BHARATHIDASAN UNIVERSITY

Tiruchirappalli-620024

Tamil Nadu, India.

Programme: M.Sc., Biomedical Science

Course Title : Medical Virology

Course Code : BM59C19MV

Unit-I

General Methods of Diagnosis and Serology

Dr.P.JEGANATHAN

Guest Lecturer

Department of Biomedical Science

General methods of diagnosis and serology

TABLE 10.2 **Specimens Appropriate for Laboratory Diagnosis of Various Clinical Syndromes**

Syndrome	Specimen
Respiratory	Nasal or throat swab; nasopharyngeal aspirate; sputum
Enteric	Feces
Genital	Genital swab, urine
Eye	Conjunctival (and/or corneal) swab
Skin	Vesicle fluid/swab/scraping; biopsy solid lesion
Central nervous system	Cerebrospinal fluid; feces (enteroviruses)
Generalized	Throat swab[a]; feces[a]; blood leukocytes[a]
Autopsy/biopsy	Relevant organ
Any	Blood for serology[b]

[a] *Depending on known or presumed pathogenesis.*
[b] *Blood is allowed to clot, then serum kept for assay of antibody.*

COLLECTION, PACKAGING, AND TRANSPORT OF SAMPLES

- Immediately after collection the swab should be swirled around in a small screw-capped bottle containing virus transport medium.
- This medium consists of a buffered balanced salt solution, to which has been added protein (e.g., gelatin or albumin) to protect the virus against inactivation, and antibiotics to prevent the multiplication of bacteria and fungi.
- The swab stick is then broken off aseptically into the fluid, the cap is tightly fastened and secured with adherent tape to prevent leakage, and the bottle is labeled with the patient’s name, date of collection, and nature of specimen.

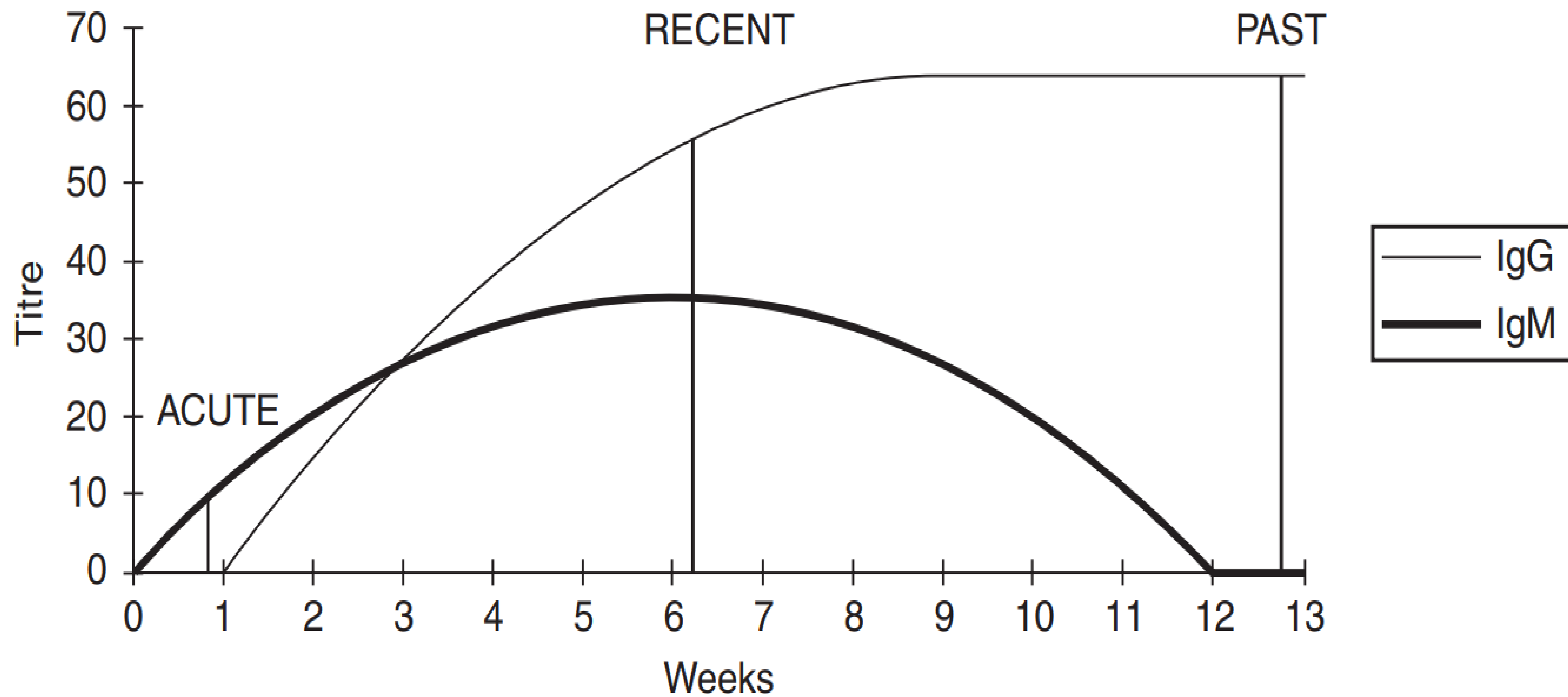
Clinical diagnosis

- Very often it is difficult
- As many viruses have clinically similar presentation (e.g. hepatitis viruses, and the same virus can have many different clinical presentations (e.g. enteroviruses)
- Experienced clinicians are not able to diagnose rubella or measles
- A specific diagnosis of a viral rash is an important, clinical management of the patient, control of infection, outbreak control and to ensure the efficacy of vaccination programs.

Serology

- Viruses cannot be easily cultured.
- ‘Serology’ means the study of serum and can be used to detect both antibody and antigen (e.g. hepatitis B surface antigen).
- Antibodies are produced as a host response to viral infection.

- Immunoglobin A is produced at the local site of infection and provides local immunity, for example in the gut or respiratory tract.
- The generalized humoral immune response is mounted by B lymphocytes and the first antibody to appear is of the IgM class, which can be detected as early as a couple of days after an acute infection.
- Some of the B lymphocyte clones then switch over to producing IgG antibody, which appears from 7–15 days after onset of infection.
- Both classes of antibodies continue to rise in response to the infection, peaking at about 6 weeks post infection.
- Viral specific IgM then declines and is normally undetectable by about 3 months after infection.
- IgG antibody persists for life and is responsible for providing lifelong immunity to the particular virus.



RECENT
PAST
ACUTE
Titre
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Weeks
IgG
IgM

Acute or recent infection

- Demonstrating the presence of virus specific IgM
- (IgG may or may not be present)
- Showing a rise in antibody titre between an acute and convalescent specimen, or a high antibody titre in a convalescent specimen.

Past infection or immunity

- Demonstration of virus specific IgG alone
- (and absence of IgM).

Principle of serological techniques

- Qualitative (e.g. give only a yes or no answer)

  Presence of IgM or IgG
- Quantitative (e.g. measure the antibody level).

  Detection of a rising or a high antibody titre
- Antibody ‘titre’ is expressed as the inverse of the highest serum dilution at which the antibody is detected.
- For example, influenza A antibody titre of 128 means that antibody to influenza A was detected until a 1 in 128 serum dilution, but not in higher dilutions.

Table 47.1. *Diagnostic uses of the serological techniques.*

Test	Example of use
Complement fixation test (CFT)	Respiratory viruses – measures total antibody, is quantitative; to diagnose recent infection acute and convalescent serum samples are required to show rise in titre.
Enzyme-linked immunosorbent assays (EIA or ELISA)	IgG/IgM antibody – rubella, measles, mumps, HIV, hepatitis A etc. Antigen – hepatitis B surface antigen in serum samples, norovirus and rotavirus antigen in faeces.
Immunofluorescence (IF)	IgG/IgM antibody – EBV, VZV. Antigen – RSV, influenza and other respiratory viruses in respiratory secretions.
Latex and gel particle agglutination	Antibody – rubella, toxoplasma. Antigen – rotavirus, norovirus.
Western blot (WB) and line assays (LIA)	Used to confirm HIV and HCV-screen positive specimens.
IgG avidity assays	To confirm recent CMV, rubella and toxoplasma infections.

Microscope- Negative staining

- Virus-containing fluid is placed on a carbon coated grid usually with prior clarification and concentration by ultracentrifugation;
- Virions adhere to the surface and become “negatively stained” when an electron dense fluid such as sodium phosphotungstate is added and surrounds the virions.
- Excess salts and proteins which affect the translucency of the specimen in the microscope.
- Characteristic virion size and structural details

Thin-section electron microscopy

- Fixed tissue sections.
- Used particularly in a research setting
- Viral etiology or where the pathology of a particular disease is being investigated

Serological techniques

- All serological techniques that detect antibody are based on the principle of adding specific viral antigen(s) to patient serum.
- If virus-specific antibody is present in the serum then it will bind to the antigen to form an antigen/antibody complex.
- An indicator system (depending on the technique) is then used to detect whether such a complex has been formed.
- These techniques can be reversed to detect the presence instead of viral antigen, such as hepatitis B surface antigen, in the patient's serum

Enzyme-linked immunosorbent assay- ELISA

- Antigen is attached to the base of a plastic microtitre well (solid phase).
- Patient's serum is added to this microtitre well.
- If specific antibody is present in the serum it will attach to the antigen on the solid phase. Excess serum is washed off.
- Anti-human antibody coupled to an enzyme is added to bind to this antibody/ antigen complex. Excess enzyme is washed off.
- A substrate for the enzyme is added, a colour change indicates a positive reaction
- Due to the action (on the substrate) of the enzyme which has been bound to the antigen/antibody complex.
- Detected by eye or measured in a spectrophotometer, and the intensity of the colour can indicate how much antibody is present in the serum.

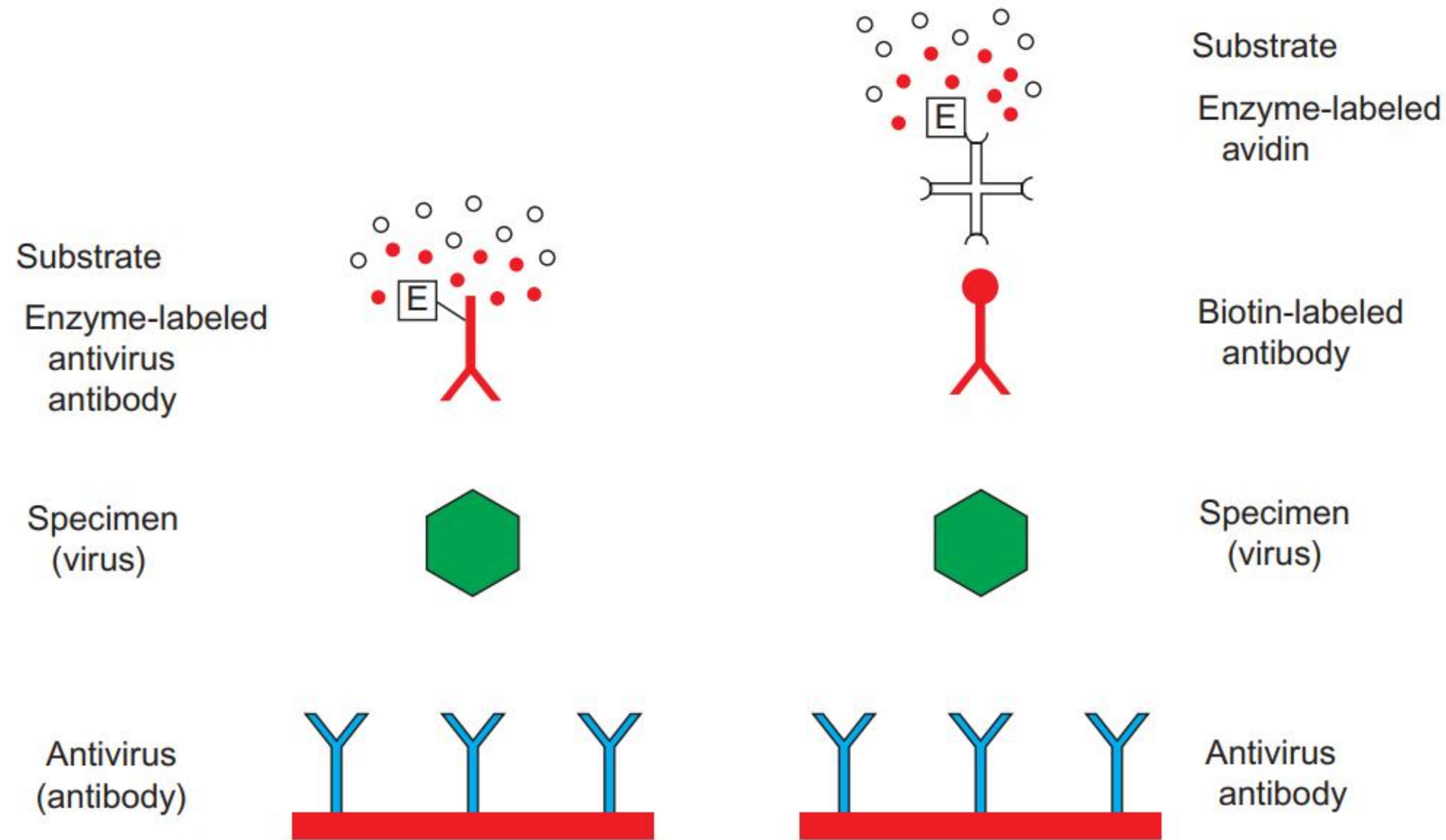


FIGURE 10.1 Enzyme immunoassays (EIA or ELISA) for detection of virus and/or viral antigen. *Left*: Direct method. *Right*: Indirect method, using biotinylated antivirus antibody, followed by enzyme- (e.g., peroxidase)- labeled avidin. In each case an enzyme substrate is then added to develop a color reaction. Note the immobilization of the capture antibody on a solid support to facilitate subsequent washing steps. *Reproduced from MacLachlan, N.J.,*

Immunofluorescence tests (IF or IFT)

- Fluorescein-labelled anti-human antibody is used to detect a positive reaction, (enzyme/substrate detector)
- Apple-green fluorescence under a light microscope.
- Cells from the patient’s secretions (e.g. nasopharyngeal aspirate) are fixed to a spot on the glass slide and fluorescein-labelled monoclonal antibody against the virus (RSV, influenza A etc.) is added.
- A mixture of these monoclonal antibodies can be added at the same time

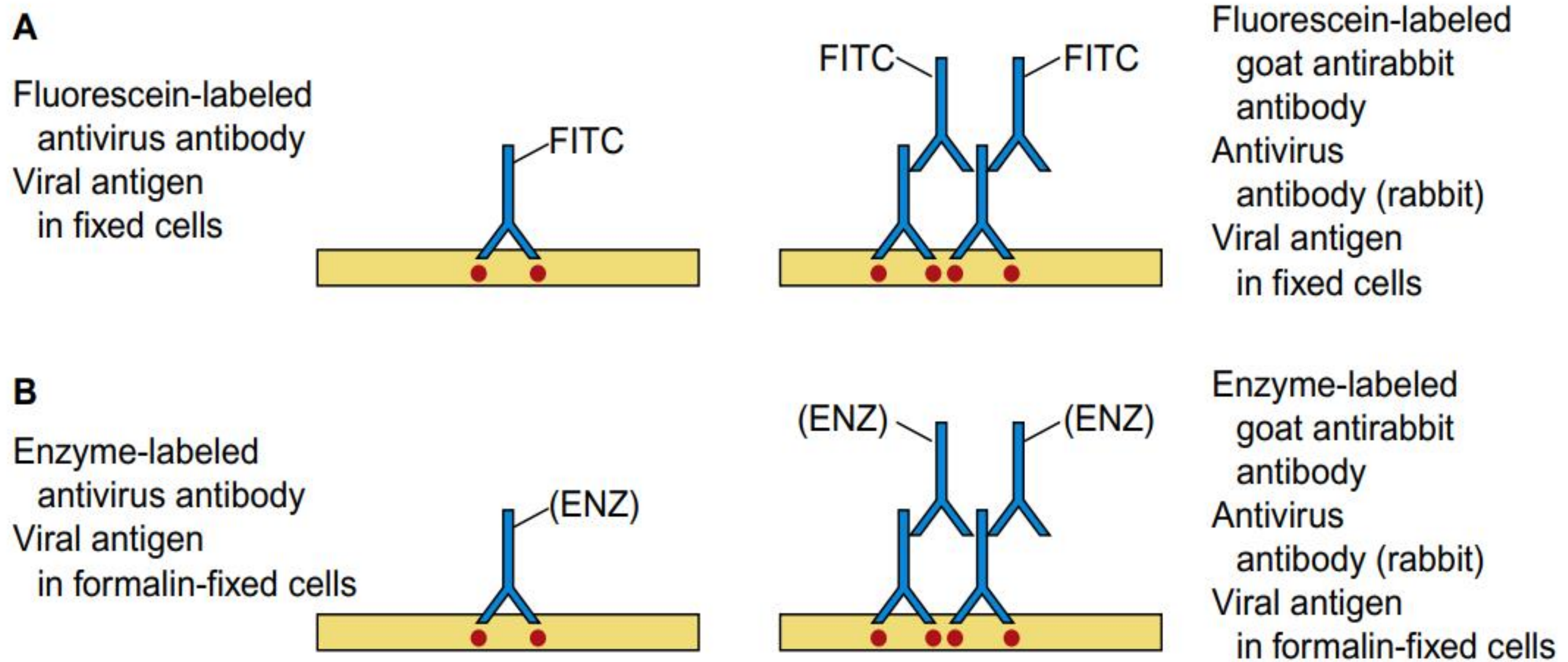


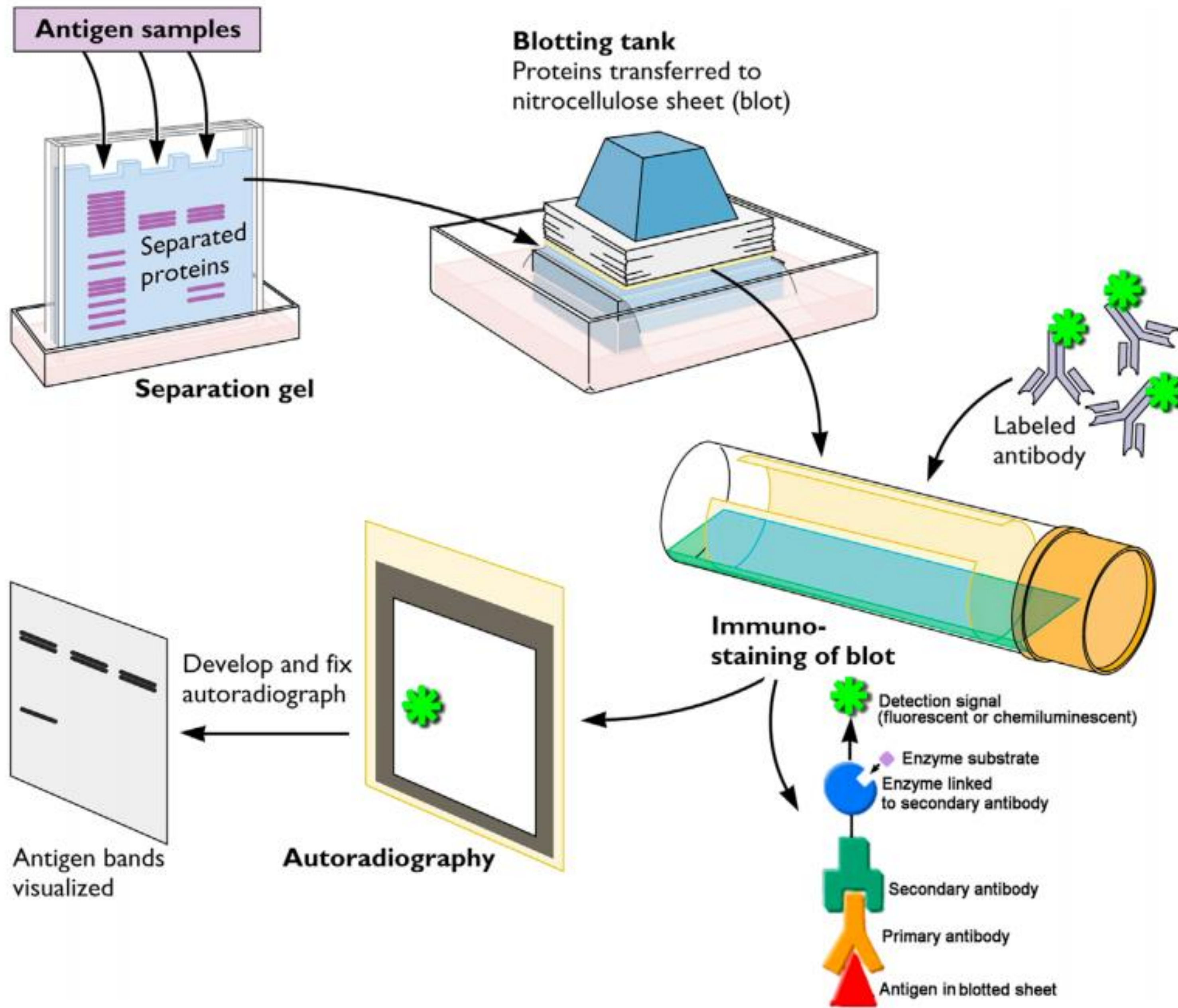
FIGURE 10.2 Detection of viral antigens in tissues or cell smears by immunofluorescence (A) and immunohistochemistry (B). In each case, th method is shown on the left, and the indirect method on the right. *Reproduced from MacLachlan, N.J., Dubovi, E.J., 2011. Veterinary Virology, fo Academic Press (*Figure 5.6*), with permission.*

Western blot (WB) or line immunoassays (LIA)

- Specific viral proteins are transferred on blotting paper either from a gel (western blot) or produced by recombination or peptide synthesis.
- Further steps are similar to those of EIA
- The viral antigen band on the blotting paper develops colour , if specific antibody to that particular antigen is present in the serum.

- A virus-containing sample is digested with the anionic detergent (SDS) and electrophoresed on a polyacrylamide slab gel (PAGE), which separates the different viral proteins according to the molecular mass.
- The bands of viral protein are then transferred ("blotted") onto a nitrocellulose membrane by capillary transfer or usually by electrophoresis in a different plane to immobilize the polypeptides.
- The unoccupied areas of the membrane are blocked ("quenched") by saturation with a suitable protein, then washed, dried, and cut into strips which can be used to test individual patient sera.
- Each test serum or plasma ("primary" antibody) is then incubated with one strip to enable antibodies to bind to the individual viral proteins.
- Following rinsing, bound antibody is detected by the addition of enzyme-labeled anti-human immunoglobulin ("secondary" antibody).
- Following another wash, the bands are revealed by the addition of a substrate chosen to produce an insoluble colored product.

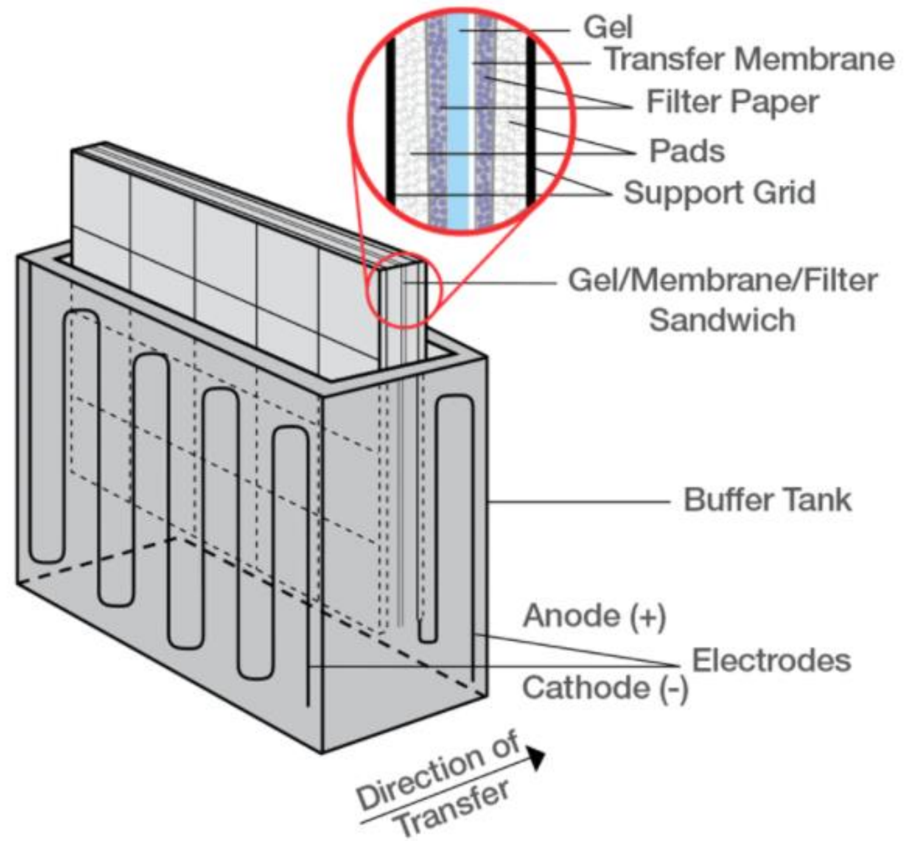
https://www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-western-blotting.html



This technique may be used with a preparation of known antigens, for example, an infected cell lysate, having been separated on a gel, to examine a serum sample containing unknown antibodies; alternatively, a known antiserum can be used to detect those antigens in an unknown sample that react after running on a gel.

Electrophoretic transfer of proteins

- Involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable, protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution.
- The process involves the use of porous pads and filter paper to facilitate the transfer.
- When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached.
- The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel.

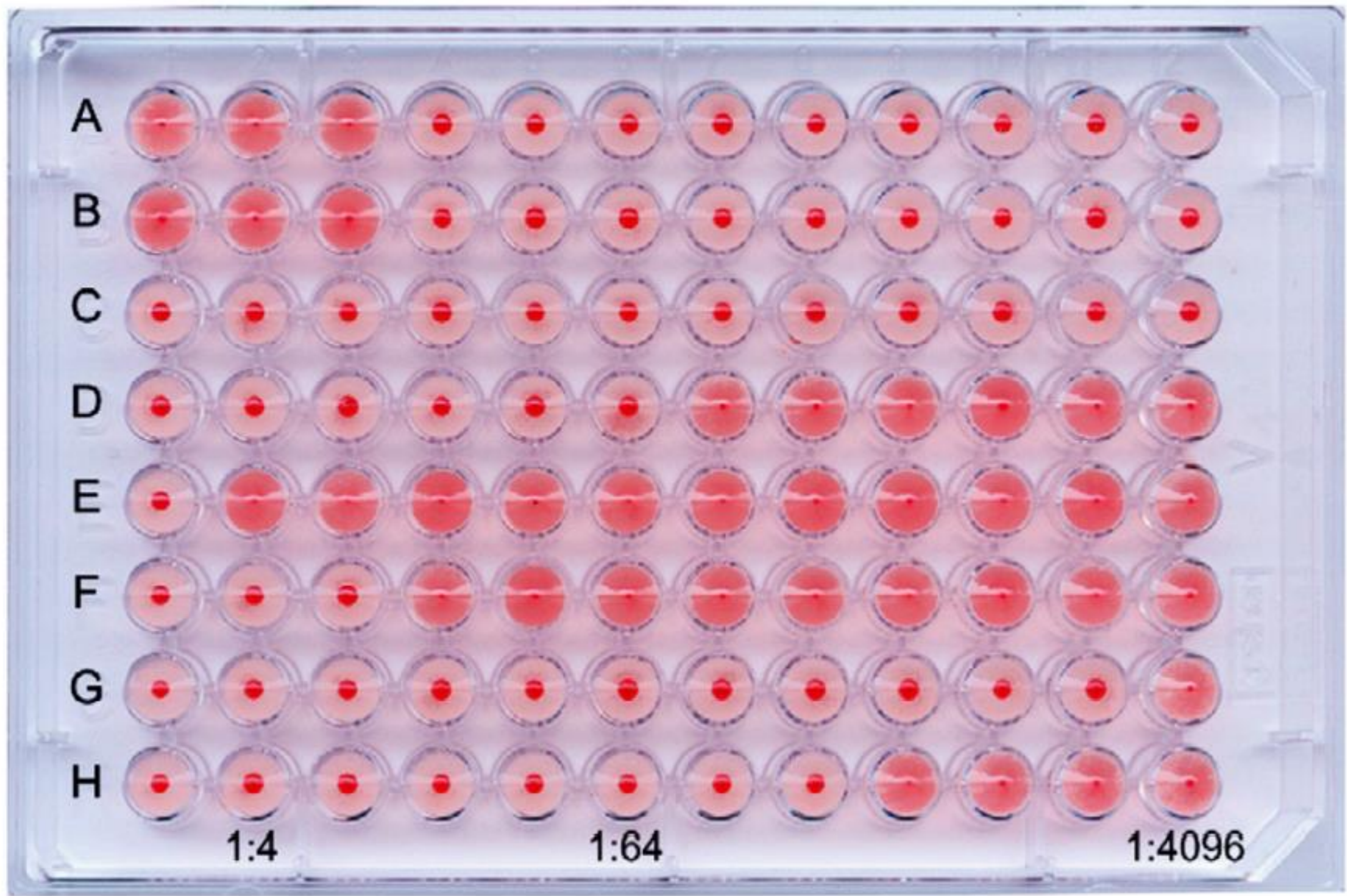


https://www.youtube.com/watch?v=7SVHqK_mFtQ

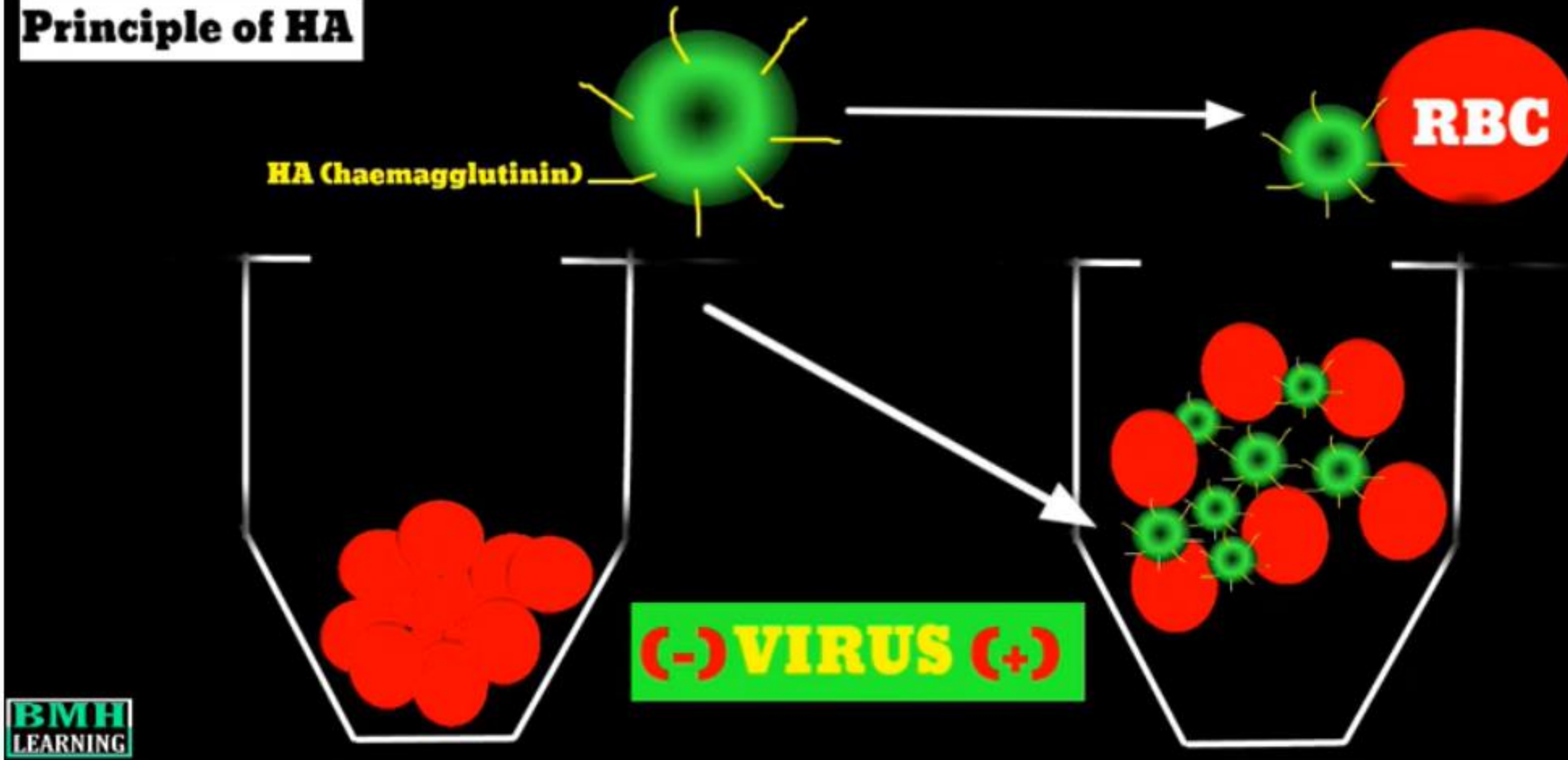
Haemagglutination (HA) and haemagglutination inhibition tests (HAI)

- These tests detect antibodies to viruses (rubella, influenza) that possess a haemagglutinin antigen.
- One of the highly pathogenic viruses - increased human-to-human transmissibility capability).
- Patient antibody inhibits agglutination of red blood cells by virus or antigen

- Sera are (A) treated to remove non-specific agglutinins and non-specific inhibitors of agglutination.
- (B) Treated sera are serially diluted (twofold) in buffered saline in rows of wells of 96-well microtiter plates.
- (C) A volume of influenza virus containing 4 hemagglutinin units is added to each well and plates are incubated for 30 minutes.
- (D) A standard volume of turkey red blood cells (0.5% suspension) is added to each well.
- (E) The plates are incubated and read when control wells show complete red blood cell settling (button)
- Where virus agglutinates the erythrocytes, these form a shield pattern; however, where enough antibody is present to coat the viral HA, hemagglutination is inhibited, and the erythrocytes settle to form a button on the bottom of the cup.



https://www.youtube.com/watch?v=nN8MBU8S4EI



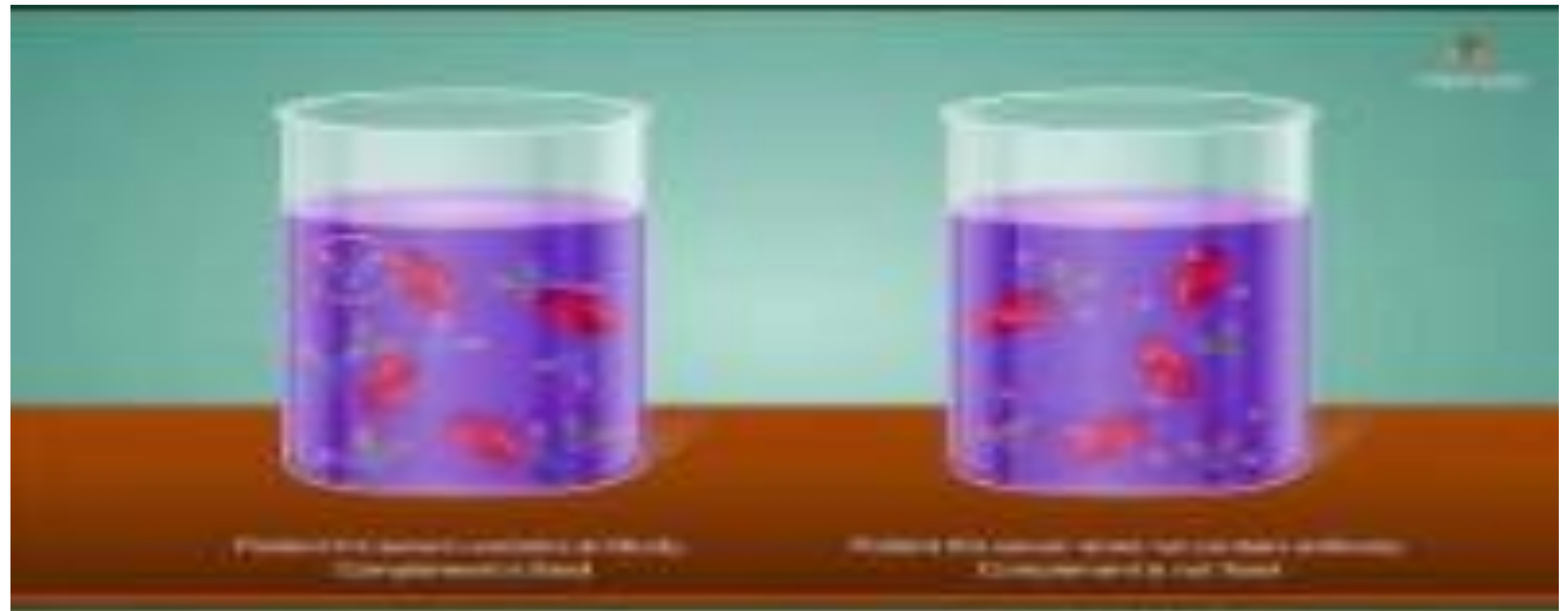
Haemagglutination Assay | HA Assay | HA Test |

Latex agglutination (LA) and gelatin particle agglutination test (GPAT)

- Here the antigen or antibody is adsorbed on an inanimate particle (latex or gelatin) and a positive reaction is indicated by agglutination of the particles

Complement fixation test (CFT)

- An antigen/antibody complex is formed it will ‘fix’ (bind) complement
- So free complement is not available to lyse sensitized red cells that are added as indicator.



DNA or RNA hybridization

- A complementary RNA or DNA probe is used to bind to the DNA or RNA viral genome.
- The DNA–RNA hybrid can then be detected using a labelled monoclonal antibody.
- This technique can be used on tissue samples (in situ hybridization),
- Viral genome can first be transferred on to a blotting paper (dot blot hybridization).

PCR polymerase chain reaction (PCR) or Nucleic acid amplification techniques (NAATs).

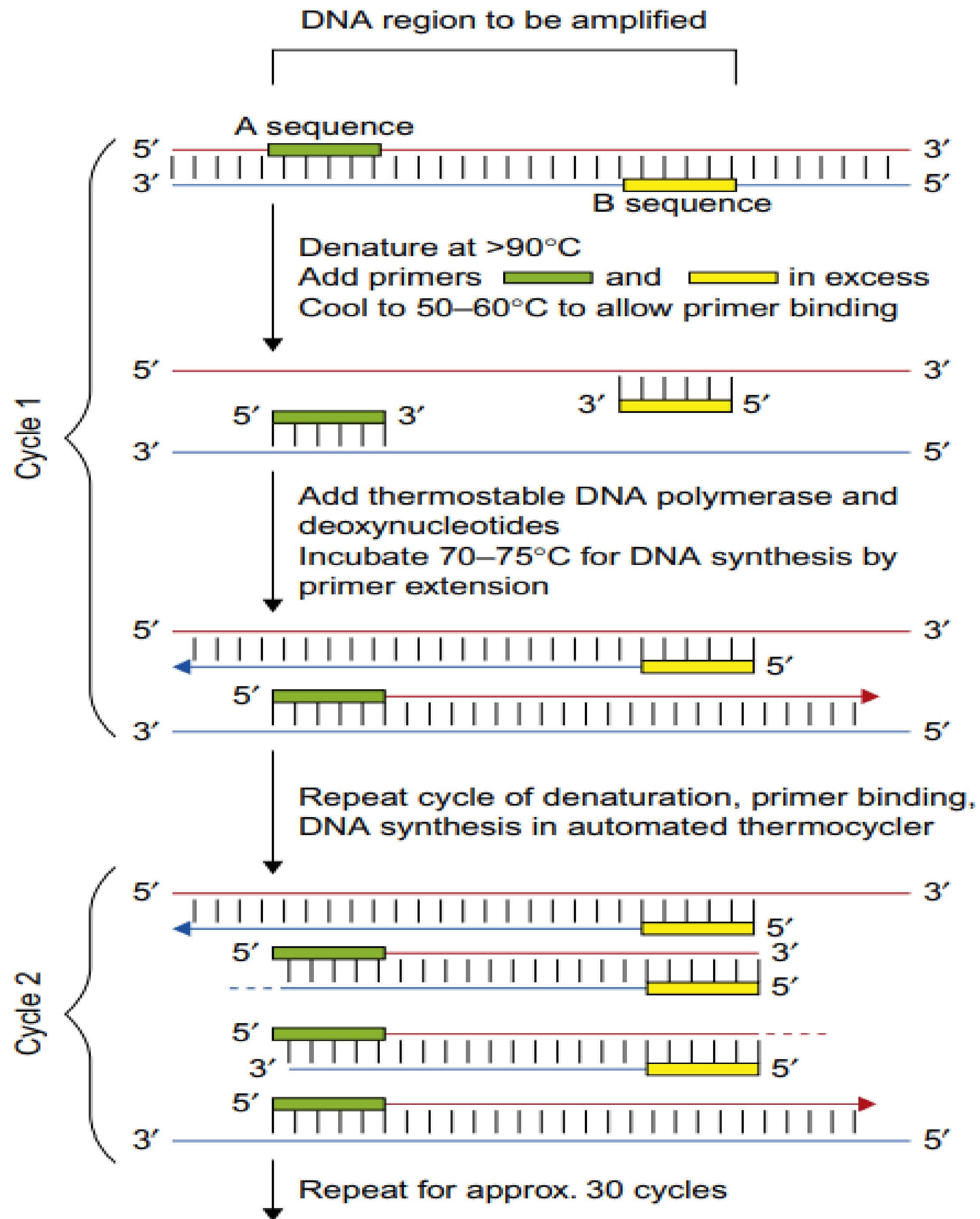
- Single copy of DNA or RNA can be amplified more than a million times.
- To detect RNA viruses, RNA has to be first transcribed to complementary DNA by means of an enzyme called reverse transcriptase; RT PCR.
- Bacterial enzyme, taq polymerase, to initiate DNA amplification.
- Extraction and denaturation of the DNA or RNA followed by amplification.

Complex chemical reactions

- Heating and cooling of the sample mix in a thermocycler.
- Each heating and cooling cycle takes only a few minutes to complete, and doubles the number of DNA copies.
- Greater than a million copies of the genome can be produced in about 40 such cycles, which take only 2–3 hours to complete.
- The amplified DNA product or amplicons can be detected by use of specific probes labelled with a chemiluminescent or fluorescent dye.

Thermal cycling

- Repeated heating and cooling to melt and re-anneal DNA along with enzymatic replication of the targeted melted strands of DNA.
- Primers (short DNA fragments) containing sequences complementary to each end of the target region of the DNA of interest must first be synthesized.
- Then, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting.
- In the second step, the temperature is lowered and the two DNA strands become templates for DNA synthesis.
- In the presence of heat-resistant DNA polymerase and deoxynucleotide triphosphates, two new copies of the desired region of the DNA are produced.
- As PCR progresses through cycles of melting, annealing, and extension, the DNA generated is itself used as a template for replication.



https://www.youtube.com/watch?v=VD5qEVTsjTc

Table 49.1. *Nucleic acid amplification techniques and their application.*

NAAT technique	DNA	RNA	Diagnostic application
Polymerase chain reaction (PCR)	Yes	Yes	Most popular of NAATs. Used to detect a wide range of viruses in different clinical specimens.
Transcription mediated amplification (TMA)	No	Yes	Messenger RNA of chlamydia.
Nucleic acid sequence based amplification (NASBA)	No	Yes	HIV, HCV.
Strand displacement assay (SDA)	Yes	No	Chlamydia.

- Nested PCR – Uses two separate amplification steps -Twice the amount of amplicons as compared to traditional PCR.
- Multiplex PCR – Can detect several different viral genomes in a single reaction mixture -Detection of several viruses at the same time
- Real-time PCR – Here the amplification and detection steps of PCR occur simultaneously (rather than sequentially) - Time shortened.
- Quantitative PCR – comparison of the amount of DNA or RNA present in the patient sample - To determine the virus quantity or viral load in the patient specimen

Viruses

- Elucidation of life's chemical foundation
- Their borderline position between living and inanimate matter for the study of basic life processes
- Viroids, a group of replicating entities still simpler than viruses
- Their study will equally contribute to our knowledge of basic biological phenomena.

VIROIDS

Viroids are low-molecular-weight RNAs

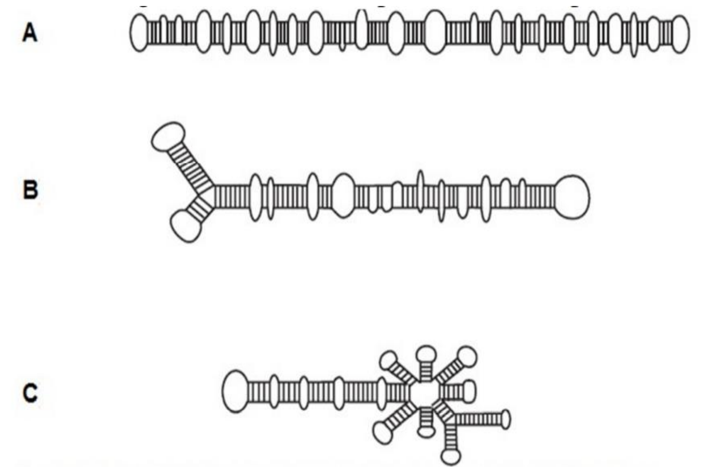
They are not found in healthy individuals of the same species, but when introduced into such individuals, they are replicated autonomously despite their small size and cause the appearance of the characteristic disease syndrome.

Thus, viroids are the causative agents of the diseases in question.

Unlike viral nucleic acids, viroids are not encapsidated

i.e., no virion like particles can be isolated from infected tissue.

Classification of Viroids



(A) Rod-like secondary structure proposed for PSTVd, the type member of family *Pospiviroidae*.
(B) Quasi rod-like secondary structure proposed for ASBVd, the type member of family *Avsunviroidae*.
(C) Complex branched conformation proposed for PLMVd.

- Two families named as Avsunviroidae and the Pospiviroidae.
- Avsunviroidae are able to catalyze self-cleavage of multimers produced during replication and do not possess a central conserved region (CCR)
- Pospiviroidae family don’t have self-cleaving properties but possess a central conserved region (CCR).
- The species are primarily defined on the basis of sequence data.

- In 1971, Theodor Diener, a pathologist working at the Agriculture Research Service, discovered an acellular particle that he named a viroid, meaning “virus like.”
- Viroids -short strand of circular RNA capable of self-replication.
- Potato tuber spindle disease, which causes slower sprouting and various deformities in potato plants
- PSTVs take control of the host machinery to replicate their RNA genome.
- Viroids do not have a protein coat to protect their genetic information.

Differ basically from those of conventional viruses

1. The pathogen exists in vivo as an unencapsidated RNA.
2. Virion like particles are not detectable in infected tissue.
3. The infectious RNA is of low molecular weight.
4. Despite its small size, the infectious RNA is replicated autonomously in susceptible cells, ie., no helper virus is required.
5. The infectious RNA consists of one molecular species only.

- Tomato planta macho viroid (TPMVd) infects tomato plants, which causes loss of chlorophyll, disfigured and brittle leaves, and very small tomatoes, resulting in loss of productivity in this field crop.
- Avocado sunblotch viroid (ASBVd) results in lower yields and poorer-quality fruit.
- Peach latent mosaic viroid (PLMVd) can cause necrosis of flower buds and branches, and wounding of ripened fruit, which leads to fungal and bacterial growth in the fruit

REFERENCES

- Principles of Virology 2nd edition by S.J. Flint, L. W. Enquist.
- Morag C and Timbury MC Medical Virology 10th edition, Churchill livingstone, London.
- Jawetz, E. Melnick J.L., Adelberg E. A. Review of Medical Microbioplogy. 19th Edition.
- Medical Virology 4th edition by David O. White and Frank J. Fenner. 14th Edition.

THANK YOU