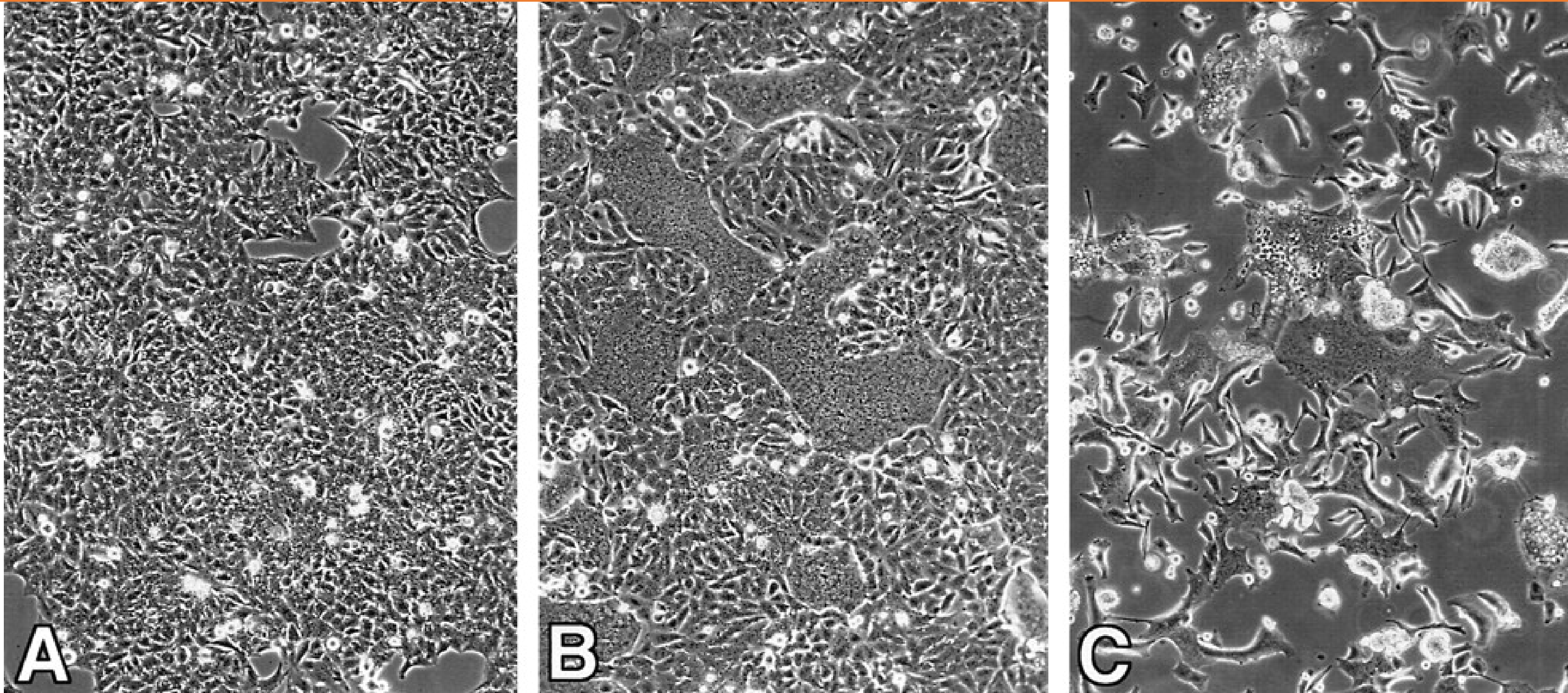


The microscopic appearance of a monolayer of uninfected **human fibroblasts** grown in cell culture (A) and the same cells after infection with herpes simplex virus (B), demonstrating the cytopathic effect caused by viral replication and concomitant cell lysis.

Various Forms of CPE

3- RSV-infected respiratory epithelial cells : (CPE, syncytia, cell death or lysis):

.
Respiratory epithelial cells (HEp-2, laryngeal carcinoma cell line) on day 1 (A),
day 3 (B), and day 5 (C) following infection with RSV. Magnification, ×400
After 1 day, the cells continue to appear healthy. After 3 days, many of the cells have fused, forming large syncytia.
After 5 days, most of the syncytia have detached from the surface and the majority of the cells are dead.



Joseph B. Domachowske, and Helene F. Rosenberg Clin. Microbiol. Rev. 1999;12:298-309

Clinical Microbiology Reviews

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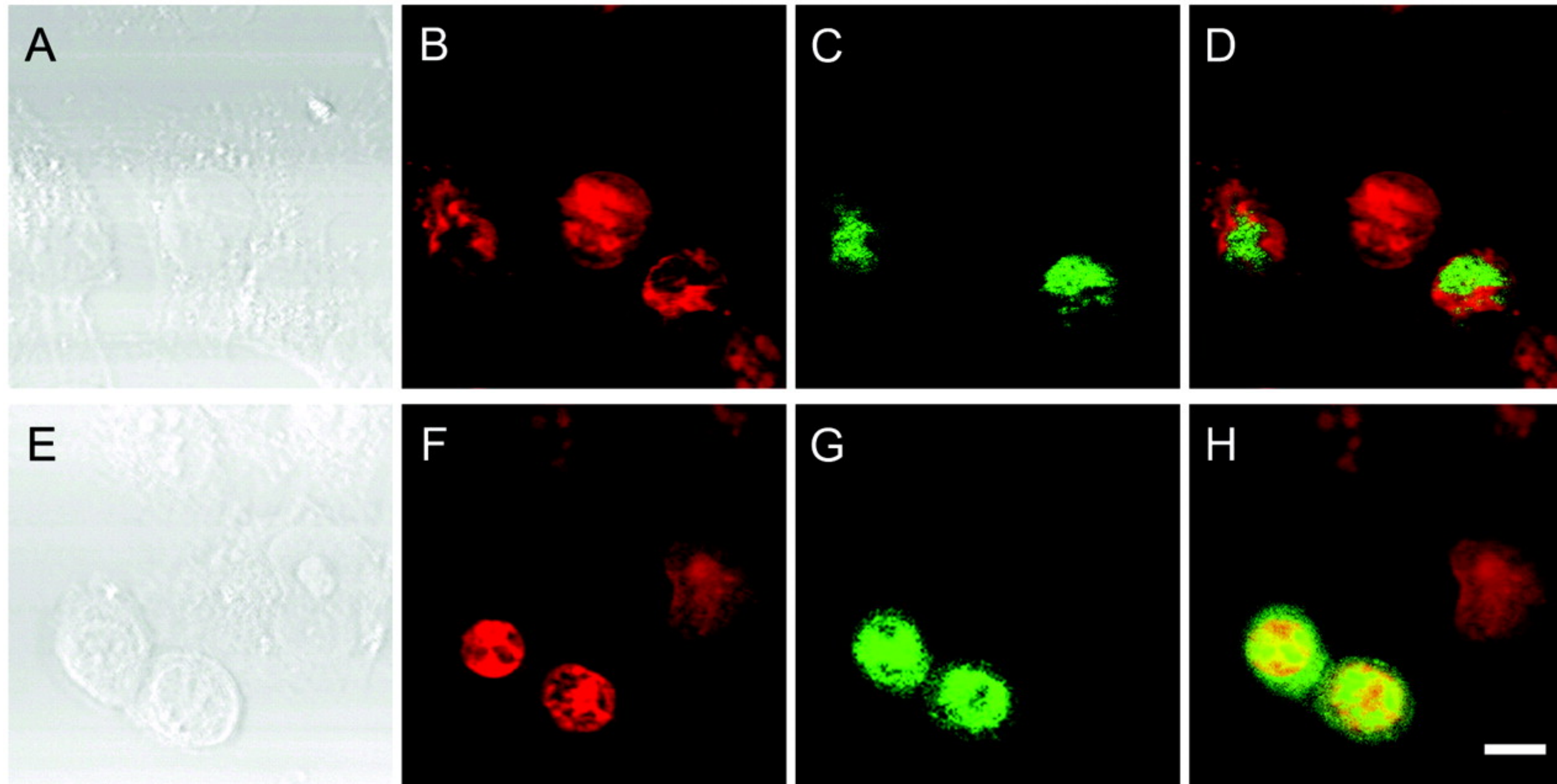
Various Forms of CPE

3- Polio virus: (CPE, refractile rounding and sloughing, cells lyse, detach from surface and leave granular debris).

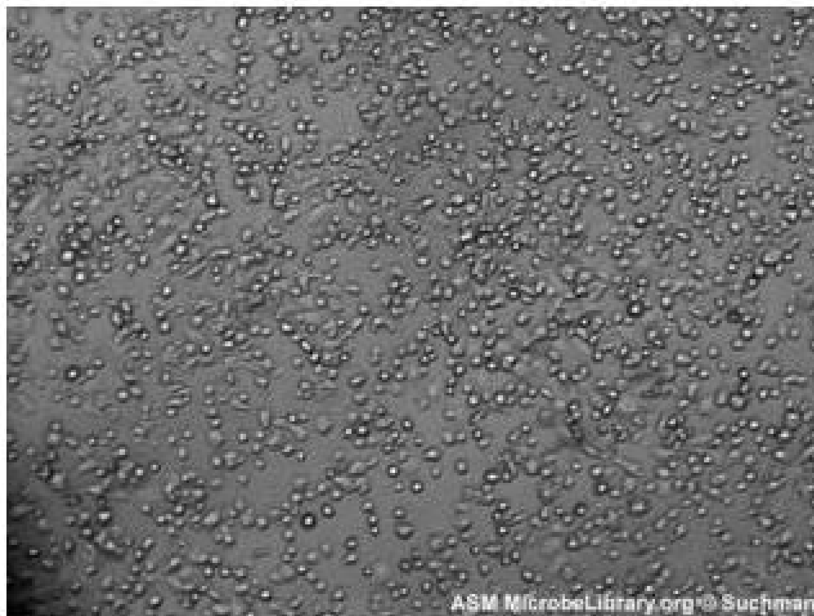


Transient expression of HA-tagged 2Apro.
Mammalian expression vector pCI-neo encoding HA-tagged 2Apro was transfected into cells, which were subjected to an immunofluorescence study.
Neural cells (A, B, C, and D) and HeLa cells (E, F, G, and H) were fixed 2 days post transfection.
Red indicates nucleic acids, and green indicates HA-tagged 2Apro.

Various Forms of CPE

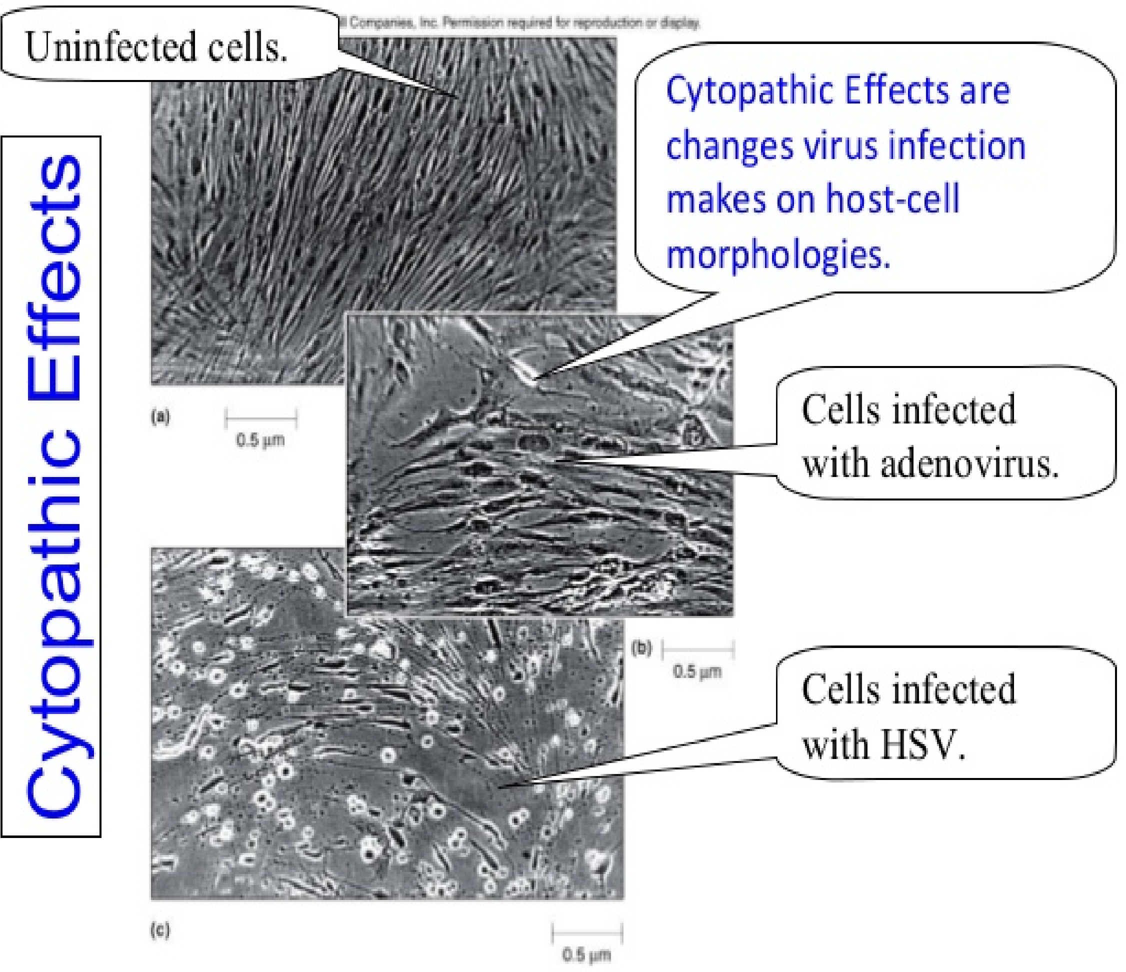
4- Cytomegalovirus (CMV): (CPE, Swelling and clumping)

Adenovirus infected cells greatly enlarge and clump together in" grape-like" clusters.



Cytomegalovirus infected cells swell and round up

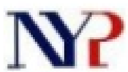
Various Forms of CPE



CPE

Different effect of different viruses in different cell lines

	Non-infected		
A549	A	B HSV-2	C Adenovirus
MRC-5	D	E CMV	F Rhinovirus
RhMK	G	H Enterovirus	I Influenza A virus
HEp-2	J	K RSV	L Monkey B virus



Tests for Virus Cultivation

Hemadsorption Test

Some viral agents insert into the membranes of infected cells surface components that agglutinate erythrocytes from various species of animals.

These viruses can be detected in cell cultures by exposing the infected monolayer to the specific type of erythrocyte.

Hemadsorption Inhibition Test

Viral agents can be positively identified by the hemadsorption inhibition test.

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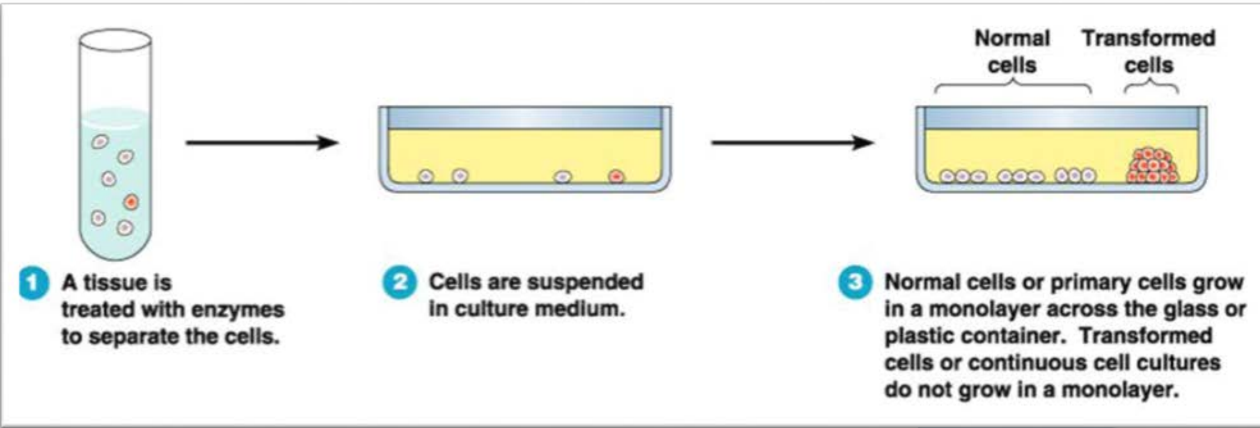
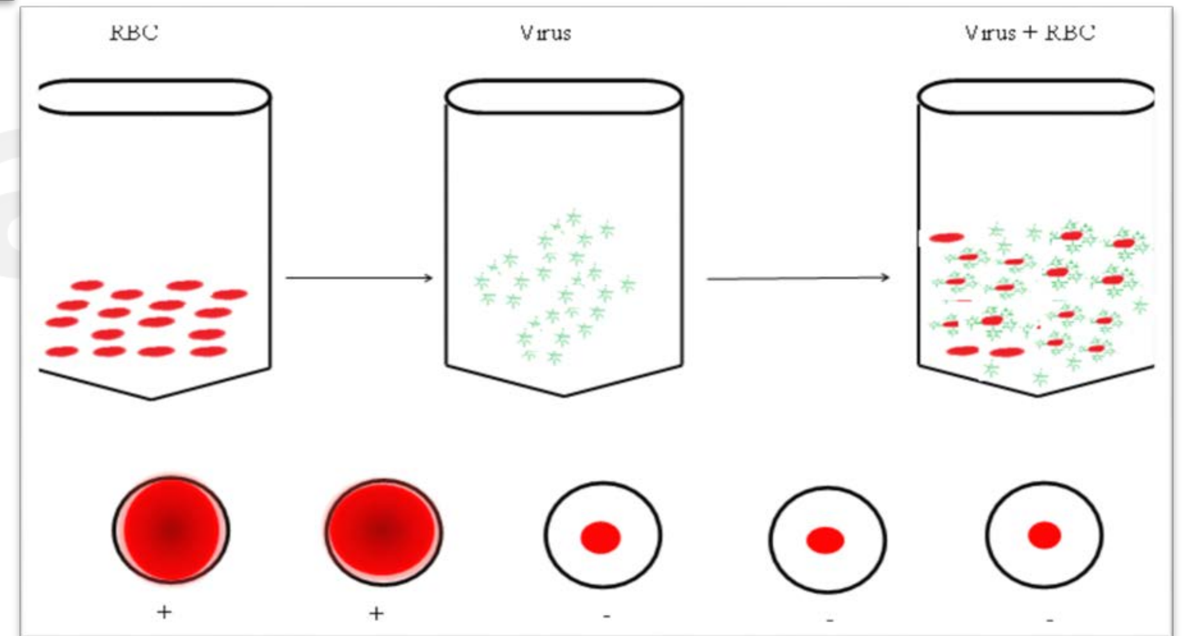
Hemadsorption as rapid test for viral diagnosis

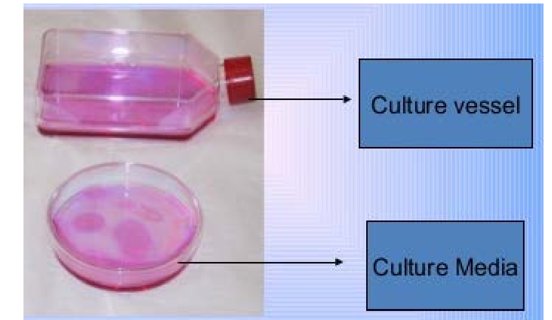




The Experiment:

Material:

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–20°C)
- Cell counter
- Inverted Light microscope
- Liquid nitrogen (N_2) freezer or cryostorage container
- Sterilizer (i.e., autoclave)
- Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multi-well plates)
- Pipettes and pipettors (Four 1-ml serologic pipettes)
- Media, and reagents (200-ml Hanks' balanced salt solution, Three ml 0.4% guinea pig erythrocyte suspension)



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The Experiment:

Material:

- Test tube holder for microscope
- Two slanted cell culture racks
- Cell lines (Uninoculated tube cell culture)
- Tube cell cultures of the common viruses listed in Study Chart 32-1)
- Sets of primary rhesus monkey kidney (PMK) cells, continuous human epithelial cell (HEP-2), and human diploid fibroblast (HDF) cultures of unknown viruses selected from the list of viruses in Study Chart 32-1
- PMK culture of influenza A virus
- Ten fresh PMK cell cultures
- 0.2 ml of the following receptor-destroying enzyme–treated antisera:
  - Influenza viruses A and B
  - Parainfluenza viruses 1, 2, and 3
  - Mumps virus

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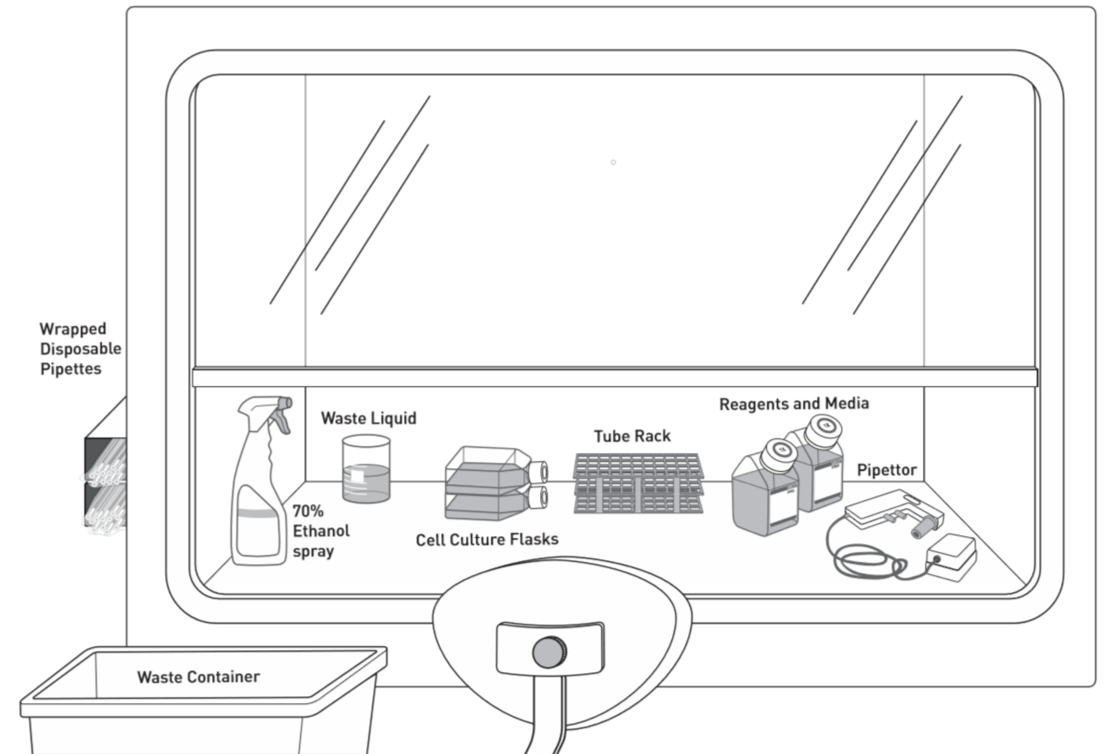
The Experiment:

Material:



Inverted Light Microscope witch has Large stage so plates and flasks can be used.

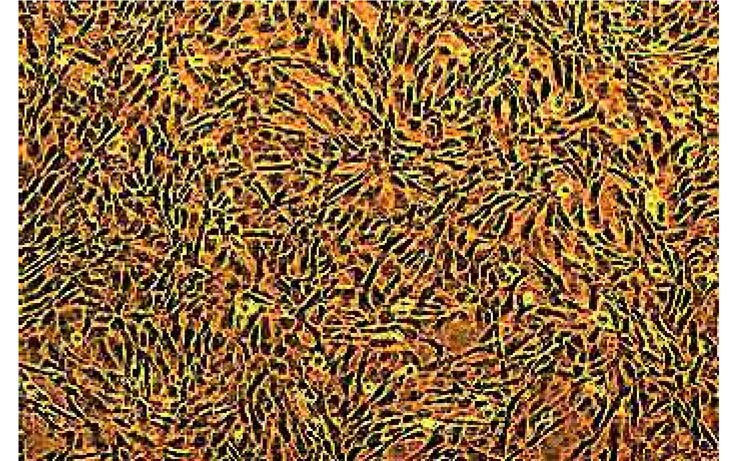
Specimen Magnification; 5X, 10X, 20X, 40X.

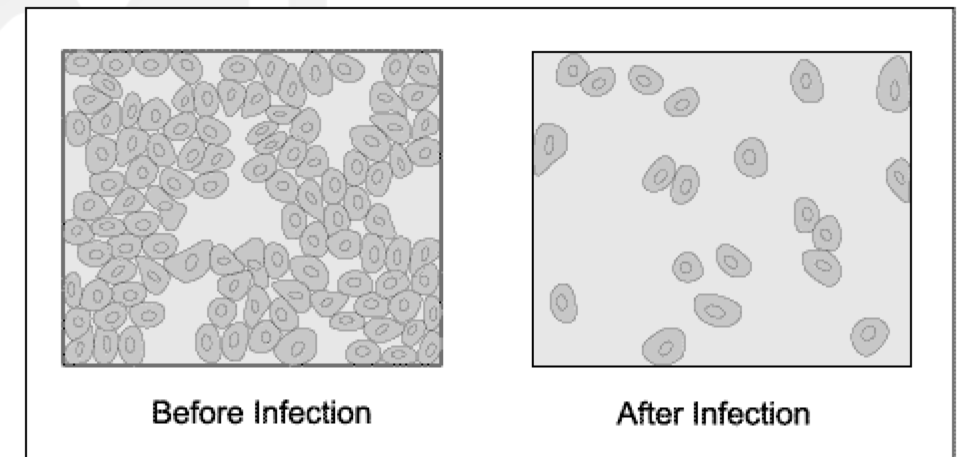
The growth of cell lines:

Cells are grown under stringent aseptic conditions, as the media that the cells are grown in is rich in a wide range of nutrients and will support the growth of a wide range of opportunistic bacterial contaminants.

***Mycoplasma* sp.** are a particular nuisance as they are hard to detect, are small and will pass through many filters, and are found widely in the environment including in plants and animals.

Cell culture media often includes a range of salts, vitamins, hormones and other growth factors, amino acids or proteins, glucose, antimicrobial agents, a buffering system, a pH indicator, and non-specific sources of nutrients such as fetal bovine serum. Examples of cell culture media include Medium M199, Eagle's Basal Medium and RPMI 1640.





Cells growing in cell culture attach to the surface of the container (glass or specialised plastic) then start to divide and grow across the surface. They form a sheet of cells one cell thick (monolayer) and when the surface is covered it is called confluent. Many cells stop growing once confluent (contact inhibition).

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The growth of cell lines:

Some cells do not attach to surfaces and are grown in a suspension culture, whilst cancer cells often keep growing and pile up forming tiny heaps of cells.

Cells may be subcultured (called passaging) by:

stripping the cells from the surface using trypsin, EDTA and some gentle mechanical motion

dispersing the cells in a suitable medium (often a 1:10 dilution is used)

finally seeding the cells into ten more containers or a container ten times the size of the original

In this way significantly large numbers of cells can be grown.

Cells are incubated at an appropriate temperature (37°C for human and mammalian cells, 28°C for insect cells) usually in an atmosphere enriched in CO_2 (to provide buffering with $NaHCO_3$ in the cell culture media) and humidified (to prevent cell cultures from drying out).

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Growth of viruses in cell culture

Appropriate cell lines are infected with sterile preparations of virus or suspected isolate (sterile means that all bacteria and fungi have been excluded) and the cell line is incubated for a number of days to allow the virus to grow in the infected cells. Remember that the virus must grow in a living cell.

The cell culture is then inspected under a low magnification inverted microscope and changes in the cells due to virus replication (cytopathic effect or CPE) are looked for. Depending on the virus and cell line used CPE can include rounding up (shrivelling) of the cell, loss of patches of cells or all the cells from the monolayer, inclusion bodies (dark aggregated viral material in the cell), or formation of large fused areas of cells (syncitia).

Immunofluorescence can be used to detect the presence of specific viral proteins in an infected cell culture using antibodies directed against the viral protein and a Fluorescence microscope.
Viruses may also be harvested and purified from the cells of the monolayer or the cell culture medium for further research or investigation.

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Examination of Cell Cultures for the Detection of the Cytopathic Effect

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- An examination of cell cultures to detect the CPE of an infecting virus is the first step in the identification of viral agents in culture. Practice recognizing and describing the CPE by following the instructions for each virus culture assigned:

1. Record the name of the infecting virus on Worksheet, and examine the monolayer of the culture as described in the previous section.
2. Review a description of the typical CPE of that virus in your textbook.
3. Find infected cells in the monolayer of the culture that demonstrate the typical CPE of the infecting virus, and draw a few representative cells on Worksheet.

Cultures of Unknown Viral Agents

- The determination of the type, time required, and selectivity of the CPE is often enough to enable presumptive identification of the infecting virus. For each set of unknown cell cultures assigned, follow the steps given here:

1. Obtain a set of PMK, HEP-2, and HFD cell culture unknowns, and record the unknown number on Worksheet.
2. Examine each type of monolayer for evidence of the CPE, and describe the appearance of any CPE on Worksheet. If no CPE is evident, then record that observation.
3. Record on Worksheet the number of days from inoculation to the detection of the CPE.

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Cultures of Unknown Viral Agents

4. For those cultures demonstrating the CPE in one or more of the cell cultures, indicate which type or types of cells demonstrated the CPE by placing a check mark in the column of the affected cell type on Worksheet.
5. Compare the description of the CPE of the unknown with the descriptions of the CPE of known viral agents.
6. Record the probable identification, if possible, on Worksheet. If no probable identification is possible, based on the evaluation of the monolayer for CPE alone, then record the further action needed to identify the viral agent or to otherwise make a conclusion about the results of the cell culture.

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The hemadsorption procedure-1

Perform the hemadsorption procedure with all appropriate cell culture unknowns by following the steps given here:

- Choose the appropriate cell cultures from the group of culture unknowns, and record the unknown numbers on Worksheet.
- Obtain a PMK cell culture of influenza A (positive-control preparation) and an uninfected PMK cell culture (negative-control preparation).
- With a clean pipette for each tube, aspirate the maintenance medium from all tubes, and discard it into an appropriate container for biohazardous waste.
- Add 0.2 ml of a 0.4% guinea pig erythrocyte suspension with a sterile pipette to each culture to be tested. To avoid cross-contamination, do not allow the pipette to contact the culture tubes, and add the erythrocyte suspension to the positive-control preparation last.

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The hemadsorption procedure-2

- Place the tubes in a slanted rack to ensure that the suspension covers the monolayer, and refrigerate at 4°C for 30 minutes.
- Remove the tubes from the refrigerator and, without delay, invert the cell culture tubes to dislodge loose erythrocytes. Microscopically examine the monolayer in the negative-control tube to ensure that no erythrocytes are adhering to the monolayer. Record the hemadsorption results on Worksheet.
- Examine the monolayer in the positive-control tube to ensure that the erythrocytes are adhering to the monolayer. Record the hemadsorption results on Worksheet.
- If the positive- and negative-control cultures show acceptable results, then examine the monolayers of the unknown cultures. Record the hemadsorption results on Worksheet 32-4 for each unknown tested.

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The hemadsorption procedure-3

- To detect hemagglutination of erythrocytes, place a drop of culture fluid on a glass slide. Add a coverslip to the slide, and microscopically examine the drop of culture fluid for agglutination of erythrocytes. Examine the culture fluid in each unknown culture, and record the hemagglutination results on Worksheet.
- Based on the hemadsorption test results, list on Worksheet the possible identifications of each unknown viral agent on that worksheet.
- Answer the questions on Worksheet .

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The hemadsorption inhibition procedure

- Perform the hemadsorption inhibition procedure with each culture of unknown hemadsorbing viral agent.
- Record each unknown number on Worksheet.
- Dislodge and suspend the infected cells of the unknown culture by scraping the monolayer off the inside of the culture tube with a sterile Pasteur pipette.
- Obtain and label nine tubes of fresh PMK culture with the appropriate unknown number.
- Subculture the hemadsorbing virus by transferring 0.1 ml of the dislodged cell suspension in the unknown culture tube to each of the nine tubes of fresh PMK monolayer.
- Incubate the PMK subcultures at 36° to 37°C for 72 hours.

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Newer cell culture formats

1. Centrifugation-enhanced inoculation and pre-CPE detection of viruses in cell culture.
2. Virus isolation in transgenic cell lines
3. Virus isolation in cocultured cells.

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– Centrifugation-enhanced inoculation and preCPE detection of viruses in cell culture.

Cytospinning (shell vials):

- In this assay cells are grown on a coverslip in a vial , infected with the specimen and submitted to low- speed centrifugation followed by over night incubation.
- Labeled antibody to early antigen is used to stain cells after 24- 48 hours.

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Detection of Herpes Virus Simplex 1 using the shell vial technique and immunofluorescence.

Inoculate with specimen

Coverslip with tissue culture monolayer in shell vial

Centrifuge to enhance infection of monolayer

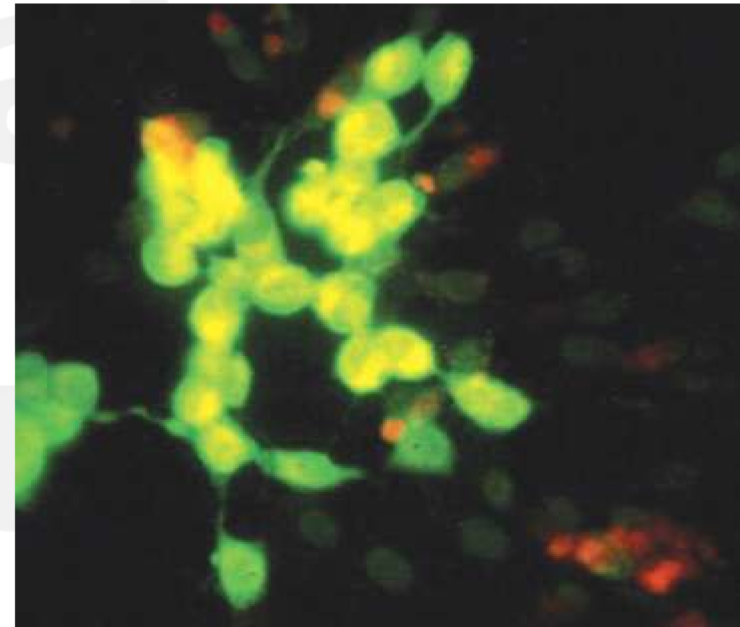
Incubate at 35 °C for 1–5 days depending on virus

Stain with anti-viral fluorescent monoclonal antibodies

Mount coverslip on slide

Read with fluorescent microscope

Modified from J. H. Shelhamer, et. al., Ann. Intern. Med. 124 (1996): 585-599.



Reproduced from Athmanathan, S., S. R. Bandlapally, and G. N. Rao, BMC Clin. Pathol. 2 (2002): 1-5.

Shell Vials Technique

- Centrifugation Culture or Shell Vial Technique is used a lot in clinical labs.

TABLE 5-4 Detection of Virus: Traditional CPE Method vs. Shell Vial Technique

Virus	Days to Detect CPE Conventional Cell Culture Method [avg(range)]	Days to Detect CPE Shell Vial Centrifugation Method [avg(range)]
RSV	6(2–14)	1–2
Influenza A	2(1–7)	1–2
Influenza B	2(1–7)	1–2
PIV 1-4	6(1–14)	1–2
Adenovirus	6(1–14)	2–5
HSV	2(1–7)	1–2
VZV	6(3–14)	2–5
CMV	8(1–28)	1–2