GENERAL AND SYSTEMIC VIROLOGY
(MICRO – 303)

PRACTICAL NOTES

FOR

DVM 5th Semester

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VIRO PRACTICAL 1

VIRUSES

These are strict intracellular parasites which replicate by using host cell machinery.
In viruses, there is no division (like bacteria) but they always replicate (formation of a number of copies similar to the parent).
Viruses holding the position in between the living and non-living organisms and therefore it can be observed that their crystals can be formed.

MAIN POINTS TO DIAGNOSE VIRAL DISEASES

Following are some essential points which are very important in diagnose point of view.
1. History
2. Signs and Symptoms
3. Postmortem examination
4. Isolation and Identification

1. HISTORY

This is very important and necessary step to go forward for a successful diagnose. For a disease diagnose, one should have to know about the age, sex, and breed, vaccination schedule, feeding habits and housing type of the diseased animal.

2. SIGNS AND SYMPTOMS

In case of viral diseases, usually nervous signs are very visible. For example; hydrophobia, excitement.
In furious form of rabies, the suffered animal chases the moving objects blindly.
Thus, the signs and symptoms lead us to a tentative diagnosis of a disease.

3. POSTMORTEM EXAMINATION

Viruses have special affinity for specific organs. For example,

- If we found hydro pericardium in birds then it can be said that this case may be of Hydro-Pericardium Syndrome (HPS).
- During postmortem exam. of birds, If we see that the bursa is swollen and hemorrhagic and the age of the bird is about 2 weeks – then we can say that this case might be of Infectious Bursal Disease (IBS).
- If there is enlargement of spleen in birds – then this case may be of New-Castle Disease.
- In FMD, animals may show fluid filled vesicles on their extremities and in buccal cavity.

4. ISOLATION OF VIRUSES

a) Sample Collection:

- The sample should be collected only from the infected organs or tissues. In case of Influenza – nasal secretions.
- The sample should be collected at the peak stage of the disease but before the antibodies (Ab) production. As we know that viral infections are time-limited thus if antibodies have produced before taking a sample, these will try to neutralize the effect of viral particles.
- Sample collection must be done by keeping in view the sterilized conditions.
- Sample must be leaked proof and labeled properly.
- If we are working in field conditions, then ice packs should be used to prevent the sample from any harsh environmental conditions.
- For transportation of sample, transport media must be 4% solution of or Hank's balance salt solution.

b) Processing of Tissue Sample: will be discussed in next practical class.
c) Purification: It is very necessary step to get viruses – containing pure suspension.

5. IDENTIFICATION OF VIRUSES

For this purpose, many diagnostic laboratory tests can be performed. Following is the list of tests usually performed for identification of viruses.

i) Haemagglutination test
ii) Haemagglutination inhibition
iii) Indirect Haemagglutination
iv) Agar Gel Precipitation Test (AGPS)
v) Enzyme Linked Immuno-Sorbant Assay. (ELISA)
vi) Fluorescent Antibodies Technique (FAT)
vii) Complement Fixation Test (CFT)
viii) Cell Culture
ix) Polymerase Chain Reaction (PCR)
x) Single Radial Hemolysis Test

VIRO PRACTICAL 2

COLLECTION OF CLINICAL SAMPLE

Collection of suitable samples at right time in a proper way is very important for laboratory diagnosis of viral infections. As we know that almost all the viruses have special affinity for specific organs, thus *collect a clinical sample from the specific organ only.*

DISEASE NAME	CLINICAL SAMPLES OF CHOICE
Hydro-pericardium Syndrome	Liver tissue
Infectious Bursal Disease (IBD)	Bursa of Fibricius and serum
Foot and Mouth Disease (FMD)	Epithelia covering the vesicles in feet and mouth
Rabies	Brain pieces particularly the cerebellum etc.
Rinderpest	Whole blood, serum samples and pieces of spleen
Bluetongue	Whole blood, serum, pieces of lymph node & spleen
New-castle disease	Pieces of spleen
Marek's disease	Feather follicle and serum

PROCESSING OF A CLINICAL SAMPLE

For isolation/purification of virus, it is important to process the sample.
The processing of a sample includes;

i. Homogenization
ii. Centrifugation
iii. Chloroform treatment
iv. Filtration
v. Ultra-centrifugation (Sucrose gradient/CsCl)

HOMOGENIZATION

It is the one of the important step towards the processing of clinical sample. The main objective of homogenization is to disintegrate and disrupt the cells so that the virus may come out of cell. This process can be carried out by;

1. Sand treatment
2. Electrical Homogenizer

1. Sand Treatment

It is an old method which is quite laborious and regarded as time consuming also.
Step1: Take a sample size of 15-20 grams from a viral-infected organ.
Step2: Chop it uniformly with a sterilized scalpel or scissors.
Step3: Mix this sample now with sterilized sand and then, homogenize in Pestle-Morter.
Conclusion

Sand particles will disintegrate the cells and they became separated. During homogenization, sand particles will also damage the cell membrane. As a result, virus will come out of cell.

2. Electrical Homogenizer

By this method, the homogenesity is more efficient. The cells do not burst. Unlike sand treatment, it is not time-consuming and can be performed much quick.

Step1: Take a sample (15-20 grams) and place in a glass beaker.

Step2: Add an adequate amount of normal saline (NS) ➔ Tissue pieces + NS

Step3: Homogenize this suspension by using an electrical homogenizer.

Step4: Keep this suspension on 3-4 shorts of homogenizer.

The duration of each short should be **20-30 sec**. It should never exceed from this limit, otherwise burning of motor may happen due to production of more heat.

Step5: After this process, now go for <Sonification>.

SONIFICATION

It is a process by which living tissue or cell can be damaged through sound waves bombardment. *Ultra-sonifier:* An instrument used in sonification.

Step1: Placed the sample in ultra-sonifier and give it 2-3 shorts of duration 10 sec each.

Conclusion: The sound waves emitting from the ultra-sonifier disrupt the cells and thus, the virus may come out of the cell.

CENTRIFUGATION

After homogenization of the clinical sample, go for preliminary centrifugation.

Step1: Make a suspension (10%) of the tissue sample in normal saline solution.

Step2: Centrifuge this suspension at 5000rmp for 3-5 min.

Step2: Collect the supernatant for further processing.

CHLOROFORM TREATMENT

After centrifugation, if the suspension is semi-transparent or quite opaque in appearance. Then we have to go for chloroform treatment. This will make the suspension more clear and transparent. We can apply this treatment again and again for a suspension until it becomes clear and transparent. **CAUTION:** This treatment is not applicable for all types of viruses.

Step1: Take equal volumes of virus-containing suspension and chloroform in a glass test tube.

e.g. If we have 4ml of suspension then, add 4ml of chloroform in it.

Step2: Centrifuge this suspension at 1500rpm for 3-5 min.

Step3: After centrifugation, we will observe three distinct layers;

i) Supernatent/uppermost (virus containing layer) ii) Middle one (coagulated protein layer) iii) Bottom layer that will be more transparent (Chloroform)

Step4: Collect the supernatant to go for filtration.

FILTRATION

In filtration, usually membrane filters are used which are of varying pore sizes. The main purpose of filtration is to remove all the bacteria and spores of fungi from the suspension.

Step1: First of all, take a filter of pore size 0.8 um and filter the suspension.

Step2: Then pass the suspension through the filter of pore size 0.45um

Step3: Finally filter the remaining obtained suspension from the filter of 0.2um.

Important Point: By passing the suspension in the filter of pore size 0.2um, the bacterium of the smallest size (0.2um) will also be trapped easily.

Hence, after implementing the filtration process, we will be able to get a semi-purified form of suspension that will be further processed under ultra-centrifugation to get a purified from.

ULTRA-CENTRIFUGATION

Ultra-centrifugation: It is some sort of centrifugation which is carried out by a special type of centrifuge run with a speed of 60,000 – 100,000 rpm.

Ultra-centrifugation can only be performed under vacuum condition. The temperature should be maintained at 4^0C throughout the centrifugation process. ***Important Note:*** The air resistance and high temperature (than 4^0C) may result into a burning process.

Step1: Prepare different sucrose/cesium chloride concentration gradient in the separated test tubes.

Important Note: The concentration gradients are prepared according to the known densities of the suspected virus in the under-process suspension.

For example, we take 4 sucrose concentrations i.e. 10%, 20%, 30%, 40%, while keeping in view the density of the virus.

Step2: Take 5ml of 10% sucrose solution and put in a glass tube.
Step3: Placing it in deep freezer and let it to freeze.
Step4: Likewise, add all sucrose concentrations one by one and freeze after each addition.
Step5: Finally after removing the tube from the freezer, Add a few milliliter of viral suspension.
Step6: Centrifuge the suspension at 90,000rpm for 2-4 hours at temperature 4^0C.

OBSERVATIONS

Virus crosses the sucrose gradient and stays at the suitable layer where its density matches.
If the suspension contains only one type of virus, then, a distinct band will be visible.
But if it possesses more than one type of virus, then according to types of virus, varying number of bands may be examined.

How to Get Purified Viral Suspension?

Step7: Use a disposable syringe and inject the needle in the clearly remarkable band.
Step8: Collect the purified viral suspension with much care.

Usage of Purified Viral Suspension

1. It can be used for virus growth/culture purposes.
2. It can be used for making a vaccination.
3. It can be used for the direct demonstration of the virus.

VIRO PRACTICAL 3

WASHING OF RED BLOOD CELLS (RBCs)

We need washed RBCs for different diagnostic tests such as;
i. Haemagglutination
ii. Haemagglutination Inhibition
iii. Indirect Haemagglutination
iv. Complement Fixation Test

Procedure: The whole process includes;
1. Collection of blood
2. Procurement of RBCs
3. Washing of RBCs
4. Preservation of RBCs

1. COLLECTION OF BLOOD

Step1: Collect the avian or mammalian blood for which we are going to making a diagnostic test.
Step2: Take a glass flask which should has contained many glass beads inside.
Step3: First of all, sterilized this flask and then, pour the blood into it.
Step4: Shake the flask gently to prevent the clotting of blood.

The shaking of blood is also termed as ***defiberinization***. It may be of two types:

a) *Mechanical defiberinization:*

In this type, gentle shaking is done by mean of hands in order to prevent the clotting of blood. It is regarded as an ideal process of shaking of blood but vigorous shaking should avoided at all.
OUTCOME OF VIGOROUS SHAKING: Glass beads present in the glass flask will cause hemolysis of RBCs.

b) *Chemical defiberinization:*

In this type; a variety of anti-coagulant substances is being added into the blood that inhibit clot formation.
Mostly used substances are; Heparin, EDTA, Sodium citrate.

2. PROCUREMENT OF RBCS

After collection of blood, the next step is to procurement of RBCs from the blood.
Step1: Fill the centrifuge tube with the blood.
Step2: Place this centrifuge tube in the centrifuge machine.
Step3: Centrifuge at 1500 rpm for 3-5 minutes.

Conclusion:

After centrifugation, we can observe the tube for three distinct layers;
First layer(supernatant) contains the plasma of the blood.
Second layer is very thin which contains WBCs.
Third one is very packed and thick layer contains RBCs. As RBCs are the heaviest particles among all, thus they will settle down at the bottom of the tube to form a distinct layer.
What we have to do?
Step4: Discard the above two layers (of plasma and WBCs) with much care
Step5: Procure the RBCs from the third layer. RBCs are known as Packed RBCs.

3. WASHING OF RBCS

After the procurement of RBCs, washing of RBCs is conducted.
Step1: Add an adequate amount of phosphate buffer or normal saline solution.
Step2: Mix it gently and then go for centrifugation.

Conclusion:

As a result of centrifugation, RBCs will settle down at the bottom and the supernantant now will be of straw colored which is due to remnants of RBCs.
Step3: Discard the supernatant (straw-colored) and seek for packed RBCs at the bottom of the tube.

Important Indication:

Wash the RBCs until we may get the clear supernatant by using the same process (→ mixed with buffer solution → centrifuge → discard the supernatant).

Result:

The clear and transparent supernatant indicates the perfect washing of RBCs.

4. PRESERVATION OF RBCS

After the procurement of washed RBCs, it is very essential to preserve the RBCs for using them in future.
After preservation, the RBCs can be used for diagnostics tests whenever they are needed.
Step1: Make a working dilution of RBCs in the normal saline solution as follows;

1% v/v (Mixed 1 ml of RBCs + 99 ml of normal saline solution)

The working dilution can be prepared of following strength; 0.8% v/v (0.8 ml of RBCs + 99 ml of NSS)

***Important Note*:** Distilled water should never be used for any process; i.e. washing, preservation. It causes a drastic change in osmolarity of the solution which results into bursting of RBCs → Hemolysis of RBCs.

VIRO PRACTICAL 4

HAEMAGGLUTINATION TEST (HAT)

Many families of viruses have the property to bind to erythrocytes (RBCs) of different species through complementary receptor sites on RBCs surface. The binding of erythrocytes produces visible agglutination, termed viral haemagglutination. This test is performed to observe the haemagglutination potential of the viruses.

Example

Newcastle disease virus (*ND virus*), Egg dropping syndrome virus (*EDS virus*) i.e. adenovirus and Influenza virus

Swine Flue Virus (H1N1) is also a best example of such viruses that may cause haemagglutination.This virus presents *two types of proteins* in the form of many short projections or spikes on virion surface.

H1 = Haemagglutinins

N1 = Nuramanidase

The infectivity of H1N1 virus mostly based on the presence of Haemagglutinin protein on the viral surface.

Interaction between Viral Proteins and RBCs

In a normal saline solution, when red blood cells (RBCs) are poured in a vial/glass flask, they gathered at the bottom and exhibit a clear "*Button*" formation.

But when a sample of haemagglutinating virus is being mixed with the RBCs (RBCs+ Normal Saline Solution), then there is sudden agglutination reaction between the virus particles and RBCs → Clump formation → RBCs remain suspended in the solution, not gather at the bottom.

No agglutination of RBCs indicates the formation of button.

Hence, the interaction of virus (in terms of viral proteins) and Cells i.e. RBCs is due to recognition of specific receptors on the cell surface. Receptor on the Cell surface is*; N-Acetyl Neuramanic Acid* (NANA)

DEMONSTRATION OF HAEMGGLUTINATION TEST

Step1: Take the spleen sample if the individual is suspected for ND-virus infection (New castle disease virus)

Step2: After taking the sample, our destination is to get the purified virus suspension so follow the following procedure which we have already discussed in the Practical 2.

Take the sample → Chop it and mix with normal saline solution → Homogenization → Sonification → Initial centrifugation → Chloroform treatment → Filtration (for obtaining clear fluid) → Ultracentrifugation

Step3: After ultra-centrifugation, we have got the purified virus suspension.

Step4: Inoculate that suspension into the embryonated chicken egg.

There are different routs of inoculation.

In this case inoculate 0.1-0.2 ml of suspension in allantoic cavity.

Step5: If the death of the embryo occurs within 24 hours of inoculation, then it should be considered that it is not due to virus infectivity. The cause of death may be any other one.

Step6: If death doesn't occur, then collect chorio-allantoic fluid after 72 hours.

Step7: Go for centrifugation in order to get the clear fluid.

Step8: Perform some tests for initial conformation of suspected virus in the fluid.

SLIDE AGGLUTINATION TEST

It is very simple and easier test; not require so many instruments.

Step1: Take a glass slide which should be free from any type of contamination.

Step2: Mark one side of slide as A and the other one as B.

Step3: Put a drop of chorio-allantoic fluid along with a drop of RBCs on one side **(***side A).*

Step4: Put a drop of chorio-allantoic fluid along with a drop of normal saline solution on other side **(side B)**

OBSERVATIONS

Side B: RBCs are accumulated at the center while in case of Slide A: There is no gathering of RBCs in the center but they are spread homogenously and present a reddish area. → So, the

initial conformation reveals that suspected virus containing fluid contains haemagglutinating virus.

MICRO-TITRATION PLATE METHOD

The micro-titration plate is basically consists of 96 small U-shaped wells (1-12 Columns and A-H Rows).

If we have only a single virus sample then we will use only the single row of micro-titration plate. For example, in this confirmatory test we will have to use Row A.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

PROCEDURE

Step1: Select Row A and perform the test by using all the wells (1-12) present against this row.
Step2: Take 50 ul of normal saline for each well and put it from well no. 1 to 12 carefully.
Step3: Add 50 ul of virus suspension in the well no.1.
Step4: Take 50 ul from well no. 1 and add it into well no.2 and similarly do this with all the well up to 11.
Step5: After addition of 50 ul of virus suspension in well no. 11, now discard 50 ul from this well.
Step6: After adding virus suspension, now add 50 ul of 1% RBCs from well no. 1 to 12.
Step7: Now, place this plate at room temperature for some time.

OBSERVATIONS

- First of all observe the well no. 12 (known as control well), there should be button formation essentially.
- Observe all the other wells for button formation and suspended clump formation.
  For example, there is no button formation from well no. 1-5
  There is clear button formation from well no. 7-12.
  And in well no.6, 50% button formation and 50% heamagglutination (HA)

CALCULATIONS

What is Titre?

It may be defined as "Reciprocal of Highest dilution which shows positive (+ve) results.

Well Numbers Vs	Dilution ratios
1	1:2
2	1:4
3	1:8
4	1:16
5	1:32
6	**1:64**

Conclusion

So we concluded from the above observations that the titre of the virus is 1:64 → 10^6
The titre of a virus only reveals the infectivity of the virus but it doesn't help us to identify the viruses.

VIRO PRACTICAL 5

HAEMAGGLUTINATION INHIBITION (HI) TEST

The principle of this test (HI) is the inhibition of haemagglutination of a virus by specific antibody.

Objective

- To detect an unknown Haemagglutination virus (HA virus) by using known antisera.
- To detect the presence of specific antibodies in a particular serum for a known virus.

We inactivate the virus by using known antibodies. These Abs hide the HA proteins on the virus coat and thus, inhibit its potential to agglutinate the RBCs.

Preparation of Desired Virus Suspension

We will have to check first HA- potential of virus, e.g. in the last experiment, we calculate the titre 1: 256 and this is also known as "1HA unit". In this experiment, we have to use the virus of titre "4HA unit". For 4 HI unit, divide the 256 by 4 → 256/4= 64.

It means that the virus we use originally whose titre was 1:256 will be taken 1ml and 63 ml of normal saline solution to achieve the 4HA unit titre for the desired suspension.

Why we can not find the exact Abs titre?

Because serum antibodies are not in pure form as viruses are, as well as no. of viruses are dominant, due to which we don't know the exact Abs titre.

PROCEDURE

Step1: Use a micro-titration plate
Step2: Take 25ul normal saline for each and add in all the wells from A1→A12.
Step3: Add 25ul serum sample in the well no.1
Step4: Then, make 2 fold dilution uptil well no. 10.
Step5: Add 25ul of 4HA unit virus from well no. A1→A11
Step6: Incubate for 20-25 minutes at 27C.
Incubation help in the formation of Abs and Virus complex, if both are specific to each other.
Step7: Add 25ul of 1% washed RBCs in all the wells (A1 → A12).
Step8: Not disturb the plate for some time.

OBSERVATION & RESULT

This test is reverse of HA because in this test, button formation will occur in the first few wells and bead formation will be there in the last remaining wells.

Button Formation

It reveals that if Abs titre is much higher in the wells, so there will be no RBCs agglutination occurs by virus. Because of high Abs titre, virus has been neutralized and RBCs are settled in the button like appearance at the bottom.

Bead Formation:

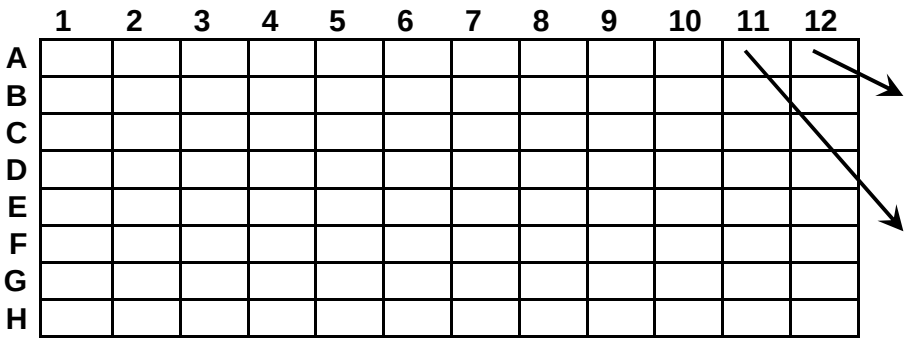
It can be observed that if antibodies are not specific to virus, then all wells should have bead formation. On other hand, due to low Abs titre in the last few wells, virus has not been completely neutralized by Abs. As a result, RBCs beads are formed in these wells.

If antibodies are not specific:

If antibodies in the serum are not specific for virus, then all wells should have beads.

This test can be applied to detect the virus specific to certain known antibodies or vice versa. This is the advantage of this test over HA, in which we can only detect the HA virus, but can not Abs.

Example: If we have a serum sample to measure Abs titre for a particular virus, e.g. ND virus, then we can determine it by using our virus sample.



Serum & Virus Control Well
There is no serum as well as virus, thus button formation should be essentially.

Serum Control Well
There should be bead formation because it contains virus + RBCs but no serum.

APPLICATION

This test gives us some idea that you should have to vaccinate the animal again or not. For example, if birds have 1:64 titre of Abs then it means birds have sufficient titre to defend against the virus, otherwise you have to vaccinate the birds again for enhancing Abs titre.

VIRO PRACTICAL 6

INDIRECT HAEMAGGLUTINATING TEST (IHAT)

This test is performed to detect those viruses which do not have ability to agglutinate RBCs directly *because* they do not have haemagglutinin protein coat, thus they can not attach directly to RBCs.

Example:

IBDV	infectious bursal disease virus
HPSV	Hydro-pericardium Syndrome virus
RPV	Rinder pest virus
IBV	Infcetious bronchitis virus

To demonstrate haemagglutination, we coat the RBCs with either antigen or specific antibody which then, may reveal HA pattern.

As a whole, we can say that, IHAT is usually used to detect level of antibodies in serum.

Application:

IHAT is applied for;

- determination of titre of antibodies in the serum
- Determination of titre of virus antigen with little modification that is called Passive haemagglutination test.

Demonstration of Indirect Haemagglutination Test (IHAT)

In this test, we first coat the RBCs with an antigen.

Sensitization of RBCs

"Coating of antigen/antibody on the outer surface of RBCs is known as "Sensitization of RBCs".

Methods of Sensitization of RBCs

There are two methods of sensitization;

i) Direct sensitization
ii) Indirect sensitization
iii)

Direct Sensitization

It is performed for those RBCs which do not have ag-determinants on their surface; i.e we can use O –ve blood group.

PROCEDURE:

1. Take blood by disposable syringe in glass tube having anticoagulant in it.
2. Centrifuge the blood afetr collection @ 15000 rpm for 3-5 min.
3. RBCs settle down at the bottom and upper straw colored fluid is plasma. There is also a white layer of WBCs formed.
4. Discard straw color fluid and WBCs layer and hence packed RBCs are obtained.
5. Wash these packed RBCs two or three times.
6. After proper washing of RBCs,
   Take 1 part of RBCs + 1 part of Ag + 2 parts of Normal saline/PBS.
   i.e. 1 ml of RBCs + 1 ml of Ag + 2 ml of N.S/PBS
7. Put this test tube having suspension in incubator and incubate at 37C for 45 min.
8. During incubation, after short intervals shake test tube gently.→ This will help RBCs to come in contact with Ag.
9. After 45 min again centrifuge @ 1500 rpm for 3-5 min to remove the free or loosely attached Ag on RBCs surface.
10. Discard straw color fluid (supernatant fluid)
11. Add Normal saline in equal amount and give one more washing.
12. Further more, to check sensitization of RBCs, we can perform slide agglutination test.

OBSERVATIONS:

If they are properly sensitized → they will attach on Abs present in the serum and will settle down. In other case, there will be homogenous reddish appearance.

NOW,

a) Make 1% suspension of sensitized RBCs.
b) Add 50ul of normal saline in all the wells of 1st row (A1 to A12)
c) Add 50ul of serum of serum sample in A1.
d) Make 2 fold dilution of serum uptill A11
e) Add 50ul of 1% sensitized RBCs in all wells.

OBSERVATION & RESULT:

At this point, we can detect the titre of antibodies present in the serum by previous practice.

Indirect Sensitization

In indirect sensitization of RBCs, we can use any RBCs group from human, sheep, poultry etc., those have ag-determinants on outer surface.

By this technique, we hide those ang-determinants by adding coupling agents i.e. Tannic acid, Chromium chloride, and Gluteraldehyde.

PROCEDURE:

1. Procure blood by using a syringe in a test tube having anticoagulant in it.
2. Centrifuge it @ 15000 rpm for 3-5 minutes.
3. Discard supernatant and get packed RBCs.
4. Wash these RBCs 2-3 times.
5. Take 1 part of RBCs + 1 part of coupling agent + 2 parts of Normal saline/PBS.
6. Incubate this suspension for 30 min at 37 C.
   Coupling agent will hide Ag-determinants and will make a layer over outer surface.
7. Add 1 ml of antigen in the suspension and incubate again at 37C for 25 min.
8. During incubation, after short intervals shake test tube gently.→ This will help RBCs to come in contact with Ag.

9. After 45 min again centrifuge @ 1500 rpm for 3-5 min to remove the free or loosely attached Ag on RBCs surface.
10. Discard straw color fluid (supernatant fluid)
11. Add Normal saline in equal amount and give one more washing.
12. Further more, to check sensitization of RBCs, we can perform slide agglutination test.

NOW,

f) Make 1% suspension of sensitized RBCs.
g) Add 50ul of normal saline in all the wells of 1st row (A1 to A12)
h) Add 50ul of serum of serum sample in A1.
i) Make 2 fold dilution of serum uptill A11
j) Add 50ul of 1% sensitized RBCs in all wells.

Observation & Result:
At this stage, we can detect the titre of antibodies present in the serum by previous practice.

PASSIVE HAEMAGGLUTINATION TEST
It is a modification of IHA test in which outer surface of RBCs is coated with Abs instead of Ag. In this way, we may able to determine the titre of antigen in a virus suspected suspension.

VIRO PRACTICAL 7

AGAR GEL PRECIPITATION TEST (AGPT)

This test is based on the interaction of specific antigen with specific antibodies inside a gel. Their interaction results into the formation of thin visible white precipitations.

Pre-requisites
For visible precipitation, following two conditions should be fulfilled;

a) Antigen (ag) must be soluble in nature.
b) Concentration of antigen and antibodies should be optimum.

Reasons:
a) If the antigen will not be in soluble form → it can not diffuse through the gel to form precipitation.
b) if concentration of both antigen and antibody is not optimum → there will not be visible precipitation to view with naked eye.

APPLICATION
For detection of virus
We can use this test for the detection of any kind of antigen as well as antibody if they fulfill above two conditions.
For detection of bacteria and protozoa
We can also use this test for the detection of bacteria and protozoa but the sample should sonicate first to separate the surface antigen from cell surface. While making a suspension, the supernatant will contain this surface antigen that should be collected for further process.

PROCEDURE
Step1: Noble agar is being recommended for this test. We need two different concentrations i.e. 0.8% and 1.5% (made in phosphate buffer solution) for this test.

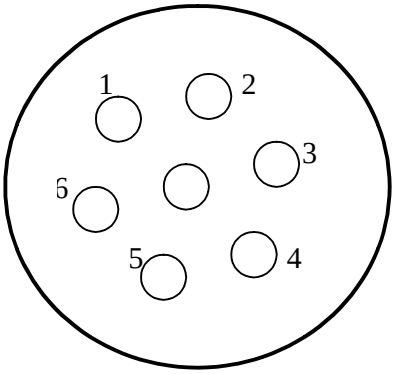
Noble agar is a purified form of agar agar. When it is dissolved in normal saline or distilled water, it appears just like a clear glass or quite transparent. This property helps us to visualize the precipitation formed in the medium. On the other hand, other medium give hazy or opaque appearance when dissolved in normal saline or water. Thus, they can create difficulty for visualization of formed precipitation.

Step2: Take a glass petri plate.

Step3: Add agar suspension of 1.5% concentration in the petri plate, to make a layer of 3-4 mm in thickness.
Step4: Allow this layer to solidify
Step5: Then, add agar suspension of 0.8% concentration to make another layer above first one of same thickness.
Step6: Again, allow this second layer to solidify.
Step7: Now we will have to make wells in the above layer of 0.8% concentration with the help of any glass rod.

Pattern of wells
There should be 1 central and 6 surrounding wells that will surround the central one in circular fashion.

Case 1: Now, suppose we have different known antibody serums and an unknown antigen sample and we are desirous to detect the antibody specific to that unknown antigen.
Step8: add antigen sample in the central well and different antibody serums in the surrounding wells.



Fill the central well with unknown antigen and surrounding wells as follows:

Well no. 1:	Antibody serum sample against ND virus
Well no. 2:	Antibody serum sample against IBD virus
Well no. 3:	Antibody serum sample against AI virus
Well no. 4:	Antibody serum sample against HPSvirus
Well no. 5:	Antibody serum sample against IB virus
Well no. 6:	Normal saline solution (NS)

Well no. 6 contain normal saline will be regarded as “control well”. We can use negative serum sample (having no antibody) instead of normal saline in this well.
*Step9***:** Place this plate in the incubator and incubate at 37C temperature, 70-80% humidity for 24-72 hours.
Why humidity is required? In the absence of humidity, the media may shrink and wells may come closer to each other results into a little or no space between wells.

OBSERVATIONS:
As antigen as well as antibodies will diffuse in all sides, thus if antigen will be specific to any antibody, a line of thin, white precipitation will be formed between them.
A single line of precipitation
Suppose, we observe a line of precipitation between central well and well no.4. It indicates that antigen is specific to antibody sample of HPS virus.
A few lines of precipitation
Instead of a single precipitation line, if antigen is specific to more than one antibody samples, many precipitation lines may be visible that will indicate the specificity of antigen to that particular antibody.

Case 2: If we have a known antibody serum and unknown antigen samples:
Then place the antibody serum in the central well and antigen samples in the surrounding wells. The observations will be the same as for Case1.

Why we make 1.5% conc. Layer in the petri plate first?
- As antigen and antibody can not diffuse through 1.5% con. layer, thus we make this layer for sealing of wells.

If we don't make 1.5% coc. layer and simply made the wells in the 0.8% conc. layer:
While making the wells by glass rod, media may disturb and raised a bit from the plate surface → create a small space between the media and plate surface. At this stage, if we will pour our antibody and antigen samples, they will diffuse in the small space instead of diffusing via medium. Therefore, 1.5% conc. layer is required strongly 1) to seal the wells so that antigen and antibody will only diffuse through the medium 2) to avoid the diffusion into the space.

Routine laboratory Practices:
Noble agar is very costly and highly precious. Thus, we want to use this medium in a very small quantity only to cope its requirement.

In lab. Practices, we make only a single layer of 0.8% conc. and don't make 1.5% conc. For sealing purposes, we add one or two drops of 1.5% conc. medium after making the wells. Drops should not be too big to fill the entire well. Thus, we economically save the medium.

DISADVANTAGES
This test requires maximum time – better for research purposes but not fair/good for commercialization.

Modification *to lessen the time of demonstration*: Counter current immuno-precipitation Test.

COUNTER CURRENT IMMUNO-PRECIPITATION TEST (CCIPT)

Procedure:
- Take a U – Shaped glass tube.
- Fill it with 0.6% Noble agar (it will be in semi-solid form).
- Add antigen suspension on one end and antibodies sample on other end and make circuit as follows:
  Antigen ------- Negative terminal (as Antigen is positively charged)
  Antibody ------- Positive terminal (as Antibody is negatively charged)
- Allow to run DC Current (5 ampere) for 15-20 minutes.

Observation:
- If antigen is specific to antibody: there will be formation of white precipitation line/band at the point of interaction.

Advantage:
- This test is very easy to perform and very less time consuming.

Consult Remaining Practicals from* "hand written notes"** ***attached with this.