Name Admn. No. Academic Session

Department of Veterinary Epidemiology & Preventive Medicine
DGCN College of Veterinary & Animal Sciences

Laboratory Manual
Veterinary Preventive Medicine-I

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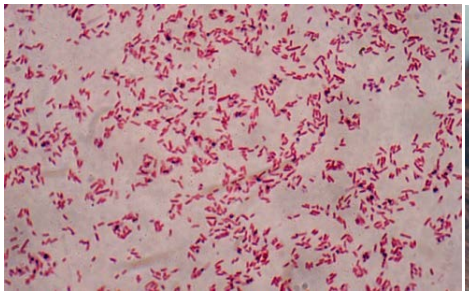
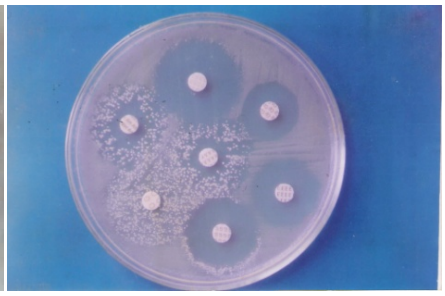
Professor & Head





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PALAMPUR 176 062

Foreword

It is a matter of immense pleasure that the department of Veterinary Epidemiology and Preventive Medicine is bringing out a "Practical Manual" on Veterinary Preventive Medicine- I course No. VEP– 422 (0+2) as per the recommendations of the Veterinary Council of India minimum standard of Veterinary Education, Regulation, 1993. Veterinary Preventive Medicine is an important discipline of Veterinary Science covering wide range of objectives viz. definition, incidence, etiology, transmission, epidemiology, pathogenesis, clinical findings, diagnosis, treatment and control as well as control and prevention of infectious diseases among livestock and poultry.

The practical part of this encompasses Rural Clinical Work that include surveillance, health tests, vaccination etc. and laboratory exercises to impart practical hand on quick and reliable diagnosis of infectious diseases; which is imperative for control and prevention of such diseases among animal populations. I am sure that this practical manual will greatly facilitate in understanding the basic concepts of Veterinary Preventive Medicine.

Prof. R. C. Katoch 15/07/06
Dean
College of Veterinary & Animal Sciences
CSK HPKV, Palampur – 176 062

Best Wishes

Brij Bihari Lal Butail
Political Advisor to
Chief Minister of HP
05-08-2006

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VETERINARY PREVENTIVE MEDICINE-I

Signature of Student

Exercise No. 1 **Date:**

Exercise: Collection, Preservation and transportation of specimens for laboratory diagnosis of animal diseases.

For prevention and control of diseases, early detection of cases and correct disease diagnosis is very important. In order to arrive at correct diagnosis, some clinical samples / specimens collected from live sick animals are analyzed in local hospital laboratory or some times, required to be send to disease diagnostic laboratories for examination. If the material selected for laboratory examination is not appropriate or the method of collection, preservation and dispatch of the material is faulty, it may not be possible to arrive at any definite conclusion. So far as the diagnosis of the disease is concerned, followings samples are mainly collected for diagnostic purposes:

1. Whole Blood samples

The blood sample should be collected in dry, clean and sterilized screw capped glass or plastic vials. Blood is collected for following purposes:

- For haematological examination in anticoagulant
- For serum separation, blood without anticoagulant.
- For bacteriological purposes in nutrient broth.

Preservatives commonly used

Material	Preservative to be used
1. Blood for haematology	
2. Blood for bacterial isolation	
3. Blood for virus isolation	

Sites for blood collection in different animals:

Sr. No.	Site for collection	Species
1.	Jugular vein	Cattle, sheep, goat, horse and large wild animals
2.	Cephalic vein	Dog
3.	Ear vein	Cat, small dogs, rabbit, and monkey
4.	Tail	Sheep, pig, cattle, rat, mouse
5.	Heart	Any animal, bird or reptile
6.	Femoral, saphenous or Tibial vessels	Dog, cat, small mammal, rat and primate
7.	Mammary vein	Dairy cattle
8.	Ant. Vena cava	Pig
9.	Wing vein or comb	Birds
10.	Retro ocular plexus	Mice, rats & guinea pigs
11.	Femoral vein	Monkeys

Clip the hair and shave site of blood collection. Disinfect the skin with 70 % alcohol, rectified spirit or tincture of iodine. Apply pressure on area to raise vein. Insert sterilized hypodermic needle (23-24 gauge in small animals & 16-18 gauge in large animals) into vein. Discard first few drops of blood. Gently aspirate the blood with syringe (in small animals) or collect directly into vial (in large animals)

2. Blood Smears

Blood smears are prepared for the demonstration of bacteria, haemoprotozoa etc. Blood smears should preferably be made from fresh blood taken directly from the animal without any anticoagulant.

- **Wet blood Smears**: A drop of blood is placed on a slide and covered with a cover slip. It is most useful for detecting the motile (live) trypanosomes.
- **Thin blood smear**: A small drop of blood is placed on a clean, grease free glass slide kept horizontally. Hold another slide / spreader slide at an angle of 30º to 40º in front of drop of blood. Bring the spreader slide in contact with drop of blood and allow it to spread. Draw the spreader slide with a smooth and firm movement. Air dry the smear and fix the smears with methanol (methyl alcohol) for 2 - 3 minutes.
- **Thick blood smear**: A large drop of blood is placed in the middle of slide and is spread with the corner of another slide or needle in an area of about half inch. It should be dried by keeping it covered with a petri-dish but should not be fixed prior to staining. It is made for detection of chronic infections or when blood parasitaemia is low. Due to opacity, thick smears should be de-haemoglobinized for better detection of parasites. Dip the slide in distilled water keeping horizontally for three minutes (till color disappears).Remove the slide from water, air dry and. Fix in absolute methanol for 3-5 minutes and again air dry.

3. Serum samples

Serum is collected for conducting serological tests against infectious disease, diagnosis of metabolic diseases and to study the biochemical profiles of sick animals.

The vials containing whole blood are kept in a slanting position undisturbed away from direct sun light to allow the blood to clot. The serum a clear straw coloured fluid that oozes from clot is transferred to clean tube/ vial. Remove RBC's, if any, by centrifugation at 1000 rpm for 10 minutes. It should be kept in mind that serologic as well as biochemical procedures are adversely influenced by haemolysis. It is transported over ice to the laboratory without preservative. If it is likely to take more time, any of the following preservative should be added. In laboratories sera are stored at -20ºC till further use.

Plasma is desirable particularly for biochemical estimations since there is less chance of haemolysis during collection and subsequent separation from the cells than serum. However, anticoagulants present in plasma may interfere with biochemical tests; therefore, serum should always be submitted unless plasma is specifically requested. Because lipemia can interfere with a number of chemistry tests.

Preservatives to be used:

Materials	Preservative to be used
1. Serological tests	
2. Biochemical tests	
3. Blood sugar	

4. Urine samples

The urine samples are collected for urinalysis, bacterial microscopy and culture or for a viable bacterial count and metabolic diseases like ketosis. Fresh urine samples are either collected during normal urination or by catheterization. A catheterized sample is preferred; however, if a sample is obtained by other means it should be a midstream collection to minimize urethral and vaginal micro flora or debris.

Sample should be collected in sterile clean screw capped glass or plastic bottle. At least 100 ml of urine must be collected. It can be preserved in refrigerator. If this is not possible, few drops of toluene to cover the surface area of urine or few crystals of thymol for 5-10 ml of urine must be added. If sample of urine is small, it should be centrifuged at 1000 rpm for 10 minutes to ensure adequate amount of sediment and the supernatant can be used for chemical tests and culture.

Urine samples should be cultured with in one hour of their collection. For dark field microscopy for Leptospira the examination should be completed within 20 minutes of collection.

Preservatives used for urine:

S. No.	Preservative	Indications
1		
2		
3		
4		

5. Faecal sample

The faecal examination is done mostly for parasitic infection (helminthes eggs and coccidial oocysts) but is also examined for bacterial and viral infections. Uncontaminated and representative sample should be collected. Faecal examination should be conducted on a fresh faecal material. Sample should preferably be taken directly from rectum of animal. Use either hand gloves or smear the hand with Vaseline before inserting the fingers in rectum and transfer to screw-capped vials or small polythene bags. For microbiological studies samples should be collected using sterile swabs directly from rectum and transport over ice in a test tube containing few drops of sterile normal saline. If collected faeces can't be examined within few hours, the sample should be refrigerated until it is tested. If faeces are to be transported over long distance, these should be fixed and preserved. For few days preservation, faeces should be mixed with equal quantity of 10% formalin and for longer periods 70 per cent alcohol may be used. Faecal samples for cultural studies of coccidia should be submitted in 2.5 % potassium dichromate solution.

6. Exudates and Uterine discharges

- **Pus**: About 3 ml of pus should be collected along with scrapings from the wall or margin of abscess. Pus at center of an abscess is often sterile. Pus collected from recently formed abscess yield best results. Pus for cultural & sensitivity test should be collected in a sterile container with the help of sterile syringe, Pasteur pipette or sterile swabs. Before collection wash the surface with soap and water and disinfect with 70% alcohol or Tr. Iodine. Pus smears can be subjected to direct microscopic examination after staining.
- **Exudates:** Exudates from open wounds, one should attempt to obtain fresh exudates from deeper parts of lesions with sterile swabs and transport over ice in a test tube containing few drops of sterile normal saline for microbiological investigations. Pleural, peritoneal and synovial fluids should be aspirated using all aseptic precautions and submitted over ice.
- **Uterine discharges**: Uterine discharges are collected from repeat breeding anf infertility cases for CST. Wash external genitalia with soap and clean water and disinfected with Candy lotion. One person should assist by opening the vulvar lips to avoid external contamination while other pass the sterile pipette fitted to a sterile syringe through cervix for collection of uterine discharges. Clean the external surface of pipette with a swab of alcohol and transfer the contents in to a sterile screw capped vial and transport over ice to the laboratory. These samples should be processed with in 24 hours especially for trichomoniasis.

7. Cerebrospinal fluid

CSF can be collected by sub-occipital or sub-lumbar puncture. CSF should be submitted for examination when clinical signs of central nervous system disease are displayed. It may help in ascertaining the cause by examining colour, turbidity, protein content, total cell count, differential cell count and bacteriological examination of CSF.

8. Milk Samples

These are mostly collected for diagnosis of mastitis. As diagnosis of mastitis depends upon the identification of bacteria so milk samples should be collected with as little contamination as possible. Milk samples should be collected as soon as possible and before treatment of animals with antimicrobial agents.

- Wash the dirty udder with soap and clean water followed by disinfection with cloth soaked in a disinfectant.
- Dry the udder, clean end of teat with a swab dipped in 70% alcohol or tincture of iodine.
- Discarded First few streaks of milk from each quarter; hold the sterile vials slightly away from the teat, remove the cap, and collect 10 ml of milk sample from all the affected quarters i.e. left fore quarter, left hind quarter, right fore quarter and right

hind quarter, respectively in already labeled screw capped vials. Transport samples over ice to the laboratory.

9. Lymph node smears

The site of suitable superficial lymph node (pre-scapular) is cleaned with a swab of alcohol. The gland is punctured with a sterilized hypodermic needle and a small amount of fluid is sucked into the syringe. A little of this fluid is ejected on to a slide for making its smear in the manner, a thin blood smear is made. Impression smears are made from lymph nodes removed from dead animals or from its small portions removed through biopsy from the living animals, suspected to be suffering from theileriosis by cutting the lymph node with a sharp knife and touching the cut surface on a clean slide at two or three places. These are dried, fixed and stained like thin blood smears. Rapid air drying of smears minimizes cell distortion, thereby enhancing diagnostic quality. These are used for detecting trypanosomes or schizonts of theileria.

10. Skin samples

If intact pustules or vesicles are present, surface should be disinfected with 70 percent ethyl alcohol, dried and material aspirated with sterile syringe and needle. If ringworm is suspected, hair should be plucked from the lesion and the edges of lesions are scraped with a blunt scalpel blade. For the diagnosis of mite infestation, deep skin scrapings should be taken. Scrapings should be made from deeper layers of skin with blunt scalpel until blood begins to ooze. In extensive lesions, always select peripheral areas. When demodectic mange is suspected, put pressure on the skin to express mites from the hair follicles. Add few drops of 10 per cent pot. Hydroxide to sample and mix with the help of tooth prick or teasing needle and also break the scabs and matted hair.

11. Histopathological samples

The tissue pieces should be collected as soon as possible after death of the animal to prevent damage by autolysis and putrefaction. Similarly, the biopsy collected from the living animal should be preserved as soon as possible. The samples should not be frozen before fixation. For histopathological examination the tissue pieces should not be more than 1cm in thickness, otherwise there will not be proper fixation. The pieces should include both the normal as well as diseased portion. The preservative for histopathological examination is 10% Buffered formalin (add 100 ml of commercial 40% formaldehyde to 900 ml of normal saline). Ratio of 10% formalin to the tissues should not be less than 20 times. The tissue should be preserved preferably in a wide mouth container.

12. Toxicological analysis

Tissues or fluids for toxicological analysis should be as fresh as possible and kept in a refrigerator or preserved chemically. For chemical preservation, 95% ethanol (@1 ml / g of sample) is satisfactory. Denatured alcohol should not be used because the denaturant introduces contamination. Formalin is usually undesirable because it interferes with

many tests. The container for packing and transporting specimens should be free of chemicals and should be prepared before use. Plastic containers, both bags and jars, are ideal. Jars with metal screw caps should be avoided, especially when metal poisoning is suspected.

Selected questions

1. Enlist the most common errors made while collecting the blood sample.

2. What precautions will you observed while collecting the blood samples?

3. What factors determine choice of anticoagulant ?

4. Which anticoagulant is best for collection of blood sample for total cell count and why?

5. When should blood sample be collected for microbiological studies from live sick animals?

6. Qualities of a good blood smear.

7. Why metal screw capped vials and jars are avoided for collection of samples for toxicological analysis?

8. What is exfoliated cytology?

9. Should samples be collected from cases of notifiable diseases?

Exercise No. 2 **Date:**

Exercise: Stains and staining of bacteria

The bacteria when examined under living conditions are transparent and colourless. For detailed study of their shape, size and structure the staining of bacteria is valuable. Certain dyes are used for staining of bacteria. A dye is an organic compound containing chromophore and auxochrome groups linked to a benzene ring.

1. Gram's staining

This is most commonly used differential method of staining. The organisms stained by this method fall into two groups(a) Gram positive and (b) Gram negative Gram positive bacteria retain crystal violet stain and do not get decolourized with alcohol and appear violet or blue in colour while Gram negative organisms are decolourized by alcohol and take counter stain (Safranin) and appear red. Many bacteria show a variable reaction. The old cultures of Gram positive bacteria also take counter stain and appear red. The Gram positive and Gram negative bacteria differ greatly in their cell wall composition. The composition of stains and other solutions used in Gram's staining is given below:

A. Ammonium oxalate crystal violet

Solution I

Crystal violet	2 gm
Ethyl alcohol (95%)	20 ml

Solution II

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Mix solutions I and II and filter through Whatman filter paper No.1

B. Lugol's iodine solution (modrant)

Iodine	1 gm
Pot iodide	2 gm
Distilled water	300 ml

Dissolve the ingredients and filter.

C. Ethyl alcohol (95%) to be used as decolourizer.

D. Safranin (Counter stain)

Safranin (2.5%) solution in 95% ethyl alcohol	10 ml
Distilled water	100 ml

Mix and filter.

Materials:

Bacterial culture / morbid materials, staining solutions and reagents.

Procedure:

i. Make bacterial smear and fix over the flame. The impression smears are fixed with methanol for 2-3 minutes.

ii. Pore ammonium oxalate crystal violet stain and allow it to act for 3-5 minute.

iii. Wash with tap water.

iv. Add Lugol's iodine and allow it to react for one minute.

v. Wash with tap water.

vi. Decolourize with 95% ethyl alcohol for about 30 seconds till no colour comes out from the smear.

vii. Counter stain for 30 seconds with safranin solution.

viii. Wash with tap water, dry and examine under the microscope with oil immersion objective. Gram positive organisms will be deep violet while Gram negative will take red colour.

2. Methylene blue staining

This staining technique is employed to demonstrate the presence of bacteria in blood and tissues by staining blood smears or tissue impression smears. It is mostly employed to demonstrate anthrax and pasteurella organisms.

Materials:

Suspected blood, morbid tissues or tissue fluid and Loeffler's methylene blue stain

Procedure:

i. Prepare impression smears from suspected tissue(s) or blood smear(s) and fix with methanol for five minutes.

ii. Flood the smears with Loeffler's methylene blue for 3-5 minutes.

iii. Wash with tap water

iv. Blot dry and examine under microscope.

The anthrax bacillus will stain blue with pink stained capsule (McFadyean reaction) while *Pasteurella multocida* will exhibit bi-polar staining.

3. Ziehl Neelsen's staining (Acid fast stain)

The acid fast bacteria belonging to genus Mycobacterium do not stain readily with simple staining methods. The organisms are stained with hot carbol fuchsin and they will resist decolourization with 3 percent hydrochloric acid in 95 per cent alcohol because of the presence of a waxy substance known as mycolic acid. The organisms are counter

stained with methylene blue or another contrasting stain. The composition of regents is give below:

Composition

(a) Concentrated carbol fuchsin

Basic fuchsin	1 gm
Absolute alcohol	10 ml.
Phenol 5% aqueous solution	100 ml.

Dissolve the dye in alcohol and add phenol solution

(b) Acid alcohol

Ethyl alcohol (95%)	97 ml.
Concentrated hydrochloric acid	3 ml.

c) Loeffler's alkaline methylene blue

Methylene blue saturated alcoholic solution	30 ml
Potassium hydroxide (1% aqueous sol.)	1 ml
Distilled water	99 ml

Add potassium hydroxide solution in distilled water and mix methylene blue solution and filter.

Materials

Morbid material and staining reagents.

Procedure:

i. Make a smear of morbid material and fix it over the flame methanol fixation.

ii. Flood the smear with carbol fuchsin and heat from the below till steam comes out. Allow the hot carbol fuchsin to act for 3 to 5 min. Do not boil the stain or allow it to dry on the slide.

iii. Wash the slide with tap water.

iv. Decolourize with acid alcohol for about 15-20 seconds until the bacterial smear appears faint pink or colour less. Wash it with tap water.

v. Counter stain with Loeffler's methylene blue for about 30 seconds.

vi. Wash with tap water, blot dry the slide.

vii. Examine the slide under microscope with oil immersion objective and make a drawing of a field under microscope. Acid fast bacteria will take pink / red colour while non acid fast stain blue.

4. Spore Staining:

Under certain un-favourable conditions spore forming bacteria develop in to spore. These are refractile bodies resistant to various physical & chemical agents. The

spores may be spherical or oval and may be central, sub-terminal or terminal in position. Spores are difficult to stain. With Gram's stain, mature spore remain unstained bodies. Ziehl Neelsen's staining method is used. The procedure is same except the decolourizer used here is 0.5 % Sulphuric acid instead of acid alcohol..

5. Gimenez staining

This technique is used for staining of Chlamydia and Rickettsia in impression smears as well as after their isolation in chicken embryos or cell culture.

Stock Stain Solution

Sol. I	10 % basic fuchsin in 95 per cent ethanol	100 ml.
Sol. II	4% aqueous phenol solution	250 ml.
	Triple distilled water	650 ml.

Mix solution I and II thoroughly before adding distilled water. Incubate at 37° C for 48 hours for maturation.

Stock Buffer solution [PBS 0.01 MPH 7.5]

Sod. di hydrogen phosphate ($Na\ H_2PO_4.\ 2H_2O$)	0.15 g.
di Sod. Hydrogen phosphate ($Na_2\ HPO_4$)	1.27 g
Sod. Chloride	8.00g
Distilled water	1000 ml.

Working Stain Solution

Add one part of stock stain solution in two parts of stock buffer, mix thoroughly and filter through Whatman No. 1 filter paper.

Decolourizer

Acetic acid	0.5 ml
Distilled water	100 ml

Counter Stain Solution

Malachite green	8.0 g
Distilled water	1000 ml.

Materials:

Morbid sample, microscopic slide, staining solutions, methanol, Bunsen burner, microscope and immersion oil (immersion oil)

Procedure

i. Prepare smears from morbid material.

i. Fix the smear on flame or with methyl alcohol.

iii. Cover the smear with Gimenez stain (working) for 5-10 min.

iv. Wash with water.

v. Decolourize with 0.5 % acetic acid till no more pink colour comes out.

Vi Counter stain with malachite green staining solution for 1 min.

vii. Wash with water and blot or air dry.

viii. Examine under oil immersion.

The elementary bodies / inclusions will appear pink and other structures greenish blue.

6. Giemsa staining:

Blood smears are stained by Giemsa method for bacterial and blood protozoan infection.

Procedure:

i. Stain the smears with Giemsa stain for 30 min (1:20 dilution of stock sol.) or over night (1:50 dilution) after fixing with methanol.

ii. Wash with buffered saline or distilled water and blot or air dry.

Examine under oil immersion.

7. Lacto phenol cotton blue:

This is used for staining mycological specimens.

Phenol	20 g
Lactic acid	20 g
Glycerol	40 ml
Distilled water	20 ml

Dissolve by heating gently in hot water. Add 0.05 g Cotton blue.

Procedure: Used for preparation of semi-permanent stained mycological specimens. Slide culture mount or small piece of mycelium teased out on a clean glass slide in lacto phenol cotton blue and sealed with nail polish.

Selected Questions

1. What precautionary measures will you adopt in the laboratory while handling and staining infectious materials or bacterial cultures?

2. Enlist Acid fast bacteria of Veterinary importance.

3. Enlist the sporulating bacteria along with shape, size and location of their spores.

4. Differentiate between Loeffler's alkaline methylene blue stain and Polychrome methylene blue stain.

5. Perform capsule staining in the laboratory.

Exercise No. 3 **Date:**

Exercise: Identification of pathogenic bacteria of veterinary importance.

Bacteriological Media

Diagnostic bacteriological media can be divided into the following categories:

Chemically defined media

In these the exact amount of each ingredient is known. They are used mainly for experimental purposes but citrate broth is an example of a chemically defined medium that is used in diagnostic bacteriology.

Basic nutritive media

These are capable of sustaining growth of the less fastidious bacteria. Nutrient agar is an example.

Enriched media

Agar media, such as blood agar for the growth of fastidious bacteria. The media are usually enriched with blood, serum or egg yolk.

Enrichment broths

Liquid media that are selective for a particular bacterium. such as selenite broth for the selection of salmonellae.

Selective media

These agar media have been made selective for the growth of a particular bacterium or group of bacteria and are used extensively in diagnostic bacteriology. They contain inhibitory substances that prevent the growth of unwanted bacterial species. Many selective media such as brilliant green and Mac Conkey's agar can also be described as 'indicator media'.

Indicator media

These are particularly useful in diagnostic bacteriology. They are designed to give a presumptive identification of bacterial colonies due to the biochemical reactions in the media. Indicator media often contain fermentable sugars plus a pH indicator that gives a colour change in the media. Mc Conkey agar contains the fermentable sugar lactose and neutral red as the pH indicator. Bacteria such as *Escherichia coli* that ferment lactose produce acidic metabolites that change the colonies and surrounding medium to a pink colour. Salmonellae that cannot ferment lactose will use the peptones in the medium with the production of alkaline metabolic products. Salmonella colonies and surrounding medium are pale straw in colour. Other indicator media may be designed to show hydrogen sulphide production (XLD agar) or aesculin hydrolysis (Edward's medium). Blood agar, although an enriched medium, may also be considered as an indicator medium as it shows the type of haemolysis of a particular bacterium.

SIM medium

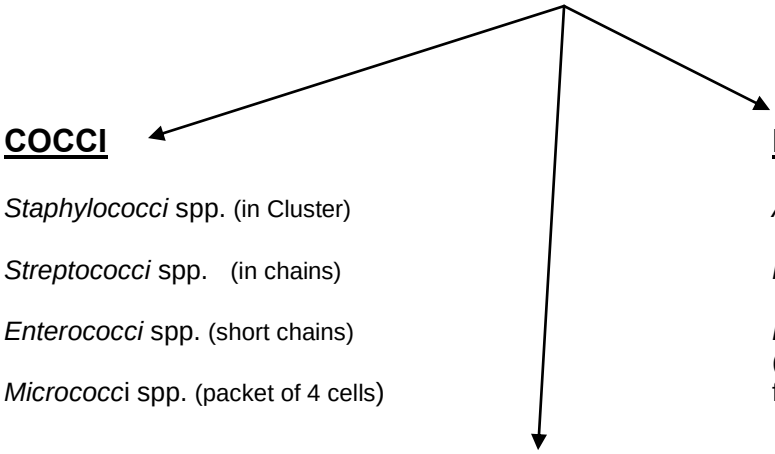
Can be used to detect motility and will also indicate indole and hydrogen sulphide

production.

Media used in diagnostic bacteriology can be obtained commercially as dehydrated powders or can often be purchased as pre-poured plates.

Gram positive bacteria

(STAIN PURPLE BLUE)



COCCI

Staphylococci spp. (in Cluster)

Streptococci spp. (in chains)

Enterococci spp. (short chains)

Micrococci spp. (packet of 4 cells)

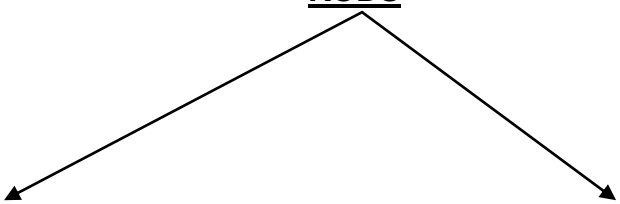
FILAMENTOUS & BRANCHING

Actinomyces bovis (MZN -)

Nocardia spp. (MZN +)

Dermatophillus congolensis
(Zoospore in tram tract formation , 2 or > across)

RODS



PRODUCES ENDOSPORES

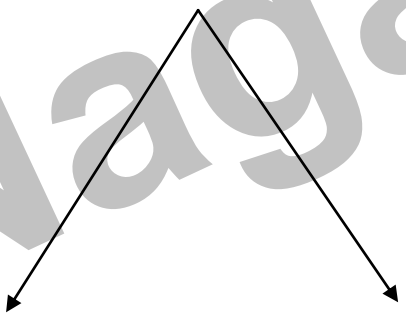
(Large G+ Rods)

Clostridium spp.(anaerobic)
Spore tends to bulge mother cell.

Bacillus spp. (aerobic)
Spore don't tends to bulge mother cell.

Bacillus anthracis blue square ended Rods in short chains

NO ENDOSPORES



ZN POSITIVE

Gram+ cell wall but don't stained by Gram's method.

Mycobacterium spp.
(Long thin beaded rods).

M. lepraemudun
(Long thin rods).

M. avium paratuberculosis
(short rods in clumps, bright red)

ZN NEGATIVE

Corynebacterium spp.
(G+ rods some spp are pleomorphic)

Actinomyces pyogenes
(G+ pleomorphic rods)

Rhodococcus equi
(G+ rods with a tendency to coccal forms)

Listeria monocytogenes
(with coccal forms in culture).

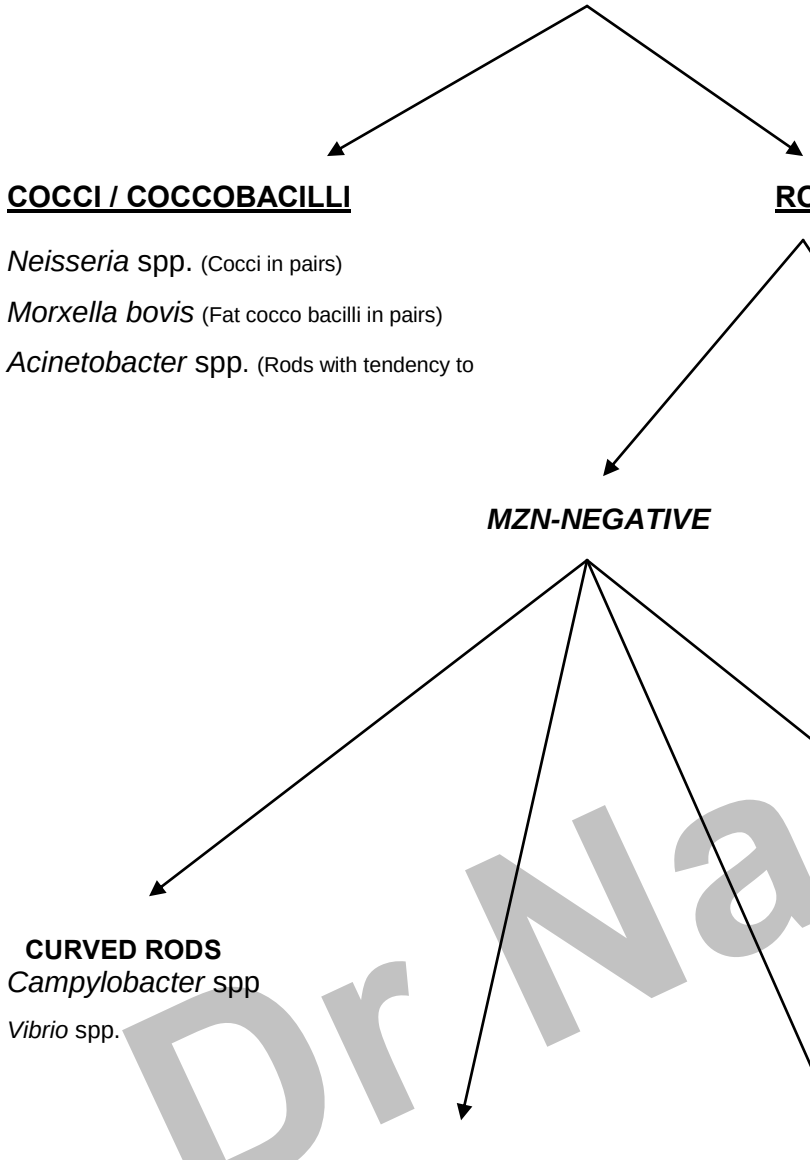
Erysipelothrix rhusiopathiae
(G+rods, rough form from chronic cases, can be in short filaments)

Staining reaction and cellular morphology of Gram positive bacteria.

MZN = Modified Ziehl-Neelsen stain
ZN = Ziehl-Neelsen stain.

Gram Negative Bacteria

(STAIN RED)



COCCI / COCCOBACILLI

Neisseria spp. (Cocci in pairs)

Morxella bovis (Fat cocco bacilli in pairs)

Acinetobacter spp. (Rods with tendency to

RODS

MZN-NEGATIVE

MZN- POSITIVE

Brucella spp. (Small coccobacilli in clumps, grows on bact. media).

Chlamydia spp. (Small coccobacilli in clumps & no growth on bact. media)

CURVED RODS

Campylobacter spp

Vibrio spp.

FILAMENTOUS

Fusobacterium (DCF & anaerobic)

MEDIUM SIZED RODS

Enterobacteriaceae

Pseudo & *Aeromonas* spp.

Bordetella spp.

Actinobacter spp.

Haemophilus spp.

Pasteurella spp.

Alcaligenes spp.

Bacteroides spp (anaerobic)

HELICAL (Spirochaetes)

Leptospira interrogans (Dark field)

Serpulina spp.(Gram's or DCF)

Treponema spp

Borrelia spp. (Grams stain)

Staining reaction and Cellular Morphology of Gram Negative bacteria

MZN = Modified Ziehl Neelsen stain
DCF = Dilute carbol fuchsin stain.

Primary Identification of bacterial isolates in laboratory

1. Oxidation- Fermentation (O-F) test

This test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium. The medium is semi-solid and usually contains glucose as the test sugar and bromothymol blue as the pH indicator. The un-inoculated medium (pH 7.1) is green and if acid is produced by the bacterium, as a result of glucose utilization, the medium becomes yellow (pH 6.0). Bacteria that can metabolize glucose under either aerobic or anaerobic conditions are facultative anaerobes and in this test are said to be fermentative.

The aerobes that require atmospheric oxygen for growth and metabolism are called oxidative. Some bacteria are un-reactive in the conventional O-F medium, either because; they are unable to grow in the basal medium or because they cannot attack glucose.

The conventional O-F medium is most suitable for non-fastidious, Gram-negative bacteria. Modifications can be made to the medium to test for:

- Fastidious bacteria unable to grow in the medium. In this case the basal medium can be enriched with 2 per cent serum and / or 0.1 per cent yeast extract.
- Staphylococci and micrococci. The formula for these organisms for O-F test medium is:

Pancreatic digest of casein	10.0 g
Yeast extract	1.0 g
Agar	2.0 g
Bromocresol purple	0.001 g
Distilled water	1000 ml

Procedure: Two tubes of the O-F medium are heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen. The tubes are then cooled rapidly under cold running water. Both tubes are stab-inoculated with the bacterium. A layer of sterile paraffin oil is layered on top of one of the tubes (sealed tube) to a depth of about one cm. The inoculated tubes are incubated at 37°C and examined in 24 hours and then daily for up to 14 days.

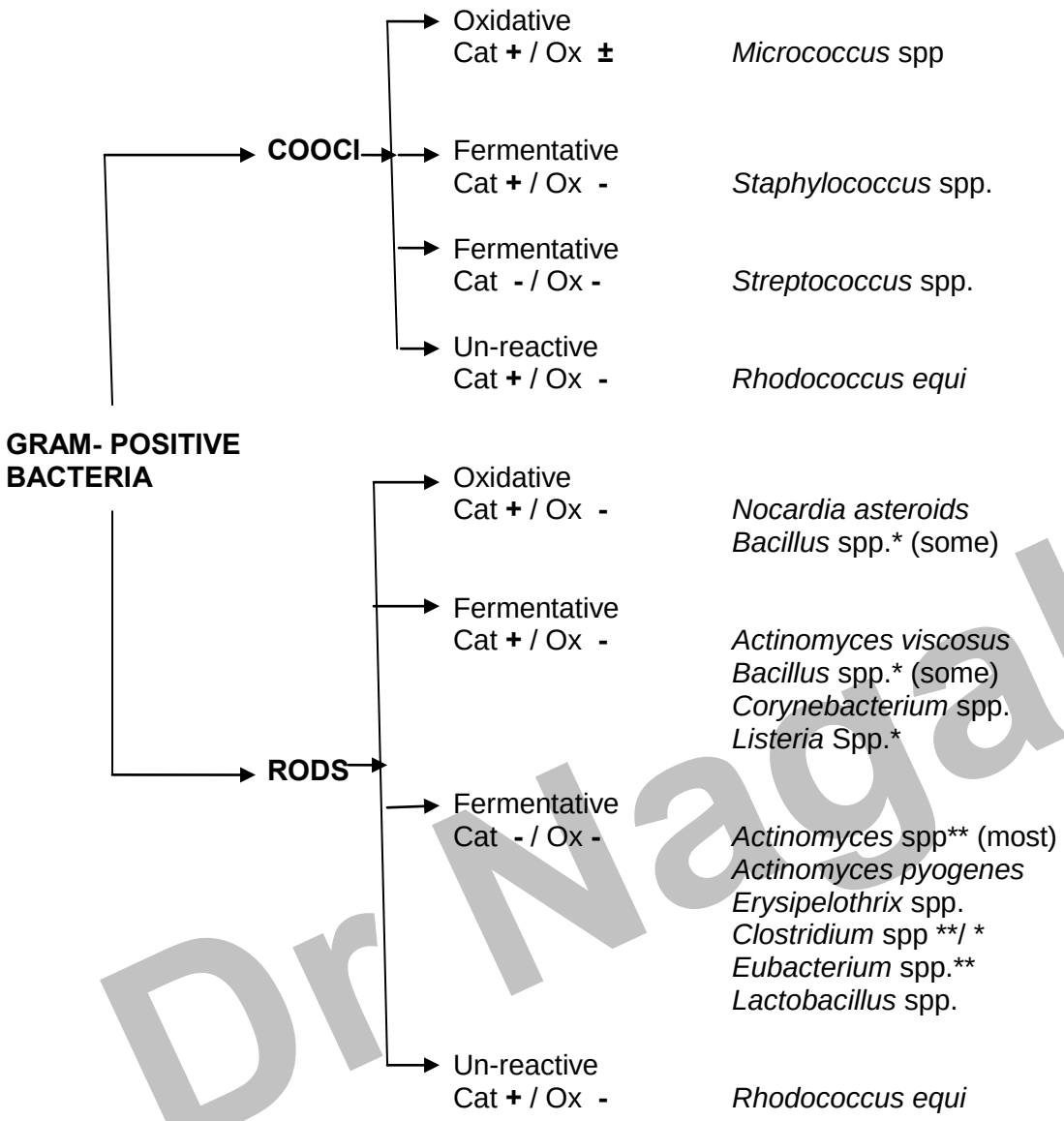
Interpretation of Results:

Results	Open tube	Sealed tube	Examples
Un-reactive	Green	Green	*Bordetella* spp.
Oxidative	Yellow	Green	*Pseudomonas* spp.
Fermentation	Yellow	Yellow	*Aeromonas* spp.

2. Catalase Test:

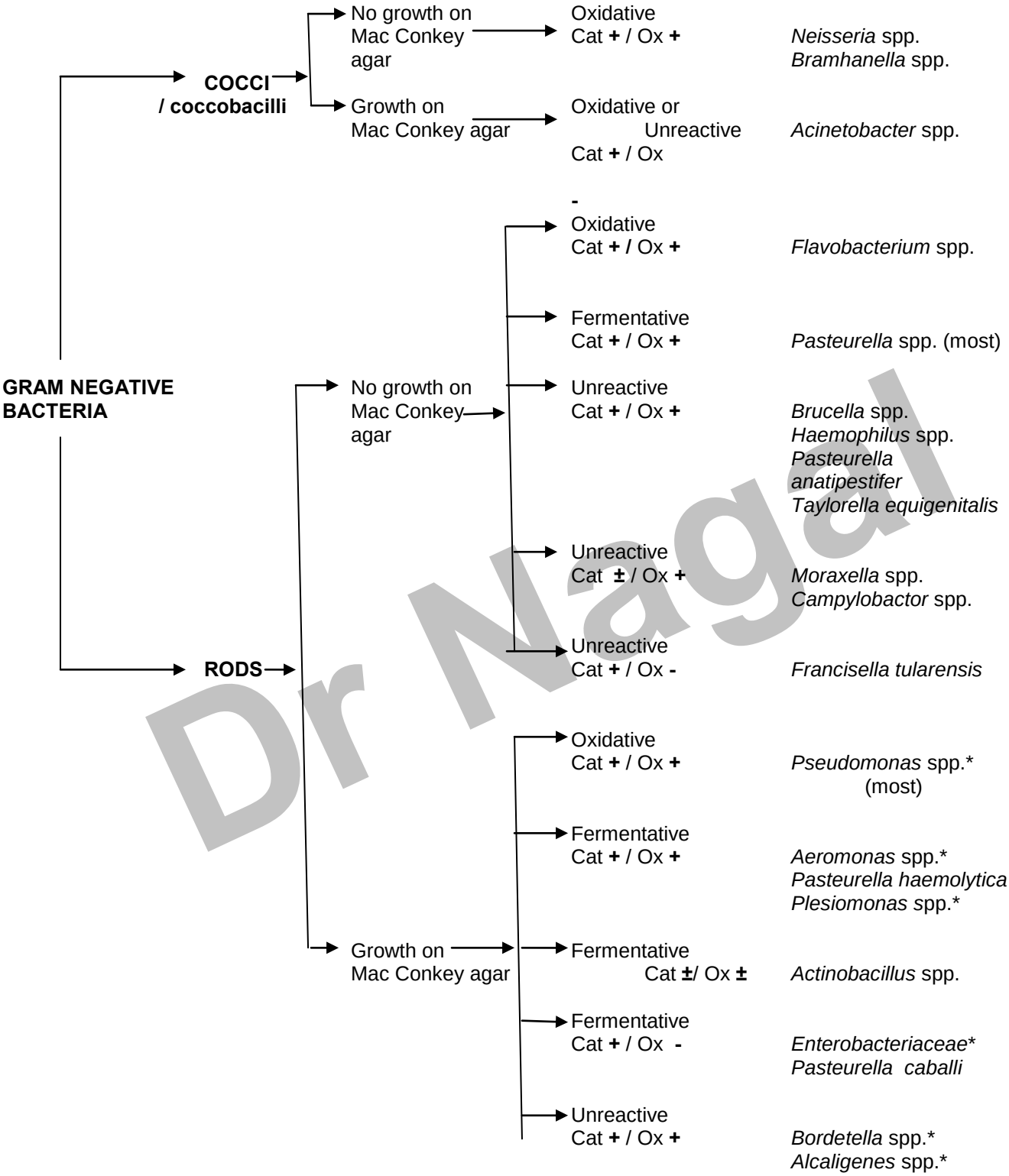
Place a small volume of H_2O_2 in a small test tube. Transfer a colony of organism to this tube. The presence of enzyme catalase in culture in indicated by release of oxygen from H_2O_2 (air bubbles in H_2O_2 = positive test).

Primary identification of Gram positive bacteria



Cat	=	Catalase
Ox	=	Oxidase
+	=	Positive reaction
-	=	Negative reaction
±	=	Variable
*	=	Motile
**	=	Anaerobic.

Primary identification of Gram negative bacteria



Cat = Catalase; **Ox** = Oxidase; **+** =Positive reaction; **-** =Negative reaction; **±** =Variable; *=Motile

Once the bacterium has been identified to a generic level, further tests can be carried to identify species.

Selected Questions

1. Differentiate between Gram positive & Gram negative bacteria?

2. Why old cultures of Gram positive bacteria after staining appear as Gram negative?

3. Within what period should a clinical sample be cultured for isolation of bacteria?

4. What is Modified Ziehl Neelsen’s staining technique and where is it used?

Exercise No. 4 **Date:**

Exercise: Cultural and Sensitivity test (CST)

Antimicrobial chemotherapeutic agents are selectively toxic; they hamper the vital biochemical processes of infecting bacteria without seriously disturbing those of the host. Main aim of CST is to find out most effective antimicrobial for the treatment of infectious disease for which the sample has been sent to the laboratory. Thereafter, the choice of drugs for therapy must always rest in the hands of a clinician, whose training qualifies him to select and evaluate antimicrobial agents in relation to the patient's condition. Sample for CST must be collected before start of therapy or at least one week after the end of last therapy.

Different Methods of antimicrobial susceptibility tests are:

1. Dilution Methods

Dilution and diffusion are the two fundamental principles underlying techniques for the determination of susceptibility to antimicrobial agents

Dilution techniques are of two types:

- Tube dilution tests and
- Plate dilution tests.

Tube dilution tests entails exposing a measured number of microorganisms to increasing concentrations of antibiotics in liquid media; the latter involves a modification in which the agents are serially diluted in solid media.

After incubation at 37°C the lowest drug concentration that prevents visible growth is determined. This endpoint is defined as the minimum inhibitory concentration (MIC), which is expressed in micrograms or units per milliliter. Organisms are regarded as susceptible to a particular agent if its usual levels in the blood are two to four times as big as the *in vitro* MIC. Dilution tests yield accurate quantitative data that allow direct comparisons between various antibiotics. They also provide information about synergistic and antagonistic effects by making it possible to test an organism with two or more antibiotics at the same time. Unfortunately, dilution tests are laborious, time-consuming, and expensive in terms of equipment and personnel efforts, so they are inadequate for routine clinical work.

2. Bauer Kirby Method

The Bauer-Kirby disc method is based on the fact that for a given antibiotic, the size of the zone of inhibition is inversely related to the minimal inhibitory concentration. In other words, the size of the inhibition zone produced by the antibiotic increases as the MIC decreases, demonstrating that zone size is a measure of the MIC. By plotting many dilution-determined MIC values against

measured zone sizes and calculating a regression line for each antibiotic, it is feasible to prepare a graph from which an approximate MIC can be determined.

3. Disc Diffusion Method

In diffusion techniques, a homogeneous film of the test organism is exposed to the known concentration of antibiotics in an agar medium. An antibiotic impregnated filter paper discs are placed or ditches (wells) are made in the agar medium, serve as reservoir from which the chemotherapeutic agent diffuses out. If the bacterium is sensitive to a particular agent than a zone of inhibition occurs around the disc. But there should be strict standardization of procedure because there are many factors which influence size of “zone of inhibition”.

Procedure

1. Prepare a suspension of pure culture in a small quantity of nutrient broth (1-2 ml).
2. Soak the sterile cotton swab in the suspension of organisms.
3. Carefully swab the entire surface of agar plate (preferably blood agar, Mueller Hinton Agar) with the organism suspension; cover the entire surface by streaking first in one direction and then rotate at 90^{o} angle from the first and repeat.
4. The plates are allow to stand for 3 to 5 minutes but not longer than 15 minutes to absorb the excess moisture of inoculum; otherwise, antimicrobial agents may get diluted beyond their scope of activity from disc.
5. Put selected discs containing the antimicrobial agents; For determination of accurate sensitivity, both high and low concentrations of antibiotic discs should be used.
6. The discs should be placed 25 mm apart (centre to centre), approximately six discs per standard 90 mm plate).
7. Each disc should be gently and firmly pressed onto the surface of agar using a sterile pointed forceps or applicator stick.
8. Invert the plate and incubate with in 15 minutes at $37^{o}C$ for 24 hours
9. Compare the size of zone of inhibition of each antimicrobial agent with the standard using a scale.
10. To each antimicrobial agent used, the bacterium is reported as susceptible, moderately / intermediate susceptible, or resistant (no zone of inhibition).

Results & interpretations:

S. N	Name of antimicrobial agent	Disc Conc.	Size of zone of inhibition	Remarks
1				
2				
3				
4				
5				
6				

Selected Questions

1. Why it is mandatory to collect sample for culture sensitivity test before start of therapy?

2. What clinical materials are commonly collected for culture sensitivity examination?

3. Are there some differences between *in vitro* and *in vivo* sensitivity?

4. Enlist the reasons for development of resistance to antimicrobial agents?

5. What are the factors that influence the zone of inhibition?

Exercise No.5 **Date:**

Exercise: General characters of moulds and yeasts and media for isolation

The fungi are composed of an interwining branching mat called mycelium. The filaments that make up this mycelial mat are called **hyphae**. Most of the mat grows on or in the surface of the nutrient medium so that it can extract nutrients; the mat is therefore called **vegetative mycelium**. Some of the mycelium mat rises upward from the mat and is referred to as **aerial mycelium**. Specialized hyphae are produced from the **aerial mycelium** and give rise to spores that are the reproductive elements of the mold.

The identification of moulds depends upon the cultural characters, especially the colour of the surface of colony, rate of growth and size, as well as the microscopic morphology. The study of the morphology of colony is done by micro culture which is made on a block of agar on slide.

Yeasts are **unicellular organisms** about five to 10 times larger than bacteria. Morphologically, they may be ellipsoidal, spherical, and, in some cases, cylindrical. Most yeast reproduces asexually by a process called **budding**. A bud is an outgrowth from the parent cell that pinches off, producing a daughter cell. Some yeasts may also undergo sexual reproduction when two ascospores conjugate, giving rise to a zygote or diploid cell. The nucleus of this cell divides by meiosis, producing four haploid nuclei called ascospores contained within the cell, which is now called the **ascus**. When the ascus ruptures, the **acospores** are released and conjugate, starting the cycle again.

The identification of the yeasts depends upon morphology and culture characters. The identification of species depends upon the capabilities the yeast to ferment and assimilate sugars and nitrate.

MEDIA FOR CULTIVATION OF PATHOGENIC FUNGI

The media required for isolating pathogenic fungi from clinical specimens needs to be selective, so as to depress the growth of faster growing bacteria and rapidly growing contaminating fungi. The most commonly used medium for isolating pathogenic fungi is Sabouraud dextrose agar (SDA). It has a pH of 5.6 (prevents growth of bacteria) and support the growth of fungi that are acid tolerant.

The medium may be made more selective by addition of cholramphenicol (antibiotic). Cyclohexamide (actidione) has an anti-fungal action against some of contaminating fungi, but can be inhibitory for some of pathogenic fungi such as Zygomycetes, *Aspergillus* sp., *Cryptococcus neoformans* and some of dimorphic fungi such as *Blastomyces dermatitidis*. Therefore, it is advisable to use media with and without selective agent cyclohexamide especially when attempting to isolate dimorphic fungi.

Enriched medium such as Brain- heart infusion agar (BHIA) with 5% blood is used for dimorphic fungi. When incubated at 37˚C, this helps conversion from mycelial to yeast phase of growth.

Emmon's modified Sabouraud dextrose agar (ESDA) with pH 6.9 has been recommended for isolation of Dermatophytes.

Yeast extract is added to the medium to provide the growth factors needed by *Trichophyton* sp. (alongwith Chloramphenicol 0.05 g/liter and Cyclohexamide 0.4 g/ liter of medium).

Using an incubation temperature of 37˚C is itself a selective procedure. This temperature is harmful to many Non-pathogenic fungi. Fungi that invade animal tissues can tolerate 37˚C. However, many potentially pathogenic fungi such as *Aspergillus* species prefer lower incubation temperature i.e. 20 – 25˚C (room temperature).

MOST SUITABLE MEDIA FOR SOME PATHOGENIC FUNGI

Fungal Pathogen	**Sabouraud dextrose**	**Sabouraud dextrose agar +C**	**Sabouraud dextrose agar + C+C***	**Emmon's Sabouraud agar + yeast extract +**	**Brain heart infusion agar + 5% blood**	**Brain heart infusion agar + 5% blood + C+C***	**Incubat-ion Temp. & Time**
Aspergillus spp.	+	**+**	**-**	**-**	**-**	-	37˚C, 1-4 days
Zygomycetes	**+**	**+**	**-**	**-**	**-**	-	37˚C, 1-4 days
Candida albicans	**+**	**-**	**+**	**-**	**-**	-	37˚C, 1-4 days
Crytococcus neoformans	**+**	**+**	**-**	**-**	**-**	-	37˚C, 1-2 weeks
Dermatophytes	**+**	**-**	**+**	**+**	**-**	-	25˚C, 2-6 weeks
Dimorphic fungi (Yeast phase)	**+**	**-**	**-**	**-**	**+**	+	37˚C, 1-4 weeks
Dimorphic fungi (Mould phase)	**-**	**+**	**+**	**-**	**-**	-	25˚C, 1-4 weeks
Fungi causing subcut. Mycosis	**+**	**+**	**-**	**-**	**-**	-	25˚C, 2-3 weeks

+ = Medium support the growth of fungi; **-** = Not suitable
C = Chloramphenicol; **C + C*** = Chloramphenicol & Cyclohexamide.

Examination of culture is to be carried out from time to time as most of the fungi have got slow growth rate. The culture slant should be incubated at least for four weeks before being discarded as culturally negative. Examination of the growth will provide clues to the special characteristics which .are essential attributes for proper identification. The materials are to be picked up carefully from the growth and examined under microscope using a mounting fluid like lactophenol cotton blue.

Exercise No. 6 **Date:**

Exercise: Principles of identification of fungi

In general, for diagnosis of fungal infection, more emphasis is placed on morphological structures. The procedures include:

1. Direct microscopic examination Appearance of fungi in clinical samples.

1. Place 1-2 drops of 10-20% Potassium hydroxide (KOH) on a microscopic slide and add a small amount of specimen to the drop of KOH and mix with a tooth prick. Gently heat over the low flame of Bunsen burner or sprit lamp but do not boil or over heat.
2. Place a coverslip and allow it to stand for 1-2 hours at room temperature or over night in a moist chamber. KOH partially clears the proteinaceous debris.
3. Examine under light microscope or phase contrast microscope.

2. Colonial characteristics on laboratory media and type of pigmentation (colony colour).

3. Microscopic morphology This includes:

a) *Type of hyphae*

b) Type of spores

c) *Type of spore bearing structure*.

a) **Type of hyphae** :

1. Septate: Presence of cross walls in the hyphal filament.
2. Aseptate: Absence of cross walls in the hyphal filament; also known as ceonocytic hypha.
3. Pseudo-hyphae: Chain of elongated budding cells that have failed to detach.

b) **Type of spores*:*** Two types of spores are produced by fungi

1. Asexual spores

i. Microconidia- Small single celled conudium.

ii. Macroconia- Large often multicellular conidium

iii. Chlamydospore- These are thick wall, resistant spores formed by direct differentiation of the mycelium e.g. *Candida albicans*

iv. Arthrospore- Formed by the disarticulation of myecelium e.g. *Geotrichum candidum*.

v. Blastospore- A spore produced as a result of budding process of the mycelium or from a single spore e.g. *Sacchromyces* spp.

vi. Sporangiospore- An asexual spore produced by cleavage in a closed, often spherical structure called sporangium e.g. *Rhizopus*

spp. The specialized hyphae bearing sporangium is known as sporagiophore and the persisting dome shaped upper portion of sporangiophore is called Columella.

2. Sexual spores

i. Ascospore- They are produced in a sac like structure called ascus. The ascospore results from the fusion of two nuclei. E.g. *Sacchromycetes* spp.

ii. Basidiospore- Produced on a specialized club like structure called basidium.

iii. Zygospore- A thick walled sexual spore produced through the fusion of two similar gametangia found in the class Phycomycetes.

c) **Type of spore bearing structure**:

1) Conidiophore: is a stalk like branch of mycelium on which conidia develop either singly or in more numbers.

2) Sporangiophore: is a specialized hyphae bearing sporangium; and the dome shaped upper portion of sporangiophore is called Columella.

3. **Bio-chemical Tests**: Can be used for yeasts and to limited extent to identify moulds e.g. *Cryptococcus neoformans*

4. **Biopsy examination**: The skin biopsy from the suspected lesions may be made and stained with fungal stains.

(a) **Haematoxylin and eosin stain**. This stain is widely used for the histopathological examination of tissues. Many fungi take this stain. But, there is limitation too as some fungi do not take this stain properly.

(b) **Special fungal stain**. These are Gomori's methanamine silver technique (GMS), Gridley's fungus stain (GF) and Periodic Acid Schiffs technique (PAS). The periodic acid and adjacent hydroxyl group of complex polysaccharides as present in fungal cell walls are oxidized to aldehyde.

In GMS procedure, the aldehydes reduce the methanamine silver nitrate complex and result in brown black staining of fungal cell walls due to the deposition of reduced silver where ever aldehydes are located. The depth of colour produced depends on the amount of aldehyde present at any particular site. The GMS procedure is regarded best for screening a tissue section because it provides better contrasts and often stains fungal elements those are refractory to GF and PAS procedure.

In the GF and PAS procedures, the aldehydes react with the Schiffs agent, colouring fungi reddish purple and pinkish red, respectively. The PAS procedure can be preceded by diastase digestion to remove glycogen. This

eliminates some of the non-specific staining of normal tissue components and cellular debris and may result greater contrast between the fungi and the background tissues.

(c) **Combined GMS and H.E. stain**. The combined stain helps simultaneous study of fungal morphology and tissue responses. This is also very much helpful for black and white photography due to increased contrast of the background tissues.

6. **Wood's Light Method**: Certain strains of *M. canis* and *M. audouinii* produce fluorescence - a positive yellow-green colour when examined under Wood's light. This fluorescence is due to tryptophan metabolite produced by the fungi that had invaded actively growing hairs. Fluorescent is not present in scales or crust in culture of dermatophytes.

5. **Serological Tests**: These tests such as precipitation, CFT and latex agglutination are carried out for some of the mycotic diseases e.g. histoplasmosis, paracoccidioidomycosis and blastomycosis..

Exercise No. 7 **Date:**

Exercise: Immunity to infectious diseases

The host employs innate and acquired immunological defenses to combat invasion by infectious agents. Both humoral and cell mediate mechanisms can be fully expressed against these invasions.

Which mechanisms of defense are employed, may be affected by the manner in which the pathogen confronts the host. Organisms that inhabit blood are more likely to evoke humoral immune responses, whereas those afflicting host tissues might preferentially elicit cellular immune responses.

Infectious organisms have elaborated several mechanisms that enable them to elude the host immune system. Generally these include means of evading detection or of modulating the nature of the host's immune response.

Several avenues are actively being explored to develop and improve vaccines not currently available (or less than ideal) that will effectively provide acquired immunity against pathogenic organisms.

Confrontation by potentially infective biological agents occurs on a regular basis. Defense against these agents is one function of the immune system.

Microbial pathogens (which include viruses, bacteria and fungi) usually produce acute infections.

Innate defenses against microbial pathogens include

(a) Anatomical barriers to the physical entry of the pathogens

(b) Non-specific defenses that seek to eradicate the intruders (e.g. natural killer cells and phagocytes) and

(c) Biochemical factors that augment cellular responses or directly impair successful infection by the pathogens (e.g. interferon and lysozymes).

Acquired immunity is antigen-specific. It is produced by specific antigens of the pathogen and is directed against particular antigenic determinants. It has the additional advantage of conferring immunological memory.

Humoral defenses against microbial pathogens lead to

(a) Neutralization or interference with binding to the host cells

(b) Opsonization,

(c) Complement mediated lysis or

(d) Antibody-dependent cellular cytotoxicity reactions.

Specific cell mediated responses against microbial infection are mediated by T_C and T_D cells. Cytolytic responses that occur can also be mediated by non-specific killers (e.g. macrophages and natural killer cells) that are specifically recruited by T helper cells.

Microbial pathogens can escape reprisal by host's immune system by either

(a) Avoiding detection (antigenic drift or modulation) or

(b) Modulating the host's immune responses (e.g. immuno-suppression).

Infections by protistan micro-parasites (protozoa) and animal macroparasites (helminths) are generally chronic.

Innate defenses against protistan and animal parasites are similar to those employed against microbial pathogens, namely mechanical defense and non-specific cellular and humoral defenses (e.g. natural killer cells and phagocytes; alternate complement pathway).

Acquired immunity against protistan and animal parasites includes

(a) The production of specific antibodies

(b) Activation of specific effector T cells and the generation of B cell and memory T cell. However, acquired immunity frequently does not confer protection against re-infection by protistan and animal parasites.

Humoral responses against protistan and animal parasites involve IgM, IgG and IgE. IgE is especially important against helminths. These defenses act against parasites in several ways. These include

(a) Physical interference (blocking attachment or obstructing orifices)

(b) Causing agglutination

(c) Promoting more active phagocytosis (opsonization)

(d) Inducing cytotoxicity and

(e) Neutralizing parasitic products.

Cell-mediated responses against protistan and animal parasites are primarily conducted by macrophages, neutrophils, eosinophils and platelets. Their effectiveness may be enhanced by lymphokines released by antigen-activated T cells. Antigenic-specific cytotoxic T cells may, nevertheless involved in direct lysis of some parasites.

Protistans and animal parasites escape immunological destruction by

(a) Anatomical seclusion,

(b) Evasion

(c) Immune suppression.

Through seclusion, parasites seek to escape detection. Evasion mechanisms attempt to avoid recognition as foreign agents (antigen masking) or elude immunological memory (antigenic variation). Immunosuppressive mechanisms endeavor to diminish the efficiency of the host's response against the parasite.

Vaccines are used as a means of generating immunity against infectious agents. Traditional approaches have used live, attenuated or killed pathogens, their antigens (purified), and toxoids.

New approaches:

(a) Make use of DNA technology to produce analogues of infectious vectors,

(b) Generate synthetic versions of antigenic epitopes and

(c) Create mimetics of particular antigens or antibodies.

Selected questions

1. Write in brief about new generation veterinary vaccines.

Exercise No. 8 **Date**

Exercise: Introduction and general guide lines for vaccination in animals.

Vaccination can confer immunity to many bacteria and viruses and to some helminthes. They are routinely used to prevent diseases. The immunity which follows a microbial infection can be achieved artificially by two types of vaccine: inactivated (killed) or live (usually attenuated).

Vaccination has been extremely successful in veterinary medicine and live vaccines have been widely used (NDV, Fowl-pox, CDV, and Rinderpest).

Certain viral diseases of farm animals are controlled by import restrictions and slaughter in some countries (e.g. FMDV, Rabies, SFV, and Rinderpest). Sometimes vaccination is not allowed because animals may become infected without clinical symptoms, serological diagnosis would not be possible and feral populations could act as reservoirs.

Vaccines have been used against diseases which kill (e.g. velogenic NDV) and are increasingly being used against diseases of production (e.g. IBV) where clinical signs are mild.

When a vaccinated animal is exposed to virulent virus (challenged) the vaccine must prevent clinical disease. Ideal vaccines should also prevent any excretion of challenge virus which may infect and induce disease in susceptible contact animals.

Most viruses infect via mucosal surfaces where the protective antibody is IgA. Many vaccines are given parenterally, but even when applied to mucosae both the production of IgA and memory for IgA production is less efficient than for serum IgG.

Safety

Inactivated or killed vaccines must not contain infective virus or bacteria during manufacture - this can be a particular problem with regard to FMDV. Live vaccines must not revert to virulence during animal passage and therefore, should ideally have been tested before releasing to vaccination under field conditions by performing animal to animal passages. Live vaccines should also have no residual pathogenic effect such as drop in egg production seen with IBV vaccines and blue-eye by CAV vaccines; nor should they contain adventitious agents e.g. MDV vaccines contaminated with avian adenovirus. Adventitious agents may also occur in killed viruses if they are less susceptible to inactivation than the vaccine virus e.g. ASFV in crystal violet inactivated SFV vaccine (in S. America 1983), and Scrapie in Louping ill vaccines (Scotland; 1960).

Timings of vaccination

It is important to try and keep unvaccinated animals isolated. The timing of vaccination varies according to the species of animal and to whether a live or killed vaccine is used. It is most important to by-pass maternal immunity and to ensure an anamnestic response.

The activity of passive antibody is determined by the level of antibody in the dam, by how much is transferred via colostrum (or more rarely the placenta), and by its capacity to neutralize the vaccine. Passive antibody wanes because circulating IgG has a half life of 10-20 days in domestic mammals (1-2 days in poultry). Thus levels are generally low by 8-16 weeks in dogs and cats; by 12-24 weeks in farm animals; but as early as 3 weeks in poultry. The action of passive immunity may also be avoided by giving vaccines via the respiratory tract where there is little maternal immunity e.g. NDV and IBV are given by aerosol to 1-3 day old chicks. On the other hand increasing passive immunity may be a deliberate part of the control programme; thus neonatal diarrhoea in farm animals might be reduced by vaccinating the dams.

If the vaccine is live only one inoculation is required because the virus replicates and thus presents a much larger antigenic mass than the original inoculum. It is usual to use inactivated vaccines in two doses with the second inoculation at 14 days apart thus evoking the anamnestic response. The use of adjuvant increases the efficacy of inactivated vaccines both in the way it is presented to macrophages and duration of presentation.

Guidelines

Not all the vaccination however, imparts life long immunity. So, it is important to know the period of immunity, age at which the animal must be vaccinated, dosages and route of vaccination and storage conditions for stocking vaccines.

For making the vaccination programme effective the following points should be kept in mind:

- Perform vaccination in healthy stock. Suitable treatment for any abnormal conditions would be beneficial before vaccination. Animals in advanced pregnancies should not be vaccinated.
- No vaccination programme should be carried out during any outbreak of disease. Attendants and fomites carry infection from diseased animals to healthy stock.
- Keep all vaccines under refrigeration until ready to use.
- At the time of vaccination, the reconstituted vaccines should be kept on ice. Also

avoid direct exposure to sun light.

- Destroy all the empty bottles and unused vaccines. Saving left over vaccine is a dangerous practice (especially poultry vaccines) as the immunizing ability of a vaccine can be readily lost in a short time.
- Clean up and disinfect all the equipment and clothings after the vaccination job is over.
- For the success of any immunization programme, it is advisable that the vaccination be performed by trained and qualified personnel.
- Strictly follow manufacturer's directions and instructions.
- Keep a record of the brand, kind and batch number of vaccine used.

The vaccination schedules are given on the following pages for different domestic animals. These schedules are only a guide lines. It must be tailored to meet specific needs and local conditions as determined by the Veterinarians. .

Exercise No. 9 **Date:**

Exercise: Veterinary bacterial vaccines.

The veterinary vaccines are preparations containing antigenic substances which on administration induce a specific and active immunity against infectious diseases produced by bacteria, viruses or other micro-organisms, parasites or toxins. The vaccines may contain micro-organisms, or inactivated parasites, antigenic fractions or substances produced by these organisms and rendered harmless, but retaining all or part of their antigenic properties.

The bacterial vaccines may be prepared from cultures grown on suitable solid or liquid media and contain inactivated or live bacteria or their immunogenic components. The identity, antigenic potency and purity of each bacterial culture used must be carefully controlled.

Bacterial vaccines, reconstituted where necessary, are liquid preparations of various degrees of opacity. Bacterial vaccines that are toxoids are either clear or slightly opalescent liquids, emulsions or suspensions.

The concentration of living or inactivated bacteria is expressed in terms of international units of opacity, or where appropriate, by viable count, total cell count or other suitable methods. For vaccines containing adjuvant, such counts are carried out before the addition of adjuvant.

Bacterial vaccines are thus categorized as killed bacterial and living bacterial vaccines.

The killed bacterial vaccines contain bacteria or their immunogenic components that have been inactivated in such a way that adequate immunogenicity is retained or they are bacterial toxins, the toxicity of which has been reduced to a low level or completely removed without destroying their immunogenicity (toxoids). The vaccines may be precipitated with alum or other suitable agent, purified by adsorption or adsorbed on aluminium phosphate, aluminium hydroxide, calcium phosphate or other adsorbent prescribed.

Living bacterial vaccines may be prepared from strains of attenuated virulence or from strains of low virulence occurring in nature, that are capable of stimulating immunity against the pathogenic strains of same or antigenically related species of bacteria. Combined (polyvalent) vaccines are mixture of 2 or more vaccines.

Living Bacterial Vaccines

1. Anthrax spore vaccine
2. *Brucella abortus* (strain 19) vaccine

Inactivated Bacterial Vaccines

1. Black quarter vaccine
2. Haemorrhagic septicaemia oil-adjuvant vaccine
3. Haemorrhagic septicaemia vaccine alum treated
4. Enterotoxaemia vaccine
5. Multi-component clostridial vaccine
6. *Salmonella abortus equi* vaccine
7. Fowl-cholera vaccine (polyvalent)
8. Fowl spirochaetosis

ANTHRAX SPORE VACCINE (LIVING)

(Avirulent anthrax spore vaccine, *Bacillus anthracis* vaccine (living)

The vaccine is a suspension of living spores of an uncapsulated avirulent strain of *Bacillus anthracis* in 50% glycerin saline. .

An avirulent strain of *B. anthracis* of known antigenicity is grown on suitable medium (nutrient agar) at pH 7.4 in Roux-flasks. After 72-96 hours of incubation at 37ºC the pure sporulated culture growth which shows 70-80% sporulation, is washed with normal saline.

Two guinea pigs each are inoculated subcutaneously with 0.5 and 0.2 ml with culture washing. Another set of two guinea pigs each are inoculated subcutaneously with 0.5 and 0.2 ml with the glycerinated culture washing (diluted 1:5 for inoculation). Add pure sterile glycerin pH 7.0 to 7.2 to the extent of 50% by weight to culture washing and keep in a cool place for 21 days. There may be some degree of swelling at the site of inoculation but no death. The death if any, may be ruled out for virulent anthrax. The spore count of the glycerinated suspension is made.

Dose

Cattle and large animals	-	2 ml containing 10 million spores
Sheep and goats	-	1 ml containing 5 million spores

It is to be inoculated subcutaneously.

***BRUCEUA ABORTUS* STARIN 19 VACCINE (LIVING)**

(Contagious abortus strain 19 vaccine (living)

Brucella abortus strain 19 vaccine is a suspension of pure smooth living culture of *Br, abortus* strain 19 of low virulence in normal saline solution. The vaccine may be supplied as liquid or freeze dried preparation that is reconstituted with normal saline immediately before use.

A 48 hours old smooth culture of *abortus* strain 19 on glycerin agar slants is inoculated on potato infusion agar medium pH 6.8 in Roux flasks and incubated for 48 to 72 hours at 37ºC. The flasks showing good pure growth are washed with buffer saline

and tested for purity. The bacterial concentration is assessed by packed-cell method with the help of Hopkins tube and diluted with buffer saline to 0.72% concentration. This is considered equivalent to 16,000 million viable organisms in 1 ml.

Dose: 5.0 ml by subcutaneous route

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BLACK QUARTER VACCINE

(Black leg vaccine or Quarter evil vaccine)

Black quarter vaccine is a culture of *Clostridium chauvoei or* a mixture of *Clostridium chauvoei* and *Clostridium septicum* grown in a suitable anaerobic fluid medium and rendered sterile and non-toxic by the addition of solution of formaldehyde in such a manner that it retains its immunizing properties.

Clostridium chauvoei organisms are grown in meat-liver-sodium thioglycollate medium (pH 8.2-8.4) at 37ºC for 35 days or until good growth is obtained. Formalin solution (10%) is added in sufficient quality to the culture to yield a final concentration of 0.5% formalin by volume in the product.

Dose

Cattle	5 ml
Sheep & goats	2-3 ml by S/C route.

HAEMORRHAGIC SEPTICAEMIA-OIL-ADJUVANT VACCINE

(Pasteurella multocida adjuvant vaccine)

The vaccine is a homogenous suspension of formalized *Pasteurella multocida* 6B strain with liquid paraffin and lanolin.

A pure growth of highly antigenic strain of *Pasteurella multocida* in phase I (Izatnagar P- 52) grown on nutrient agar medium containing 0.5% yeast extract is washed with 0.5% formal saline. The pooled suspension is diluted with formal saline to match tube no. 6-7 of Brown's opacity tube (2,100 millions organism / ml). The safety of this adjusted suspension is tested on albino mice and observed for 3 days; before it is mixed with liquid paraffin and lanolin in suitable proportions.

The vaccine should be inoculated intramuscularly and the site of inoculation should be given a gentle massage. The bottle of vaccine should be shaken well before use.

Dose

Cattle and buffaloes up to 140 kg	2 ml
Above 140 kg	3 ml
Sheep and goats	2 ml

HAEMORRHAGIC SEPTICAEMIA VACCINE ALUM TREATED

(Pasteurella multocida vaccine alum treated)

The vaccine is a formalized culture of a virulent strain of *P. multocida* in a nutrient

broth treated with potash alum.

A highly potent antigenic strain of *Pasteurella multocida* in phase II (Izatnagar P-52) is grown in nutrient broth at 37ºC. The pure growth is inactivated by the addition of formalin solution to a final concentration of 0.5%. This is treated with potash alum to give a final concentration of 1 %.

Dose

Cattle & Buffaloes 5 ml S/C route.

ENTEROTOXAEMIA VACCINE

(Clostridium perfringens type D, Formol culture, Pulpy kidney vaccine)

Enterotoxaemia vaccine is a culture of a highly toxigenic strain of *Clostridium perfringens* type D, grown in an anaerobic medium rendered sterile and atoxic by the addition of formaldehyde solution in such a manner that it retains its immunizing properties.

Selected toxigenic strain of *Cl. perfringens* type D is grown in a liquid medium under conditions which ensure maximum epsilon toxin production. The culture is checked for purity and toxicity in mice. Solution of formaldehyde is added in suitable concentration and the formalized culture is kept at 37ºC till product is sterile and non-toxic.

Dose

Sheep and goats 2.5 ml by S/C route; repeated after 14 days with same quantity.

The second injection is timed about 10 days before the disease is expected to occur. Lambs and sheep vaccinated during the previous year, require only one injection in the subsequent year. Pregnant ewes can also be safely vaccinated, the second injection being given about 3 weeks before lambing / kidding. Lambs / kids above 3 months of age can be vaccinated without any untoward reaction.

MULTI COMPONENT CLOSTRIDIAL VACCINE

It consists of highly antigenic components containing the toxoids of *Clostridium perfringens* type D. *Cl. perfringens* type C, *Cl. novyi. Cl. septicum* toxoids are prepared in double strength and then combined in such proportion as would invoke adequate antitoxic response in the vaccinated sheep against each antigen incorporated in the vaccine.

The above strains are grown separately in suitable liquid media under conditions which ensure maximum toxin production. The cultures are checked for purity and toxicity in mice. Solution of formaldehyde is added to a 0.5% final concentration and formalized cultures are kept at 37ºC till the product is sterile and atoxic. The formalized anacultures are pooled, precipitated by the addition of aluminium chloride 20% solution

in distilled water to have a final concentration of 1 % of the chemical and pH adjusted to 6.0. The sedimented toxoid is reconstituted to half its original volume in thiomersal saline. With the minimum prescribed toxin yield, the ratio of antigenic components will be as follows: *Cl. perfringens* type D, 0.75 ml; *Cl. perfringens* type C, 1.25 ml; *Cl. septicum,* 2.5 ml; and *Cl. navyi.* 0.5 ml.

Dose

Two doses of 5 ml each are inoculated subcutaneously at an interval of 14-21 days in the first year. Later in subsequent years annual vaccination is repeated with single dose only. The dose is the same for lambs and adult sheep. The bottle should be shaken before use.

SALMONELLA ABORTUS EQUI VACCINE

(Equine abortion vaccine)

It is a mixture of equal parts of pure formalized cultures of smooth laboratory strains of *Salmonella abortus equi* (S-128, S-129, S-130).

One of the above strains are grown on plain agar at 37ºC for 24-28 hours. The pure growth is washed with normal saline solution and pooled. The suspension is standardized to contain approximately 600 million *Salmonella abortus equi* organisms per ml and inactivated with formalin solution. Potash alum is added to give a final concentration of 1 %. Since the above strains are antigenically identical, anyone of the 3 strains can be used for the production of vaccine.

Dose

The immunity conferred by the inoculation of a single dose of vaccine is of low degree and of short duration lasting about 2 months. It is therefore, recommended that to enhance the degree of immunity at the time of first vaccination, the animal should be given a course of 3 injections of 10 and 20 ml at interval of 10 days. These injections will accordingly be given just before service. To maintain the degree of immunity at a useful level, it is necessary to repeat the vaccination at fairly frequent intervals. It is recommended that these subsequent inoculations be carried out at the fourth month after service and again during the six and eight months of pregnancy. Vaccination should not be carried out later than the beginning of the ninth month pregnancy. Each dose is to be given intramuscularly. A slight swelling may appear at the site of inoculation but this disappears in 3 or 4 days.

FOWL CHOLERA VACCINE

(Pasteurella multocida (polyvalent) vaccine (avian strain)

It is a formalized pure broth culture of virulent strains of *Pasteurella multocida* (avian).

The strains are grown separately in nutrient broth for 48 hours at 37ºC. The pure

growth is inactivated with a solution of formaldehyde in a suitable concentration. The cultures are mixed in equal proportions and the final vaccine is dispensed in suitable containers. Dose**:** 0.5 ml I/M

FOWL SPIROCHAETOSIS VACCINE

(Tick fever vaccine)

The vaccine consists of a merthiolated suspension of chorioallantoic membrane, internal viscera and blood of chick embryos infected with a vaccine strain of spirochaetes and freeze dried.

Developing chick embryos, 11 days old, are injected with 0.2 mi of sterile fresh blood containing spirochaetes via the chorioallantoic membrane. The inoculated embryos are incubated at 37°C and candled daily. The dead ones are discarded. On the seventh day the living embryos are chilled in a refrigerator for 2 hours. The chilled embryos are harvested separately and necrotic lesions in liver noted. Representative samples of blood should be examined for teaming spirochaetes. The internal viscera, chorioailantoic membranes and blood are collected and pooled, weighed and held in deep freeze at -20°C to -50°C for one week. Thereafter, the material is blended with equal quantity of merthiolate (final concentration of merthiolate in the suspension should be 1: 10,000) thoroughly for 3 times and the vaccine is ampouled in 2 ml quantities and freeze dried.

Dose: 0.5 ml Intramuscularly of reconstituted vaccine. (1 ampoule of vaccine reconstituted with 10 ml of cold distilled water).

Vaccination schedule of bovines

Age	Vaccine	Revaccination
1 month	**FMD**	Booster to be given at 4th month
2 month	**Theileriosis**	Annual
3 months or above	**Rabies**	Annual
4 months	**1st booster FMD**	
6 months	i) **HS** & **BQ** combined ii) **HS alum** precipitated	Every 6 months or as per manufacturer's Instructions
7 months	***Brucella abortus*** strain 19	Effective up to 5th gestation
10 months	**2nd booster FMD**	As per manufacturer's Instructions.
13 months	**FMD, HS, BQ**	
Lactating animals	**Mastitis** (Hipramastivac) Booster after 1 month	6 months

Note: Vaccines against anthrax and brucellosis are not routinely administered; except in areas where any of the disease is endemic. For Rabies prophylactic vaccination is not done.

1. Vaccination Schedule for Large Animals
Bacterial Vaccines

S No	Name of Vaccine	Manufacturer	Vaccine type / Contents	Dose & Route	Vaccination Schedule	Packing	Remarks
	I. Anthrax*						
1	Anthrax spore vaccine*	Institute of Animal Health and Vet biologicals (IAHVB) Bangalore	Living spores of uncapsulated avirulent Sterne strain of *Bacillus anthracis* in 50% glycerin saline.	1 ml S/C route	Primary: 6 mo of age Revaccination: Annual	100 doses	Immunity within 14 days; can be used in outbreaks
	II. Brucellosis*						
1	*Brucella abortus* strain 19 vaccine*	I V R I, Izatnagar; IAHVB, Bangalore	Suspension of smooth live culture of *Br. abortus* in NSS	5 ml S/C	At 6-9 mo of age	1 or 5 doses	Effective up to 5th gestation; Not to be used in male calves & advanced stage of pregnancy.
2	*Br. abortus* strain 45/20 vaccine*	-do-	Killed vaccine used along with adjuvant		Animals of all ages; protect for 18 mo; Two doses at 6-12 wk interval	-	Does not interfere in sero-surveys to detect prevalence of brucellosis.
	III. Haemorrhagic septicaemia (HS)						
1.	HS alum precipitated vaccine	Pb. Vet. Vac. Inst. Ludhiana	Formalized suspension of *Pasteurella multocida* in potash alum	5ml S/C	Primary: 6 mo. of age Revaccination : 6 mo	80 doses	Immunity within 7 days; can be used in the face of outbreaks
2.	Raksha HS vaccine	Indian Immunologicals Ltd. Hyderabad	Formalized suspension of *P. multocida* adsorbed on alum hydroxide gel.	2ml S/C	Primary: 6 mo. of age Revaccination: Annual	50 doses	-do-
3.	HS oil adjuvant vaccine	IAHVB, Bangalore	Formalized suspension of *P. multocida* in liquid paraffin and lanolin.	Up to 300 lbs : 2ml I/M > 300lbs: 3ml I/M	Primary: 6 mo. of age Revaccination: Annual	100 doses	Immunity after 21days; therefore, not used in outbreaks
	* Not routinely administered except in endemic areas.						

	Name of Vaccine	*Manufacturer*	*Vaccine type / Contents*	*Dose & Route*	*Vaccination Schedule*	*Packing*	*Remarks*
	IV. Black quarter (BQ)						
1	BQ vaccine	Pb.Vet Vac. Inst. Ludhiana	Formalized culture of *Clostridium chauvoei* & *Cl. septicum.*	Cattle: 5ml Sh / G: 2-3 ml S/C	Primary: 6 mo. Revaccination: 6 mo.	50 doses	Can be used in the face of outbreaks.
2	Raksha BQ vaccine	Indian Immunologicals Ltd. Hyderabad	Formalized culture of *Cl. chauvoei* adsorbed on Aluminium Hydroxide gel	C, B, Calf: 2ml S/C	Primary: 6mo Revaccination: Annual	50 doses	-do-
	V. Enterotoxaemia						
1	Raksha ET	Indian Immunologicals Ltd. Hyderabad	Inactivated culture of anaerobically grown *Cl. perfringens* type D organisms adsorbed on Alum. Hydroxide gel.	Sh / G: 2ml S/C	Primary: 4 mo Revaccination: annual	50 doses	-do-
2	Enterotoxaemia vaccine	Pb.Vet Vac. Inst. Ludhiana, IVRI, IAHVB, Bangalore.	Formaldehyde inactivated *Cl. perfringens* type D grown in anaerobic medium.	2.5-3ml S/C	Primary: 3mo Booster: 14 days Revaccination: annual	100 doses	Immunity within 10 days of second injection.
	VI. Tetanus						
1	Tetanus toxoid	Haffkin Institute Bombay	Contains purified tetanus toxoid and thiomersal 0.01% preservative	Horse: 10 ml Cattle: 1-2 ml S/C.	Horses: Primary 5-8 wk Booster: 30 days Revaccination: Annual.	1, 5 or 10 ml	Mares vaccinated 6 weeks before foaling.
	VII. Mastitis						
1	Hipramastivac	Hipra Labs, Spain	Inactivated *Streptococcus agalactiae, Strept. dysgalactiae, Strept. uberis, Staphylococcus pyogenes, Staph. aureus, E. coli, Pseudomonas aeruginosa* and *Corynebacterium pyogenes.*	3 ml I/M	Primary at any time; Booster: 4 wks; Repeat: 6 mo.	1; 5 or 30 doses	Treat sub-clinical mastitis, if any, before vaccination.

1. When using any vaccine, its manufacturer's instructions should be strictly followed.
2. All the vaccines should be stored at +2 to +8°C. Freeze dried vaccines should be preferably stored at –20°C (deep freeze).
3. For a specific place or situation, the schedules given may be adapted or modified as per the requirements.
4. Vaccines against anthrax & brucellosis are not routinely administered, except in areas where any of the disease may be endemic.
5. For rabies prophylactic vaccination is not done.

Exercise No. 10 **Date:**

Exercise: Veterinary viral vaccines.

Viral vaccines are sterile suspensions or freeze-dried powder containing modified living or inactivated virus particles which in their original un-altered stage cause disease. The vaccine derives its name from the disease. It is prepared from the blood or tissue of a suitable host in which it has been grown *in vivo* or from tissue-culture.

Immunity may be induced artificially by a variety of preparations of viral antigen. The use of virulent living viruses has disadvantages. These to a large extent could be overcome by using attenuated viruses.

The fowls were vaccinated against infectious laryngotracheitis by injecting virulent virus into the cloaca, a route that did not cause serious disturbance (unusual route). It had the risk of spreading the disease among healthy birds or animals.

Another method was to give virulent virus and antiserum together to induce active immunity. This method was used successfully for some time in protection against cattle plague, swine fever, canine distemper and sheep-pox, but it entailed the risk of spreading the disease.

A modification of the method was to give antiserum after the live vaccine was presumed to have established itself in tissues. This procedure, being uncertain in its effect, is no longer in use.

While preparing live vaccine, advantage is taken of the fact that the virulence of a virus can be attenuated without loss of immunogenicity.

Attenuation may be effected in various ways, such as serial passage through an animal of a different species or in cell-culture by repeated passages and selection by plaque techniques. An example of the former method is the use of a strain adapted to goats and rabbits for the protection of cattle against rinderpest, or a strain passaged through pigeons for protection against fowl-pox and various attenuated strains for protection against African horse sickness, Rift valley fever of sheep, and Bluetongue of sheep.

With attenuated or live modified vaccines the amount of virus given is fairly small, but it is introduced by a route that ensures its further multiplication. When live vaccines are used, only one inoculation is needed. The vaccinia virus is inoculated by scarification or puncture into the deeper layers of the dermis only. After multiplication at the site of the lesion, it passes into the body. Mixed or polyvalent vaccines containing 3 or more viruses may at times be used with advantage.

The use of modified living viruses as vaccines has become popular, especially in countries where these diseases are endemic, e.g. in India where considerable economic losses are being sustained due to virus diseases like Ranikhet, and some other poultry diseases.

The important viral vaccines being manufactured / used in India are:

1. Foot-and-mouth-disease vaccine
2. Rabies vaccine (inactivated) 5% BPL
3. Rabies vaccine (inactivated) cell-culture
4. Rabies vaccine (living)
5. Canine distemper vaccine
6. Canine hepatitis vaccine
7. African horses sickness vaccine (living)
8. Swine-fever-vaccine lapinised (living)
9. Sheep-pox vaccine (living) cell-culture
10. Sheep-pox vaccine (inactivated)
11. Fowl-pox vaccine-chick embryo virus (living)
12. Ranikhet disease vaccine (living)
13. Ranikhet disease vaccine-F strain (living)
14. Fowl-pox vaccine-pigeon-pox virus (living)
15. Marek's disease vaccine (living)
16. Infectious bursal disease vaccine
17. Avian infectious bronchitis vaccine (living)
18. Avian encephalomyelitis vaccine (living).

FOOT & MOUTH DISEASE VACCINE

Inactivated tissue-culture vaccine

Foot-and-mouth-disease (FMD) vaccine is a liquid preparation containing one or more types of FMD virus, inactivated in such a way that its immunogenic property is maintained. It may also contain an adjuvant.

The vaccine is described as monovalent, bivalent, trivalent or polyvalent depending on the number of types of virus used. It protects cattle against FMD to homologous type / subtype of virus.

The virus is propagated in monolayer cell-culture of calf, goat or pig kidneys or in mono layers, suspension-cum-mono layers or suspension cultures of BHK_{21} cell-line. The virus is harvested and cellular debris removed by filtration. Inactivation is carried out by formaldehyde solution. The adjuvant may be aluminium hydroxide and /or sap-onin. In inactivated gel vaccine, the antigen is concentrated by sedimenting the product at +4°C and drawing out the supernatant and leaving behind the product in a concentrated form. For preparing polyvalent vaccine, monovalent vaccines are mixed together in equal volume.

Dose

The dose varies depending upon the preparation. Each dose should

contain 3 PD_{50} antigen against which protection is claimed.

SHEEP POX VACCINE (LIVING CELL-CULTURES)

i) Attenuated sheep-pox vaccine

It is a freeze-dried preparation obtained by growing attenuated sheep-pox virus in a suitable cell culture system, viz. lamb kidney/testicular/thyroid-cell culture.

The virus is grown in primary suitable cell culture monolayer cultures viz. lamb kidney/testicular/thyroid cell culture in Roux flasks. The sterile virus contents are harvested by 3 cycles of freezing and thawing; 6 to 7 days post-infection and when more than 80% cells, show CPE; are then pooled. The cellular debris or cell aggregates are removed by filtration. Equal quantity of preservative comprising 50% lact-albumin hydrolysate (LAH) and 10% sucrose or any other suitable stabilizer is added before freeze drying.

The vaccine may be prepared with RM/65 strain, RF strain, Romanian, Fennar or Ranipet strain of sheep-pox virus. The vaccine prepared with RM/65 strain does not evoke either thermal or local cutaneous reaction following vaccination. The vaccine prepared either with sheep-pox RF strain or Ranipet, shows 'takes' after a week after vaccination.

Dose

1.0 ml containing 10^3 $TCID_{50}$ virus intradermally in the caudal fold.

ii) Inactivated sheep pox vaccine

Sheep-pox vaccine is a formalin -inactivated gel-treated tissue vaccine.

Healthy susceptible Sheep, 8-12 months of age, are inoculated subcutaneously with 500 ml of 1: 100 dilution of the Russian virulent sheep-pox virus. Skin of the abdomen along with the oedema fluid is collected 7-8 days post-inoculation. The infected tissues are homogenized in 10% concentration in phosphate buffer (pH 7.4-7.6) which after the extraction of the virus is mixed with sterile gel and buffer.

Dose

Sheep	5 ml S/C	in femoral region on inner aspect.
Lambs	3 ml	-do-

A slight swelling at the site of inoculation may develop that gradually subsides.

RABIES VACCINE

i) Antirabic inactivated vaccine

It is a brain tissue suspension of animals infected with a suitable strain of rabies-fixed virus, inactivated with phenol, beta-propiolactone or some other suitable agent.

Young healthy sheep are inoculated intracerebrally or intrathecally with 0.5 ml of

1: 100 to 1:200 dilution of seed virus having mouse infectivity titre of 10^5 LD_{50} per 30 µl or more, and are kept under close observation for 10 days. The inoculated sheep are sacrificed as soon as the usual prodromal symptoms develop (definite signs are paralysis / clinical encephalitis). Before the animals become dead, the heads are separated and skin over the head is removed. Then a diamond-shaped opening is made in the skull, brain is taken out and placed in a sterile container.

All the brains are paired and adjusted to weigh 160 g each. Each pair is blended in sodium potassium phosphate buffer to make 10% suspension W / V and filtered through muslin cloth. To each flask thiomersol solution is added

For inactivation of 10% viral suspension, β-propiolactone (BPL) is added and inactivation of virus is checked in mice. Phenol is added as preservative. On establishing sterility, materials of all the sterile flasks are pooled and diluted with buffer to make a 5% suspension.

Dose

It is intended for use in animals of all species either for pre or-post-exposure, prophylaxis against rabies. The vaccine may be injected into any part of the body subcutaneously, where the subcutaneous tissue is loose, e.g. loose skin on the back or / and flank. The bottle should be shaken before use. The dosage is given below:

Species of animals	**Dosage if the animal has never been immunized against rabies**	**Dosage if the animal has some time previously been immunized against rabies**
Animals weighing under 15 kg. e.g. dog, pup, cat, monkey	2 ml daily for 14 days	2 ml daily for 7 days
Animals weighing 15 – l00 kg e.g. dog, calf, sheep. goal, deer	5 ml daily for 14 days	5 ml daily for 7 days
Animals weighing 100-800 kg, e.g. buffalo, bullock, cow, horse	15 ml daily for 14 days	15 ml daily for 14 days
Camel, elephant	30 ml daily for 14 days	30 ml daily for 7 days

ii) Tissue-culture inactivated rabies vaccine

Cell culture inactivated rabies vaccine is freeze dried preparation of a 'rabies fixed virus' adapted to and propagated in cell-culture, and inactivated by suitable method.

Strain of virus: PV strain of fixed rabies virus adapted to BHK_{21} clone13 cells is used. Infect cell monolayer with cell-line-adapted virus (using 1-5 PFU/0.1 - 0.5 LD_{50} per cell) Harvest of the virus After 3 days of infection, collect the maintenance medium containing virus and freeze it at -60ºC. Replace the maintenance medium and continue to incubate, harvest on 6^{th} day post-infection and so on.

Add BPL and keep at 3°C for 4 hours for inactivation of virus. After inactivation, sodium thiosulphate at a final concentration of 2% is added. Adjust pH to 7.4 to 7.6.

Dose

It is 1 ml subcutaneously or intramuscularly for dogs.

Prophylaxis: At the age of 4 months and above 1 ml. Booster after 1 year.

Schedule: For post-bite vaccination schedule irrespective of age, species, size or breed of animals is on days 0, 3, 7, 14, 30 and 90.

iii) Rabies vaccine (living) chick embryo

It is a freeze-dried suspension of chick embryo tissue infected with a suitable attenuated strain of rabies virus (Flury strain).

Active chicken embryos, 7 days old, injected with 0.25 ml of 20% of suitable attenuated "Flury strain" of rabies virus into yolk-sac and incubated at 36.5ºC for further 9 days. On day 9 post-inoculation only living embryos are harvested and chilled. Embryo yolk-sac, extra-embryonic tissues and fluid are discarded. After cutting their beak and claws embryos are homogenized. The blended suspension is cleared by light centrifugation and filtered through muslin cloth. To the filtered suspension antibiotics are added (penicillin 1,000 IU and streptomycin 2,000 mg/ml). The processed product is dispensed in 3 ml quantities, in suitable vials and freeze dried, and this constitutes a single dose for dog.

Dose

The dried vaccine is reconstituted with 3 ml of chilled sterile distilled water. The contents are thoroughly mixed. Inject 3 ml of the reconstituted vaccine intramuscularly. Pups vaccinated at 4 months should be revaccinated at 1 year of age.

This vaccine is intended for use only as a prophylactic agent and not for post-exposure treatment.

Rinderpest Vaccine

RP Vaccination has been discontinued under National Rinderpest Eradication Programme as per the instructions of Govt. of India. Currently it is not manufactured in India. However, Veterinarians have been advised to keep watch on occurrence of cases of RP under field conditions.

2. Vaccination Schedule for Large Animals
VIRAL VACCINES

S No	*Name of Vaccine*	*Manufacturer*	*Vaccine type / Contents*	*Dose & Route*	*Vaccination Schedule*	*Packing*	*Remarks*
	I. Foot & Mouth Disease (FMD)						
1	Raksha FMD vaccine	Indian Immunologicals Hyderabad	Tissue culture aziridine inactivated FMD virus strains O, A, C, Asia 1 adsorbed on Aluminium hydroxide gel and saponin adjuvant.	C, B, Calf: 3 ml Sh / G: 1 ml S/C route	Primary: 4 mo; Booster 2-4 wks Revaccination: every 6 mo	30 ml 90ml	Maintain cold chain; Slight swelling at the site of injection.
2	FMD vaccine-concentrated, tetravalent	Intervet Lab. Ltd. Hyderabad	Tissue culture, formaldehyde inactivated, FMD virus strains, O, A_{22}, C, Asia1 adsorbed on Al. hydroxide gel and saponin adjuvants.	C, B: 10 ml Sh / G: 5ml S/C route	Primary: 6-8wk; Booster 4-6 wk Revac. 6 m	100 ml	-do-
3	FMD vaccine-super-concentrated tetravalent	Intervet Lab. Ltd. Hyderabad	Do	C, B: 5 ml Sh / G: 2.5ml S/C route	Do	50 ml, 100 ml	-do-
4	Clovax FMD oil adjuvant vaccine	Intervet Lab. Ltd. Hyderabad	Binary ethylamine (BEI) inactivated FMD oil adjuvant vaccine	C, B: 3 ml S/Cor I/M Pig: 3 ml I/M Sh / G: 2 ml S/C, I/M	*Young animals*: Primary: 3 wk; Booster: 12-14 wk; Booster: after 24 wk of first; Revac: annual *Adult animals*: Primary: any age; Booster: after 24 wk; Revac: annual	30 ml, 90 ml	-do-
5	Raksha-ovac FMD oil adjuvant vaccine	Indian Immunologicals Hyderabad	Aziridine inactivated FMD virus strains O, A, C, Asia1 with mineral oil as adjuvant	C, B, Calf, Pigs: 2 ml I/M; Sh / G: 1 ml I/M	Primary: 4 mo; Booster: after 9 mo; Revac: annual	10 ml, 30 ml	-do-
	II. Infectious Bovine Rhinotracheitis (IBR)						
1	**Ibrivax**	Intervet Lab. Ltd. Hyderabad	Inactivated oil adjuvant	2 ml I/M	Primary vac.: 6-8 mo Revaccination : annual	20 ml 10 doses	Safe for pregnant animals

S No	Name of Vaccine	Manufacturer	Vaccine type / Contents	Dose & Route	Vaccination Schedule	Packing	Remarks
	III. Rabies						
1	Raksharab	Indian Immunologicals Hyderabad	Rabies strain propagated in BHK_{21} cell line, inactivated with aziridine; Alum hydroxide gel as adjuvant.	1 ml S/C or I/M	Primary: 3 mo. or above; If primary given below 3 mo, booster at 3 mo Revac: Annual	1 ml, 5 ml and 10 ml	Post bite vacci sch (Therapeutic) : 0, 3, 7, 14, 28 & 90 days
2.	Nobivac Rabies	Intervet Lab. Ltd. Hyderabad	Pasteur strain of rabies virus grown in BHK_{21} cell line and inactivated by β- propiolactone; Aluminium (Al.) phosphate adjuvant	1 ml S/C or I/M	Primary: Cattle, Horse- 6 mo, Revacination: Annual	10 ml	-
	IV. Sheep Pox						
1	Sheep pox tissue culture formal gel vaccine	IAHVB, Bangalore	Ranipet strain of sheep pox virus 40-50 passages in secondary lamb testicular culture; inactivated with formalin adsorbed to Al. hydroxide gel.	1 ml S/C on inner aspect of thigh	Repeat every six months	-	Immunity develop within 7-14 days; can be used in outbreaks.
2	Raksha SP	Indian Immunologicals Hyderabad	Derived from cell cultures infected with attenuated "Romanian strain" of sheep pox virus.	1 ml I/M	Primary: 3 mo	50 doses	Avoid in advanced pregnancy.
	V. Swine Fever						
1	Swine Fever vaccine (Live)	IVRI, Izatnagar; Pb.Vet Vac Inst. Ludhiana	10% spleen suspension of rabbits infected with modified rabbit adapted swine fever virus. The suspension made in yolk buffer and freeze dried.	1 ml I/M	At any age	3-5 doses	1
	VI. Polyvalent Vaccines						
1	Raksha Biovac	Indian Immunologicals Hyderabad	FMD inactivated antigens against O A C Asia1 strains & formalin killed *P. multocida* culture. These are mixed with light mineral oil and homogenized to form oil suspension.	C, B: 3 ml Sh / G: 1 ml I/M route	Primary: 4 mo; Booster: after 9 mo Revac: annual	30 ml, 90 ml	Protect against HS & FMD
2	Raksha Triovac	-do-	In addition to biovac vaccine contains inactivated *Cl chauvoei* culture	-do-	-do-	-do-	Protect against HS, FMD and BQ
3	Raksha HS+BQ	-do-	Formaline inactivated cultures of *P. multocida* and *Cl. chauvoei* adsorbed on Al hydroxide gel.	C, B, Calf : 3ml S/C.	Primary 6m or above; Revacc: annual	90 ml	Protect against HS and BQ

Parasitic vaccines

Theileriosis

S No	*Trade Name*	*Manufacturer*	*Vaccine type/ contents*	*Dose & route*	*Vaccination schedule*	*Packing*	*Remarks*
1	Rakshavac T	Indian Immunologicals Hyderabad	Attenuated tissue culture *Theileria annulata* schizont vaccine	3 ml S/C	2 mo of age; no booster Revaccination: Annual	5 doses	Avoid in advanced pregnancy; Immunity develops 6weeks after vaccination

3. Vaccination Schedule for Dogs

AGE	SCHEDULE
3 weeks	Deworm the pup for parasitic infestations such as roundworms and hookworms one week before vaccination.
4 weeks	Vaccination against CD and CPV (if bitch was not vaccinated)
6 weeks	Vaccination against CD and CPV (if bitch vaccinated otherwise act as booster)
8-10 weeks	Vaccination against CD, CPV, CAV-1, 2, Leptospirosis, Coronavirus, Parainfluenza and Bordetella.
12-13 weeks	-Booster of above-
3 months or above	Rabies
5-6 months (optional)	Vaccination against CD, CPV, CAV-1, 2, Leptospirosis, Coronavirus, Parainfluenza and Bordetella and repeat annually.
8 months	Rabies booster and repeat annually.

Vaccines for dogs

Name of vaccines	Protects against diseases				
	Rabies	CD	ICH	Leptospirosis	Parvovirus
Candur DHL	-	+	+	+	-
Candivac	-	+	-	-	-
Canine distemper (chick embryo cell Culture)	-	+	-	-	-
Canine hepatitis (CBCC)	-	+	+	-	-
Canine distemper (I.V.R.I)	-	+	-	-	-
Canulin DH	-	+	+	-	-
Candur DHLP	-	+	+	+	+
Parvodog	-	-	-	-	+
Nobi-vac Puppy DP	-	+	-	-	+
Rabisin	+	-	-	-	-
Nobi-vac RL	+	-	-	+	-
Nobi-vac-Rabbies	+	-	-	-	-
Pentadog	+	+	+	+	-
Raksha Rab	+	-	-	-	-
A.R.V. (20%)	+	-	-	-	-

Some Commercial Vaccines for Dogs

S. No.	*Diseases*	*Trade name of Vaccine*	*Age at vaccination*	*Dose & Route*	*Revaccination*	*Manufacturer*
1	**Rabies**	Raksharab	3 mo. & booster after 28 day.	1 ml S/C or I/M		Indian Immunologicals
		Defensor	3 months;	1 ml S/C		Pfizer Animal Health
		Rabisin	4 weeks but 11 weeks in pups from immunizes bitch;	1 ml S/C or I/M		Serum Institute of India
		Candur-R [inactivated cell culture vaccines]	7 weeks but 12 weeks weeks in pups from immunizes bitch.	1 ml S/C or I/M	Annually	Hoechst Roussel Vet.
2	**Parvovirus**	Parvodog	7-8 wks, booster at 12 wks	1 ml S/C	Annually	Serum Institute of India
3	Distemper, Hepatitis & Leptospirosis	Polyvalent vaccine Novivac-DHL, Caniffa-DHL etc.	6-8 weeks & booster at 12-14 weeks of age	2 ml S/C	Annually	
4	Distemper, Hepatitis, Leptospirosis & Parvovirus	Magavac-6	-do-	Reconstitute the freeze dried vaccine with liquid vaccine just before vaccination. 1 ml S/C	Annually	Indian Immunologicals
		Vanguard				Pfizer Animal Health
		Candur DHL+P				Hoechst Roussel Vet.
5	Distemper, Hepatitis, Leptospirosis & Rabies	Pentadog	-do-	-do-	-do-	Serum Institute of India

4. Vaccination Schedule for Poultry

Recommended for healthy chicks / birds and is not for clinically sick or severely debilitated birds under conditions of severe stress.

For Commercial Layers

S.No	AGE	NAME OF VACCINE	DOSE	ROUTE
1	Day-old chick	Marek's Vaccine at Hatchery	0.2 ml	I/M
2	3-7 days	Gumboro Live (Lukert strain) + IBD (Killed)	0.2 ml	I/O I/M
3	8-9 days	LaSota + N.D Killed	1 drop 0.2 ml	I/O I/M
4	14-18 days	Gumboro (Intermediate strain)	0.1 ml	D/W
5	20-21 days	Debeaking		
6	24-26 days	I.B. Live	0.1 ml	I/O or D/W
7	28-32 days	Gumboro (Intermediate strain)		D/W
8	33-35 days	LaSota (Booster)		D/W
9	42-46 days	Gumboro (Intermediate strain)		D/W
10	7th week	Fowl Pox	0.1 ml	W /w I/M or S/C
11	8th week	Coryza (Killed) or Fowl Cholera (Killed) or Both	0.5ml	I/M Breast
12	10th week	R_2B (Live)	0.5 ml	I/M Breast
13	12th week	Coryza (Killed) or Fowl Cholera (Killed) or Both	0.5ml	I/M Breast
14	13th week	LaSota + IB (Booster)		
15	14th week	Fowl Pox (Booster)	0.1 ml	W /w I/M
16	15 th week	Debeaking and Grading		
17	16th week	Deworming		
18	17th week	R_2B or ND (Killed) Booster	0.5 ml	
19	40th week	Repeat LaSota at 12 weeks interval		D/W

For Commercial Broilers

S.No	AGE	NAME OF VACCINE	DOSE	ROUTE
1	Day-old chick	Marek's Vaccine at Hatchery	0.2 ml	I/M
2	3-7 days	IBD Lukert strain + IBD killed	0.2 ml	I/O I/M
3	8-10 days	LaSota N.D Killed	1 drop 0.2 ml	I/O or I/N I/M
4	14-18 days	IBD Intermediate Strain	1 drop	I/O or D/W
5	28-30 days	LaSota (Booster)	1 drop	I/O or D/W

NOTE: **I/N**: Intranasal; **I/O**: Intraoccular; **S/C**: Subcutaneous injection; **I/M**: Intramuscular injection; **D/W**: Drinking water

* IBD (Killed) at 17th - 19th day is recommended in areas where the disease is prevalent.

Coryza (Killed) and /or Fowl Cholera (Killed) vaccines need to be used in areas where either or both diseases are prevalent.

** In case of broiler parents IB (Killed) and N.D+IBD (Killed) vaccines need to be administered in 22nd and 23rd week, respectively.

Following vaccines are to be used in endemic areas for the healthy flocks

1	Infectious coryza (killed) vaccine	8th week and repeated after 4 weeks.
2	Polyvalent fowl cholera (killed) vaccine or FC+IC combined killed vaccine	8th week and repeated after 4 weeks
3	Spirochaetosis (killed) vaccine	8th week onwards.

Exercise No. 11 **Date:**

Exercise: Vaccination failure in livestock and poultry.

There are many reasons for vaccination failure. These include:

Factors associated with manufacturer: The method of manufacture may have destroyed the protective epitopes, or there may simply be insufficient antigen. Such problems are relatively uncommon and generally can be avoided by using vaccines from reputable manufacturers.

In other cases, the vaccine may not be effective because it contains strains of organisms or antigens that are different from the disease-producing agent.

Factors associated with vaccinator: An effective vaccine may fail due to unsatisfactory administration. For example, a live vaccine may be inactivated as a result of improper storage, use of antibiotics in conjunction with a live bacterial vaccine, chemical sterilization of syringes, or excessive use of alcohol on the skin. Administration by non-conventional routes may also affect efficacy. When vaccine is administered to poultry or mink by aerosol or in drinking water, the aerosol may not be evenly distributed throughout a building, or some animals may not drink adequate amounts. Also, use of chlorinated water may inactivate vaccines.

Factors associated with host: If an animal is incubating the disease before vaccination, the vaccine may not be protective; vaccination against an already contracted disease is usually impossible

The immune response, being a biologic process, never confers absolute protection nor is equal in all individuals of a vaccinated population; because the response is influenced by many factors. In a random population, it tends to follow a normal distribution: the response will be average in most animals, excellent in a few, and poor in a few. Those with a poor response may not be protected by an effective vaccine; therefore, it is difficult to protect 100% of a random population by vaccination.

The size of this unresponsive population varies among different vaccines, and its significance depends on the nature of the disease. For highly infectious diseases, in which herd immunity is poor and infection is transmitted rapidly and efficiently (eg. foot-and-mouth disease), the presence of unprotected animals can permit the spread of disease and disrupt control programs. In contrast, for diseases that are inefficiently spread (eg. rabies), 60-70% protection in a population may be sufficient to effectively block disease transmission within that population and therefore, may be satisfactory from a public health perspective.

The most important cause of vaccine failure in young animals is the inability of an antigen to impart immunologic memory in the presence or absence of passive maternal antibodies. Vaccines also can fail when the immune response is suppressed, eg. in heavily parasitized or malnourished animals (such animals should not be vaccinated).

Stressors: Stress, including pregnancy, extremes of cold and heat, and fatigue or malnourishment, may reduce a normal immune response, probably due to increased production of glucocorticoid.

Selected questions

1. Adverse effects of vaccines.

Exercise No. 12 **Date:**

Delayed type hypersensitivity test (DTH)

Exercise: Tuberculin test.

This test is widely used through out the world for the diagnosis and control of tuberculosis in cattle and to lesser extent in other animal species. The basis of this test is that animals infected with tuberculosis develop a DTH reaction to *Mycobacterium* spp. So that when an extract of the organism i.e. tuberculin is subsequently injected into the skin. A localized DTH reaction occurs at the site of inoculation characterized by inflammation, swelling and pain.

It is a herd test gives only an indication of the number of sensitized animals and may thus be used only as a preliminary test prior to the initiation of a control programme

Requirements : Bovine tuberculin (PPD), sterile tuberculin syringe, 26 gauge needle, spring caliper, razor, 70 % alcohol, face mask, gloves and cap etc.

PPD of bovine tuberculin: Prepared from *M. bovis* strain AN 5, contains 1 mg PPD per ml equivalent to 2000 Tuberculin Units (TU) per 0.1 ml and preserved with 0.5% phenol.

There are 4 methods to perform tuberculin testing:

1. **Single intradermal test (SID) :**

Procedure: Test sites used for tuberculin testing vary in sensitivity and between countries and include the neck region, anal, caudal fold at the tail base or vulvar lip.

Shave 2 x 2 inch area of skin about the middle third of neck approximately mid way between upper and lower edge and disinfest with 70 % alcohol. Pinch up a fold of the shaved skin of neck region and measure its thickness by Varnier / Spring caliper and record the reading. Now hold the skin fold firmly between the thumb and fore finger of the left hand and insert into it the needle of the tuberculin syringe containing 0.1 ml of tuberculin. The needle should be 26 or 27 gauge and 1.25 cm long. The depth to which the needle is inserted into the skin is of great importance. It will vary some what with the thickness of the skin. The reaction produced after a deep injection made into the dermis is more marked than that observed after an injection made superficially just under the epidermis. The needle should not be allowed to pass too deeply so as to penetrate into the subcutaneous tissue. When the needle has been correctly inserted, inject the tuberculin into the dermis for which the application of some pressure may be necessary. The proper injection of tuberculin becomes evident by the appearance of a small pea like nodule in the skin. Measure the thickness of the skin fold 72 hours after the injection. The initial measurement gives information concerning the normal thickness of the skin and, serves as a guide to the depth at which the injection must be made. Care should be taken to exert a uniform degree of pressure on the skin with the calliper so as to obtain

comparable readings.

Interpretation of Results:

- Palpate the swelling at the site of injection and note the presence or absence of warmth, tenderness and consistency of swelling. An increase in skin thickness 4 mm or more is considered positive. In animals that are not infected by the disease usually no change in thickness is observed. Sometimes, a small swelling is produced, but the increase in skin thickness does not usually exceed a few millimeters. Also-palpation reveals absence of local heat and tenderness.

- In animals that are infected, there is usually a considerable increase in skin thickness. Palpation reveals an appreciable degree of local heat and tenderness with a variable degree of diffuse, oedematous infiltration; any of these characteristics give the feeling of a peculiar sensation to the touch, which is different from that felt on palpating the skin of a animal which is not infected. The most characteristic feature of a positive reaction is the presence of diffuse oedema.

Demerits of SID:

1. Lack of specificity because of presence of non visible lesion reactors. It is due to the sensitization of animals with other harmless *Mycobacterium* spp. *Nocardia farcinicus* causing farcy in bovines sensitize animals to this test.
2. Failure to detect cases of minimal sensitivity, viz. in early stages of disease, in advanced stages of disease, in recently parturated and in old animals. Mammalian tuberculin is not specific to differentiate infection with *M. bovis, M. avium*, *M. tuberculosis* and *M. avium paratuberculosis*.

2. Stormont test :

This test is devised to detect the cases of tuberculosis that are poorly sensitized. Procedure is same as single I/D test. In this test second dose of tuberculin is injected at the same site (same dose and route) seven days after first injection. After 24 hours of 2nd injection, examine the area. If it is hot, painful, swollen and increase in thickness of 5 mm or more, then reaction is considered positive for tuberculosis.

The increased sensitivity to Stormont test is thought to be due to attraction of T-cells to the site by the first injection. The increased sensitivity begins at day 5th, reaches its peak on 7th day and ends on 12th day post injection. Disadvantage of this test is that three visits are required to perform this test.

3. Short thermal test:

Four ml of dilute tuberculin is injected subcutaneously in neck area of cattle with rectal temperature not more than $39^{O}C$ or $102^{O}F$ at the time of injection and for 2 hours later. The temperature is again noted at 4, 6 and 8 hours after injection. If temperature rises above $40^{O}C$ or $104^{O}F$ within this time period, then reaction is considered positive for tuberculosis. In infected animals the temperature peak usually occurs between 6-8 hours and is generally $41^{O}C$.

Test is able to detect advanced cases of disease. Highly efficient test to detect spreader case(s) giving negative reaction to SID. Sometimes there may be death due to anaphylactic reaction.

4. Comparative test:

Procedure is same as single I/D test. In this test avian and mammalian tuberculin are injected simultaneously 12 cm apart on the same side of neck area one above the other. Test is read after 72 hours. Greater of two reactions will indicate organism responsible for causing sensitization. This test is not meant for primary screening but only to follow a non-reactor to determine infecting organism.

The recently developed interferon-γ assay used on blood lymphocytes stimulated with *M.* bovis antigen shows promise as an alternative to the widely used SID test.

Desensitization:

When the suspicious reactor is encountered then the question of when to re-test is complicated by phenomena of sensitization caused by absorption of tuberculin and other foreign proteins. Period for desensitization is more marked and of longer duration after subcutaneous than intradermal injection. It is shortest for SID i.e. 30 days but it is recommended not to retest for 60 days. For Stormont test it is 6 months.

If tuberculin testing is done during this period, no skin reaction will occur. The possible reasons are:

- Suppressor T-cells in response to mycobacterium antigen.
- T-cell tolerance or anergy.
- Specific or non-specific trapping of antigen sensitive T-cells in the lymph nodes.
- Depressed CMI response due to old age, concurrent diseases, malnutrition and /or advances stage of tuberculosis.

Selected Questions

1. Are DTH tests specific and sensitive?

2. What are the causes of false positive tuberculin test?

3. What are the reasons for false negative tuberculin test?

4. What do you mean by Non-Visible lesion reactors (NVL)?

5. What are other laboratory test to confirm tuberculosis in animals?

6. Enlist the diagnostic antigen preparations prepared by the Biological Product division of IVRI, Izatnagar.

Exercise No. 13 **Date:**

Delayed type hypersensitivity test (DTH)

Exercise: Johnin test.

The test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of avian PPD tuberculin or Johnin (avian tuberculin and Johnin are of comparable sensitivity and specificity) into skin. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species.

Cutaneous hypersensitivity test or single intradermal test: The test is carried out by the intradermal inoculation of 0.1 ml of avian PPD tuberculin or johnin into a clipped and shaven site (2x2 inch), usually on middle third of the neck. The skin thickness is measured with calipers before and 72 hours after inoculation. Hot painful, oedematous swelling along with increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. However, sensitization to the *M. avium* complex is widespread in animals, and neither avian tuberculin nor Johnin are highly specific. This test is widely used and is still prescribed for certification testing by some countries.

1. Intravenous test

The intravenous test has been reported to be superior but requires more Johnin than intradermal test. Since the test is expensive and impractical, and improvement in diagnostic accuracy is doubtful, test is seldom used.

- **Intravenous test using avian tuberculin:** This test is performed by injecting 10 ml of PPD (avian tuberculin) intravenously. Rise in temperature by $1^{O}C$ or $2^{O}F$ at 6-8 hours after injection indicates positive reaction. Systemic reaction may also include anorexia, depression, dysponea and erection of hair coat.
- **Intravenous test using Johnin*:*** This test is performed by injecting 2-4 ml of Johnin intravenously. Rise in temperature to $39.5^{O}C$ or $103^{O}F$ within 3-8 hours after injection is considered as positive reaction.

Exercise No. 14 **Date:**

Delayed type hypersensitivity test (DTH)

Exercise: Mallein test.

It is an allergic test similar to tuberculin test is employed for diagnosis of glanders in equines.

Requirement: Mallein PPD, tuberculin syringe, hypodermic needle 26 gauge ½ inch long, 70 % alcohol.

Mallein: Standard strain of *Burkholderia mallei* is grown in synthetic media for 4-6 weeks. The culture is then steamed for one hour. Contents are filtered through muslin cloth and filtrate is evaporated in porcelain dish to half of its original volume on a steam bath. 5% phenol solution is added in the ratio of 1:9 of mallein. The product is then sterilized through Seitz filter and dispensed in rubber capped vials.

Glanders can be diagnosed by one of the following methods.

1. **Intradermopalpebral Test**: Most commonly use test. Clean the eye lids with cotton swab soaked in 70% alcohol or warm water. Inject 0.1 ml of mallein PPD with tuberculin syringe intradermally in to the cutaneous tissue (closely attached to dermis above, the so called tatrsal plate near the rim of orbit) of the lower eye lid. If the injection has been properly made, there will appear a minute circumscribed bleb at the site of injection, after the needle has been withdrawn.

   Interpretation of Reaction: In an infected animal: A series of well marked changes appear in the conjunctiva and subcutaneous tissues. There is watery discharge from the eye which later became muccopurulent. In positive cases the palpebral tissues become swollen all around the orbit resulting in to marked distortion in the out line of the region, that can be readily judged by comparing with the other eye. There is severe conjunctivitis. The pain is manifested in the injected region by frequent blinking of eye lids. The eye lashes become matted together with discharge and usually well observed with in 24 hours post injection. But some times, these changes do not become distinct between 24 to 48 hours. These changes persist for 3-4 days.

   In normal healthy animal: No changes are observed. Mallein in healthy horses do some times produce extensive swelling during the first few hours post injection. The swelling subsides within 2-10 hours after injection.

   This test can be repeated in a few days using opposite eye for re-testing.

2. **Cutaneous Test**:

- **Intradermal Skin Test:** Inject 0.1 ml of mallein I/D in the skin of middle third of neck after site preparation. A positive reaction develops as a delayed oedematous swelling at the site of injection.

- **Subcutaneous Skin Test / Short thermal test:** Animal to be tested should get rest for sometime, so that their rectal temperature become normal. Inject 1.0 ml of diluted mallein PPD subcutaneously in the middle third of neck. Rectal temperature of animal is recorded at 9, 12, 15, 18, 20 and 24 hours post injection. If temperature begins to rise 18 hours afterwards and reaches 104ºF or more with or without inflammatory changes at the site of injection indicates positive reaction. Local reaction is manifested by hard, painful swelling, which tends to increase 24 hours PI and persists for 3 to 4 days.

3. **Ophthalmic Test**: Two to three drops of mallein PPD are instilled in conjunctival sac of one of the eye. A positive animal will develop symptoms similar to intradermopalpebral test 6 to 8 hours post instillation. The changes persist for 2-3 days. This test is not very much reliable, since many external factors can induce conjunctivitis.

Selected question

1. What are the other laboratory procedures that can be used for the diagnosis of glanders?

2. What precautions will you take while collecting samples for laboratory diagnosis of glanders or while making clinical observations among equine populations?

Exercise No. 15 **Date:**

Delayed type hypersensitivity test (DTH)

Exercise: Brucellin Skin test.

An alternative to immunological test is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of LPS) and standardized antigen preparation is used. It is essential to use a standardized, defined brucellin preparation that does not contain LPS antigen, as this may provoke nonspecific inflammatory reactions or interfere with subsequent serological tests. One such preparation is brucellin INRA prepared from a rough strain of *Brucella melitensis* that is commercially available*. .

The brucellin skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals. Also, results of this test may aid the interpretation of serological reactions thought to be false positive serum reactors due to infection with cross-reacting bacteria, especially in brucellosis-free areas. However, not all infected animals react therefore, this test alone cannot be recommended as the sole diagnostic test or for the purposes of international trade.

Procedure: A volume of 0.1 ml of brucellin is injected intradermally into the caudal fold, the skin of the flank, or the side of the neck. The test is read after 48–72 hours. The skin thickness at the injection site is measured with vernier / spring callipers before injection and at re-examination.

Interpretations: A strong positive reaction is easily recognized by local swelling and induration. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction.

Although the brucellin intradermal test is one of the most specific tests for brucellosis in unvaccinated animals, diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd, but should be supported by other reliable serological test.

Desensitization: The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore, an interval of 6 weeks is generally recommended between two tests on the same animal. .

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* Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.

Exercise No. 16 **Date:**

Exercise: Abortus bang ring test (ABR) or Milk Ring Test

The milk ring (MR) test is a simple diagnostic test for use on bulk milk supplies. It provides a very useful screening method for detecting herds of dairy cattle infected with brucellosis. It is equally useful in surveys, eradication or in surveillance. Originally, the test was designed to be used on milk collected in 50-litre cans, but it can also be used effectively on much larger volumes of mixed milk such as tanker collections. The MR test can be integrated with quality control tests carried out on milk received at collecting centers or dairies. For brucellosis surveillance it is recommended that the milk from each herd be tested by the MR test every three months.

Principles: The test consists of adding a drop of haematoxylin-stained antigen to 1.0 ml of milk in a narrow tube, mixing and allowing the milk to stand until the cream has risen to the surface. In a positive sample the antigen is agglutinated and adheres to the cream globules and rises to the surface with them, thus colouring the cream layer blue. The column of skim milk below the cream regains, partially or completely, its original white colour. In a negative sample the antigen remains dispersed in the skim milk column colouring which is blue and the cream layer is white.

Requirements: *Antigen*: The antigen is a suspension of heat-killed *B. abortus* cells, stained with haematoxylin and adjusted to a cell concentration of 4 per cent. The pH is adjusted to between 4.0 and 4.3.

Dropper: A dropper delivering 0.03 ml of antigen is often provided with the antigen. If necessary a micro pipette delivering 0.03 ml can be used.

Before the sample is taken, the milk contained in cans or holding tanks must be thoroughly mixed to ensure that the cream is evenly dispersed. About 5 to 10 ml of milk samples are collected in a small test tubes or screw capped vials. If milk samples are not to be tested on the same day, then 0.5 ml of formalin is added as a preservative, It prepared by adding 7.5 ml of 37 % formalin to 1 liter of distilled water.

Procedure

1. Arrange a series of small tubes of at least 2 ml capacity.
2. Mix the milk sample to ensure even distribution of the cream.
3. Add 1 ml of each sample to succeeding tubes.
4. Add one drop (0.03 ml) of antigen to each tube.
5. Thoroughly mix the antigen and the milk by inverting the tube several times.
6. After mixing the antigen and milk in the last tube, inspect all the tubes and ensure that thorough mixing of antigen and milk has been achieved.
7. Incubate the tubes in water bath at 37°C for 1 hour.

Results: If the blue colour in the cream layer is more intense than in the lower (skim milk) column, the test is positive. Various degrees of positivity may be awarded. If the

intensity of colour in the cream layer is equal to or less than that in skim milk, the test is considered negative.

Interpretation of the result: A positive result is evidence that one or more animals contributing to the milk sample is infected with brucellosis. There are two principal causes of false positive results.

- Presence of cases of mastitis.
- When cows are in drying of period.

Such problems occurs, when sample is derived from small number of animals.

Adjusting the milk ring test for herd size: When milk sample is collected from bulk tankers; it may be necessary to adjust the test to compensate for the dilution factor. ABR / MR test on milk from bulk tankers is adjusted according to herd size over 2 years of age by adding 30 µl of antigen to below mentioned quantities of milk:

No. of animals contributing milk	Volume of milk for ABR
< 200	1 ml
201-500	2 ml
501-900	3 ml

Limitations:

- The test is not effective on pasteurized milk.
- Not suitable for homogenized milk
- The most reliable and reproducible results are obtained when milk is tested after cool storage for 48-72 hours.
- The test is designed for use on bulk, i.e. diluted milk. Milk from individual animals needs to be diluted in negative milk before it can be tested by the MR test; however, serological tests are generally more suitable for testing individual cows.
- The standard test is effective on bulk milk from herds containing up to 200 cows.

Observations and results:

Exercise No. 17 **Date:**

Exercise: Rose Bengal Plate Test (RBPT).

The Rose Bengal plate test (RBPT) is a simple test that is effective in the diagnosis of bovine brucellosis when used as a screen test. For diagnosis in individual cattle it may be somewhat oversensitive, especially in those vaccinated with strain 19 vaccine. Cattle negative to the RBPT may be considered to be brucellosis-free but it is advised that sera sample positive to the RBPT should be retested by a definitive test, usually the complement fixation test (CFT).

The RBPT may be used for testing sheep and goats in the same way as cattle but is less proven in these species. For testing pigs for *Brucella suis* infection the RB test may be used as a definitive test.

Principles: The RBPT is a rapid agglutination test in which a drop of serum and a drop of antigen are mixed together and observed for agglutination. RBPT detects all the three main anti-brucella immunoglobulin isotypes (IgM, IgG_1 and IgG_2) normally searched for in ruminant serum.

The antigen deteriorates rather rapidly at room temperature and because of this; its transport may present problems. The test is also affected by the temperature at which it is carried out. For these reasons and for more accurate and reproducible results the RBPT should be used as a laboratory rather than a field test.

Requirements: Micropipette or dropping pipette (30µl drop size), tooth pricks,

Plates: It is recommended that the test be done in WHO haem-agglutination (HA) plates, or on a flat glass plate or microscopic slides or ceramic tile divided into 15 mm squares. In addition, a variety of plates, usually of plastic material, some with shallow cavities, can be used.

Antigen: This is a killed suspension of smooth *B. abortus* cells stained by Rose Bengal and adjusted to an 8 % cell concentration by the packed cell volume method and buffered to a pH of 3.6. Antigen should be stored at about 4°C and should never be frozen.

Reading apparatus: A white translucent surface, plastic or glass, illuminated from below such as X-ray viewer.

Control serum: A control serum that gives a minimum positive reaction should be tested before each day's tests are begun. This serum should be stored frozen in small aliquots and brought to room temperature before use.

Procedure:

1. Bring the serum samples and antigen to room temperature.
2. Place one drop of serum samples on to successive squares on the test plate (30 µl for flat plates, 25 µl for HA plates).
3. When all the serum samples have been dispensed, Place one drop of antigen (30

µl / 25 µl) over each drop of serum sample.

4. Mix the contents of each reactant thoroughly with different tooth pricks. Tilt the slide in all directions for 4 minutes.
5. Record the results immediately over translucent illuminator or x-ray viewer as.

Agglutination – Serum sample is positive for brucella antibodies.

No agglutination - Negative reaction, similar to known negative serum sample.

More detailed evaluation:

0== No agglutination, no rimming, uniform pink colour

1 = barely perceptible agglutination and/or some rimming

2 = fine agglutination, definite rimming, some clearing

3 = coarse clumping, definite clearing

Interpretation of the results: This will depend on whether the test is being used as a screen test (cattle), as a definitive test (pigs), or as a presumptive test in other species.

As a screen test: Any degree of positivity, i.e. a 1+ reaction or greater should be retested by the definitive (CF) test

As a definitive test: Definite agglutination indicates a positive result.

In the HA plate method; a 1+ reaction depending on the presence of a rim without detectable agglutination should be regarded as suspicious/doubtful.

Observations and results

Exercise No. 18 **Date:**

Exercise: Serum tube agglutination test (SAT) for diagnosis of bovine brucellosis.

Serum tube agglutination test (SAT) has been used with success for many years in surveillance and control programmes for bovine brucellosis. This test detects IgM and IgG_2 but fail to detect IgG_1. This test has not been recognized as a prescribed or alternative test for diagnosis of brucellosis. The animals positive for this test should be re-examined by CFT or ELISA tests prior to their culling.

Requirements:

- *Brucella abortus* plain antigen: It is a suspension of a pure, smooth culture of *Brucella abortus* strain 99 or *Br. melitensis* in phenol saline.
- *Brucella abortus* known positive serum
- Test sera sample
- Phenol Saline Solution (NSS containing 0.5% phenol).
- 1 ml serological pipette with 0.01 graduation
- Glass test tubes (110 x10 mm)

Procedure:

Arrange 10 serological tubes in a test tube stand.

Tubes No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Saline (ml.)	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
↑	↑									
Serum (ml)	0.2→	0.5→	0.5→	0.5→	0.5→	0.5→	0.5→	0.5→	0.5→	0.5 ↓

Mix contents in tube No.1 and transfer 0.5 ml to tube 2. Continue till tube 9 & discard 0.5 ml from tube No.10

Antigen (ml) in each tube.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Final serum dilution	**1:10**	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**	**1:640**	**1:12801:2560**		**1:5120**

Mix thoroughly by rolling between the palms and incubate in water bath at 37°C 20 hrs.± 1 hour. Read the results. The last tube showing 50% agglutination will give the end titer.

Antigen Control tubes:

For each days work, prepare a set of control tubes for comparing the results of test samples.

Antigen Control tubes	0.5% phenol saline	Antigen	Degree of agglutination
Tube I	Nil	2.00 ml	No agglutination
Tube II	1.25 ml	0.75 ml	25% agglutination
Tube III	1.50 ml	0.50 ml	50% agglutination
Tube IV	1.75 ml	0.25 ml	75% agglutination
Tube V	2.00 ml	nil	100% agglutination

Incubate the antigens control tubes at 37°C 20 hrs.± 1 along with the test samples. Results of agglutination should be noted after keeping the tubes for an hour or two at the room temperature.

Examine all the tubes and compare with the antigen control tubes. Note the degree agglutination for each sample of serum as under:

The degree of agglutination is to be judged by opacity of the supernatant fluid."

+ + + +	Comparable with tube V of the antigen Control series
+++	Comparable with tube IV of the Antigen Control series
++	Comparable with tube III of the Antigen Control series
+	Comparable with tube II of the Antigen Control series
No agglutination	Comparable with tube I of the Antigen Control series.

50% agglutination(++) should be considered as end point.

Titer of serum sample: The highest dilution of sera sample showing 50% agglutination is considered as its titer.

Interpretation of results:

- A titer of 1:40 (80 IU) or above should be considered positive for brucellosis in cattle, buffaloes and humans.
- A titer of 1:20 (40 IU) or above should be considered positive for sheep goats and breeding bulls. But to be considered doubtful in cattle, buffaloes and humans.
- A titer of 1:10 (20 IU) or more to be considered doubtful in sheep, goats and breeding bulls.

Observations and results

Sera sample √	Cow	Breeding bull	Buffaloe	Sheep	Goat	Human

Selected questions

1. What is agglutination test? Enlist their types & applications in diagnostic laboratory.

2. What is prozone phenomenon? How it can be prevented in sheep and goat sera to be tested by SAT?

3. Why SAT gives false positive results in bovines?

4. Which surveillance test is best for brucellosis and why?

5. Can be use tetrazolium to stain *B. abortus* organism for ABR test?

Exercise No. 19 **Date:**

Exercise： Strategies for control and prevention of diseases in animals.

Appendix

Brief Bio-data of student (optional)