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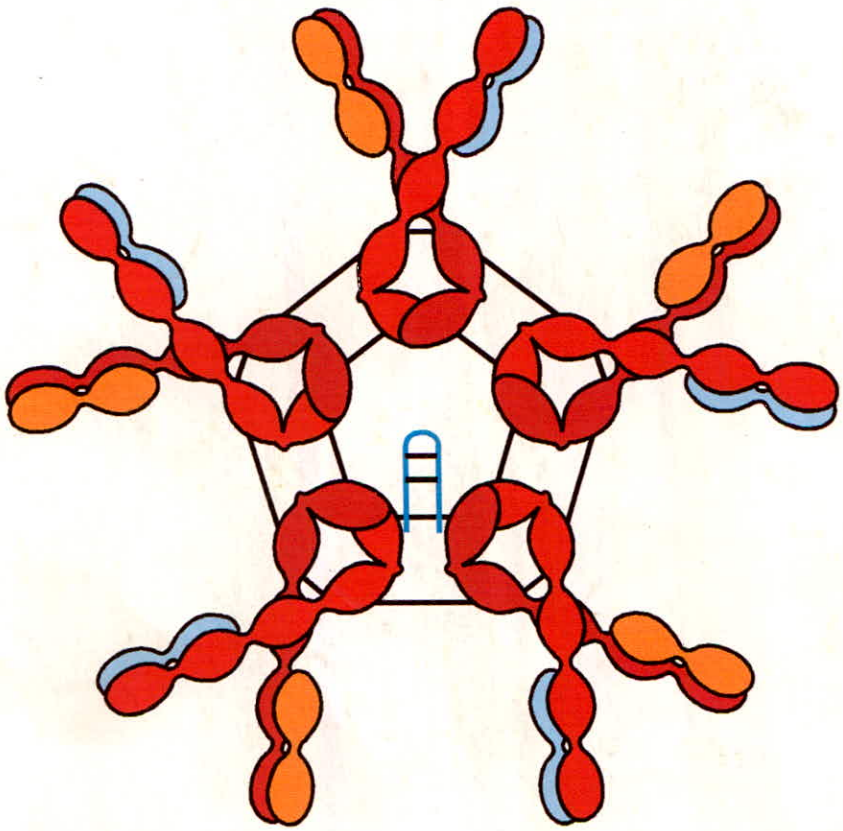
Laboratory Mannual

VETERINARY IMMUNOLOGY AND SEROLOGY



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PREFACE

Veterinary Council of India course curriculum has been introduced in almost all Veterinary colleges in the country. It is inbuilt in this course curriculum that for every course the course faculty should develop a lecture schedule for imparting theory teaching and at the same time they should also come up with a laboratory manual for practical classes in each course. Hence, the release of "Laboratory Mannual in Veterinary Immunology and Serology" meets the VCI requirement for practical classes at Under graduate level. Additionally, this manual also provides a baseline material for Post graduate students both at MVSc and Ph. D levels. While developing the manual, sufficient care has been taken to give emphasis on principles under each topic rather than details. The study questions provided under the topics will be helpful in enhancing the mental horizon and competence in the techniques. Sufficient space has been provided under each topic for recording the observations/findings. In this way it is hoped that the students will be able to acquire necessary technical and hands on learning experiences. As the topics in the practical schedule have been designed to develop the concept of the basics and the theoretical aspects of the course,the practical exercises under taken by the students will ultimately help in developing the concept of the subject. Further, while compiling the mannual efforts have been made to take care of the latest development in the field of immunology. Inclusion of the topics like Western Blotting Technique, PAGE and the like demonstrates an attempt in this direction. It is hoped that this mannual will prove to be a good teaching aid to the course faculty to impart practical training systematically and within the defined frame. The protocols, tables and methods included in the text will enable the students to do the practical and the learning exercises at their own.

At the end the Department expresses its gratitude to one and the all who have contributed in development of this mannual in any form.

DURG
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FOREWORD

It gives me immense pleasure to record that the Department of Veterinary Microbiology has come up with a **"Laboratory Manual of Veterinary Immunology and Serology"**. I understand this manual has been developed in the frame of undergraduate teaching in accordance with the Veterinary Council of India (VCI) guidelines. Additionally, this manual will also act as ready reckoner for both M.V.Sc. and Ph.D. scholars. Further, immunology being the component of course curricula of several subjects in the different Departments, all of them will be benefited from this endeavour. Sufficient efforts have been made to keep the students abreast of the latest information on methods and techniques in the field of immunology. This will certainly go a long way in empowering the students with skills and inculcating confidence and competence in the application of the subject for disease diagnosis and prophylaxis. The topics, methods and techniques in practical classes are designed to assist the students in developing the concept of the subject, providing them with skill, methods and techniques which they can apply in giving quick and timely diagnosis of the disease as well as planning a suitable strategy for their control. As such this manual will prove to be of immense help in acquiring underlying principles and developing the intellectual and technical skills to use them to solve the practical problems.. Efforts taken by the faculty of the department is commendable. I wish this manual should meet the aspirations of the under graduate students in particular and all those associated with disease diagnosis and immunopriphylaxis in general.

Dr. Sudhakar Jogi
DEAN

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Exercise No.-1

Collection of Blood in Laboratory Animals

The techniques and sites of blood collection vary with the animal species and the quantity of blood required. Common bleeding sites of various laboratory animals and practicable volume of blood obtainable from laboratory animals to be discussed in the class.

Rabbits and Guinea Pigs:

1. **Ear vein puncture:** It is commonly used in Rabbits. The area around the marginal ear vein is shaved and disinfected with 70% alcohol before the vein is nicked with a razor blade. Blood may also be collected directly into a syringe.

2. **Cardiac puncture:** The rabbit/guinea pig is laid down in supine position on a dissecting table or especially designed boards to hold the head of the animal as far backward so as to avoid side-ways movement of the skull. Hind limbs are also stretched to get a 'barrel chest'. Hairs are shaved off from chest area avoiding any abrasion of skin or thoracic nipples. Insert 21 G needle 1¼ inches long loaded on the 10 ml syringe through the gap between last sternal rib on the left side of the midline and the xiphoid process. Penetrate downward for a few millimeter, slant forward at an angle of 30 degree to the chest wall till heart beats are felt. Push forward approximately 3-6 mm and simultaneously retract the plunger of the syringe. If blood doesn't flow at once, first pull the needle back slightly rather than advancing it deeper, otherwise start a fresh.

3. **Bleeding from the orbital sinus:** The rabbit/guinea pig is restrained by an assistant and held on its side with limbs fully extended. The head is firmly held with one hand and the eye lids are partially retracted with the thumb and fore finger. The tip of the heparinised microhaematocrit tube broken to 50 mm is placed in the dorsal junction of the bulbar and palpebral conjunctivas, midway between the medial and lateral canthi of the eyes. The long axis of the capillary tube is directed somewhat ventrally and caudally in relation to the skull. The fibrous conjunctiva is penetrated by thrusting the capillary tube inward while rotating it between the thumb and the fore finger. The capillary enters the

venous sinus which is at or behind the equatorial region of the eye ball adjacent to the bony orbit. An adequate blood flow into the tube can be obtained by seeking optimal effects of gravity.

Rats and Mice:

1. **Tail and toe clipping:** Small quantity (0.2-0.3 ml) of blood can be collected by clipping the tail or toe and aspirating the blood directly from the site. The animal has to be under anaesthesia during the method. The animal, including the tail should be kept warm by means of a lamp and gently restrained.

2. **Tail vein puncture:** The rat is placed on a flat surface under a bright light. The tail is grasped between the thumb and the index finger. The tail is first swabbed with xylol and then with 70% alcohol and dried with a sponge. Two ml syringe fitted with 25-27 G and ½-1 inch needle is used. The needle is introduced near the distal portion with the level up. This allows one to observe directly as the needle enters the vein and gentle aspiration can be applied. This is difficult technique and requires much practice to gain proficiency.

3. **Incision technique for rat:** Under anaesthesia the jugular vein can be entered after a ventrolateral neck incision. The small saphenous vein or the femoropopletial vein can be punctured by applying the incision over the caudolateral aspect of the rear leg for collection of small quantities of blood. The femoral vein may be easily entered by incising the skin of the groin at the junction of the leg and the abdomen.

4. **Jugular vein puncture for mice:** The hair on the neck and upper thorax region is removed and both jugular veins are easily seen. A 26 G needle is used to enter the distended vessel.

5. **Cardiac puncture:** Large blood sample can be collected by cardiac puncture. This is performed under anaesthesia and the animal is restrained in dorsal recumbence by a restraint device. The heart is located under the 5th to 6th ribs by palpation with the index finger of the left hand, the left thumb on the rat's right side. A 2-5 ml syringe with a 19-25 mm (¾-1 inch), 24-26 gauge needle is used for bleeding. The needle is inserted at 45 degree angle into the thoracic cavity up to the point where heart is felt

Reference:

Talwar, G.P. (1983). A Handbook of Practical Immunology. Vikas publishing House Pvt. Ltd. New Delhi.

Study questions:

1. Discuss the use of small animals/laboratory animals in experimental biology.
2. Discuss the ethical issues about the use of laboratory animal in biology.
3. Write down the technique for collection and preservation of serum from blood.

Exercise No.-2

Methods of Inoculation in Laboratory Animals

Laboratory animals are used for the production of antiserum. An antigen can be administered into animal body by various routes. The selection of route of inoculation depends upon the form and the nature of the antigen. The various routes of inoculation of antigen are mentioned below-

1. **Intradermal:** This route is used for injecting viscous and slowly dispersing forms of an antigen such as antigen emulsified with Freund's adjuvant. It provides rapid access to the lymphatic. Intra dermal inoculation is made into the flank or back of rodents, guinea pigs and rabbits.

2. **Subcutaneous:** This route is suitable for emulsions, precipitates and viscous materials and the antigen spread a little more as compared to intra dermal mode of injection. However, antigen is absorbed slowly into circulation as compared to intra peritoneal injection.

3. **Intramuscular:** It is one of the most frequently adopted routes of immunization and suitable for alum precipitated and adsorbed antigens. The antigen is deposited in the muscular layer e.g. thigh muscles. The needle is inserted from the rear at right angles to the skin surface, at a point half way along the femur, so that its point lies within the muscle. The inoculation is then made, needle withdrawn and the site gently massaged.

4. **Intraperitoneal:** Intraperitoneal injection of antigen leads to immediate attraction of macrophages and monocytes. Therefore, it is more suitable for complex and particulate antigen, such as cell suspensions or antigens which required processing. Hold the mouse as described for subcutaneous injection and insert the needle (23 or 21 G) into the abdomen to a depth of about 6 mm. Deeper insertion may damage other organs. Inoculate the required volume, withdraw the needle and punch the site of needle entry.

5. **Intravenous:** This route is good for particulate antigens such as cells and bacteria and is not advisable for viscous and non aqueous

antigens. In case of rodents tail vein is used for intravenous inoculation. If success is not achieved in first attempt, the other tail vein or same vein at higher point toward base can be tried after re-warming. Though 0.1 to 0.2 ml dose is recommended through this route but up to 1.0 ml can be injected. This route requires extra care as antigen is directly entering the blood circulation which may cause anaphylactic shock and death.

Dorsal vein of penis can be used for intravenous inoculation in case of male rats and guinea pigs. In case of rabbits ear vein is used for intravenous inoculation.

6. **Foot Pad:** This method can be used to inoculate particulate and cellular antigens in mouse, guinea pigs and rabbit. Inoculation is done only in the hind foot as fore paws are used by the animal for manipulating food. The foot pad is cleared with 70% alcohol and needle can be inserted from either the distal or proximal direction to a depth of about 5 mm into the pad. In guinea pig and rabbit the needle is inserted through inter digital space to a depth of about 10 mm. A volume of 0.25 ml can be injected by this method.

Reference:

Kadian, S. (2003). Production and use of instructional material on Veterinary Immunology and Serology. ICAR Center of Advanced Studies on Vet. Microbiology, CCS Haryana, Agriculture University, Hisar.

Study questions:

1. Draw well labeled diagram to illustrate various routes of inoculation in poultry.
2. Enlist the diagnostic procedure in which intradermal route of inoculation is used.
3. Give in a tabular form the name of various laboratory animal species alongwith the site of intravenous injection.
4. What should be the preferred route(s) for adjuvanted vaccines? Give examples.

Preparation of Antigens form Bacteria

Bacteria contain many antigens which are distributed within their structural framework. Isolation and purification of these antigens is of interest for a variety of purposes. Suspensions of bacteria that have only one antigen ('H' or 'O') exposed on their surface are also of great utility. The agglutinable O and H bacterial suspensions can be prepared by methods given below -

Somatic Antigen ('O' antigen):

Cell wall of many gram negative bacteria contain somatic antigen. Somatic antigen is present both in motile and nonmetal bacteria. These are complex of lipo protein, polysaccharides and lipid. The lipopolysaccharides have the toxic pyrogenic and immunologically specific properties of the 'O' antigen of smooth colony gram negative bacteria. The 'O' antigens are resistant to prolonged boiling at 100^0C and treatment with alcohol and acid.

Procedure:

1. Harvest the bacteria from the surface of agar with minimum amount of sterile normal saline.
2. Place the bacterial suspension in a boiling water both for two and half hours.
3. Centrifuge at 1400 x g for half hour. Discard the supernatant. Resuspend the bacteria in 0.3% formalinized saline.
4. Check the sterility of bacterial suspension by inoculating a tube of suitable broth. Incubate for 48 hrs at 37^0C and examine for the growth.
5. Adjust the concentration of bacterial suspension to the desired value by using nephelometer.

Flagellar Antigen ('H' Antigen):

'H' antigens are found only in motile bacteria. Flagellar antigen are protein in nature and is a fibrous protein called flagellin. Flagella have high differentiation characteristics for different strains of bacteria and therefore used for diagnostic purposes. 'H' antigens are destroyed by heating at 100^0C for 2.5 hours or by treatment with alcohol for 4 hours, centrifuge and resuspend in saline.

Procedure:

1. Inoculate 500ml of nutrient broth with 1ml of actively motile; 18 hr culture of bacteria. Incubate for 24 hrs at 37^0c.
2. Add equal volume of 0.6% formalinzed saline to the culture medium. Allow to stand at room temperature for three days.
3. Check the sterlity of bacterial suspension.
4. Centrifuge the suspension at 1, 400 x g for ½ hour. Discard the supernatant. Resuspend the bacteria in 0.3% formalanized saline.
5. Adjust the concentration of the bacterial suspension to the desired value by using a nephelometer.

Standardization of bacterial suspension by Nephelometry:

The bacterial suspension need to be standardized for use in serological tests or for immunization of animals. For this purposes most simple and convenient method is the use of Mc Farland's Nephelometer.

In this technique the turbidity of a test suspension is compared with the respective turbidity of a series of ten standard tubes containing suspension of barium sulphate. The turbidities of these standard tubes corresponds to varying concentrations of bacteria as shown in table -

Standard Tube No.	1% $BaCl_2$ (ml)	1% H_2SO4 (ml)	No. of bacteria (value listed x 10^6 per ml)
1.	0.1	9.9	300
2.	0.2	9.8	600
3.	0.3	9.7	900
4.	0.4	9.6	1200
5.	0.5	9.5	1500
6.	0.6	9.4	1800
7.	0.7	9.3	2100
8.	0.8	9.2	2400
9.	0.9	9.1	2700
10	1.0	9.0	3000

Reference:

Talwar, G.P. (1983). A Handbook of Practical Immunology. Vikas publishing House Pvt. Ltd. New Delhi.

Study questions:

Q. Explain the following terms. Give example of each.

1. Antigenicity and Immunogenicity
2. Heptan and carrier
3. H. antigen and O antigen.
4. Primary and Secondary immune response.
5. Particulate and soluble antigen.
6. Vaccine, bacterins and toxoid.

Preparation of Hyperimmune Serum

Antigen is used to induce a detectable immune response. Immune response is expressed either in form of activated cells (Cell Mediated Immune response) or as antibodies (Humoral Immune response). When the interest is to raise antiserum against an antigen, the objective is always to have an antiserum containing maximum amount of antigen specific antibodies.

The magnitude of immune response depends on the following factors. Therefore, before initiating immunization programme, careful consideration should be given to each of these factors.

i. Choice of animal.
ii. Form and dose of antigen.
iii. Adjuvant
iv. Route of immunization
v. Immunization schedule

i. Choice of animal

Rabbit, Goats, sheep and horses are generally employed for production of antibodies. In random bred immunized animals, the antibody titer may vary by as much as 500 times amongst individuals. To overcome this problem a group of animals should be immunized (5 to 10 animals) and animals producing higher antibody titer with desirable characteristics should be selected. Alternatively inbred strains of animals can be employed to avoid the variation in immune response among individuals.

ii. Form and dose of antigen

Good antibody response is usually obtained when particulate antigen are injected intravenously without an adjuvant. Soluble antigens are readily diluted out, catabolized and hence stimulate poor immune response. To over come this problem primary dose should be given with adjuvant and by a route which retain the antigen for long enough time to activate immune response: eg. Subcutaneous, Intradermal routes. Proteins can usually be given in a wide dose range (1 µg to 1 mg/ animal) without inducing high or low dose tolerance. Antigen may be injected in fluids state,

absorbed to insoluble particles such as alumina or incorporated in matrix material such as agar, Freund's Adjuvant etc.

iii. Adjuvants

Freund's complete adjuvant is a mixture of oil (Bayol F) and detergent (mannide mono-oleate), containing killed *Mycobacterium tuberculosis*. The incomplete adjuvant is a mixture of oil and detergent alone. It is most effective non-specific immunopotentiator. Saponin a mixture of water soluble triterpene glycosides extracted from the bark of a South American tree, is highly surface active and forms stable complexes with proteins released from viral envelopes. It is a safer immunostimulating agent used in veterinary vaccines.

iv. Route of Immunization

The various routes used for Immunization are as mentioned below-

a. Intradermal route:

Intradermal route provide rapid access to the lymphatic but causes itching and dermatitis, therefore it is generally avoided. The antigens by this route are inoculated in flank or back of rodents and Guinea pig, forearm in higher primates such as monkeys and baboon. Pea like swelling at the site of injection indicates a true intradermal injection. The route is preferred for injecting viscous and slowly dispersing antigens.

b. Subcutaneous route:

Subcutaneous route is suitable for emulsions, precipitates and viscous materials. The antigen inoculated by the this route spread a little more as compared the intradermal route.

c. Intramuscular route:

Intramuscular route is suitable for alum precipitates and adsorbed antigens and generally inoculated in thigh muscles.

d. Intraperitoneal route:

Intraperitoneal injection of antigen causes immediate attraction of macrophages and monocytes.

e. Intravenous route:

Intravenous route requires precaution as antigen is directly entering into blood circulation which may cause anaphylatic shock and ultimately results in death of the animal.

Immunization schedule:

It is impossible to recommend any single ideal immunization schedule for all antigens.

Materials:

- Soluble antigen (Bovine serum albumin, 1 mg/ml)
- Particulate antigen (20% sheep RBC suspension)
- Freund's complete and incomplete adjuvants
- Rabbits - 2 for each antigen
- 5 ml syringe along with 24g needle
- PBS, Alsever's solution

Procedure:

A. For particulate antigen:

The immunization schedule for preparation of antibodies against sheep RBC is as follows -

1. Prepare 20% suspension of sRBC in PBS after giving three washing to one week old sheep blood preserved in Alsever's solution.
2. Inject 20% suspension of sRBC on various days as per protocol given below.

Days	Dose and Route*
1	1.0 ml I/V
5	0.5 ml S/C & 1.5 ml I/V
10	0.5 ml S/C & 2.0 ml I/V
15	0.5 ml S/C & 2.5 ml I/V
20	0.5 ml S/C & 3.0 ml I/V

3. Bleed the animal to collect antiserum after 9 days of last injection by cardiac puncture.

* I/V inoculation should be given 15 min after S/C inoculation

B. For soluble antigen:

1. Prepare bovine serum albumin (BSA) in PBS at a concentration of 1 mg/ml.
2. Inject the antigen solution as such or after preparing water in-oil emulsion either in Freund's complete (FCA) or Freund's incomplete (FIA) adjuvant.
3. Inoculate rabbits as per the protocol given below -

Days	Dose	Route
0	1.0 ml emulsion in FCA	S/C
21	1.5 ml emulsion in FIA	i/m
36	1.0 ml antigen solution	i/v

4. Bleed the rabbits to collect antiserum 3-4 days after the last injection.

Reference:

Husdon, L. and Frank, C.H. (1991). Basic techniques, In: Practical Immunology 3rd Edition. Black well Scientific Publication, Oxford.

Study questions:

1. Define the following-

a. Cell mediated immunity, Humoral immunity

b. Memory cells, Plasma cells, Macrophage

c. Antiglobulin, Immunoglobulin

d. Constant region, variable region, domain

e. Hinge, F (ab), F (c)

2. Classify adjuvants. Name at least 10 adjuvants used in veterinary practice.
3. Differentiate between complete and incomplete adjuvants. What is usefulness of these adjuvants for submit/synthetic and recombinant protein vaccines ?

Exercise No.- 5

Purification and Concentration of Immunoglobulins

Hyperimmune serum contains immunoglobulins (Ig) alongwith non specific serum protein molecules. It is desirable to work with comparatively enriched or totally pure immunoglobins in a large number of serological techniques.

Materials:

- Ammonium sulfate solution (saturated)
- Sodiumchloride solution
- Dialysis bag, etc.

Procedure:

1. Prepare saturated solution of ammonium sulfate (700 g/L at 0^0C or 760 g/L at 25^0C) by dissolving it in hot distilled water and cooling at room temperature.
2. Adjust the pH of clear supernatant to 7.0 using ammonia solution and store in presence of ammonium sulfate crystal.
3. For precipitation, take 40 ml of saturated ammonium sulfate solution and add gradually to 60 ml of serum under constant stirring at room temperature.
4. Resultant suspension is allowed to stand for 30 min with occasional stirring.
5. Collect the precipitate by centrifugation at 3000 rpm for 20-30 min.
6. Dissolve the precipitate in 30 ml distilled water and precipitate again by gradual addition of 20 ml of saturated ammonium sulfate solution. Repeat the cycle twice.
7. Dissolve the final precipitate in 15 to 20 ml of 0.15 M sodium chloride solution.
8. Ammonium sulfate is removed either by dialysis or by gel filtration on a column of sephadex G-25.

Reference:

Talwar, G.P. (1983). A Handbook of Practical Immunology. Vikas Publishing House Pvt. Ltd., New Delhi.

Study questions:

1. Explain the principle of purification of protein by salt fractionation.
2. What are other methods of purification of proteins.

Agglutination Test

Agglutination test is one of the oldest methods for demonstrating the occurrence of immune reaction *in vitro*. It is the interaction between antibody and a particulate antigen, resulting in visible clumping. The antibodies that produce such reaction are called agglutinins. The particulate or insoluble antigen can be bacteria, cells of higher plants or animals, micro fungi or rickettsciae. Synthetic polymer particles like latex (Polystyrene), bentonite and Collodian can also be agglutinated after attachment of antigens on their surfaces. To produce visible reaction the particles should have a diameter greater than 200-250 nm. Reaction time for agglutination is short, from a few minutes to a few hours at the most. The reaction is sensitive enough for demonstration of antibody.

Principle:

Agglutination is the linking together (lattice formation) of different particles or cells by antibody molecules that specifically attach to the antigenic determinants on the surface of the particles or cells. There is formation of large lattices through the cross linking of the antibody particles. These lattices sediment readily due to the large size of clump.

A. Tube Agglutination Test

Agglutination is performed in test tube. In this test, one can also determine antibody titer of the given serum sample.

Materials:

- *Brucella abortus* plain antigen
- *B. abortus* antiserum
- Normal saline Solution (NSS)
- Serological tubes
- Graduated 1 ml. pipettes

Procedure:

1. Arrange serological tubes in a rack, number them 1 to 10.
2. Take 0.8 ml. NSS in tube No.1 and 0.5 ml. in tube no.2 to 10.

3. Add 0.2 ml. of *B. abortus* serum in tube no.1, mix thoroughly and transfer 0.5 ml. to tube no. 2.
4. Mix the content of tube No.2 and transfer 0.5 ml. to tube No. 3, continue mixing and transferring upto tube No.9.
5. Discard 0.5 ml. from tube No.9. Tube No. 10 will act as antigen control.
6. Add 0.5 ml. *B. aborts* plain antigen into tube No.1 to 10. Mix the diluted serum-antigen thoroughly and incubate at 37^0c for 24 hours.
7. After incubation, examine Antigen control (Tube No.10) for uniform bacterial suspension. Agglutination is observed as clumps or flakes in a clear surrounding medium.
8. Results are expressed in titre, which is the reciprocal of highest dilution showing complete agglutination.

Prozone phenomenon:

First few tubes which contain high amounts of antibody do not show agglutination. This is called prozone phenomenon. This can be explained as that there are enough antibodies which can bind to all possible antigens on the surface independently, with both their valances instead of one antibody molecule linking two antigenic determinants on two neighboring particles and hence preventing the formation of lattice. A similar phenomenon is the postzone non-agglutination which takes place in excess Antigen because of similar reason. i. e. Antibody bind to the antigen but are not capable of cross linking the particle.

B. Plate Agglutination test

It is a simple and rapid test that can be easily carried out in field for the diagnosis of bacterial diseases such as brucellosis, salmonellosis, pasteurellosis, etc.

Materials:

- *Brucella abortus* colored antigen
- Suspected serum
- Glass slides
- NSS, etc.

Procedure:

1. Take one drop each of antiserum and NSS and place them separately far apart on the glass slide.
2. Add one drop of coloured antigen to each drop and mix it properly.

3. Wait for two minutes and look for agglutination reaction.
4. In positive case clumping of antigen is observed, while no reaction is observed in drop containing NSS (Negative Control).

C. Abortus Bang Ring Test (ABRT):

This test is also known as milk ring test (MRT). It is employed to test milk from brucella infected cows/buffalo.

Materials:

- Suspected milk sample
- *B. abortus* Antigen (Milk ring test antigen)
- Test Tube.

Procedure:

1. Take 2 ml. Milk sample, add 1 drop of Brucella antigen and mix thoroughly.
2. Incubate the milk for 1 hour at room temperature or for 30 min. at 37^0c in a water bath.
3. Examine the tube for agglutination.

Interpretation:

In positive samples the clumps of agglutinated organisms are present on the surface of milk along with fat globules. The remaining column of milk will remain clear. A negative test will show colored milk throughout, with an uncolored cream layer on top.

Reference:

Talwar, G.P. (1983). A Handbook of Practical Immunology. Vikas Publishing House Pvt. Ltd.

Study questions:

1. Enlist various applications of Agglutination test in the field of diagnostic microbiology?
2. What are the factors affecting the agglutination reaction of antigen with specific antibody.
3. How far the agglutination test can be used in detection of cross reacting antigen & antibody? Discuss.
4. Draw illustrative diagram to explain mechanism of agglutination reaction.

Exercise No.-7

Micro Haemagglutination Test

Haemagglutination is indirect measure of virus titre and it does not indicate the infectivity of the virus. The HA assay is done by end point titration. It is non serological test.

Principle:

Many viruses contain multiple copies of viral protein in the capsid or envelope that can bind to receptors on erythrocytes of some species. The erythrocytes of species that can be agglutinated vary with the virus and the conditions used, however the mechanism remain the same. The virus particle is multivalent and attaches to more than one erythrocytes. If enough virus is present, the erythrocytes are bound into lattice- like aggregates. These aggregates then settle to the bottom and form a uniform layer. Non aggregated erythrocytes also settle to the bottom, however, they slide to the center of the bottom to form a pellet.

Materials:

- 96 well 'U' bottom polystyrene plate
- Dilutors of volume 25 and 50 µl
- PBS/NSS
- Virus antigen
- 0.8% erythrocyte suspension
- Centifuge tubes
- Alsever's solution

Procedure:

1. Take 0.05 ml PBS from well No. 1 to 12 in 96 well 'U' bottom polystyrene plate.
2. Add 0.05 ml of virus material in well No. 1 and make a two fold serial dilution with the help of dilutors.
3. In control well add only 0.05 ml PBS.
4. Add 0.05 ml of 0.8% RBC suspension to all the wells.
5. Shake the plate gently for mixing and uniform distribution of erythrocytes.
6. Keep the plate at room temperature for 40 minutes.

7. In positive reaction erythrocytes will appear as diffused sheet over the surface of well. Whereas in negative reaction erythrocytes will settle in the center of bottom of the well.
8. Record the HA pattern with the aid of reading mirror and result of HA test are expressed in titre which is reciprocal of the highest dilution showing complete agglutination of erythrocytes

To detect the presence of virus in test material spot HA performed by using 10% RBC suspensions.

Reference:

Shakya, S. (1989). Studies on EDS-76 virus strains with reference to seroprevalence, Adaptation is embryonated chicken eggs and in tissue culture system and their antigenic relationship. M.V.Sc. thesis, J.N.K.V.V., Jabalpur.

Study questions:

1. What are the steps employed for preparation of 0.8% SRBC suspension? What precautions you will take?
2. What is HA unit? How is it different from titre?
3. How will you prepare Alsever's solution? What are its advantages.
4. Give in a tabular from the list of haemagglutinating viruses alongwith species of red blood cells agglutinated.

Micro Haemagglutination Inhibition Test

The haemagglutination Inhibition (HI) test is performed to measure the level of inhibiting antibodies in a serum which are capable of preventing agglutination of susceptible erythrocytes by viruses.

Materials:

- 96 well 'U' bottom polystyrene plate.
- Micropipettes
- Dilutors of Volumes 25 µl and 50 µl
- PBS/NSS
- 0.8% erythrocyte suspension
- Virus antigen suspension
- Test or known hyperimmune serum.

Procedure:

1. On the basis of HA titre per 0.05 ml virus antigen, dilute the virus to give 8 HA units / 0.05 ml from which 0.025 ml (4 HA units) are used for carrying out the HI test.
2. Take 0.025 ml of PBS from well No 1 to 12 in polystyrene plate.
3. Add 0.025 ml of test serum in well No. 1 and make two fold serial dilutions with the help of dilutors.
4. Add 0.025 ml of virus antigen (4HA) to each serum dilution.
5. Incubate the plate at room temperature for 30 min.
6. Add 0.05 ml of 0.8% RBC suspension to each well containing serum-virus mixture.
7. Shake the plate gently and keep at room temperature for 40 min.
8. In this test a known positive and negative serum sample are included as control.
9. Record the results with the help of reading mirror. The reciprocal of the highest serum dilution showing complete inhibition of haemagglutination can be taken as HI titre.

Reference:

Shakya, S. (1989). Studies on EDS-76 virus strains with reference to seroprevalence, Adaptation is embryonated chicken eggs and in tissue culture system and their antigenic relationship. M.V.Sc. thesis, J.N.K.V.V., Jabalpur.

Study questions:

1. Discuss advantages of Micro HA test over conventional test.
2. Name the diseases where HI test is routinely used.
3. Explain virus elution and its significance.

Passive Haemagglutination test

Passive haemagglutination (HA) has been used as a very sensitive tool for detecting small amount of antibodies against several bacterial and viral diseases. It is agglutination of red cells by antibodies specifically directed against soluble antigen that have been previously adsorbed or otherwise attached to red blood cell surface. Red cells are very popular as inert particles because of their uniform size and shape. The most important feature of these particles is that they are red in color and their agglutination is very easy to observe. Treatment of RBC's with tannic acid renders them absorbable to various proteinacious antigens.

Materials required:

- Sheep erythrocytes in Alsever's solution
- Tannic acid
- Antigen
- Suspected serum samples.

Procedure:

(A) Preparation of sensitized sheep RBC

1. Collect the sheep blood in Alsever's solution. Separate the erythrocytes by centrifugation at 2500 rpm for 15 min.
2. Wash the erythrocytes thrice in phosphate buffer saline (PBS, pH 7.2) and prepare a 2.5 % (v/v) suspension.
3. Treat the erythrocytes with tannic acid by adding 15 ml of : 20,000 dilution of tannic acid to 15.1 ml of 2.5 % erythrocytes. Mix the suspension gently and incubate at 37^0C for 15 min in a water bath.
4. Separate the erythrocytes by centrifugation at 2500 rpm for 5 min. After three washings in PBS, finally prepare a 2.5% suspension in PBS.
5. For sensitization of sheep erythrocytes, mix 7.5 ml of 2.5 % suspension of tannic acid treated erythrocyte with 1.9 ml of virus suspension and 35 ml of PBS, pH 6.4. Incubate with intermittent shaking for 1 hr at 37^0C.
6. Centrifuge at 2500 rpm for 8 min and wash with normal rabbit serum diluent
7. Resuspend the cells in 7.5 ml of normal serum diluent and store at 4^0C till further use.

(B) Test Proper:

1. Prepare two fold serial dilutions of the test serum starting with 1:10 in Perspex plate using PBS.
2. Add 0.1ml (2.5%) of sensitized sheep erythrocytes to each well containing 0.5 ml of serum dilutions.
3. Mix by rotating the plate gently.
4. In control wells, take sensitized cells with normal serum as well as the sensitized cells with hyper immune serum.
5. Keep the plate at room temperature and record the aggregation after 3 and 12 hours.
6. Note the serum end point which is expressed as the highest dilution at which cells agglutinated. A compact mass of cells forming a smooth edge button is considered as negative.

Study questions:

1. Is passive HA is same as indirect HA? Name the diseases where this test is commonly employed.
2. List the differences between passive HA and direct HA.
3. Indicate the nature of antigen used in this test. In case it is soluble antigen, then, how does it differ from precipitation test?
4. Find out the other inert particles which may be used to convert soluble antigen into particulate form by chemical linking.

Reverse Passive Haemagglutination Test (RPHA)

RPHA is widely used for detection of antigen in tissue homogenates and tissue culture fluid.

Material required:

- Microtitre plate
- Sheep erythrocyte
- Alsever's solution
- Glutaraldehyde
- Phosphate buffer saline (PBS)
- Test antigen / tissue homogenate
- Hyper immune serum
- Horse serum etc.

Procedure:

1. Collect the sheep blood in equal volume of Alsever's solution, mix thoroughly and store at 4^0C for 2 days before use.
2. Wash erythrocyte thrice with phosphate buffer saline (PBS, pH 7.2) and resuspend the erythrocytes to make 2.5% (v/v) suspension in PBS.
3. Mix 1 ml of 0.6% glutaraldehyde with 9 ml of 2.5% erythrocyte suspension, shake constantly for 10 min at room temperature.
4. Separate the cells by centrifugation at 2000 rpm for 10 min and wash thrice with PBS. Adjust again to make 2.5 % conjugated erythrocyte suspension.
5. Sensitize the conjugated erythrocytes by mixing 4 ml of PBS, 0.4 ml of 2.5 % suspension of conjugated erythrocytes and 1.0 ml of hyperimmune serum. Keep the suspension at 37^0C for 30 min.
6. Again separate the cells by centrifugation at 2000 rpm for 10 min, wash thrice with PBS containing 1% horse serum and finally make 1% suspension in PBS.

Test Proper:

1. For test proper, make 2 fold serial dilutions of test antigen in microtiter plate using PBS.
2. To 0.25 ml volume of each dilution add equal volume of 1 % sensitized erythrocyte suspension and mix gently.
3. Incubate the plate at 37^0C for 2 hrs.
4. Known antigen and PBS without antigen included as positive and negative controls, respectively.
5. The complete agglutination of cells is considered as positive result and titre is expressed as the reciprocal of the highest dilution showing haemagglutination.

Study questions:

1. List some applications of this test.
2. List the differences between PHA and RPHA tests.

Exercise No. -11

Detection of Antigen by latex agglutination Test

Latex agglutination test employs polystyrene beads of <1.0 µm diameter for non specific adsorption of antibodies or antigen, which can be used for detection of antigen or antibodies in laboratory and clinical samples. Like other agglutination-based tests, it is simple to perform and rapid in providing results. In addition, its sensitivity is more.

Material Required:

- Latex beads suspension 10 % (W/V)
- Bovine serum albumin
- Sodium azide
- PBS
- Specific antibodies (Rabbit anti-Rota virus)
- Antigen (Faecal extract 20% as rota virus antigen)

Procedure:

Preparation of Latex Beads:

1. Wash 500 µl Latex beads suspension (0.2 µm diameter) twice by adding 15 ml PBS and centrifugation at 12000 x g for 15 min at 4^0C and finally resuspend in 10 ml PBS to make 4% suspension.
2. Add equal volume of washed latex suspension and partially purified Rabbit anti-Rota virus antibodies (10mg/ml)
3. Shake the suspension for 2 hr. at 37^0C.
4. Wash the antibody coated latex beads thrice with PBS by centrifuging at 12,000 x g for 15 min at 4^0C.
5. To block unbounded or uncoated latex sites resuspend the beads in 10 ml of PBS containing 0.1% sodium azide and 0.1% bovine serum albumin for 10 min at room temperature.
6. Centrifuge at 12000 x g for 15 min and resuspend the pellet in 10 ml PBS containing 0.1% sodium azide and store at 4^0C till further use.

Test proper:

1. Prepare two fold dilutions of the test antigen (20% faecal extract).
2. Mix 25 µl of each antigen dilution with 25 µl coated latex beads on a glass slide.
3. Rock gently for 2 min and read agglutination visually, illuminating the slide from the side, against a dark background.
4. For control mix the beads with rota virus negative samples.
5. The results are interpreted as strongly positive (4+), moderately positive (2+) weakly positive (+) and negative (-).

Reference:

Husdon, L. and Frank, C.H. (1991). Basic techniques, In: Practical Immunology 3rd Edition. Black well Scientific Publication, Oxford.

Study questions:

1. Identify the immunoglobulin (Ig) class involved in latex agglutination test.
2. Explain the role of latex particles size in this test? Give the size of commonly used latex particles.

Exercise No.-12

Coagglutination Test

The coagglutination test using protein A containing staphylococcus Cowan I strain has been used successfully for the diagnosis of bacterial (eg. Brucella) and viral (eg. Goatpox virus, New Castle disease virus, Avian leukosis virus, Infectious bursal disease virus) antigens, typing of pneumococci, identification of mycoplasma strain, etc. The test is easy to perform, specific, sensitive and does not require sophisticated laboratory equipments.

Principle:

The interaction of IgG antibodies with surface of protein A containing *Staphylococcus aureus* through their Fc region, followed by interaction of the Fab region of these same antibody molecule with surface antigens of bacteria/ virus for which they are specific. Thus when the appropriate reagents are present coagglutination will take place in which the Y shaped antibody molecule will serve as the bridge between staphylococcus and the coagglutinated micro-organism for which it is specific. The reaction of protein A with antibodies provide a unique configuration to expose the Fab parts of immunoglobulin molecule outwards and thus enabling the antibody to combine with relevant antigen without any steric hindrance.

Material Required:

- *Staphylococcus aureus* Cowan I strain
- Brain Heart infusion broth.
- Phosphate buffer saline (pH 7.4), (PBS)
- Formaldehyde 0.5%
- Known hyper immune serum and unknown Antigen.
- Sodium azide 0.1 %
- Glass slides etc.

Procedure:

1. Inoculate the culture of *Staphylococcus aureus* Cowan I stain in brain heart infusion broth. Incubate at 37^0C for 24 hrs.
2. Harvest the bacterial cells by centrifugation at 2,500 rpm for 10 min.
3. Wash the pelleted bacterial cells twice with PBS.

4. Treat the washed bacterial cells with 0.5% formaldehyde at room temperature for 3 hrs.
5. Wash the formaldehyde treated bacterial cells thrice with PBS.
6. Adjust the bacterial cells to a concentration of 10% (v/v) in PBS
7. Keep the bacterial suspension in water bath at 80^0C for 30 min.
8. Store the suspension at 4^0C till further use.
9. Take 0.1 ml (10 mg/ml concentration) of inactivated immunoglobulin and mix with 1 ml of 10% suspension of heat treated Staphylococci and allow to react for 30 minute.
10. Wash the suspension of antibody coated Staphylococci twice with PBS and resuspend to give a 10% (v/v) suspension.
11. Add the sodium azide (0.1%) to give a final concentration of 1%.
12. Store the reagent at 4^0C until used.
13. For the coagglutination test proper, 2 drops of sample are mixed with an equal volume of coagglutination reagent on a glass slide and mix thoroughly.
14. The positive reaction is observed with in few seconds. The coagglutination reaction is scored as very strong (4+), strong (3+), medium (2+) and weak (+) based on intensity of reaction and time taken for clear agglutination.

Reference:

Shakya, S. and Joshi, R.K. (1997). Coagglutination test for rapid detection of IBD virus antigen Tropical Animal Health and Production. 29: 105-107.

Study questions:

1. Explain the mechanism of coagglutination taking into account protein A as well as species and subclass of immunoglobulin (Ig).
2. Mention important applications of this test.
3. Name the culture media and their composition which is used for the propagation of *Staphylococcus aureus* (Cowan I Strain).
4. Plan slide coagglutination test for field diagnosis of H.S.
5. Draw a typical diragram of IgG indicating both Fc and Fab regions.

Precipitation Test

Principle:

The technique is widely used for characterization and quantitation of antigen or antibody. The tests are easy to perform, give extremely valuable information and do not require sophisticated instruments. The ability of antibodies to form precipitin lines with its specific antigen in the gel is the basis of this technique.

When a given antigen in soluble form is mixed with its corresponding antiserum in the gel, the two reactants combine and form precipitating aggregates. The gel (1% agarose/noble agar/agar solution) are semi solid media that provide the pore size sufficiently large for the free diffusion of immunoreactants until antigen and antibody reach the optimal concentration for lattice formation. Presence of unrelated substances in test solution is tolerated and does not interfere with the reaction. The various factors that affect the diffusion rate are molecular weight and size of the antigen, temperature, gel viscosity and interaction between gel matrix and reactants.

Types of immunoprecipitation technique -

The technique can be divided into two main categories-

A Immunodiffusion (ID)

1. Simple / single immunodiffusion.
   a. One dimension (tube) eg Oudin technique
   b. Two dimension (plate) eg Single Radial Immuno Diffusion
2. Double immunodiffusion (DID)
   a. One dimension (Tube)
   b. Two dimension (plate) eg Ouchterlony technique.

B. Gel electrophoresis-

1. Counter current immuno electrophoresis (CIEP)
2. Immune electrophoresis (IEP)
3. Rocket immuno electrophoresis (RIEP)

Agar Gel Precipitation Test

The Agar Gel Precipitation Test (AGPT) or Ouchterlony technique is widely used for characterization and quantitation of antigens and antibodies. The test is easy to perform, give extremely valuable information and do not require sophisticated instruments.

Materials:

- Agarose,
- Normal saline solution (NSS)/PBS
- Microscopic slide
- Amido black Stain
- Antigen and antibodies etc.

Procedure:

1. Take a good quality agar/agarose and prepare a 1.0% suspension in normal saline solution, boil and cool at 60^0C and add sodium azide to a final concentrations of 0.01 %.
2. Select a clean scratch free, grease free microscopic slide and pour 5 ml molten agar over it and allow it to solidify.
3. Keep the slide at 4^0C for 1 hr for complete setting.
4. Punch out the wells by using gel cutter, keeping 3-4 mm distance between each well.
5. Seal the bottom of the wells with little of molten agar. This will prevent leakage of antigen or antibody between gel and the slide.
6. Add 10-20 µl known antiserum in the central well and same volume of unknown antigen or field samples in peripheral wells.
7. Incubate the slides in a moist chamber at room temperature for about 24 hrs.
8. After incubation the antigen antibody reaction can be seen in the form of precipitation arc / line. The intensity of the arc or line is proportional to the concentration of the antigen or antibody.

Staining of Precipitin lines:

The precipiting bands can be stained for considerable improvement in sensitivity and for permanent records.

1. Wash the gel plates with 1.5 % sodium chloride solution for about 4 days, change the solution three times a day to remove the non precipitated proteins.
2. Remove the salt by keeping the gel in distilled water for 1 hr.
3. Dry the gel at 37^0C after putting a wet filter paper over them.
4. After complete drying remove the filter paper and clean the slides for few seconds in running water.
5. Prepare the Amido black stain by using 1 % amido black in 7% acetic acid. Stain the slides for 15-30 min. Destain with 5 % acetic acid.
6. After complete destaining, dry the slides in air and record the results.

Reference:

Talwar, G.P. (1983). A Handbook of Practical Immunology. Vikas Publishing House Pvt. Ltd.

Study questions:

1. How doses the quality of agar affect this test? Name the types of agar generally used for this purpose. What is endosmosis/electro endosmosis?
2. Illustrate diagrammatically the line of identify between antigens. Explain its significance.
3. Explain the relationship between two antigens which demonstrate spur formation in precipitation test.
4. List the diseases where this test is routinely used.
5. What about the salt concentration of buffer solution when avian serum samples are used?

Counter Imuno Electrophoresis

Principle:

The immuno electrophoresis involves the migration of charged protein particles in an electric field. The rate of migration depends on the magnitude of electric charge over the particles and some other factors like voltage of the current, pH of the buffer etc. At pH 8.6, antigens migrate towards anode while antibody, towards cathode and at equivalence precipitation will occur. The electric current shortens the time required for development of precipitation and that is why this method is faster than double immuno diffusion.

Materials:

- Agarose
- Veronal buffer
- Antigen
- Antiserum
- Whatmen filter paper No. 1
- Microscopic slides
- Electrophoresis apparatus etc.

Procedure:

1. Prepare the veronal buffer as follows-

Sodium barbital	-	10.31 gm
Barbituric acid	-	1.84 gm
Sodium acetate	-	6.80 gm
Disttled. water to make	-	1 lit
pH	-	8.6

2. Prepare 1% agarose gel in veronal buffer and pour about 5 ml over microscopic slide.
3. After complete setting of the gel, punch out the wells 3-4 mm apart.
4. Place the suspected antigen sample on cathode side well and antiserum in the anode side well.
5. Place the slide in electrophoresis apparatus tank and connect it with buffer tanks with the help of paper wicks.

6. Supply current of 7-8 mA / slide for 1-2 hrs.
7. By the end of electrophoresis observe for precipitation lines.
8. Wash, dry and stain the slides as described under AGPT.

Reference:

Shakya, S. (1989). Studies on EDS-76 virus strains with reference to seroprevalence, Adaptation is embryonated chicken eggs and in tissue culture system and their antigenic relationship. M.V.Sc. thesis, J.N.K.V.V., Jabalpur.

Study questions:

1. Explain about the
   a) Electrophoresis
   b) Immunoelectrophoresis
   c) Counter immunoelectrophoresis
   d) Radial immunodiffusion
2. Name the diseases which can be diagnosed by using CIEP test.

Complement Fixation Test

Principle:

The test depends on the fixation of complement by an antigen-antibody system. Two systems are used. (i) The test system containing the known type specific serum and the infected tissues/cell culture fluid to be typed or quantified for antigen or the test system containing the viral antigen and the serum to be tested for antibody and (ii) the indicator or hemolytic system containing sheep red blood cells and rabbit anti-sheep red blood cell serum or hemolysin. The complement in the test serum must be inactivated by heat.

The test is performed in two steps: (i) The serum to be tested, complement and antigen re allowed to react at a given temperature and (ii) sheep red blood cells sensitized with hemolysin are added and the mixture incubated. If the serum tested contains antibody to the antigen, complement is fixed when the combination of antigen and antibody occurs. The complement therefore, does not participate in the hemolytic system for lysis of the sensitized cells. The absence of lysis indicates positive test for identification of antibody. Lysis indicates a negative test i.e., the serum did not contain antibody to the antigen employed and complement was not fixed. Complement was then available for participation in the hymolytic system. The complement fixation test may also be used to detect antigen using a serum known to contain antibody.

Materials:

- Microplates
- Dropping pipettes, 0.025 ml capacity
- Virus antigen
- Hyper immune serum
- Complement
- Haemoloysin
- Incubator/Water bath
- Reading mirror

Preparation of reagents:

Virus antigen: Any material containing the virus may be used as antigen. It could be infected tissue, fluid and cell culture. For use in the test, virus suspension should be centrifuged to remove cellular debris. Sometimes infected tissue material may require treatment with chloroform, fluorocarbon, trypsin and polyethyleneglycol. If antigen gives anti-complement activity, it should be appropriately treated and diluted.

Antibodies: It may be known specific antiserum or serum from infected and convalescing animal. The serum or antiserum is heated at 56^0C for 30 minutes to remove complement effect.

Complement: Guineapig fresh serum constitutes the complement. Freeze-dried complement is also available commercially. Healthy guineapigs are bled by cardiac puncture of bled from the throat and blood is collected in clean sterile tubes and allowed to clot at room temperature for 1-2 hours. Tubes are then kept at 4^0C for 4-6 hours and serum is separated. It is centrifuged and then distributed in small quantities in glass vials and stored deep frozen (20^0C or below). The titre of complement is determined before use.

Haemolysin: Rabbit antiserum against sheep erythrocytes can be prepared in laboratory or obtained from commercial sources.

Sheep erythrocytes: Sheep blood is collected aseptically in equal volume of Alsever's solution (appendix) and allowed to age at 4^0C for at least 4 days. A small volume of blood is taken and centrifuged at 1,500 rpm for 15 minutes. The supernatant is discarded and cells are resuspended in Veronal buffer solution (VBS) and centrifuged as above. Two more washings are done in Veronal buffer solution. Then red blood cells are suspended in saline solution and packed by centrifugation at 3,000 rpm for 15 minutes. The erythrocyte sediment is resuspended to contain a final concentration of 1% RBC.

Titration of complement and haemolysin: (to be discussed in the class)

Test proper:

1. Deliver 25 µl of antiserum per well
2. Deliver 25 µl antigen per well
3. Deliver 50 µl complement (so as to contain 3 HD/50µl) per well
4. Add 25µl µl Veronal buffer in serum control and antigen control, 50µl Veronal buffer in complement control and 75µl Veronal buffer in rbc control.
5. Shake the plate to mix the reagents, seal with tape and incubate in water bath at 37^0C for 30 minutes.
6. Prepare the Haemolytic system, which should contain 4MHD/ml haemolysin and 1% sheep erythrocytes. Keep the also in the water bath at 37^0C for 30 minutes for sensitization along with plates.
7. Remove the plate and ad 50µl or Haemolytic system per well, mix by shaking the plate, seal with the tape and again keep at 37^0C for 30 minutes.
8. Remove the plate, centrifuge in a microspin centrifuge for 5 minutes and take the reading. Alternatively, keep the plate in a refrigerator at 4^0C and take preliminary reading after 2 hours and final reading the next morning.

Interpretation:

The degree of fixation is read in terms of amount of setting of rbc for which a score in the scale of 0 to 4 is given.

0= No rbc settled (100% haemolysis)

1= 25% rbc settled (75% haemolysis)

2= 50% rbc settled (50% haemolysis)

3= 75% rbc settled (25% haemolysis)

4= 100% rbc settled (0% haemolysis)

A reading of 2 of above in a particular type specific serum is indicative of the type to which the virus antigen belongs. Ideally all controls, antigen control, sera control, complement control would show a score of 0 i.e. 100% haemolysis. However, if some setting is observed in antigen, sera control due to anti complementary activity, this score should be deducted from the test proper scores.

Reference:

Bansal, R.P. (1996). Techniques in Diagnostic Virology. Published by ICAR, New Delhi

Study questions:

1. Define Complement. What do you mean by inactivation of complement?
2. Prepare a flow chart to demonstrate sequence of reactions involving components of complement leading to bacteriolysis/haemolysis.
3. Develop a protocol for titration of haemolysis and complement.
4. Why only guineaping serum is used as a source of complement?

Exercise No.-16

Fluorescent Antibody Technique

Immunohistochemical technique using fluorescence as a tracer has achieved wide recognition in the study of both cell surface and intercellular antigens. Such method can be used to determine quantitatively or qualitatively presence of antigen in various cells and tissues.

Principle:

Antibodies that are bound to cells or tissue sections can be visualized by tagging the antibody molecules with a fluorescent dye or fluorochrome. In this technique, the most commonally used fluorescent dye are fluorescein isothiocynate and rhodamine. Both dyes can be conjugated to the Fc region of an antibody molecule without affecting specificity of the antibodies. Each of these dyes absorbs light at one wavelength and emit light at a longer wavelength. Fluorescein absorb blue light (490 nm) and emits an intense yellow green fluorescence (517 nm). Rhodamine absorbs the yellow green range (515 nm) and emits a deep red fluorescence. The emitted light is generally viewed with a fluorescence microscope which is equipped with a UV light source and excitation filters.

The Fluorescent antibody staining of cell membrane molecules or tissue sections can be direct or indirect. In direct staining the specific antibody (called primary antibody) is directly conjugated with fluorescein dye. In indirect staining the primary antibody is unlabeled and is detected with an additional fluorochrome labeled reagent.

Materials:

- Capripox infected kid testes cell culture monolayer.
- Goat anticapripox virus antibodies.
- Rabbit antigoat FITC conjugate.
- PBS (pH 7.4)
- Glycerol.
- Fluorescent microscope, etc.

Procedure:

1. Wash the monolayer on coverslip/smears with pre-warmed PBS (pH 7.4) and then fixed in chilled acetone for 20 min at 4^0C.
2. Place the coverslip on a glass slide with cell facing upward and pour two drops of diluted hyperimmune serum (1:50) on it.
3. Incubate in humid chamber for 1 hr at 37^0C.
4. Wash the coverslips thrice with PBS each of 5 min.
5. Flood the coverslip with rabbit antigoat FITC conjugate (1:100 diluted with PBS and filtered by 0.22 µm membrane filter)
6. Incubate for 1 hr in humid chamber at 37^0C.
7. Wash the coverslips thrice with PBS and mount in fluid consisting of 9 parts of glycerol and 1 part of PBS.
8. Control coverslips were processed similarly and examined under fluorescent microscope.
9. Observe under the fluorescent microscope for fluorescence.

Reference:

Shakya, S. (2001). Identification and molecular characterization of Immunogenic proteins of capripox virus. Ph.D. Thesis, G.B. Pant University of Agriculture & Technology, Pantnagar.

Study questions:

1. Draw illustrative diagram to explain the principle of FAT.
2. Write down the advantages of indirect immuno fluorescence staining over direct staining.
3. Discuss various applications of FAT in the field of Vet. Diagnostic Microbiology.
4. Write down the precautions in FAT to avoid non specific fluorescence.

Exercise No.-17

Enzyme Linked Immunosorbant Assay

Enzyme linked immunosorbant assay, commonly known as ELISA (or EIA), have had an important impact on the immunodiagnosis of infectious diseases. Although ELISA originally described the detection of antibody, modifications of the procedure have become numerous.

Principle:

ELISA are based on the use known enzyme antibody conjugate (E-Ab). Substrates that yield easily detected coloured or fluorescent products measures the enzyme and thereby its linked antibody. The enzyme used are alkaline phosphatase, horse radish peroxidase etc. Because of their sensitivity specificity and reactivity, enzyme immunoassays have been extensively used as aids to clinical diagnosis and for research applications.

Indirect ELISA:

Antibodies can be detected or quantitated with indirect ELISA. In this ELISA, wells are coated with the antigen and in second step, the test antibodies raised in mice are allowed to bind the antigen. Test antibodies bound to the antigen are then incubated with anti-mouse horse radish peroxidase (HRPO) conjugate. Anti-mouse HRPO conjugate bound to the test antibody gives a colour reaction when incubated with its chromogen substrate. In a negative reaction, the conjugated antibodies are not immobilised in the wells because they don't find their antigen (Test serum antibodies) in the wells and hence colour does not develop.

The test is performed with a purified antigen and therefore, optimum dilution of the anti-mouse HRPO conjugate only needs to be determined.

Materials:

- 96 well ELISA plate
- Aliquot of test antiserum (1/10 dilution)
- Aliquots of the antigen and the mock antigen (BSA, Milk powder)
- Anti-mouse HRPO conjugate (Aliquot of 1/10 dilution)
- Coating buffer - 10ml
- Washing buffer-500ml
- Diluent-15 ml
- Orthophenylene diaminodihydrochloride (OPD) 6mg, substrate buffer (10ml) and hydrogen peroxide
- 1 M H_2SO_4 10 ml
- Few 10 ml and 1 ml pipettes
- Single channel and multichannel pipettes (40-200 µl)
- Incubator at 37^0C
- ELISA reader
- Disinfectant for the antigen
- Wad of filter papers

Procedure:

1. Mark the plate as shown in fig.
2. Dilute the antigen in coating buffer to give about 10 µg/ml concentration. Prepare about 6 ml of the dilution of the antigen.
3. Aliquot 50 µl of the diluted antigen in each well of the plate except the well A1. To this, aliquot 50 µl of the mock antigen. The well A1 would be kept as negative control. Either leave the plate at 4^0C overnight or incubate at 37^0C for 2 hr.
4. Decant the antigen in an appropriate disinfectant. To wash the ELISA plate, fill the wells of the plate with the washing buffer and discard. Repeat it 5 x. Blot dry the plate by tapping on a wad of filter papers.
5. Pipette 100 µl of the test antiserum (1/10 diluted stock) into 1 ml of the diluent to prepare 1/100 dilution of the test antiserum.
6. Aliquot 100 µl of the 1/100 diluted test antiserum into wells A1 to H1. Aliquot 50 µl of the diluent in all the remaining wells of the plate.

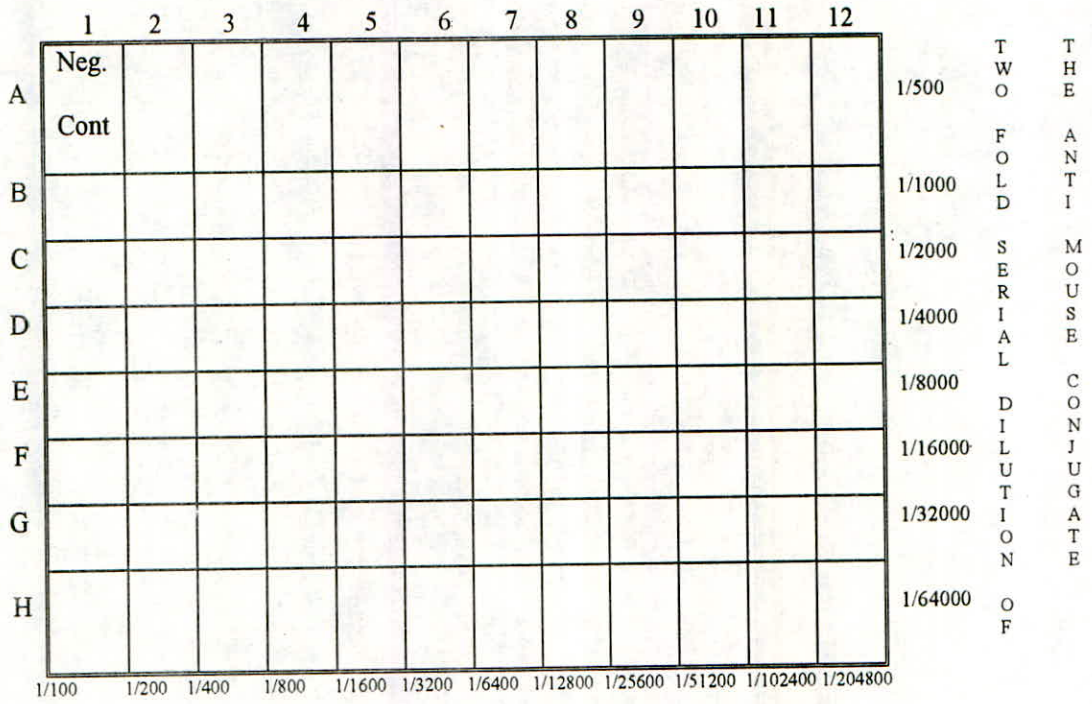
7. Set a 8 channel pipette to 50 µl. Transfer 50 µl from wells A1-H1 to A2-H2. Mix by pipetting in and out 2-3 times and then again transfer 50 µl from A2-H2 to A3-H3. Like-wise go across the plate to column 12th of the plate. Starting from 1/100, two fold serial dilutions of the test antiserum are thus made in 50 µl volume.
8. Incubate the plate at 37^{0}C for 1 hr.
9. Wash and dry the plate as described above.
10. Pipette 40 µl of the 1/10 stock of the anti-mouse HRPO conjugate into 2 ml of the diluent to make 1/500 dilution of the anti-mouse HRPO conjugate.
11. Aliquot 100 µl of the 1/500 diluted anti-mouse HRPO conjugate into wells A1-A12 and 50 µl diluent into remaining wells of the plate.
12. Mix as described earlier and transfer 50 µl with a 12 channel micropipette from A1-A12 to B1-B12. Like-wise go across the plate upto H row of the plate (H1-H12). Two fold serial dilutions starting from 1/500 in 50 µl volume of the anti-mouse HRPO conjugate are thus made.
13. Incubate the plate at 37^{0}C for 1 hr, washed and dry as described above.
14. Add 10 ml of the substrate buffer to 6 mg OPD. Mix and add 20 µl of H_2O_2.
15. Aliquot 50 µl of this OPD solution into all wells of the plate.
16. Keep it for about 10 mm. at 37^{0}C for colour development.
17. Stop the reaction by adding quickly 50 µl of 1M H_2SO_4 to all the wells.
18. Set the optical density (OD) of the well A1 as blank and then read the OD of each well of the plate in ELISA reader at wave length of 492 nm.

Observation:

More intense colour should develop towards top left side corner of the plate, lesser at top right and bottom left corners and least colour intensity at bottom right corner. Colour development in all rows should show a pattern of gradation of more intense colour at left side to lesser colour towards the right side of the plate.

Interpretation:

Select the dilution of the anti-mouse HRPO conjugate that gives OD of 0.3 or above at or above dilution of 1/5,000 or close to dilution 1/5,000 of the test antiserum.



NEGATIVE CONTROL-MOCK ANTIGEN+ THE ANTISERUM (1/100) + ANTI MOUSE HRPO CONJUGATE (1/500)

FIG:- INDIRECT ELISA-LAYOUT OF A 96 WELL ELISA PLATE

Precautions:

- Purified antigen should be used to get only specific antibody - antigen binding positivity.
- Impurities in the antigen preparations might decrease the amount of antigen coated in the wells because other impurities would also become coated along with the antigen.
- Antigen might not be stable at very high alkaline pH of the coating buffer or might change structurally when getting coated to the surface of the plate wells.

Reference:

Kumar, A. (1997). Laboratory Manual- Murine Monoclonal antibody production. ICAR Center of Advanced Studies on Vet. Microbilogy. CSS Haryana Agricultural University, Hisar.

Study questions:

1. Define the followings in relation to ELISA:
   i. Enzymes
   ii. Substrate (s)
   iii. Stopping solution
   iv. Indicator system
   v. Haemolysin
2. Give a protocol for preparation of HRPO and antibodies conjugate.
3. List the conjugates available in the market with sources.
4. Name the diseases where ELISA is routinely used for diagnosis. List the merits and demerits of this test.
5. Differentiate between Direct and Sandwich ELISA.

Exercise No.-18

Sandwich ELISA

The principle of sandwich ELISA is similar to that of indirect ELISA. In this ELISA, the antigen is not coated directly on the surface of ELISA plate wells but is trapped by antibodies which have been coated on the surface of ELISA plate well.

Optimum dilution of the trapping/coating antibody and antimouse HRPO conjugate are determined in the checker board titration. A fixed appropriate concentration of the antigen is used in the test.

Materials:

- Trapping antibody raised against the purified antigen in any species other than the mouse.
- All other materials are the same as described for indirect ELISA.

Procedure:

1. Mark the plate as shown in fig. to divide it into four blocks of the wells. Each block has 4x6 wells.
2. Pipette 60 µl of the trapping antibody (1/10) into 3ml of coating buffer to make 1/500 dilution of the trapping antibody.
3. Aliquot 100 µl of the 1/500 dilution of the trapping antibody into wells A1-A12 and E1-E12. In remaining wells of the plate, aliquot 50ml of the coating buffer.
4. Set a 12 channel micropipette to 50 µl, mix well by pipetting in and out and transfer 50 µl to row B (B1-B12) from the wells of row A. Go across the plate upto row D (D1-D12) and discard 50 µl from wells of row D to keep the volume 50 µl in all wells of the row D. Similarly, transfer 50µl from wells of row E (E1-E12) to wells of row F (F1-F12) and go across the plate to row H (H1-H12). Discard 50µl from wells of row H to keep the volume 5Oµl in all wells.

   This way two fold serial dilution starting from 1/500 to 1/4,000 dilution of trapping antibody in 50µl volume are made in rows from A-D and similar two fold dilutions in rows E-H.
5. Incubate the plate at 37^0C for 2 hr or leave it overnight in refrigerator at 4^0C.
6. Wash and dry the plate as described earlier.

7. Add 50 µl of the antigen to all the wells except A1, A7, E1 and E7. Add 50 µl of the mock antigen to these wells to act as negative control wells for their respective block.

8. Incubate at 37^0C for 1 hr for trapping of the antigen.

9. Wash and dry the plate.

10. Pipette 40µl of the 1/10 stock of test antiserum into 2 ml of diluent to make 1/500 dilution.

11. Aliquot 100µl of 1/500 dilution of the test antiserum into wells of column 1(A1-H1), wells of column 7 (A7-H7). Aliquot 50 µl of the diluent to all other wells of the plate.

12. Mix as described above and transfer 50 µl from wells of column 1 to wells of column 2. Go upto column 6. Discard 50µl from column 6 to keep the volume 5Oµl. Similarly, Go across the plate from column 7 to 12. Discard 50 µl from the wells of column 12.

    This way two fold serial dilution of the test antiserum starting from 1/500 to 1/16,000 (six steps) are made in wells of column 1 to 6 and similar dilutions in wells of column 7 to 12 in 50µl volume.

13. Incubate the plate at 37^0C for 1 hr.

14. Wash and dry the plate.

15. Make four dilutions of the conjugate. The dilutions should be chosen on the basis of expected activity of the conjugate. Four dilutions 1/1000, 1/2000, 1/3000, 1/4000 are described here. These dilutions are expected to give end point in most applications. In case, more number of conjugate dilutions are required to be included then another plate could also be used and the plate in the similar manner be divided into four blocks of the wells.

16. Pipette 15µl of 1/10 stock into 1.5 ml of diluent for 1/1000 dilution of the conjugate, 8µl into 1.6ml for 1/2000 dilution, 5µl into 1.5ml for 1/3000 dilution and 4µl into 1.6ml for 1/4000 dilution of the conjugate.

17. Aliquot 50µl of 1/1000 dilution of the conjugate into the wells of block 1 (A1-D1 to A6-D6), 50µl of 1/2000 into wells of block 2 (A7-D7 to A12-D12), 50µl of 1/3000 dilution into wells of block3 (E1- H1 to E6-H6) and 50µl of 1/4000 dilution into wells of block four (E7-H7 to E12-H12).

18. Incubate the plate at 37^0C for one hour.

19. Wash and dry the plate as described above.

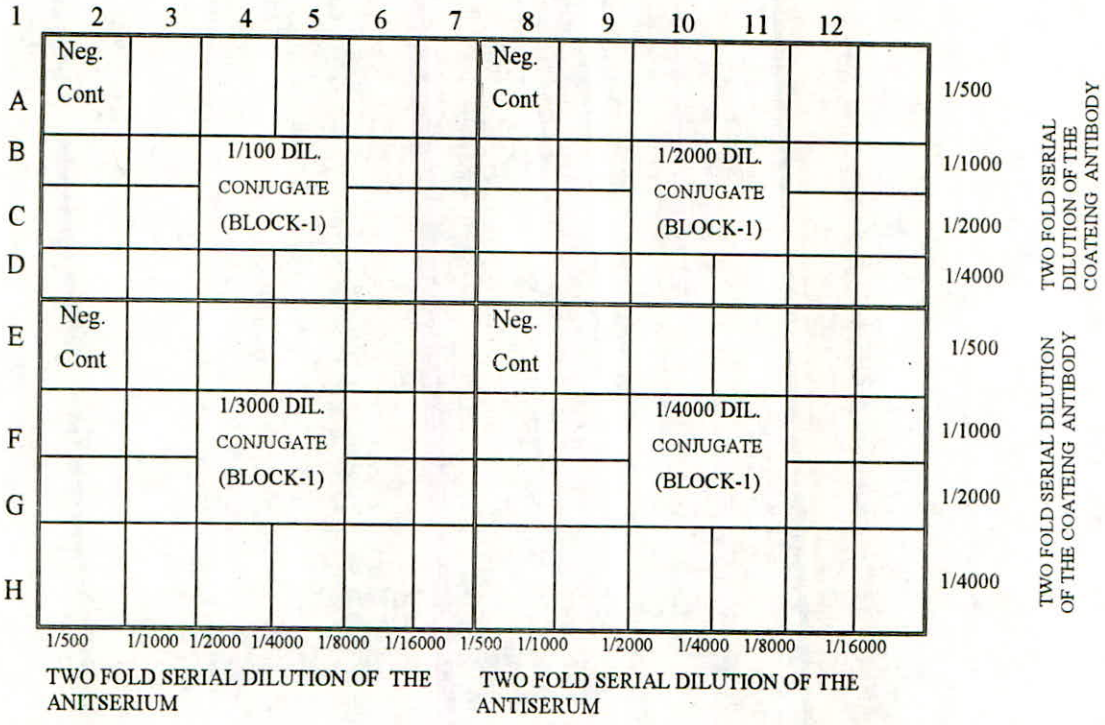
20. Add 10 ml of the substrate buffer to 6mg aliquot of OPD. Add 20 µl of H_2O_2 and then pipette 50 µl of this OPD solution into all wells.

21. Incubate the plate at 37^0 C for about 10 min.

22. Stop the reaction by pipetting 50µl of 1M H_2SO_4 to all the wells.

23. Set all wells as blank and then read the OD values of all the wells in ELISA Reader at 492 nm.

Top left side of all the blocks should have more intense colour development. Wells A1, A7, E1 and E7 are negative controls therefore very little colour, if at all, should develop in these wells.



NEGATIVE CONTROL- COATING ANTIBODY (1/500) + MOCK ANTIGEN + THE ANTISERUM (1/500) + ANTI MOUSE HRPO CONJUGATE (DIL. AS PER RESPECTIVE BLOCK)

FIG: SANDWICH ELISA-LAYOUT OF A 96 WELL ELISA PLATE

Interpretation:

Select the highest dilution of trapping antibody and its corresponding conjugate dilution which gives OD value of 0.3 or above in 1/5, 000 dilution, above or close to 1/5, 000 dilution of the test antiserum. Further more, the negative well of that conjugate block should also have little or no background colour development.

Precautions:

- This ELISA gives more specificity and sensitivity because trapping antibodies trap only the antigen and not any other contaminating proteins in the antigen preparations. Antigen is selectively trapped therefore it doesn't have to compete with other contaminating protein in coating. This way optimum antigen could be made available to test antibody to bind.

Reference:

Kumar, A. (1997). Laboratory Manual Murine Monodonal antibody production. ICAR center of Advanced studies on Vet. Microbilogy. CSS Haryana Agricultural University, Hissar.

DOT-Immunobinding Assay

DOT-immunobinding assay is an alternative test for plate ELISA requiring less time. It involves the direct application of antigen on nitrocellulose paper (NCP) and detection of Ag-Ab complexes by development of coloured dot using chromogenic substrate.

The fast brown coloured dot developed during the test remains visible for several years, which could be used for comparative and retrospective studies in the positive sera of animal suffering from the disease.

Materials:

- Protein antigen
- Known antiserum
- Bovine serum albumin
- PBS containing 0.05% Tween-20 (PBS-T buffer)
- Nitro cellulose dipsticks
- Rabbit antichicken HRPO conjugate
- 3, 3 Diaminobenzidine tetrahydrochloride, DAB
- Hydrogen peroxide
- Microtiter syringe
- Foetal calf serum

Procedure:

1. Wash the nitrocellulose dipsticks with PBS for 1-2 min and dry in air.
2. Coat the dipsticks with 1-2 µl of optimum concentration of antigen (prepared in carbonate buffer pH 9.6).
3. Dry the spots and wash thrice each for 1-2 min with PBST and then place in 2% BSA for 30 min for blocking.
4. Wash the strips 3 times with PBST and incubate in 10 fold diluted antiserum at 37^0C for 30 min.
5. Again wash the strips thrice with PBST and then incubate in rabbit antichicken horse radish peroxidase conjugate (1:5000 dilution) at 37^0C for 30 min.

6. Wash with PBS, dry at room temperature and incubate in freshly prepared horse radish peroxidase precipitable substrate solution (0.1% Diamino benzidine tetra hydrochloride in 0.1% citric acid phosphate buffer pH 5 and 5 µl H_2O_2) for 30 min.
7. The appearance of brown spot indicated the positive reaction whereas no coloured dot developed in negative reaction. On the basis of intensity of the colour reaction, it can be graded as 1+, 2+, 3+, etc.
8. Known serum and PBS can used as positive and negative controls, respectively.
9. To resolve the titres of antisera checkerboard studies with plate ELISA can be performed with this test also.

Reference:

Shakya, S. (2001). Identification and molecular characterization of Immunogenic proteins of capripox virus. Ph.D. Thesis, G.B. Pant University of Agriculture & Technology, Pantnagar.

Study questions:

1. Why blocking is necessary in DOT immunobinding assay.
2. Write down the advantages of DOT immunobinding assay over plate ELISA.
3. Write down the application of DOT immunobinding assay in diagnosis of diseases of vet. importance.

Western Blotting of Viral Proteins

Western Blotting is extremely useful technique for the identification and quantitation of specific polypeptides in a complex mixture of proteins. The technique involves the transfer of electrophoratically separated proteins from gel to solid support (eg. Nitrocellulose, Poly vinilidine fluoride, PVDF etc) and subsequent detection of the proteins with specific antibodies. These antibodies (polyclonal or monoclonal) react specifically with the epitopes displayed by target proteins over the solid support. Western blotting has sensitivity of immunoassays. Further more because electrophoretic separation of protein is always carried out under denaturing conditions, the problem of solublization, aggregation and co-precipitation of target proteins with adventitious protein are eliminated.

Western blotting involves below mentioned steps

1. Preparation of sample.
2. Resolution of sample by SDS-PAGE.
3. Transfer of proteins onto membrane.
4. Immunodetection of proteins on membrane.

1. Preparation of Sample:

i. Take secondary kid testes cell culture grown capripox virus. Purify it by sucrose density gradient method.

ii. Add 25 µl of purified virus preparation with same volume of 2 x sample buffer and 2% mercaptoethanol.

iii. Heat the suspension at 80^0C for 5 min and centrifuge at 8000 rpm for 2-3 min. Take 50 µl sample (containing 40 µg protein) for single well.

2. Resolution of sample by SDS – PAGE:

i. Assemble the vertical slab gel electrophoresis apparatus (Atto, Japan) using clean glass plates of 14 x 14 cm with 1.5 mm spacer.

ii. Prepare the 10% resolving and 4% stacking gel solution as mentioned in appendix.

iii. Pour the resolving gel solution upto the arrow mark. Overlay a layer of water or isopropanol over the running gel solution and allow to polymerize the gel for 15-30 min.

iv. Remove the overlay water layer and fix the comb over resolving gel solution and then add stacking gel solution and allow to polymerize the gel for 10-15 min.

v. Assemble the gel in electrophoresis tank.

vi. Load the samples and molecular weight marker in the wells and run the gel at 60 V till the sample entered the resolving gel and after that run at 80 volts till the tracking dye reached the bottom of gel.

vii. Remove the electrophoresed gel and carefully cut into 2 parts.

viii. Stain one part in Coomassie Brilliant Blue for one hour followed by destaining in 40% methanol-10% acetic acid. The staining helps to check the proper separation of polypeptide.

ix. Use second part of the gel for transfer onto membrane.

3. Transfer of proteins onto membrane:

Polypeptides separated on SDS-PAGE using discontinuous buffer system can be transferred onto PVDF membrane (0.45 μm pore size) by semi-dry method of electrobloting.

i. Soak two sheets of 3 mm thick Whatman filter paper in anode buffer I and place onto center of graphite anode electrode plate.

ii. Soak another sheet of filter paper in anode buffer II solution and place on top of the first two sheets of filter paper.

iii. Soak PVDF membrane (earlier treated with 100% methanol for 15 second followed by distilled water for 2 min.) in anode buffer II and place on the top of filter papers.

iv. Place the gel on membrane followed by stacking of three sheets of filter, soaked in cathode buffer.

v. Cover the assembled transfer stack with cathode plate and apply current of 0.8 mA/cm^2 for one hour.

vi. Take out membrane from sandwich and mark it to follow the orientation of the transfer.

vii. To assess the quality of transfer, cut the lane corresponding to molecular weight marker from the membrane and stain with 0.1% Coomassie Brilliant Blue for 2 min. followed by destaining in 50% methanol, 7% acetic acid for 10 min.

4. Immunodetaction of proteins on membrane:-

i. Dry the protein blotted membrane by soaking in 100% methanol for 10 second then place on filter paper for 15 min. for evaporation of methanol.

ii. Incubate the blotted membrane for 1h with specific immunoglobulins (ammonium sulfate precipitated hyperimmune serum) diluted 1/50 in blocking buffer.

iii. Wash the membrane twice with PBS each for 5 min and incubate for 30 min with rabbit antigoat HRPO conjugate (diluted 1 : 2000 in blocking buffer).

iv. Wash the blotted membrane as mentioned above and transfer into freshly prepared 50 ml of substrate solution (containing 10 mg of diamminobenzidine tetra hydrochloride and 20 ml of 30%, V/V, H_2O_2 for few minutes.

v. Stop the reaction by washing with distilled water.

vi. Dry the membrane and store in a dark place.

Reference:

Shakya, S. (2001). Identification and molecular characterization of Immunogenic proteins of capripox virus. Ph.D. Thesis, G.B. Pant University of Agriculture & Technology, Pantnagar.

Study questions:

1. Diagrammatically explain the various steps involved in western blotting.
2. Explain-
   Southern blotting
   Northern blotting
3. Write down the application of WB in diagnosis of diseases.
4. Write down the advantages of WB over other immunodiagnostic techniques.

Exercise No.-21

Evaluation of Phagocytosis : Carbon Clearance Test

Phagocytosis is one of the most important host immune defense mechanisms against invading micro organisms (bacteria, viruses, fungi, parasites and protozoa). The two major categories of cells participating in this process are (i) Granulocytes (Polymorphonuclear phagocytes) and (ii) Mononuclear phagocytes (Monocytes and macrophages). There are many methods / techniques to determine function of macrophages *in vitro* and *in vivo* system. Among them carbon clearance test is used for the measurement of *in vivo* phagocytosis in mice.

Principle:

Microorganisms or their experimental equivalent (carbon particles) are readily engulfed by circulating and tissue fixed phagocytes. The cells of reticulo endothelial system are all capable of ingesting foreign materials and degrading it by means of intracellular enzymes in phagolysosomes. The carbon particles are stable in the blood stream and unlike Indian ink do not cause thrombosis in lung. These particles are uniform in size and measure 2500 nm in diameter. When this preparation in injected intravenously, the carbon in removed by the fixed macrophages mainly of liver and spleen. The rate of removal of carbon particles from the blood stream is a measure of reticulo endothelial activity. If the blood concentration of carbon is plotted against time, the removal follows an exponential curve and therefore, rate of phagocytosis is dependent on initial concentration is proportional to the concentration in blood and inversely proportional to the amount of carbon already phagocytosed. The logarithm of blood concentration against times gives a straight line, the slope of which (K) measures the rate of phagocytosis i.e. phagocytic index.

Materials:

- Colloidal carbon suspension:- Melt the stock solution in boiling water and thoroughly shake before use. Concentration of stock solution is 64mg/ml. Dilute it in NSS to give a working concentration of 16mg/ml that is used in mice.
- Micropipette (20 µl)
- A stop watch
- A glass and metal tuberculin syringe
- Test tubes
- 1% (v/v) acetic acid
- Spectronic–20
- Glass slide
- Mice, etc.

Procedure:

1. Take 4 ml of 1% v/v acetic acid in three tubes and labeled them as 0, 3 and 15.
2. Weigh the mice.
3. Collect 20 µl of blood by venipuncture and hemolysed the blood in tube no. 0.
4. Now inject colloidal carbon suspension into the tail vein. Divide the weight by 100 to obtain the amount of colloidal carbon (16mg/ml) in ml e.g. for 21g animal the does is 0.21ml.
5. Twenty µl of blood is then collected by venipuncture at 3 and 15 min. post inoculation of colloidal carbon. The blood is then hemolysed in 4 ml of 1% acetic acid by adding it in test tube no. 3 and 15, respectively.
6. Kill the mice and take out liver and spleen on a filter paper.

Observations:

Body wt. of the mice (W) and weight of liver and spleen (WLS)

The amount of carbon in blood samples at 3 and 15 minutes is determined by measuring optical densities with the help of spectronic 20 at 650 nm, using zero minute blood sample as blank for a particular mice.

Calculations:

1. The phagocytic index (k) is determined for each mice by the following formula

$$K_{16} = \frac{\text{Log 10 O.D. at 3 min - log 10 O.D. at 15 min}}{12}$$

2. Because a carbon dose of 8mg/100g mouse weight is commonly used it is convenient to reduce all results of K_8 as follows :

$$K_8 = K_{16} \times 2$$

3. For allowing the differences in body wt. and organ (liver and spleen) weights, the corrected phagocytic index (a) is derived by the following formula.

$$a = \frac{W}{WLS} \sqrt[3]{k_8}$$

Reference:

Kadian, S. (2003). Production and use of instructional material on Veterinary Immunology and Serology. ICAR conter of Advanced studies on Vet. Microbiology CCS Haryana, Agriculture University, Hissar.

Study questions:

1. Diagrammatically explain the various steps of phagocytosis.
2. Explain the difference in phagocytosis caused by neutrophils and Macrophages.
3. Name three bacteria which are not destroyed by phagocytosis.

Exercise No.-22
Contact Hypersensitivity: An Assays for Cell Mediated Immunity

The host immune system responds to any kind of foreign antigen via inducing humoral and/or cellular immune response. Besides their role in protective immunity, these responses have formed the basis of diagnostic immunology. Largely, the diagnostic procedures are based upon detection and quantitation of antibodies, the use of cellular immune responses as a diagnostic tool has limited applications because these are relatively short lived, difficult for automation, more expensive, time consuming and require critical expertise. However, their application in developing vaccines particularly against the intracellular pathogens and for monitoring the immune status, is of paramount importance.

Principle:

Contact hypersensitivity is a simple assay of cell-mediated immune function. In this assay a reactive chemical (allergen) is applied over the shaved part of skin during initial phase (**Sensitization phase**) during which surface molecules of Langerhan cells are chemically modified (creating a neo-haphten). The modified Langerhan cells migrate to regional lymph node and initiate a primary immune response to the hapten. During the subsequent exposure (**elicitation phase**) to the same hapten the surface antigen of Langerhan cells present 'neo-antigen' in association with MHC class-II molecules and recruit and activate T-cells specific to that hapten. The cytokines produced by these (T-cells + APCs) further recruit a larger population of mononuclear cells including non-specific cells resulting into DTH reaction.

Materials:

- Adult mice, 6-12 weeks old (Swiss albino).
- Allergen:
  - * DNCB [1-chloro 2, 4, Dinitrobenzene (Hi media), Molecular wt.- 202.55, $C_6H_3ClN_2O_4$.
  - * DNFB: 2, 4, dinitro-1-fluoro benzene
  - * TNCB: 2, 4, 6-Trinitrochlorobenzene (Picryl chloride)
  - * FITC: Fluorescein-isothio-cynate

- Acetone
- Olive oil
- Razor
- Thickness gauge, etc.

Procedure:

Sensitization phase:

1. Shave the abdominal area of 5 mice. Keep three mice as control.
2. Sensitize 5 mice by epi-cutaneous application of 50µl of 0.5% DNCB prepared in a mixture of acetone and olive oil 4:1 (V/V). [It is better to use in multiple steps].
3. Before putting the mice in the cage let the solvent evaporate over the skin.

Elicitation phase:

1. On the 5^{th} day take the base line ear thickness of right ear of the mice.
2. Apply 0.25% DNCB epi-cutaneously (prepared as above in a mixture of acetone and olive oil) in 10-µl volume on each side of right ear.
3. After 24 and 48 hours record the increase in thickness of right ear vis-a-vis left ear in the sensitized mice and control mice.
4. The tissues were collected for histopathological examinations and interpretations of the observations.

Precautions:

- Since most allergens are irritant and potentially sensitizing for humans, gloves must be worn-while handling them.
- While measuring ear thickness one has to take all cautions as per the requirement of the instrument, take measurement at the first attempt and include adequate controls and minimum of five experimental animals in each group.

Reference:

Kadian, S. (2003).Production and use of instructional material on Veterinary Immunology and Serology. ICAR conter of Advanced studies on Vet. Microbiology CCS Haryana, Agriculture University, Hissar.

Study questions:

1. Mention the sites for inoculation of DNCB in different animal species and poultry.
2. Measure the thickness of skin from atleast 10 animals of a particular species and present your findings in a tabular form (the site should be same as mentioned also for that species).
3. List the sites for tuberculin testing in different animal species and poultry.
4. Compare the findings of DNCB and tuberculin tests in chicken.

APPENDIX

Reagents for ELISA

1. Coating buffer (Carbonate-bicarbonate buffer, pH 9.6)

Sodium carbonate	0.318g
Sodium bicarbonate	0.568g
Sodium azide	0.040g
Distilled water to make	200 ml

2. Blocking solution

Bovine serum albumin	1.0g
PBS-tween 20	1000 ml

3. Washing buffer (pH 7.4)

Tween-20	0.5 ml
PBS	1000 ml

4. Phosphate-citrate buffer (pH 5.0)

Solution A	Citric acid	1.921 ml
	Distilled water	100 ml
Solution B	Disodium hydrogen phosphate	2.84g
	Distilled water	100 ml

Mix. 24.3 ml of solution A to 25.7 ml of solution B and make the volume upto 100 ml with distilled water.

5. Substrate for ELISA

Orthophenylene diamine dihydrochloride	10 mg
Phosphate citrate buffer	25 ml
Hydrogen peroxide (30%)	0.5 ml

Reagents for Dot-ELISA

1. Phosphate citrate buffer (pH 5.0)

Citric acid	7.3g
Disodium hydrogen phosphate	11.86g
Distilled water	1000 ml

2. Substrate solution

Diaminobenzidine tetrahydrochloride	5.0 mg
Phosphate citrate buffer	10 ml
Hydrogen peroxide	5 µl

SDS-PAGE Reagents

I. Stock solutions

1. 2x Sample buffer (pH 6.8)

Tris base	1.51g
SDS	8.0g
Glycerol	30 ml
Bromophenol blue	2 mg
Distilled water	64 ml

Warm the solution to dissolve and allow to cool at room temperature. Adjust the pH to 6.8 and make the total volume of 100 ml. store at 37^0C.

2. 10% Ammonium persulphate

Ammonium persulphate	0.1g
Distilled water	10.0 ml

3. 2x separating gel buffer (pH 8.9)

Tris base	45.4 g
SDS	1.0 g
Distilled water	460 ml

Add concentrated HCl dropwise until pH falls to 8.88. Make volume upto 500 ml and store in plastic bottles at 4^0C.

4. Stock acrylamide for separating gel

Acrylamide	75 g
Bisacrylamide	0.6g
Distilled water	181 ml

Make the volume 250 ml and store in dark bottle at 4^0C.

5. 2x separating gel buffer (pH 6.8)

Tris base	6.06g
SDS	0.4g
Distilled water	190 ml

Adjust the pH with HCl. Make the volume 200 ml and store at 4^0C.

6. Stock acrylamide for stacking gel

Acrylamide	15 g
Bisacrylamide	0.4g
Distilled water	36 ml

Make the total volume upto 500 ml and store in dark bottle at 4^0C.

7. 10x Electrode buffer (pH 8.3)

Tris base	30.3 g
Glycine	144.2 g
SDS	10 g
Distilled water	885 ml

Make the volume to 1 liter and store at room temperature. For use 1 part of this solution is mixed with 9 parts of water.

8. CBB staining solution

Solution a-	Coomassie Brilliant blue	0.25g
	Distilled water	25 ml
Solution b-	Trichloroacetic acid	60 g
	Distilled water	720 ml
	Methanol	180 ml
	Glacial Acetic acid	60 ml

Mix solution a and b with constant stirring. Make the volume upto 1 litre and store in dark bottle at room temperature.

9. CBB destaining solution

	NaCl	150 g
	Distilled water	5 litres
Or	Glacial acetic acid	50 ml
	Methanol	280 ml
	Distilled water	670 ml
	Store at room temperature	

II. Working solution

1. Separating gel (60 ml for 2 gels)

	10%	12.5%
2% separating gel buffer	30 ml	26 ml
Stock acrylamide for separating gel	20 ml	25 ml
Distilled water	10 ml	9 ml
TEMED	100 µl	100 µl
10% Ammonium per sulphate	100 µl	100 µl

2. Stacking gel

	3%	4%
2% stacking gel buffer pH 6.8	2.25 ml	2.25 ml
Distilled water	1.75 ml	1.60 ml
Stock acrylamide for stacking gel	0.5 ml	0.6 ml
TEMED	10 µl	10 µl
10% Ammonium per sulphate	30 µl	30 µl

Buffer for protein transfer by semidry system of blotting

1. Anode buffer I pH (10.4)

Tris	3.63 g
Methanol	10 ml
distilled water to make	100 ml

2. Anode buffer II (pH 10.4)

Tris	0.30 g
Methanol	10 ml
Distilled water to make	100 ml

3. Cathode buffer (pH 9.4)

Tris base	0.30 g
Glycine	0.3 g
Methanol	10 ml
Distilled water to make	100 ml

I. Staining of protein blotted membrane

Coomassie brilliant blue R	0.1 g
Methanol	50 ml
Acetic acid	7 ml
Distilled water to make	100 ml

J. Blocking buffer for Western blotting

Bovine serum albumin	1.0 g
Sodium chloride	0.9 g
Sodium phosphate	0.178 g
PBS	100 ml

Alsever's Solution

Dextrose	2.05 g
Sodium citrate	0.80 g
Sodium chloride	0.42 g
Distilled water	100 ml

pH adjusted to 6.1 with 10% citric acid before autoclaving at 10 lbs for 15 min.