APPENDICES

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Appendix I

TECHNIQUES OF POST-MORTEM EXAMINATION (NECROPSY)

Necropsy is examination of animal after death. It helps in diagnosis of diseases and their control. It is said that “Necropsy is a message of wisdom from dead to living”. Necropsy include systemic examination of dead animal, recording of pathological lesions, their interpretation to make diagnosis of disease. Sometimes it is difficult to arrive any conclusion merely based on gross examination of dead animal. Then one should seek the help of laboratory examinations such as Histopathology, Microbiology, Immunology and Toxicology for confirmation.

Necropsy examination is an integral part of disease investigation. Therefore, veterinarian must have the knowledge of the techniques of post-mortem examination, recording of lesions, collection of proper material for laboratory and most importantly their correlation to arrive at conclusive diagnosis. The technique of post-mortem examination is as under:

**POST-MORTEM EXAMINATION OF LARGE ANIMAL**

- Place animal on left side (Ruminants) (Fig. 22.1).
- Place horse on right side and dog on vertebral column (Fig. 22.2 & 22.3).
- Make midventral incision with knife from chin to anus.
- Surround the prepuce, scrotum/mammary gland.
- Remove skin dorsoventrally. Remove skin at face, neck, thorax and abdomen.
- Cut the muscles and fascia in between scapula and body; remove fore legs.
- Raise hind legs, cut the coxofemoral ligament.
- Examine s/c tissue, muscles, superficial lymphnodes- prescapular, prefemoral supramammary, etc.
- Open abdominal cavity by cutting muscles and peritoneum.

Fig. 22.1. Diagram showing post-mortem examination of ruminant (A) position of cow and the marking for incision (B) after removal of skin and (C) after exposure of abdominal cavity.
• Open thoracic cavity by cutting xiphoid cartilage at sternum; lift ribs and press them to break at joints with vertebral column.

• Examine the visceral organs in both cavities:
  Thorax : Heart, Lungs, Trachea, Oesophagus, Mediastinal lymphnodes, Diaphragm
  Abdominal cavity :
  Ruminants : Rumen, Reticulum, Omasum, Abomasum
  Other animals : Stomach
  In all animals : Liver, Pancreas, Intestines, Mesenteric lymphnodes, Spleen, Kidneys, Ureter
  Pelvic cavity : Urinary bladder, uterus

**POST-MORTEM EXAMINATION**
(PoulTRY, Fig. 22.4 to 22.21)

• Dip the dead bird in antiseptic solution or in water; to avoid feather contamination.
• Keep the bird on post-mortem table at vertebral column and look for any lesion or parasite on skin.
• Examine the eyes, face and vent.
• Remove skin through a cut with knife and with the help of fingers. Expose thymus, trachea, esophagus in neck.
• Break the coxofemoral joint by lifting the legs. Examine the chest and thigh muscles.
• Cut on lateral side of chest muscles. Lift the chest muscle dorsally and break bones at joints with thorax. Cut bones at both sides and remove muscles, bones to expose thorax, abdomen.
• Examine different organs.
• Cut proventriculus and pull the organs of digestive tract out. Separate liver, spleen, intestines, caecum, proventriculus, gizzard, etc.
• Expose bursa just beneath the cloaca.
• Cut beak at joint, examine mouth cavity and expose esophagus and trachea.
• Remove skin of head and make a square cut on skull to expose brain.
• Take a forceps and place in between thigh muscles, remove fascia and expose the sciatic nerve.

• Separate each organ, examine them for the presence of lesion.

Fig. 22.2. Diagram showing post-mortem examination of horse (A) position of horse and marking for incision (B) after removal of skin and (C) after exposure of abdominal cavity
Fig. 22.3. Diagram showing post-mortem examination of dog (A) position of dog and marking for incision (B) after exposure of thoracic and abdominal cavity

Fig. 22.4. Photograph showing Position of bird on post mortem table

Fig. 22.5. Photograph showing external examination for presence of lice, mites & ticks

Fig. 22.6. Photograph showing external examination of eyes

Fig. 22.7. Photograph showing examination of vent
Fig. 22.8. Photograph showing removal of skin

Fig. 22.9. Photograph showing breaking of coxofemoral joint

Fig. 22.10. Photograph showing exposure of muscles for examination

Fig. 22.11. Photograph showing removal of breast muscles

Fig. 22.12. Photograph showing cutting of neck bones

Fig. 22.13. Photograph showing exposure of internal organs

Fig. 22.14. Photograph showing kidneys, ovary, oviduct after removal of digestive system and heart
Fig. 22.15. Photograph showing examination of mouth cavity

Fig. 22.16. Photograph showing examination of intestines including caeca and proventriculus

Fig. 22.17. Photograph showing examination of trachea, bronchi and lungs.

Fig. 22.18. Photograph showing examination of female genital tract.

Fig. 22.19. Photograph showing examination of testes

Fig. 22.20. Photograph showing examination of nervous system (A) brain  (B) sciatic nerve

Fig. 22.21. Photograph showing (A) Heart, (B) Spleen (C) Bursa of Fabricious and (D) Thymus
Appendix II

STEPS IN POST-MORTEM EXAMINATION

Post-mortem examination should be conducted only after receiving a formal request from the owner of animal having details of anamnesis and date and time of death. Without formal written request, one should not do post-mortem examination of animal. The post-mortem record includes the aspects of animal identification, illness, therapeutic and preventive measures adopted and date and time of death. This information provided by the owner or person requesting post-mortem, which helps in post-mortem examination and recording of lesions to make a conclusive diagnosis.

Various steps in post-mortem examination are as under:

1. External examination

Animal should be examined externally before opening the body for the presence of lesions on body surface. Eyes, ear, anus, vulva, mouth, nares etc. should be specifically examined for the presence of blood and any other lesion. If the blood is coming out from natural orifices, it should be examined for the presence of anthrax bacilli and such carcasses must not be opened for post-mortem examination. Following points should be taken into consideration while conducting external examination.

- Trauma, wound, fracture, cuts, etc.
- Fungal infection e.g. ringworm
- Parasitic infestation e.g. mange, lice, ticks
- Side of animal is lying down on earth.
- Discharges from openings.
- Burn, ulcers, erosions etc.

2. Subcutaneous tissue and musculature

Examine the subcutaneous tissue and musculature after removal of skin for the presence of lesions such as:

- Congestion, haemorrhage, oedema, nodule, anemia, icterus.
- Fat deposits
- Necrosis on muscles, hardening, calcification.

3. Abdominal and thoracic cavity

Just after opening the carcass, one should observe the presence of any lesion in abdominal and thoracic cavity and following points must be kept in mind.

- Accumulation of fluid (serus, serosanguinous, blood, pus etc.)
- Fibrinous or fibrous adhesions.
- Parasites
- Abscess, tumor etc.

4. Respiratory system

Organ/tissues to be examined

External nares, nasal passage, larynx, trachea, bronchi, lungs, air sacs (poultry) mediastinal lymphnodes.

Lesions to be observed

- Discharge from external nares.
- Growth (granuloma/polyp) in nasal passage if there is blood mixed nasal discharge.
- Trachea and Bronchi- Congestion, haemorrhage, presence of caseous exudate, frothy exudate etc.
- Lungs- Congestion, consolidation, nodules, presence of exudate on cut surfaces, oedema, atelectasis, emphysema, haemorrhage, necrosis.
- Mediastinal lymphnodes- Oedema, hardening, calcification, congestion, haemorrhage.

5. Cardiovascular system

Organ/tissues to be examined

Heart, aorta, arteries, veins and lymphatics

Lesions to be observed

- Fluid, blood, pus etc. in pericardial sac
- Adhesions, fibrin, fibrosis
- Congestion, haemorrhage, necrotic foci
- Hardening of blood vessel, obstruction, thrombi
- Presence of parasites
- Post-mortem clot/thrombi.
6. Digestive system
**Organs/tissue to be examined**
Mouth cavity, esophagus, crop, proventriculus, gizzard (poultry), rumen reticulum, omasum, abomasum (ruminants), stomach, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), cloaca, vent (poultry), anus, liver, pancreas, gall bladder, mesenteric lymphnodes etc.

**Lesions to be observed**
- Erosions, ulcers, vesicles
- Congestion, haemorrhage, oedema
- Necrosis
- Icterus
- Abscess/pus
- Perforation, needles or hard objects in reticulum.
- Intussusception, torsion, volvulus
- Parasites
- Atrophy, hardening, nodules
- Contents, catarrhal, blood mixed, digested/undigested feed material, thickening of wall of intestines.
- Cut surface of liver for parasites, lesions in bile duct.

7. Cardiovascular system
**Organs/tissue to be examined**
- Kidneys, ureter, urinary bladder, urethra

**Lesions to be observed**
- Congestion, haemorrhage, infarction, oedema.
- Necrosis, hardening, nodules
- Deposition of salts, calculi
- Obstruction

8. Genital system
**Organs/tissue (female)**
- Ovaries, oviduct, uterus, cervix, vagina

**Male**
- Testicles, Epididymis, penis, prepuce

**Lesions to be observed**
- Cysts in ovary
- Congestion, haemorrhage, oedema
- Foetus in uterus, pus, fluid
- Necrosis, overgrowth, nodules
- Atrophy, adhesions, granularity

9. Immune system
**Organs/tissue to be examined**
- Spleen, lymphnodes, bursa and thymus (poultry), bone marrow
- Peyer’s patches, GALT, RALT

**Lesions to be observed**
- Size, shape, atrophy, hardening.
- Oedema, congestion, haemorrhage

10. Nervous system
**Organs/tissue to be examined**
- Brain, spinal cord, nerves, meninges

**Lesions to be observed**
- Congestion, haemorrhage, hematoma
- Oedema, swelling
- Abscess
- Hypoplasia

11. Miscellaneous observation
- Adhesions in pleural/peritoneal cavity
- Any other left over information pertinent to post-mortem examination/diagnosis

12. Post-mortem diagnosis
- Diagnosis should be made on the basis of above findings which involve any system or organ. The most involved organ based diagnosis should be written with suggestion of etiological factors or etiology based diagnosis.
Appendix III

WRITING OF POST-MORTEM REPORT

Post-mortem report consists of two parts post-mortem record and post-mortem examination as given in the format on next page. The first part i.e. post-mortem record is having information related to animal and is supplied by the owner or person requesting post-mortem examination. Actually, it is a part of request form of the case for post-mortem examination. This is necessary for the identification of animal. It should be filled in before conducting post-mortem examination. The proper record will be helpful in establishing accurate diagnosis based on post-mortem examination.

POST-MORTEM RECORD

1. **Species:** Here one should write the species of animal such as bovine, porcine, equine, poultry, etc.

2. **Date:** Date of the post-mortem examination.

3. **Case no.:** The serial number of your post-mortem book. It shows cumulatively how many animals are examined by you in necropsy.

4. **Breed:** Mention the breed of animal, if known or supplied in the request form, such as Murrah buffalo, Jersey cattle, etc.

5. **Age/Born:** Age of animal or its date of birth. In case the exact age is not known then mention young, adult or chick, grower, adult in case of poultry.

6. **Sex:** Sex of animal (male or female).

7. **Identification number/mark:** It must be filled with utmost care; the number (tattoo number or brand number) should be the same as on animal. If the identification number is not available/illegible then write the characteristic mark of animal.

8. **Owner:** Here, the name of owner with complete address must be filled clearly. The address should be complete enough so that the report can reach the owner through post also.

9. **Referred by:** In this column, the name of Veterinary Officer/any other officer who referred the case for post-mortem examination should be written. Sometimes owner himself/herself is interested in post-mortem examination of animal; in such case the name of owner should be written.

10. **History of the case:** This includes the clinical illness of animal, duration of illness, epidemiological data, tentative diagnosis, therapeutic and preventive measures adopted. This is very important and information of this column has an important role in making the diagnosis.

11. **Reported date and time of death:** It should have the exact date and time of death of animal. Sometimes, it is difficult to note the exact time then one can write morning, noon, evening, midnight etc. to approximate the timings of death of animal. In some large farms, it is very difficult to record information with regard to each individual animal/bird so here one can write “previous night” as time of death.

12. **Date and time of post-mortem examination:** Pathologist conducting post-mortem examination should write here the exact time and date of the post-mortem examination.

The above information is very important to arrive any conclusive diagnosis. The correct information enhances the specificity of post-mortem diagnosis. Some points might be looking like insignificant but one should not overlook them and write as correct as information he/she can gather from the owner’s request letter/form.
POST-MORTEM REPORT

POST-MORTEM RECORD

1. Species:  
2. Date:  
3. Case No.:  

4. Breed:  
5. Age/Born:  
6. Sex:  

7. Identification No.:  
8. Owner with address:  
9. Referred by:  
10. History of the case:  
11. Reported date & Time of Death:  

12. Date and Time of post-mortem examination:

POST-MORTEM EXAMINATION

1. External appearance  
2. Subcutaneous tissue and musculature  
3. General observations after opening the carcass  
4. Respiratory system  
5. Cardiovascular system  
6. Digestive system  
7. Urinary system  
8. Genital system  
9. Immune system  
10. Nervous system  
11. Miscellaneous observations  
12. Post-mortem diagnosis

Signature of officer conducting post-mortem

Date:
Place:
POST-MORTEM EXAMINATION

It includes the observations made by the pathologist conducting post-mortem examination. This part of report should be filled in as soon as possible after the post-mortem examination. It is advisable that one should record some points on a small paper or diary during post-mortem examination and fill them in report after the conduct of post-mortem examination.

1. **External appearance**: Record the lesions observed in intact animal before its opening. One should place on record the side of animal lying down, lesions on skin, external parasites, trauma etc.

2. **Subcutaneous tissue and musculature**: The observations made after removal of skin, on subcutaneous tissue and muscle should be included in this column.

3. **General observations after opening the carcass**: It contains the general information or lesions present in abdominal and thoracic cavity such as accumulation of fluid, pus, blood, clot of blood, post-mortem changes such as pseudomelanosis, etc.

4. **Respiratory system**: Record the lesions observed in respiratory system right from external nares, nasal passage, trachea, bronchi and lungs along with mediastinal lymphnodes.

5. **Cardiovascular system**: Record the lesions present in heart, aorta, arteries, veins and lymphatics.

6. **Digestive system**: Record the lesions observed in digestive tract from month cavity, esophagus, crop, proventriculus, gizzard (poultry), rumen, reticulum, omasum abomasum (ruminants), stomach, intestines, rectum, anus, cloaca, vent (poultry), liver, pancreas, gall bladder etc.

7. **Urinary system**: Place on record the lesions present on kidneys, ureter and urethra.

8. **Genital system**: Record the lesions present in ovaries, uterus, oviduct, cervix and vagina in females and testes, penis etc. in males. Be careful in recording lesions in this column as it should match with the sex of animal written in post-mortem record section.

9. **Immune system**: Record the lesions present in spleen, bursa, thymus, lymphnodes, respiratory associated lymphoid tissue (RALT), gut associated lymphoid tissue (GALT) etc. Careful recording of lesions in these organs will be helpful in diagnosis.

10. **Nervous system**: Place on record the lesions present in brain, spinal cord and nerves. Most of the pathologists overlook this system and often not taken pain to examine the brain. It should not be done and every effort should be made to examine and place on record the lesions present in this system.

11. **Miscellaneous observations**: Here one can record any missing observation which has not been covered above.

12. **Post-mortem diagnosis**: This is very important. Based on the history and lesions present in different systems, pathologists by using his experience and conscience conclude the diagnosis. He/she may also write suggestions along with diagnosis or some points to suggest the diagnosis and/or contain the disease in other animals.

13. **Signature of officer conducting post-mortem**: Each and every report must be signed by the officer doing post-mortem examination. Without signature of competent officer, it has no validity.

14. **Place and date**: The person signing the post-mortem report must also write date and place of post-mortem examination.
Appendix IV

COLLECTION, PRESERVATION AND DISPATCH OF SPECIMENS FOR LABORATORY DIAGNOSIS

Tissue samples are collected from dead or live animals for laboratory examination to confirm the tentative diagnosis.

**Purpose**
- Diagnosis of disease or for identification of new disease.
- Confirmation of tentative diagnosis.
- Prognosis
- To observe the effect of treatment and give directions for future therapy.

**Precautions**
- Collect the tissues as early as possible after death of animal.
- Representative tissue/sample should be collected.
- Sharp knife should be used for cutting
- Collect the tissues directly in fixative.
- Size of tissue should not be more than 1 cm for histopathology in 10% formalin.
- Hollow organs should be taken on paper to avoid shrinkage.
- Hard organs like liver, kidneys etc. should be collected along with capsule.

**COLLECTION OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION**
- Collect the tissues under sterile condition.
- Sterilize knife/ scalpel/ spatula on flame or in boiling water.
- Surface sterilized by hot spatula
- Cut with knife and collect sample from inner tissue.
- Body fluids/blood should be collected in sterilized syringe or in Pasteur pipette.
- Specimens should be collected directly in media (liquid media-nutrient broth, peptone water, tetrathionate broth or even in normal saline solution/phosphate buffer saline).
- Seal, pack and transport the collected material to laboratory in ice/under refrigeration conditions.

**BACTERIAL DISEASES**

**Abscesses**
- Swab in sterile conditions/pus in vials
- Collect material from margin of abscess

**Actinobacillosis/ Actinomycosis**
- Tissues from affected parts in 10% formalin.
- Pus in sterile test tube/from edge of lesion
- Slides from Pus for sulphur granules.

**Anthrax**
- Blood smear from tip of the ear
- Blood for cultural examination
- Muzzle piece for biological test.
- Mark the specimen as “Anthrax suspect”

**Black Quarter/Black leg**
- Smear from swelling
- Affected muscle piece in ice.

**Brucellosis**
- Serum after 3 weeks of abortion
- Foetal stomach tied off
- Swabs from uterine discharge
- 5 to 10 ml milk in ice

**Glanders**
- Smear from discharge
• Lung, liver and spleen in 10% formalin
• Serum

**Johne’s disease**
• Bowel washings in sterile bottle
• Smear from rectal mucosa
• Mesenteric lymphnode in 10% formol saline

**Leptospirosis**
• Serum 21 days after abortion
• Milk/urine in vials (1 drop of formalin in 20 ml)
• Liver, kidney tissue in 10% formalin

**Listeriosis**
• Half brain in ice
• Half brain in 10% formalin

**Mastitis**
• 10 ml milk in sterile vial in ice

**Pasteurellosis**
• Heart blood
• Lung, spleen and mediastinal lymphnodes in ice.
• Affected tissues in 10% formalin.

**Salmonellosis**
• Liver, spleen, kidney and intestine tied off in ice.

**Strangles**
• Smear, swab of pus in ice.

**Erysipelas**
• Blood
• Spleen, kidney, liver in ice.

**Vibriosis/Campylobacteriosis**
• Foetal stomach tied off
• Vaginal mucosa in ice.

• In pig, intestine and liver in 10% formalin.

**Colibacillosis**
• Heart blood in sterile vial.
• Tissues from intestine and lymphnodes in 10% formol saline.

**Tuberculosis**
• Lungs, mediastinal and bronchial lymphnodes in ice and in 10% formalin.

**COLLECTION OF SPECIMENS FOR VIROLOGICAL EXAMINATION**
• Collect tissue under sterilized condition
• Body fluids/ blood in sterilized syringe or in Pasteur pipette
• Tissues in buffered glycerin
  • PBS pH 7.2-50%
  • Glycerin- 50%
• Avoid samples in glycerin from sensitive viruses *e.g.* Rinderpest, canine distemper
• Seal and mark the specimen bottle and transport to laboratory.

**VIRAL DISEASES**

**Foot and mouth disease**
• Tongue epithelium, vesicular fluid, saliva, pancreas in 50% buffered glycerine
• Serum

**Hog cholera/ swine fever**
• Serum under refrigeration
• Spleen, liver, kidney in 50% glycerin/ice
• Tissues from intestine, mesenteric lymphnode and half of the brain stem in 10% formol saline.

**Infectious Canine Hepatitis**
• Several pieces of liver, gall bladder and kidney in 10% formol saline.

**Pox**
• Scabs in ice and in 10% formol saline.
Rabies
- Intact head should be soaked in 1% carbolic acid.
- Fracture the skull with hammer.
- Remove skin and bones
- Half brain in 10% formalin
- Half brain in 50% neutral glycerin.
- Tissues from cerebellum and hippocampus in Zenkers fluid for 20 hrs, wash in tap water for 24 hr and keep in 80% ethyl alcohol for Negribodies.

Ranikhet disease
- Liver, spleen in 50% neutral glycerin
- Proventriculus in 10% formalin
- Brain in ice.

Rotaviral enteritis
- Faecal sample
- Intestinal tissue in 10% formol saline.

Gumboro disease
- Bursa of Fabricious, kidney, muscles in 10% formol saline.
- Bursa, kidney in 50% buffered glycerine.

SYSTEMIC DISEASES
Diarrhoea/Enteritis
- Faecal sample in sterile vial
- Serum
- Tissues of intestine, mesenteric lymphnodes in 10% formol saline.

Abortion/Metritis
- Faetal stomach content tide off or in sterile vials.
- Serum of dam after 21 days of abortion.
- Vaginal discharges in sterile conditions.
- Tissues of placenta, foetal liver, stomach, kidney in 10% formol saline.

Pneumonia
- Nasal discharge/nasal swabs.
- Lung tissue/pieces in sterile vials.
- Lung tissue and mediastinal lymphnode in 10% formol saline.

Dermatitis
- Skin scrapings in 10% KOH.
- Skin tissue in 10% formol saline.

Encephalitis
- Cerebrospinal fluid in heparinised vials.
- Brain tissue in 10% formol saline.
- Brain tissue in 50% glycerol.

Nephritis
- Urine sample in sterile vial.
- Kidney tissue in 10% formol saline.

COLLECTION OF SPECIMENS FOR TOXICOLOGICAL EXAMINATION
- Stomach/intestinal contents
- Liver, kidneys, heart blood
- Urine
- In clean glass jars
- In ice/refrigeration without any preservative
- Seal, label, transport to laboratory.
- In veterolegal cases all specimens must be collected in presence of police.
- Type of poison suspected along with detailed history, signs, lesions/treatment etc. should be written on letter with specimens.

TOXICOSIS/POISONING
Heavy metal Poisoning
- Hg, Pb, Bi, Ag
- Liver, kidney, stomach content in ice in separate containers.

Alkaloids
- Liver, stomach contents and brain tissue in ice.
Nitrate
- Fodder
- Stomach contents, blood in ice

Strychnine poisoning
- Stomach contents, intestinal contents, urine, liver, kidney in ice.

Hydrocyanic acid
- Plants
- Stomach contents, blood, liver
- Preserved in 1% solution of mercuric chloride.

Pesticides
- Fatty tissue, liver, stomach contents, blood in ice.
- Subcutaneous, omental, mesenteric fat.

COLLECTION OF SPECIMENS FOR IMMUNOLOGICAL EXAMINATION
- Heart blood in syringe/ Pasteur pipette
- CSF/Synovial fluid /peritonial fluid
- Tissues in formol sublimate or in buffered formalin
- Blood/serum/others should be sent to laboratory under refrigeration conditions.

- Add one drop of 1:10000 merthiolate in 5 ml serum as preservative.

DISPATCH OF MATERIAL
Following points must be kept in mind while dispatching the material to laboratory for diagnosis.
1. Describe the clinical signs, lesions, tentative diagnosis and treatment given to animal in your letter. Also mention the type of test you want with your tentative diagnosis.
2. Write correct address on letter as well as on the parcel preferably with pin code, if the material is sent through post.
3. Mark the parcel ‘Biological Material’, ‘Handle with care’, ‘Glass material’, ‘Fragile’ etc. in order to avoid damage in parcel. Also mark the side to be kept on upper side with arrows.
4. Seal the container so that it should not leak in transit.
5. Try to send the material as soon as after its collection from animal.
6. Keep one copy of cover letter inside the parcel and send another copy by hand or post in a separate cover.
7. Keep adequate material like thermocol etc. in the parcel which will save the material from outside pressures/jerks.
8. Use dry ice, if available otherwise use ice in sealed containers.
Histopathology is the branch of pathology which concerns with the demonstration of minute structural alterations in tissues as a result of disease. Most of histopathological techniques simulating to those of applied for study the normal histological structures. For the demonstration of minute histological changes, the tissue must be processed in such a manner that it will provide maximum information. The histopathological diagnosis is an overlooked area specially in Veterinary Sciences. Many times it has been observed that the procedures are not properly followed or the qualified person trained for histopathology is not available, which in turn affects the interpretation and/or diagnosis. Histopathological procedures are described for the benefit of readers which will help them in diagnostic laboratory.

Scope
Though the histopathological techniques are labour intensive, cumbersome and time consuming, particularly when there are automation equipments are not available; however, their use in diagnosis of diseases is unequivocal. Some of the areas where histopathological diagnosis is helpful are described as follows:

- This is useful in establishing the pathogenesis and pathology of any disease caused by bacteria, virus, chlamydia, rickettsia, mycoplasma, parasite, toxin, poisons etc.
- There are certain diseases in which histopathological examination of tissues is the only alternative to diagnose the disease. e.g. Bovine spongiform encephalopathy. The agent of this disease takes a very long incubation period and very difficult to isolate and there is no immune response and inflammation in animal. Therefore, histopathology remains the only alternative for confirmatory diagnosis.
- In some cases, the tissues from dead animals are only available material for laboratory diagnosis. This may occur either due to lack of time or due to negligence for not collecting the material for serological tests or isolation studies. Sometimes the transportation of material from remote areas destroys the other material and the tissues fixed in formalin only remains for making diagnosis. In all such cases the histopathological examination has its pivotal role.
- The histopathological procedures produce permanent slides, which can be stored for a longer period and one cannot manipulate the findings; therefore, it is considered best reliable technique.
- The histopathological techniques are useful in carrying out the retrospective studies. The unstained slides and blocks can be stored for indefinite period; which can be examined even after many years for further studies.
- The presence of causative agents can also be demonstrated in tissue sections using routine histopathological techniques or special stainings. In this Gram’s staining procedures are used for demonstration of bacteria while viral inclusions are demonstrated using hematoxylin and eoxin or other staining techniques like Macchiavello’s stain or Mann’s methylene blue eosin method. The Negri bodies are demonstrated by Seller’s stain in case of rabies in animals. In such cases, the isolation of causative agent or their serological examination does not require; since the presence of causal agent in infected tissues gives a confirmatory diagnosis.
- The detection of chemicals in tissues like enzymes, lipids etc. is included in histochemical examination; which not only describe the structural changes but also gives idea about the functional status of the organ.

Histopathological procedures
The microscopic examination of tissues or organs can be achieved by their smears or using vital
staining or by sectioning; the latter method being more commonly used in histopathological laboratories.

**Smears**

The microscopic examination using smears of any organ/tissue/cells is very rapid method which gives the results within hrs. A drop of blood is placed on clean glass slide and with the help of another slide, the smear is prepared (Fig. 22.22). In this the tissue pieces from organs are cut using a sharp knife and the cut surface is mildly touched with clean glass slides with some gentle pressure. Which gives an impression on the slide (Fig. 22.23 & 22.24). This is also known as impression smear; generally 2-5 smears are prepared on a slide. If the collected tissue material is too less then it is being pressed between two slides and the impression thus obtained on both the slides are used for study. The wet smears are fixed with methanol and can be stored or transported to laboratory for examination.

The impression smears of hippocampus, cerebellum and cerebrum of brain are very useful for demonstration of Negri bodies in rabid animals for diagnosis of rabies. The impression smears are stained with seller’s stain for few seconds, washed and, air dried and examined under oil immersion microscope for the presence of inclusion bodies also known as Negri bodies. These inclusions are characterized by intracytoplasmic, eosinophilic appearance with basophilic granules and round to oval in shape with a clear halo.

In case of pox infection in animals, the impression smears are prepared from scab or pustule for demonstration of intracytoplasmic inclusions. Sometimes the viral inoculum is inoculated on chorioallantoic membrane (CAM) of embryonated eggs; the impression smears of CAM may yield the viral inclusions. In certain bacterial diseases like haemorrhagic septiemia and enterotoxaemia, it becomes very difficult to demonstrate the organism in blood or in tissues. For confirmatory diagnosis, the material is inoculated in laboratory animals like mice, guinea pigs etc. The impression smears are then prepared from liver, spleen and other relevant organs of laboratory animals for demonstration of the organism.
Fig. 22.26. Photograph showing collection of tissue in fixative

Fig. 22.27. Photograph showing the collection of intestine on a piece of paper for fixation.

Fig. 22.28. Photograph showing the dehydration of blocks in ascending series of ethanol

Fig. 22.29. Photograph showing (A) Mould (B) Tissue capsule and (C) Block holder.

Fig. 22.30. Photograph showing section cutting on microtome

Fig. 22.31. Photograph showing lifting of tissue section from floatation bath

Fig. 22.32. Photograph showing staining of tissue sections

Fig. 22.33. Photograph showing mounting of slides with DPX
Vital Staining

Vital staining procedures are not much in use directly in the diagnosis. However, for detection of phagocytic cells in body the vital stains are used. In the living animals when vital staining procedures are used for localization of phagocytic cells, these are known as **intravital**. In **in vitro** use of vital stains is being done for the live and dead lymphocyte count in leucocyte migration inhibition test (LMIT), lymphocyte stimulation test (LST), macrophage migration inhibition test (MMIT) and macrophage function tests (MFT).

Routine Histopathological Techniques of sectioning

The tissue pieces from morbid animals should be collected properly and fixed in a suitable fixative. Then these are processed and sections of 4-5 microns are cut and taken on slides. These sections are stained and mounted to make the permanent preparations of slides. The different steps required for making the tissue slides are described briefly as follows.

1. Collection of tissue

The collection of tissues is an important step, which is many times not given proper attention. The whole diagnostic process depends upon the collection of tissue pieces. A representative tissue should have been collected carefully and should have the normal as well as abnormal (lesion) part. The tissues must be collected by qualified person after a thorough examination of each organ/system. Some times it has been observed that the collection of tissues is performed by attendants or rudely by the qualified persons and proper attention is not paid. It should be kept in mind that a representative tissue sample will only give the correct diagnosis which cannot be remedied/altered afterwards. At the time of tissue collection following points must be kept in mind which will be beneficial for making a correct diagnosis.

- The tissue pieces from morbid animal should be collected as early as possible after the death of animal. Once the autolytic changes started in the dead body; it will not give true picture of microscopic lesions due to autolysis.
- At the time of tissue collection, it should be kept in mind that the representative tissue piece should include the part of lesion and a part of normal tissue, which facilitates the identification of organ/tissue at the time of microscopic examination.
- The tissue pieces should be cut with sharp knife and using only one stroke. Blunt edge knife may require many attempts for cutting, which destroys the normal architecture of tissues.
- Tissue pieces for histopathological examination should be collected from all the organs. Some times it has been noticed that the tissue sample is taken from those part of body which shows gross lesions; merely absence of gross lesion does not mean that there will not be microscopic alteration. In many disease conditions only microscopic changes occur which do not exhibited grossly. Such selective collection of tissues gives a biased interpretation, so it is better to have tissues from all the organs for proper interpretation and unbiased conclusions of histopathological studies.
- Tissues should be collected directly in the fixative and not in any other pot or water (Fig. 22.25 & 22.26). Sometimes it has been observed that at the time of post mortem examination, the tissue samples are collected in petridishes or in bottle and bring to the laboratory, then fixative is added. This seems to be a wrong practice. The tissue bottles filled with 2/3 fixative must be available at the time of necropsy and tissue pieces should be collected directly in the fixative.
- The size of tissue piece should not be more than 5 mm; it facilitate the homogenous and smooth fixation. Large size tissues do not get fixed properly and in the middle, the tissue gets autolysed.
- The tissue pieces from hollow organs like intestines, oviduct etc should be cut
transversely and placed on a hard paper, then it should be cut longitudinally in such a way that the serosal layer sticks to paper and mucosal layer gets free. Thereafter, it should be placed in fixative along with paper. This allows a good fixation and avoids the shrinkage and folding of tissue (Fig. 22.27).

- At the time of post-mortem examination, it has been noticed that the faecal matter is removed from the intestines by pressing / squeezing them or after opening the lumen by sharp objects like knife, slides etc.; which causes damage in the mucosal layer. The representative tissue should not be collected from such damaged portions.

- The tissues from encapsulated organs should be collected along with capsule or covering. Like brain should be collected along with meninges; kidneys and liver should be collected with their capsules. The coverings of such organs also yield useful information on histopathological examination.

2. Fixation

The fixation of tissues is required for preventing the post-mortem changes like autolysis and putrefaction by saprophytes, preservation of cellular constituents in life like manner and for hardening of tissues by way of conversion of semisolids to solid material. For a proper histopathological preparation and their interpretation, the role of fixative is very crucial. Any faulty fixation cannot be remedied at any later stage. An ideal fixative should be one that fixes the tissues quickly and should not interfere with the refractive index of the tissue components.

The choice of fixative depends on the type of investigation required, the formol saline (10% formaldehyde in 0.85% sodium chloride solution) is considered best fixative for routine histopathological studies. The buffered formalin has certain advantages over formol saline and now a days it is recommended for routine use in histopathological laboratories. The buffered formalin can also be used for immunopathological studies. Buffered formalin is widely used and preferred because of its tolerance; tissues can be left for longer period without excessive hardening or damage and sectioned easily. Since it has neutral pH, the formalin pigment is also not formed in the tissues. However, for immunopathological studies like immunoperoxidase staining techniques the fixative of choice is formol sublimate. But in the absence of that buffered formalin may also be used. The time required for proper fixation is 6-12 hrs for 5 mm thick block of tissue.

3. Washing

The tissue pieces after 6-12 hr fixation are taken out from fixative and cut into 2-3 small pieces of 2-3 mm size blocks. These blocks are, then, kept in tissue capsules (Fig. 22.29 B) or in a gauge tide off with the help of thread. The identification marks written by copying pencil are also kept along with tissues. These capsules/gauge-containing tissues should be kept in running tape water overnight for at least 12 hrs.

4. Dehydration

In routine practice, the dehydration is done in ascending series of graded ethanol. The tissue blocks are kept in 50% ethanol and then in 70%, 80%, 90%, 95% absolute ethanol I and absolute ethanol II for one hour each. These ethanol graded series should be kept in tight glass stoppered bottle or in screw cap jars to prevent the evaporation. In last bottle of ethanol II sometimes the copper sulfate is layered in the bottom, covered with filter paper, which increases the life of ethanol as it absorbs the water from alcohol. But the care should be taken, as soon as the copper sulfate begins bluish due to absorption of water, the ethanol should be changed (Fig. 22.28).

To increase the process of dehydration, the tissue blocks should be agitated either mechanically in an automatic tissue processor or by shaking the container periodically. The volume of alcohol should be at least 50 times more than the tissue placed for dehydration.

5. Clearing

Usually the clearing of tissue blocks is done in xylene. Like ethanol, xylene should also be kept in
tightly stoppered bottle to prevent the evaporation. After dehydration the tissue blocks should be kept in ethanol and xylene (1:1) mixture for one hr, then the blocks are transferred to xylene I and xylene II for one hr each. If xylene is not available then benzene may be used for 3 hr as its action of clearing is slower than xylene. On complete clearing, the tissue becomes transparent, then they should be transferred in paraffin wax for impregnation.

6. Impregnation
For the impregnation of tissue blocks, the paraffin wax in used either in paraffin embedding bath or in oven fixed at 60-62°C temperature. Both the oven and embedding bath are electrically operated with thermostat to adjust the desired temperature. At the time of transfer of tissue blocks from xylene II, the paraffin wax must be kept at 60-62°C in liquid form for impregnation. Three changes are given in paraffin wax; each of one hr duration. The paraffin wax should be free from dust or other gross impurities; which can be removed by filtration through muslin cloth.

7. Casting of blocks
After 3 hr impregnation of tissue blocks in paraffin wax, the blocks are formed in moulds using molten wax. The tissues are placed in moulds (Fig. 22.29A) in such a way that desired surface should remain down ward, which should be on the base of mould. The sections are cut from this surface, so care must be taken to keep the tissue in a proper manner which should be cut into sections homogenously. The mould is then filled with molten paraffin wax and then the blocks are cooled either at room temperature or in cold water. Various types of moulds like ‘L’ shaped or ring shaped can be used. If the moulds are not available, the blocks can be prepared in glass petri-dishes or in empty slide boxes. But care should be taken to lubricate the surface of such petri-dishes and other moulds with liquid paraffin or glycerine which facilitates the easy removal of blocks after cooling and hardening of paraffin wax.

8. Trimming
The blocks are removed from the moulds and are cut so as to give the one tissue per block and the wax is trimmed by knife or by rubbing on a hot plate in order to remove the extra wax on the either side of tissue. The tissue is exposed, which facilitate the side determination on which the section is to be cut. The identification of tissue should by fixed on one side of the block by touching the block with the small paper kept on it with hot forcep or knife, which bears the number. Then the blocks are fixed on block holder (Fig. 22.29C). Care should be taken that the number of marking of block should be kept on upper side at the time of trimming of the block on microtome to remove the extra wax and expose the whole surface of tissue. The trimming of blocks is done at 10-15µ and a separate knife should be used for trimming and section cutting.

9. Section cutting
Before the sectioning, the tissue blocks are cooled on ice or by keeping them in refrigerator. The tissue floatation bath should be cleaned and filled with water having a temperature of about 60-70°C. The blocks along with block holders are fixed in the microtome (Fig. 22.30) in such a way that the marking number will be on upper side, giving the similar position to the blocks as it was during trimming. Usually the sections are cut at 4-6µ thickness on rotary microtome using a plain edge knife. The knife should be sharp enough that it should cut the desired thickness sections in the form of a ribbon and will not cause damage to the tissue. By using a brush and forceps, the ribbon of tissue sections are placed in tissue floatation bath (Fig. 22.31). The tissue sections will spread here due to melting of paraffin wax and will take the shape similar to the tissue of that block. One can make out the selection here; the best looking sections 1-5 can be lifted on a sticky glass slide, which should be kept in a tray at an angle so that the water is removed. The glass slides are made sticky by applying a sticky material on clean glass slides, which consists egg white and glycerine in 1:1 (V/V) ratio. The sticky material facilitate the
Flow Chart Showing Processing of Tissue for Histopathology

- Collection of tissues in 10% formal saline 1-2 days
- Cleaning in water 10 - 12 hrs
- 50% ethanol 1 hr
- 70% ethanol 1 hr
- 80% ethanol 1 hr
- 90% ethanol 1 hr
- 95% ethanol 1 hr
- Absolute ethanol 1 hr
- Alcohol + Xylene 1 hr
- Xylene I 1 hr
- Xylene II 1 hr

- Dry in incubator
- Lift section on slide pasted with adhesive
- Place in floatation bath 62° C
- Cut sections 4-5 μm
- Trimming to expose tissue
- Fixing of blocks on block holders
- Numbering on blocks
- Trimming of blocks
- Casting of blocks
- Paraffin III 62° C 1 hr
- Paraffin II 62° C 1 hr
- Paraffin I 62° C 1 hr
sticking of sections on slides, which will not be damaged or removed during further processing of staining. Generally, 4-5 slides are made from each block and air dried in incubator or at room temperature. The following precautions should be taken at the time of section cutting:

i. Adjust the microtome gauge at right place, generally it is adjusted at 4-5µ for routine histopathological examinations.

ii. Knife should be properly fixed with the help of screws at an angle of about 45 degree. Ensure that all the fittings are tightly fixed.

iii. The knife should be sharp enough to cut sections free from nicks. If the nicks are present on sections, the position of knife should be changed or the knife should be properly stropped.

iv. The temperature of tissue floatation bath should neither be low nor it should high than the prescribed. In low temperature, the tissue will not spread properly and its compressions and creased will not be removed, while at high temperature the paraffin wax of tissue will melt quickly making the tissue fragments destroying the original shape of section.

v. Lift the tissue sections on slide at an angle (45°) of slide so that the air bubbles should not appear in between the slide and section.

vi. Use little sticky material on slide, if it is more then drying process will take more time.

vii. If the ribbon of sections is large then it should be cut at the junction of two sections with a sharp knife or blade and small pieces are made.

viii. During summer when temperature is above 40°C, the tissue sections should be cut either in a room or laboratory having air conditioner or desert cooler. If such facilities are not available then make moisture in the environment by sprinkling of water on ground. It is necessary because at high environmental temperature, the tissue sections stick to the knife and the ribbon is not properly formed.

ix. Drive the microtome smoothly in a regular speed; jerks should not be given.

x. For marking the slides, use the diamond pencil and marking should be done at the time of section cutting itself.

10. Staining
   (A) Routine procedure
   After drying the slides are kept in slide cabinets. One slide of each block is selected for staining using the following procedures (Fig. 22.32):

   (a) Removal of paraffin
   The slides are slightly warmed either in incubator or at the flame of a spirit lamp and are placed in jar having xylene. Replace the xylene after 10-15 min with fresh xylene for another 10-15 min. This removes the paraffin from the tissue sections.

   (b) Rehydration
   After removal of paraffin, the slides are kept in descending series of alcohol. For this first they should be kept in absolute ethanol and xylene (1:1) mixture for 5 min; then in absolute ethanol, 95%, 90%, 80%, 70%, 50% ethanol for 5-6 min in each dilution. After that the slides are taken in water.

   (c) Cleaning of slides
   With the help of muslin cloth, clean the slides from both the sides. Leave only 1 or 2 section on a slide and remove the extra sections and/or paraffin wax. Wash the slides in running tape water.

   (d) Staining in hematoxylin
   Place the slides in Harris hematoxylin or Meyer’s hematoxylin for 10-15 min. Shake the slides 2-3 times for proper staining. Remove the hematoxylin solution and wash the slides in running tape water, then dip in acid alcohol for few seconds, which helps in differentiation. Wash in tape water and place the slides in ammonia water for few seconds for blueing and place in running tape water in order to remove the ammonia.

   (e) Staining in eosin
   Place the slides in 2% aqueous eosin or alcoholic eosin for 2-5 min. After staining in eosin, quickly proceed for dehydration.
Flow Chart Showing Staining Procedure

1. Deparaffinize the sections on flame / hot plate
   - Xylene I 10-15 min
   - Xylene II 10-15 min
   - Xylene + ethanol 5 min
   - Absolute ethanol 5 min
   - 95% ethanol 5 min
   - 90% ethanol 5 min
   - 80% ethanol 5 min
   - 70% ethanol 5 min
   - Water / cleaning
   - Hematoxylin 10-15 min
   - Water
   - Dip in acid alcohol

2. Mounting on DPX
   - Xylene II 10-15 min
   - Xylene I 10-15 min
   - Xylene + ethanol 5 min
   - Absolute ethanol 5 min
   - 95% ethanol 5 min
   - 95% ethanol 5 min
   - 90% ethanol 5 min
   - 80% ethanol 5 min
   - 70% ethanol 5 min
   - Eosin 2-5 min
   - Water
   - Dip in Ammonia water
(f) Dehydration
The slides are placed in 70%, 80%, 90% 95% Absolute ethanol for dehydration atleast for 5 min in each solution; then place them in absolute ethanol: xylene mixture (1:1) for 5 min.

(g) Clearing
Clear the sections in xylene and give 2 changes at least for 10-15 min each. The clearing in xylene II can be extended for even upto one hour.

(h) Mounting
Mount the slides with coverslip using Canada balsam or DPX mountant. For this the cover slips of desired size and shape are kept on filter paper and one or two drop of mountant is placed on coverslip. Takeout the slides from xylene and place on coverslip in such a way that the section is touched with mountant, press gently and lift the slide (Fig. 22.33). Remove air bubble, if any, by pressing the coverslip with fine forcep and keep the slides in horizontal position in a tray for drying.

(i) Cleaning and labelling
After drying, clean the slides with muslin cloth and xylene. Remove the extra mountant using a blade. Label the slide with a piece of paper and stick it on one corner of slide using gum or other adhesive. At the time of examination, the histopathologist should put the name of organ, main changes in sections/disease condition with other remarks on this label for future identification of the slide.

(j) Examination
On hematoxylin and eosin staining, the nuclei of the cells take blue stain while the cytoplasm is pink or red. Examine the tissue section using 10 x objective and if required then in high power or oil immersion. Precautions and important tips which should be considered at the time of staining:

i. Check the sections for staining after blueing in ammonia water for hematoxylin stain and after dehydration for eosin stain. If under stained then repeat the process or in case of overstained, the sections can be differentiated for some more time in acid alcohol to remove the excess hematoxylin and in ethanol for removing the excess eosin.

ii. Clean the slides thoroughly in water and remove all patches/spots of paraffin; which gives a good look to slides.

iii. If on clearing in xylene, the cloudyness appears then repeat the dehydration process in absolute ethanol for 10-15 min. The cloudyness appears due to presence of water in the sections which reacts with xylene.

iv. At the time of mounting, ensure that the tissue section is not get dried. So to eliminate the chance of drying, proceed fast. Ensure the proper mounting of section on slides. Sometimes the opposite side of the section is mounted and section becomes dry. To ensure the proper mounting, one should feel/touch the diamond pencil marking present on the same surface, then mount the sections. This can also be checked by touching the slide on reverse side for the presence/absence of tissue sections.

v. Labelling with paper should be done on same side, at which the section is present; which will be helpful at the time of examination.

(ii) Special procedures
In histopathological techniques, one can demonstrate bacteria, fungus, chlamydia, rickettsia or viral inclusions in the tissue sections by using special staining procedures. These special staining techniques, however, requires specific expertise but can be used in diagnostic laboratory as routine methods. Some important special staining techniques are described as under:

B. Staining for acid fast bacilli
The acid-fast bacilli are demonstrated in tuberculosis or Johne’s disease in animals. The tissues are collected in formol saline or buffered formalin and processed in same manner as for routine histopathological techniques. For special staining of acid fast bacilli following procedures are followed:
1. Deparaffinize the sections and hydrate in descending series of ethanol as described earlier.
2. Clean the slides in water and give a wash in distilled water for 5 min.
3. Place the slides in carbol fuchsin solution and keep the chamber of slides in a water bath at 56°C for 1 hr.
4. Thereafter, remove the slides from water bath and keep at room temperature for few min, wash in running tape water. Dip in acid alcohol for differentiation till the colour of tissue become pale pink.
5. Wash in running tape water.
6. Place the slides in methylene blue working solution for few seconds, wash in tape water till the colour of sections becomes pale blue.
7. Dehydrate in ascending series of ethanol, clear in xylene and mount in dPX as described earlier in histopathological procedures.

Examine the slides under oil immersion. The acid fast bacilli will be of bright red in colour with a light blue back ground.

8. Precautions
(a) Care should be taken that at 56°C for 1 hr, the stain may get dry so it is always advisable to keep it in a covered jar in water bath to prevent drying.
(b) Differentiation with acid alcohol is very crucial step and should be controlled carefully; it depends on experience of a histopathologists to stain the slides properly.

II. Demonstration of Gram positive/gram negative bacteria in tissue sections
i. Deparaffinize and hydrate the sections to water, clean them.
ii. Stain the slides with crystal violet for 2 min.
iii. Wash in distilled water.
iv. Keep the slides in Gram’s iodine solution for 5 min.
v. Wash in distilled water.
vi. Differentiate in cellosolve (Ethylene glycol monomethyl ether) until blue colour is no longer comes out from sections.
vii. Wash in distilled water.
viii. Place in basic fuchsin for 5 min and wash in distilled water.
ix. Place the slides in differentiating solution for 5 min., wash in distilled water and blot dry.
xi. Dip the slides in tetrazine for few seconds.

III. Demonstration of spirochaetes
1. During post-mortem examination, cut about 1 mm thick slice of tissues from several sites of an organ and fix it in 10% buffered formalin for 24hrs, wash in running tape water overnight and place in 95% alcohol for 24hr.
2. Transfer the tissues in distilled water and keep till the tissues sinks to bottom.
3. Stain in silver nitrate at 37°C in dark for 3-5 days and change the solution daily.
4. Wash in distilled water and place the tissues in reducing solution for 1-3 days.
5. Rinse in distilled water and dehydrate in ascending series of ethanol.
7. Impregnation/embedding is done in paraffin wax as in case of routine histopathology, cut sections at 4-5µ, dry and deparaffinise in xylene (3 changes of 5 min each).
8. Clean the slides, remove artifacts and spots of paraffin wax.
9. Mount 1-2 sections per slide with DPX.
10. Examination is done under microscope; the spirochaete will be of black colour with yellow to light brown background.

IV. Demonstration of Fungi
1. Collect the tissues in formol saline or buffered formalin and process the samples in a same way as in routine histopathology and cut the section at 4-5µ, deparaffinize and hydrate to water.
2. Place the slides in 4% chromic acid for 1 hr.
3. Wash in running tape water and keep the slides in 1% sodium bisulfite solution for 3-5 min.
4. Wash in running tape water and then in distilled water.
5. Stain with methanamine-silver nitrate working solution at 60°C in water bath till sections become yellowish brown.
6. Wash in distilled water and place in gold chloride solution for 5 min.
7. Wash in distilled water and place in sodium thiosulfate solution for 5 min and wash in running tape water.
8. Stain with light green for 1 min, wash in water; dehydrate in ascending series of ethanol, clear in xylene and mount in DPX.
9. Examine the sections under microscope, the fungi will take a black colour, mycelia and hyphae will be of rose coloured with a pale green back ground.

V. Demonstration of rickettsia
1. Tissues are fixed in formol saline or buffered formalin and processed in same manner, sections of 4-5µ thick are cut, dried, deparaffinize and hydrated to water.
2. Place in methylene blue solution for overnight and decolourize in 95% ethanol for few seconds or till blue colour is lost.
3. Wash in distilled water and place the slides in basic fuchsin solution for 30 min.
4. Decolourize in citric acid solution for 1-2 sec.
5. Differentiate in absolute ethanol for few min, clear in xylene and mount in DPX.

Examine the slides, the rickettsia will be of bright red colour and nucleus of the cell will take blue colour.
The post-mortem examination of veterolegal cases is performed as described in previous sections. However, following points must be kept in mind while doing post-mortem examination and preparing the report.

1. For veterolegal cases, post-mortem request should be signed by a police officer not below the rank of inspector or by magistrate; without which no post-mortem examination should be done.

2. Always collect maximum information on history, date and time of death of animal and treatment given. Use self knowledge and experience to determine the time of death such as rigor mortis, autolysis, putrefaction, pseudomelanosis etc.

3. Animal identification including species, breed, age and number or mark must be clearly established before conduct of post-mortem examination. It is specially necessary in insured animals as well as in religiously disputed cases.

4. All the lesions present on skin surface should be clearly defined as laceration, wound, trauma, incision, erosion, vesicle, ulcer and if there is suspected sharp edge wound or bullet injury also state its depth and width (diameter) as the case may be. Also mention the side on which the animal is lying down (ventral portion touching earth).

5. In case of dispute over still birth and calf born alive, a piece of lung should be placed in water. The lung piece will sink in water in case of atelectasis neonatum while it will float if the calf born alive.

6. If the case is suspected for toxic condition/poisoning, try to mention the type of poison in your report. This will help the police authorities to establish/confirm the type of toxin/poison in forensic laboratory.

7. The post-mortem examination of wild animals should be conducted as a special case. One should conduct the post-mortem examination only when DFO or higher officer is making request for post-mortem examination. It should be noted on the report that all the viscera including skin, bones, teeth, etc. are returned to the person requested for the necropsy and no item should be left behind.

8. Fill the post-mortem report clearly with neat hand writing and in clear language and avoid ambiguity in presentation. Avoid to write general sentences. Be specific to your findings and conclusions. Sign the report with date and must keep a copy of that with you for record and future evidences in the court of law.

9. Post-mortem examination should be conducted in day light. In darkness where the pathologist is not able to recognize the lesions, the post-mortem examination should not be conducted.

10. At the time of post-mortem examination outsiders should not be allowed. To avoid them and wild birds and animals, post-mortem examination should be done in close premises.
Appendix VII

COLLECTION, PRESERVATION AND DISPATCH OF MATERIAL TO FORENSIC LABORATORY

The collection, preservation and dispatch of different tissues/organs, fluids and viscera should be done as described in section 4 of appendix. However, in veterolegal cases, these materials should be sent to forensic laboratory under sealed packings.

- In the suspected cases of toxic condition or poisoning, the stomach and intestinal contents should be sent after proper ligation at both the ends and sent it in ice to avoid putrefaction. Besides, samples of blood, liver, spleen and kidneys should be sent in separate container.
- All the materials should be collected in leak proof glass or plastic bottles.
- Tissues for histopathology must be collected in 10% formalin or formol saline, this can be sent to laboratory under normal temperature.
- The materials suspected for toxicity should be sent in ice without adding any preservative.
- The bottles or containers should be sealed and labelled properly indicating the name of owner, identification of animal (number, name, mark etc.), type of tissue collected and preservative used. The examination requested and disease or poisoning suspected should also be written.
- A copy with details of post-mortem report and containing above information should be sent separately under separate cover.
- The address of the forensic laboratory should be clearly written.
- All the containers should be packed with cloth and sealed with sealing wax and should preferably be sent through person in order to avoid any breakage in transit.
- One copy of the forwarding letter should be kept in file for future reference and one copy should accompany the material and one copy should be sent by post. The forwarding letter bearing number and date should have the information about materials sent, type of preservative used, type of examination requested and identification of animals including other details of owner.
Appendix VIII

EXAMINATION OF BLOOD, URINE AND FAECES

BLOOD EXAMINATION

TOTAL ERYTHROCYTE COUNT
- Clean New Bauer’s counting chamber/hemocytometer counter and place clean coverslip on ruled areas.
- Suck fresh or anticoagulant mixed blood in RBC diluting pipette (red ball in bulb) upto 0.5 mark and fill the pipette with RBC diluting fluid upto 101 mark.
- Hold pipette in horizontal position and remove rubber tube. Mix the contents by rotating the pipette in between palms.
- Discard first few drops from pipette and then place a drop near the edge of cover slip to fill the space between cover slip and counting chamber.
- Keep counting chamber 1-2 min for settling of the cells.
- Count the cells under high power of the light microscope.
- Cells are counted in 5 medium squares of the central large square or 80 tertiary squares.
- Cells on top of square or left side are included in count.
- Calculate RBC per µl of blood by multiplying 10,000 to the total number of cells counted in 80 tertiary squares. It can be converted into ml by further multiplying with 1000 and in liter by 10,00,000.

TOTAL LEUCOCYTE COUNT
- Hold the pipette in horizontal position and remove rubber tube. Mix the contents by rotating the pipette in between palms.
- Discard first few drops from pipette and then place a drop near the edge of cover slip to fill the space between cover slip and chamber.
- Keep counting chamber 1-2 min for settling of the cells.
- Count the cells under low power in four large/primary corner squares of the ruled area.
- Cells on top of square and left side are included in count.
- Calculate WBC per µl of blood by multiplying 50 to the total number of cells counted in 4 primary squares. It can be converted into ml by multiplying 1000 and in liter by 10,00,000.

PACKED CELL VOLUME (HEMATOCRIT VALUE)
- Clean and dry the wintrobe tube.
- With the help of a long needle (6”) and syringe fill the blood in wintrobe tube upto mark 100.
- Take precaution that there should not be any air bubble in the tube.
- Centrifuge the wintrobe tube at 3000 rpm for 30 min.
- Record the reading of packed cell volume in percent i.e. mass of erythrocytes settled down in tube.

ERYTHROCYTE SEDIMENTATION RATE
- Clean and dry Westergren pipette.
- Suck anticoagulant mixed blood in Westergren pipette upto mark ‘O’ and fix it in stand in vertical position.
- Leave this for one hr at room temperature.
- Record the reading on pipette, it is the mm fall of erythrocytes per hr.
HEMOGLOBIN

- It is measured by using Hellige- Sahli hemoglobinometer.
- Clean and dry the graduated tube of the hemoglobinometer.
- Take 5 drops of N/10 Hydrochloric acid in tube.
- Suck the anticoagulant mixed blood in pipette upto 20 marks.
- Place the pipette in tube containing N/10 HCL and transfer the blood into acid.
- Suck acid in pipette and leave in tube.
- Keep the tube for 5 min in dark.
- Add distilled water in the tube drop-by-drop using dropper, mix with stirring rod and match the colour with standard. Add water till the colour matches with standard.
- Read the scale on tube; it is the value of hemoglobin gram per 100 ml of blood.

DIFFERENTIAL LEUCOCYTE COUNT (DLC)

- Prepare a thin blood smear on clean glass slide. Place a drop of blood on one end of slide and spread as smear with the help of another slide using its edge at 45° angle.
- Dry the smear in air and mark identification number in the thick portion of smear.
- Fix the smear in methanol for at least 5 min and dry in air.
- Stain the smear with Giemsa stain diluted to 1:10 in distilled water for 30 min or with Leishman’s stain without fixing the smear.
- Wash the slide, dry in air and examine under oil immersion microscope. Count at least 200 cells by battle ment/zigzag method. Cells counted are lymphocytes, neutrophils, monocytes, eosinophils and basophils. Cell count is presented in percent.
**ABSOLUTE LYMPHOCYTE COUNT (ALC)**  
The absolute lymphocyte count is calculated by using the data of DLC and TLC through following formula:

\[
\text{ALC} = \frac{\% \text{ Lymphocyte} \times \text{TLC} \times (10^3/\mu l)}{(10^3/\mu l)} \times 100
\]

**ABSOLUTE NEUTROPHIL COUNT (ANC)**  
The absolute neutrophil count is calculated by using the neutrophil percentage of differential leucocyte count and total leucocyte count using following formula:

\[
\text{ANC} = \frac{\% \text{ Neutrophils} \times \text{TLC} \times (10^3/\mu l)}{(10^3/\mu l)} \times 100
\]

**MEAN CORPUSCULAR VOLUME (MCV)**  
Mean corpuscular volume is determined by dividing the packed cell volume (PCV) by the total erythrocyte count in millions/\(10^6\) and multiplied by 10. The MCV is expressed in cubic microns.

\[
\text{MCV} = \frac{\text{PCV}}{(\text{Cubic} \ \mu)} \times 10
\]

**MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (MCHC)**  
Mean corpuscular hemoglobin concentration is calculated by dividing the hemoglobin in gm per 100 ml of blood by the PCV and multiplied by 100. It is expressed in percent.

\[
\text{MCHC} = \frac{\text{Hb}}{(\% \text{ PCV})} \times 100
\]

**MEAN CORPUSCULAR HEMOGLOBIN (MCH)**  
Mean corpuscular hemoglobin is calculated by dividing hemoglobin in gm per 100 ml by TEC in millions per \(10^6\) of blood and multiply by 10.

\[
\text{MCH} = \frac{\text{Hb}}{(10^{12} \ g)} \times 10
\]

**ALTERATIONS IN HEMATOLOGICAL AND BIOCHEMICAL ATTRIBUTES IN VARIOUS DISEASE CONDITIONS OF ANIMALS**

A. Hematological profile
1. **Erythrocytosis**  
Brucellosis, Campylobacteriosis, Leptospirosis, Rinderpest, haemorrhagic septicemia.

2. **Erythropenia**  
Leukemia, Haemorrhage, Aflatoxicosis, Theileriosis, Babesiosis, Anaplasmosis.

3. **Leucocytosis**  
Pyogenic infections, Rabies, Tuberculosis, Strangles, Leptospirosis, Theileriosis Babesiosis, Anaplasmosis, Haemorrhagic Septicemia.

4. **Leucopenia**  
Canine distemper, Infectious canine hepatitis, Swine fever, Brucellosis, Tuberculosis, Infectious bovine rhinotracheitis.

5. **Neutrophilia**  
Acute inflammation, Pyogenic infections, Pyometra.

6. **Neutrophiliamth (shift to left)**  
Leptospirosis, metritis, Traumatic reticulopericarditis (TRP), Canine distemper, Glanders.

7. **Neutropenia**  
Pasteurellosis, Infectious canine hepatitis.

8. **Lymphocytosis**  
Leukemia, After vaccination, viral infections.
9. Lymphopenia
Canine distemper, Infectious canine hepatitis, Infectious bovine rhinotracheitis, Foot and mouth disease.

10. Eosinophilia
Allergy, Parasitic diseases.

11. Hypohypoglycemia
Anemia, Theileriosis, Strangles, Anaplasmosis, Degnala disease, Fasciolirosis.

12. Increased ESR
Carcinoma, Nephritis, Chronic granulomatous infection, Tuberculosis, Canine distemper, Trypanosomiasis.

13. Increased Hematocrit Value/PCV
Dehydration

14. Decreased hematocrit Value/PCV
Anemia, Theileriosis, Strangles, Anaplasmosis, Blue tongue.

B. Biochemical attributes
1. Hyperglycemia
Diabetes mellitus, Chronic nephritis.

2. Hypoglycemia
Hepatic insufficiency, Ketosis.

3. Hyperproteinemia
Shock, Dehydration, Plasmacytoma, Infectious diseases.

4. Hypoproteinemia
Burn Diarrhoea, Renal dysfunction, Hepatic disorders, Tuberculosis.

5. Hyperglobulinemia
Dehydration, Leukemia, Bacterial, Viral and parasitic infections.

6. Hypogammaglobulinemia
Anemia, Haemorrhage, Immunodeficiency.

7. Hypercalcemia
Hyperparathyroidism, bone cancer, Nephrolithiasis.

8. Hypocalcemia
Hypoparathyroidism, Rickets, Osteomalacia, Ketosis.

9. Hyperphosphatemia
Renal failure, Hyperparathyroidism, Healing of fracture.

10. Hypophosphatemia
Chronic diarrhoea, Pica, Rheumatism like syndrome, Hemoglobinuria. Hyperparathyroidism.

11. Increased levels of Blood urea nitrogen
Renal impairment, nephritis, Urinary obstruction.

12. Decreased levels of BUN
Acute hepatic insufficiency, nephrosis, Chronic wasting diseases

13. Increased level of creatinine
Severe nephritis, urinary obstruction, severe toxic nephrosis

14. Hypermagnesemia
Chronic infection, Oxalate poisoning

15. Hypomagnesemia
Grass tetany, Lactation tetany, Wheat pasture poisoning.

16. Increased levels of SGOT
Hepatic necrosis, Myocardial infarction, Muscular degeneration/necrosis in dog and cat, Azoturia.

17. Increased levels of SGPT
Hepatic necrosis, Infectious canine hepatitis

18. Increased levels of Alkaline phosphatase
Obstructive jaundice, hepatitis, Hyperparathyroidism.

19. Decreased level of Alkaline phosphatase
Chronic nephritis.
20. Increased level of Acid phosphatase
Prostate carcinoma, Leukemia.

21. Increased level of Lactic dehydrogenase
Malignant lymphoma.

22. Increased level of Serum isocitric dehydrogenase
Hemolytic anemia in horses

23. Increased level of Ornithine carbamyl transferase
Liver disorders in dogs.

URINE EXAMINATION

PHYSICAL EXAMINATION

1. Colour:
   - Note the colour of urine as
     o Watery/colorless
     o Amber colour
     o Red
     o Brown
     o Yellow/Yellowish green
     o Black
     o Pale

2. Odour
   - Record the smell of the urine
     o Uremic
     o Sweetish/ Fruity
     o Fetid

3. Turbidity
   - Look for the presence of suspended material in urine
     o Clear
     o Turbid +, ++, +++
     o Cloudy

4. Foaming
   - Shake the urine in a test tube
     o No/Slight foams
     o Yellow/Green foams
     o Red/brown foams

5. Specific Gravity
   - This is measured by urinometer
     o Urine is filled in cylinder and urinometer is left in the urine
     o Record the specific gravity in urinometer.

CHEMICAL EXAMINATION

1. Reaction
   - Reaction is determined by using pH strips or pH meter.
   - For this take a pH strip and dip in urine
   - Read the change in colour on scale given with pH strips.

2. Glucose
   - Take 0.5 ml urine in a clean and dry test tube.
   - Mix 5.0 ml Benedict’s reagent in the urine and keep it in boiling water bath/flame for 5 min.
   - Remove the tube and cool them on test tube stand.
   - Record the changes of colour in tube as follows:
     o Blue (-) No glucose
     o Blue to green (+) mild glucose
     o Yellow with heavy sediment (+++) moderate glucose
     o Orange with heavy sediment (++++) highly positive for glucose

3. Protein
   - Take 2 ml of urine in a clean and dry test tube.
   - Place 2 ml Robert’s reagent over urine.
   - If protein is present in urine, then a white ring will appear at the interjunction of two fluids. It is graded as follows:
     o No ring (-) negative
     o Mild ring (+) mild positive
     o A wide ring (+++) moderate positive
     o Heavy ring (++++) positive
     o Very heavy ring (++++) highly positive
KETONE BODIES

1. Acetone
   - Take 1.0 gm mixed powder of sodium nitroprusside and ammonium sulfate (Sod. Nitroprusside 1 part, Amm. Sulfate 100 parts) in a test tube.
   - Add 5 ml urine in the salts and mix them properly.
   - To this slowly overlay 20% ammonium hydroxide solution.
   - Record the colour at the interjunction of two fluids.
   - If it is red to purple then it is acetone positive.

2. Acetoacetic acid
   - Take 10 ml urine in a clean and dry test tube.
   - Add 5 drops of Lugol’s iodine and 3 ml chloroform, mix them and allow to stand.
   - Record the colour of urine
     - Colour less : positive
     - Red/ violet colour : negative

3. Beta hydroxybutyric acid
   - Take 20 ml urine in a small beaker and add 20 ml distilled water and few drops of acetic acid.
   - Boil the contents over flame till it remains 10 ml, add distilled water to make it 20 ml and place in two test tubes 10 ml in each.
   - In one test tube add 1 ml H₂O₂ and warm it for 1 min, cool it.
   - Add 1 ml glacial acetic acid, 1 ml freshly prepared sodium nitroprusside solution in both tubes, mix thoroughly.
   - To this overlay strong ammonia water and allow to stand for 3-4 hrs.
   - Record the change in colour in H₂O₂ added tube if it is purple colour ring then it is positive.

Bile salts
   - Take 4-5 ml urine in a test tube and shake it. If persistent foams are present then it is positive for bile salts.

   - Add sulphur granules over surface of urine. In case of positive, sulphur granules will sink in urine.

Blood
   - Take 2 ml urine in a test tube I.
   - Take 1 ml saturated solution of Benzidine in test tube II. Add 1 ml 3% H₂O₂ and mix well
   - Mix the contents of tube I and II.
   - Record the development of colour. In positive case a green to blue colour will appear.

Hemoglobin/Myoglobin
   - Take 5 ml urine in a test tube.
   - Add 2.8 gm ammonium sulfate
   - Shake well and allow to stand for few min.
   - If urine become clear/ watery in colour. Then it is positive for hemoglobin. If colour remains same as before the test then it is positive for myoglobin.

Microscopic examination
   - Take 5-10 ml urine in a centrifuge tube and centrifuge it at 1000 rpm for 10 min.
   - Discard supernatant and place a drop of sediment on clean, dry glass slide.
   - Cover it with a cover slip and examine it under microscope for the followings:
     - Epithelial cells
     - Leucocyte
     - Erythrocytes
     - Microorganisms
     - Casts

FAECAL EXAMINATION

GROSS EXAMINATION
   - Collect faeces in clean and dry petridish or in small sample bottle.
   - With clean spatula and glass rod spread the faeces and note the followings:
     - Colour
     - Consistency
     - Odour
Presence of blood
- Presence of parasite/segments of parasite

**Microscopic Examination**

**Direct Smear method**
- Place a drop of distilled water on clean and dry glass slide.
- Add small amount of faeces in distilled water on slide.
- Mix with glass rod/tooth pick/ matchstick.
- Place a cover slip on it.
- Examine under microscope for the presence of parasitic ova.

**Qualitative concentration method**
- Take about 1.0 gm faeces and mix it in small amount of distilled water.
- Mix with glass rod/tooth pick/ matchstick.
- Place a cover slip on it.
- Examine under microscope for the presence of parasitic ova.

**Qualitative concentration method (Simple floatation method)**
- Take about 1.0 gm faeces and mix it in small amount of distilled water.
- Filter it through sieve/muslin cloth.
- Filterate is mixed with 4-5 ml of saturated salt solution.
- Place the mixture in a tube or cylinder and fill it upto the top.
- A clean coverslip or glass slide is placed on the mouth of tube/cylinder.
- Keep it for 30 to 60 min at room temperature.
- Remove the coverslip or slide and examine it under microscope for parasitic ova.

**Qualitative concentration method (Centrifugation floatation method)**
- Take about 1.0 gm faeces and mix it in small amount of distilled water.
- Mixture is filtered through fine sieve/muslin cloth.
- Mix the filterate with saturated salt solution (1:3) in a centrifuge tube.
- Centrifuge it at 1500 rpm for 5 min.
- Take a drop of superficial contents on a clean glass slide and examine under microscope.
- Sediment is examined for eggs of liver flukes.