DIAGNOSIS OF VIRAL DISEASES

There are well over 1000 known viruses of vertebrate and it is impossible for a single laboratory to have all the resources required for the diagnosis of all these viruses. Laboratories tend to specialize on particular viruses and serve as reference laboratories.

Stages of viral disease diagnosis:

1. Clinic: presumptive diagnosis based on clinical signs and history
2. Pathology: Observed lesions and pathological changes at gross and histopathological levels. History may provide clues.
3. Microbiological diagnosis: confirmatory diagnosis

Principles of microbiological diagnosis:

i. Isolation of viruses
ii. Detection of viral nucleic acid/specific genes
iii. Detection of viral antigen
iv. Detection of specific virus-induced antibody

Sample collection:

a. Samples must be collected at from the right site and at the right time
b. Samples to be collected must relate to the clinical signs and pathological changes observed
c. Samples must be collected as soon as clinical signs are observed
d. Knowledge of the pathogenesis of the disease may dictate the type of sample to be collected
e. Proper labeling of samples for identification and to avoid confusion
f. Samples must be sent to the laboratory with history and tentative diagnosis
g. Transport samples with ice packs (4 ^{O}C) if transit time is less than 24 hours
h. For transit above 24 hours, use dry ice at -70 ^{O}C.

i. Long term storage is achieved with liquid nitrogen at -196 ^{O}C.

j. Use transport medium containing buffer (isotonic saline) with bovine albumin/foetal calf serum (protein to prolong virus survival), antibiotic and antifungal agent (to prevent contaminants)

Samples for diagnosis of viral diseases:

- Respiratory tract infection: nasal swab, tracheal swab, nasopharyngeal aspirate, lung tissue.
- Enteric infection: Faeces, rectal swab.
- Genital tract infection: prepucial washing, semen, genital swab.
- Eye infection: Conjunctival swab.
- Skin infection: Vesicular fluid, epithelial scrapings, biopsy of solid lesions.
- Central nervous system: cerebrospinal fluid, faeces, nasal swab, brain tissue.
- Generalized infection: Nasal swab, faeces, blood leukocytes.
- Post mortem examination: relevant organ.
- Every case: Blood for serum to be used in serology.

Virus isolation:

Advantages:

- It allows for further studies on the virus isolate
- It is required for preparation of vaccines
- It is required for preparation of antigens for rapid diagnostic kits
- It is highly sensitive and reliable

Disadvantages:

- It is slow and time consuming
- It is labour intensive
- It is expensive to acquire and maintain required facilities

- Some viruses may not grow
- Selection of appropriate media may require critical consideration

Methods of virus isolation

1. Virus isolation in cell culture
2. Virus isolation in embryonated egg
3. Virus isolation in laboratory animals
4. Virus isolation in susceptible host
5. Virus isolation in arthropods

Virus isolation in cell culture

- This is the most commonly used method of virus isolation.
- It involves inoculation of sample onto confluent tissue culture monolayer.
- The inoculated tissue culture monolayer is incubated at 35-37 ^{O}C.
- The culture is examined daily for evidence of virus growth (cytopathic effect, interferon, haemadsorption and antigen detection).
- Virus is harvested from the culture by freeze-thawing and low-speed centrifugation
- The supernatant is used as antigen for virus identification
- The effect of the growing virus in cell culture can be detected by light microscopy

Types of tissue culture

Primary tissue culture: this is made directly from tissue. It may involve one or two passage. In primary tissue culture, there are mixed cell types. It is good for isolation of influenza virus, parainfluenza virus and enteroviruses. Three types are recognized: monolayer, suspension and organ culture.

a. Monolayer: derived from tissue taken directly from the susceptible host (monkey kidney, mouse embryo). The tissue is cut into very small pieces and digested into cells by proteolytic enzymes such as trypsin or collagenase. The cells are grown on glass or plastic surface as monolayer in as artificial medium containing growth factors supplemented with foetal calf serum and antibiotics.

Type of artificial medium:

- Growth medium: for cell cultivation. Its constituents include salts at physiological concentration, glucose, amino acids, vitamins, antibiotics and antifungal agents and 10-20% foetal calf serum. It is maintained at pH 7.2-7.4. After formation of confluent monolayer, the growth medium is changed to maintenance medium.
- Maintenance medium: contains similar constituents as growth medium except that the foetal calf serum is 2 -5% for survival of cells and no further division.

b. Suspension culture: cells are grown in form of suspensions in growth medium and not monolayer. Lymphocyte cultures are cultivated in suspension.
c. Organ culture: organ culture are not trypsinized into cell but grown as whole. Examples include tracheal ring organ culture.

Secondary culture: they are obtained from primary culture by passages or subcultivation. Repeated passages of primary culture will produce cell lines with almost homogenous cell type. Types of secondary culture include semi continuous cell line and continuous/established cell line.

a. Semi continuous cell line: this is derived from fibroblastic cell of animal or human foetal tissue. They have been subcultured through about 50 passages (generations of repeated

subcultivation). They have diploid number of chromosome. They are used for vaccine production. They have limited life span. Examples include HDCS, MRC-9, WI-38. This type of cell culture is good for herpes simplex and rhinovirus.

b. Continuous cell line: this is also called established cell line. Hey are widely used fro diagnostic purposes. They are derived from neoplastic cells or from normal cells that have been transformed by repeated subcultivation to behave like tumour cells. Continuous cell line can grow indefinitely. They have heteroploid (aneuploid) chromosome (variable/abnormal number of chromosome). It is good for vaccine production and research purposes. Examples include Mardin Darby bovine kidney (MDBK), MDCK, Vero cell (African green monkey kidney cells), Hep, HeLa, Crandell feline kidney (CRFK).

Detection of viral growth in cell culture

- Observation for cytopathic effect: evidence of growth of virus in cell culture is by the detection of cytopathic effect (CPE). Cytopathic effect is defined as degenerative changes caused by the growth of viruses in cell culture or virus-induced damage in cell culture. The following are examples of cytopathic effect which are usually observed by microscopy:

1. Cell lysis or cell disintegration
2. Syncythial formation: formation of multinucleated giant cell due to cell fusion. This is found with lentivirus, herpesviruses, paramyxoviruses
3. Rounding up of cell or cellular transformation (change from spindle shape to spherical shape)
4. Formation of intranuclear (adenovirus, herpesvirus, parvovirus) or intracytoplasmic (poxvirus, rhabdovirus, reovirus) inclusion bodies.

Viruses can be categorized into *burster* or *creeper* viruses based on the type of CPE produced.

Burster (lytic) viruses: these induce cell lysis and cellular transformation in cell culture

Creeper viruses: these induce formation of multinucleated giant cells.

- Plaques: Macroscopic/gross observation of CPE on monolayer cell by overlaying with molten agar. Plaques are foci of dead virus-infected cells which do not take up the stain (acridine dye) when stained and so appear as clear spots in a stained monolayer.
- Haemadsorption: adherence of erythrocytes to monolayer cells because of the growth of haemagglutinating virus in the cells. Haemagglutinin is expressed on the cell membrane of cells in monolayer and this attracts and binds erythrocytes. Feline panleukopaenia virus haemagglutinate porcine erythrocyte while porcine parvovirus haemagglutinate chick, guinea pig, monkey, human and cat erythrocytes.
- Immunofluorescence
- Interference
- Virus neutralization test
- Haemagglutination inhibition test

Virus isolation in embryonated egg

- This is no longer widely used
- However, it remains the most preferred for isolation of influenza A virus and avian viruses
- 10 to 12 day old embryonated eggs are used
- Eggs must be from specific pathogen-free flocks

Route of administration into embryonated egg: this is determined by tissue affinity of the particular virus and includes

- Allantoic cavity

- Amniotic cavity
- Yolk sac
- Chorioallantoic membrane (CAM)
- Intravascular inoculation in well-developed chick embryo

Evidence of virus growth in embryonated egg:

- Death of the embryo
- Dwarfing/stunting of embryo
- Formation of pock on the CAM
- Virus isolation in laboratory animals

Virus isolation in laboratory animals

Suckling mice: suckling mice is used for cultivation of arthropod-borne viruses. Suckling mice is inoculated intracerebrally and observed for signs of disease and death. Virus is identified from specimen collected from infected animal by complement fixation test, virus neutralization test or by other rapid diagnostic method.

Virus isolation in susceptible host

This involves inoculation of natural host species of the virus. This is used for evaluation of vaccine, production of polyclonal antibody and for pathogenicity testing/verification of Koch's postulate.

Virus isolation in arthropods

This is not a common method and is obsolete. Example includes isolation of Dengue virus in adult *Toxorhynchites*, male *Aedes aegypti* and *Aedes albopictus* mosquitoes by intrathoracic inoculation.

RAPID DIAGNOSTIC TECHNIQUES

These include:

1. Electron microscopy
2. Viral nucleic acid detection
3. Serology/detection of viral antigen and antibody

1. Electron microscopy

This is used to demonstrate the presence of virus in clinical specimens and to study the morphology or symmetry of the virus. With electron microscopy, it is possible to recognized mixed viral infection. It is also used for detection of non-viable viruses and those that cannot be grown *in vitro*. However, equipment required for the procedure is rather expensive. Besides, large number of viral particles in excess of 10^6/ml must be present in the sample before they can be detected. It is difficult to differentiate viruses with similar morphology especially those from the same family with electron microscopy.

Method:

- Homogenize sample.
- Centrifuge at low speed to remove large particulate debris.
- Ultracentrifugation to sediment available virus particles.
- Negative staining with heavy metal compound such as phosphotungstic acid or uranyl acetate. (negative staining stains the background to increase contrast so that bright virion stand out against a dark background).
- Addition of immune serum (immunoelectron microscopy) to increase sensitivity by clumping/agglutinating virus particles and enhance recovery following centrifugation).
- Observe at X13,000-100,000 magnification.

2. Detection of viral nucleic acid:

Oligonucleotide probes are used for the detection of viral DNA or RNA. Insufficient viral nucleic acid in sample is increased by amplification using polymerase chain reaction. Probes anneal to the targeted viral nucleic acid sequence which can be the whole genome, specific gene or nucleic acid segment. Variable or conserved sequence can be targeted. Double stranded genomes are first separated by heating. Oligonucleotide probes are labeled with radioactive isotopes such as ^{32}P or ^{35}S to allow viewing. Non-radioactive labels such as alkaline phosphatase fluorescein and horse radish peroxidase can be used for direct viewing whilebiotin and digoxigenin are used for indirect viewing.

I. Dot-blot hybridization

- Nucleic acid, usually DNA is extracted from sample
- Extracted nucleic acid is spotted directly onto charged nylon or nitrocellulose membrane
- Nucleic acid binds firmly onto membrane after baking
- Fluorescent dye- or radioisotope- labeled probe is added
- The membrane is washed to remove unbound materials
- Binding of probe to targeted nucleic acid is detected by autoradiography or by colour precipitation

II. In-situ hybridization

Viral nucleic as is detected in frozen section of infected cells with the aid of labeled oligonucleotide probes. Intracellular location of viral nucleic acid is revealed by autoradiography or immunoperoxidase cytochemistry

III. Southern blot hybridization

- Restriction enzymes are used to cleave DNA into short oligonucleotides
- Oligonucleotides are separated by agarose electrophoresis or acrylamide gel electrophoresis
- Separated oligonucleotides are transferred by blotting onto nitrocellulose membrane or nylon
- Probes are added and reaction detected by autoradiography or by colour development

IV. Northern blot hybridization

RNA hybridization similar to southern blot

V. **Western blot**

Application to protein identification

VI. Polymerase chain reaction

- Extraction of DNA or RNA nucleic acid from sample
- Amplification of the extracted nucleic acid in a series of repeated cycles of denaturation, primer annealing and polymerization using heat-resistant Taq (*Thermus aquaticus*) polymerase. Specific primers recognize and bind to the targeted gene to initiate the amplification reaction.
- Amplified sequences are stained with ethidium bromide and separated by electrophoresis
- The separated sequence is viewed under ultraviolet transillumination.
- This procedure is very specific and highly sensitive

3. Serology

For the detection of viral antigen or virus-induced antibody in the serum of the host

I. **Immunodiffusion**

Immunodiffusion is the simplest and most direct method for detecting antigen-antibody reaction. It is cheap and easy to perform but of low sensitivity. It is used for the detection of reaction of an antigen with its antibody by formation of precipitation. This reaction is influenced by the relative concentrations of antigens and antibody. Optimal precipitation is seen in the region of equivalence and decreases in the zones of antigen excess or antibody excess. Immunodiffusion can be categorized as single or double.

i. Single radial diffusion

This was introduced by Mancini in 1965. In single immunodiffusion, either antigen or antibody is static and the other reactant remains free to migrate and complex with the static component. Single radial immunodiffusion can be used to quantify the amount of serum protein or immunoglobulin in a sample.

Method:

- Molten agar mixed with a specific antibody to a particular antigen is poured into a Petri dish
- After the agar solidifies, a well is made at the centre
- A precisely measured amount of sample containing the antigen is poured into the well
- The antigen is allowed to diffuse radially from the well for 24 – 48 hours.
- Where the antigen meets its antibody, a precipitin ring is formed.
- The diameter of the ring is measured by a precision viewer (a calibrated microscope)
- The diameter of the ring correspond to the concentration of the antigen in the sample
- A ‘standard’ is prepared for comparison
- To prepare the standard, a serial 1 in 2 dilution of known quantity of standard antigen is prepared.

- About four wells are created in the agar and each dilution of the standard placed in different wells
- This is allowed to react and form precipitin rings at the same time interval as the test sample
- The amount of antigen in the test sample is calculated and compared with those observed in the different dilutions of the standard.

ii. Double diffusion in agar (Ouchterlony test)

In this technique, both the antigen and the antibody are free to move towards each other through a semisolid medium (agar) and form detectable precipitate (immune complex). This method permits the comparison of the relatedness of antigens in different samples.

Method

- Molten agar is poured into a Petri dish or layered on a glass slide.
- The agar is allowed to solidify and small wells of equal diameter punched in the agar
- One central well and 6-8 peripheral wells a few millimeter apart are thus made
- Known antibody is put in the central well
- Different test samples containing antigens or different serial dilutions of a particular sample are placed in the peripheral wells
- These are allowed to diffuse towards one another and react to for precipitin line in a moist chamber for 18 – 24 hours
- Observe for precipitin lines
- The concentrations on the antigen and the antibody determine the thickness of the precipitin line and its distance between the wells.
- The types of patterns of precipitin lines shows the relatedness (complete identity, partial identity and non-identity) of the antigens in the peripheral wells

II. Immunofluorescence (IF) (Direct method)

- Smear of clinical sample is made of microscope slide
- The sample is reacted with specific antiserum coupled with fluorescein isothiocyanate (FICT) fluorescent dye
- Excess antiserum is washed away
- View under ultraviolet microscope
- FICT-labelled antigen-antibody complex is visible as a green fluorescence in positive sample

Immunofluorescence (indirect method)

- Similar to the direct IF except that the specific antiserum is not labeled
- Fluorescein-labelled antiglobulin is added after the addition of the unlabelled specific antiserum to detect the presence of antigen-antibody complex
- This method is called the 'sandwich' method because there are three layers instead of two as seen in the direct method. The three layers are: the specimen to be tested which is the source of antigen, unlabelled virus specific antiserum (prepared in rabbit) and fluorescein isothiocyanate-labelled antirabbit antiserum
- The advantage of this method is that only one labeled serum (antispecies antiserum) is required to test for many viruses.

Note: Immunoperoxidase (enzyme) can be used as the label. It will react with its substrate to produce a precipitate that can be detected by light microscope without the need for ultraviolet microscope.

III. Enzyme Linked Immunosorbent Assay (ELISA) and Radioimmuno Assay (RIA)

- Polystyrene wells of a microtitre plate are coated with specific capture antibody
- Specimen from which antigen is to be detected is added
- Excess is washed away

- Enzyme-labelled specific antibody (detector antibody) is added. Enzyme can be horse radish peroxidase or alkaline phosphatase
- Excess is washed away
- Substrate that will react with the bound enzyme on the labeled specific antibody is added
- Reaction of the substrate with the enzyme produces a visible colour change which is detectable by spectrophotometry
- RIA is similar to ELISA but the label is radioactive iodine (^{125}I). Bound antibody is measured by a gamma counter. This technique is now obsolete because of health hazard associated with radioactive label used and has been superseded by ELISA.

IV. Virus neutralization test

- In microtitre plate wells, make 1:2 serial dilution of serum from which antibody is to be detected
- Add constant amount of stock virus of interest to each of the wells
- Add equal amount of susceptible cell to the wells
- Observe for CPE
- There will be no CPE in positive serum sample with high antibody titre because of the neutralizing effect of the antibody on the stock virus
- End-point is where virus neutralization stops (that is the well just before the onset of CPE)

V. Haemagglutination Inhibition (HAI) Test

- This assay is specific, easy and reliable
- Make twofold dilution of test serum
- Add known concentration of stock virus of interest (usually 4 haemagglutinating/HA unit)
- Add 0.5% erythrocyte
- Observe for haemagglutination

- There will be no haemagglutination in positive serum sample with high antibody titre because of the inhibitory effect of the antibody on the stock virus
- The highest dilution that inhibit erythrocyte agglutination is the HAI titre
- Non-specific inhibition of haemagglutination in sera can be inactivated by heating at 56 ^{o}C for 30 minutes or by treatment with kaolin, trypsin periodate or bacterial neuraminidase

VI. Complement fixation test

This method is no longer widely used. It is based on the principle that the reaction of an antigen with its antibody results in the formation of immune complex which activates and fixes complement. Free (unfixed) complement causes haemolysis while fixed complement does not cause haemolysis. The technique is not very sensitive and large amount of virus/antigen is required in the sample.

Method

- A known antiserum is heat-treated to destroy complement
- The test sample is added to the known antiserum
- The mixture is incubated and allowed to react
- A precise amount of guinea pig complement is added
- If the test sample is positive (presence of virus/antige), antigen-antibody complex is formed with the known antiserum. This will fix the guinea pig complement
- Sheep red blood cells treated with specific rabbit antibody are added to the reaction as indicator to detect complement fixation
- Unfixed guinea pig complement will cause lysis of the sheep red blood cells indicating a negative reaction
- Fixed complement will not cause lysis of the sheep red blood cells indicating a positive reaction.

INTERFERENCE

Interference occurs when infection of a host cell with one virus prevents superinfection of the same cell with another virus. This is a situation that plays out when the multiplication of a particular virus in a host cell population leads to resistance against superinfection and multiplication of another virus in the same host cell population.

Types of Interference

Heterologous: Interference: Interference between two completely different viruses.

Homologous: Interference: Interference between two related viruses or two strains of viruses.

Autointerference: Interference between a virus and its defective particles**.**

Mechanism of Interference

1. Change on receptor sites thereby preventing the attachment of the second virus
2. Competition for cellular sites and enzymes
3. Altering the metabolic pathway for the replication of the second virus
4. Inducing the production of interferon

INTERFERONS

Interferons were first discovered by Lindenmann and Isaacs in 1957. Interferons are non-viral proteins with molecular weight of about 20 Kilo Dalton (KDa) produced by cells (especially leukocytes and fibroblasts) in response to virus infection and stimulation by natural or synthetic double stranded RNA and Chlamydiae.

Classes of Interferons

1. Type I interferons: These are alpha and beta interferons (IFN-α and IFN-β). Alpha IFN is sunthesized by virus-infected monocytes, macrophages, epithelial cells and fibroblasts while

IFN-β is produced by epithelial cells and fibroblasts. They have common receptor and are stable at pH 2.0. Type I interferons are activated by natural killer cells and they enhance antigen processing and presentation to T- and B- lymphocytes.

2. Type II interferon: Gamma interferon (IFN-□). This is produced by activated T-cells in response to previously encountered antigen. It is unstable at pH 2.0.

Properties of Interferons

i. They are produced very early in the course of virus infection (usually within 48 hours)
ii. They are not produced by virus but by virus-infected cells
iii. They are not virus specific: Interferon produced against a virus is equally effective against another virus
iv. They are species specific: Interferon produced in one species of animals is not effective in another species
v. They do not directly kill viruses
vi. They do not act as antibody
vii. Double stranded RNA are the principal inducers of interferons

Major functions of Interferons

i. They inhibit viral replication
ii. They inhibit cell division
iii. They modulate immune response to infection

- They enhance the display of histocompatibility antigens on cell surfaces which helps in antigen-driven activation of T-cells
- They modulate B- and T- cells activities
- They enhance the cytotoxicity of natural killer (NK) and cytotoxic T cell (T_C)

Interferons have been used as adjuncts to immune-therapy and chemotherapy of cancer because of their immunomodulatory action and inhibitory effect on cell division.

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