

Special Issue:

Manual guidance of veterinary clinical practice and laboratory

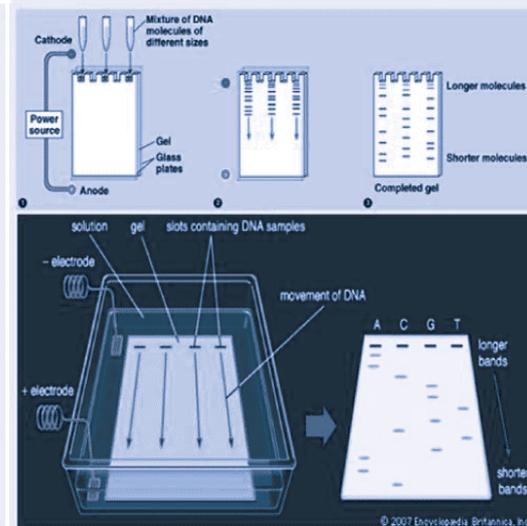
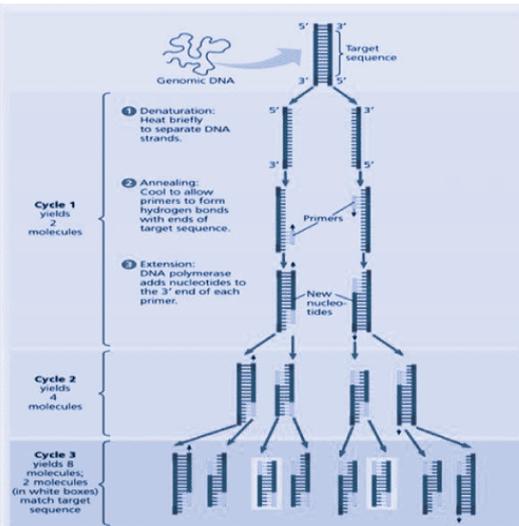
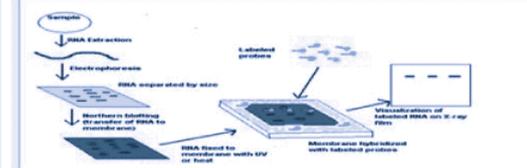
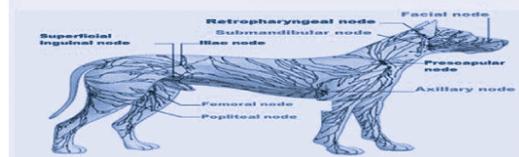
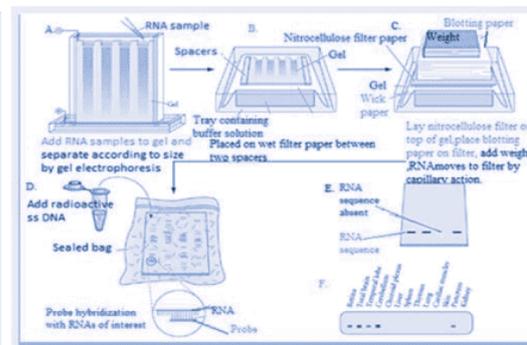
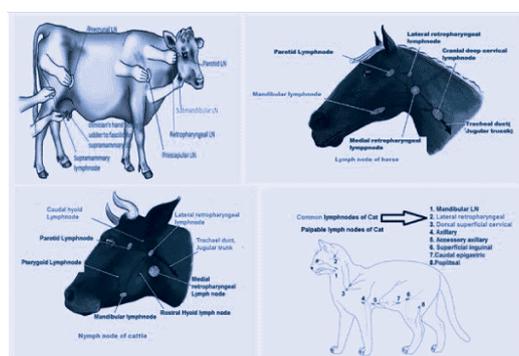
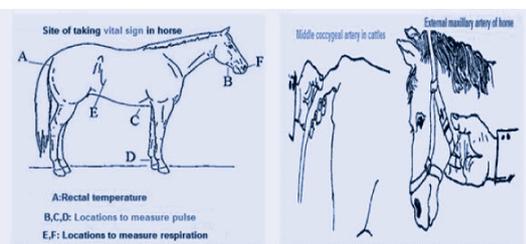


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Research Article

Veterinary Clinical Practice and Diagnosis

Introduction

Clinical examination is a fundamental part veterinary diagnosis. It provides the veterinarian with the information to determine the disease or diseases producing the clinical abnormalities. Additionally, the information derived from the clinical examination should assist the veterinarian in determining the severity of the pathophysiological processes. Without a proficient clinical examination and an accurate diagnosis, it is unlikely that the treatment, control, prognosis and welfare of animals will be optimized [1-5]. The organs or systems involved, the location and type of lesion present, the pathophysiological processes occurring and the severity of the disease can be deduced from the information gained during the clinical examination. The success of clinical examination relies heavily on the knowledge of the clinician and usually assumes a single condition is responsible for the abnormalities. Many clinicians begin their examination by performing a general examination which includes a broad search for abnormalities [6,7]. The system or region involved is identified and is then examined in greater detail using either a complete or a problem oriented examination. For this sound knowledge of Anatomy, Physiology, Pathology and Animal behavior, skills in the methods and techniques of clinical examination, knowledge of etiology, clinical sign and pathogenesis of the diseases are the basic requirements for clinician to make diagnosis [8].

The diseased animals which are presented to veterinary clinic can be analyzed by veterinarian or clinician, the clinician approach through asking the owners complaint, which request for professional assistance by giving animal history. The clinical examination ideally proceeds through a number of steps. The owner's complaint, the history of the patient, the history of the farm and the signalment of the patient are usually established at the same time by interview with the owner or keeper of the animal 9. This manuscript can assist veterinary clinicians and technicians by giving clear informations and procedures, and

also guides veterinary students and clinicians how to operate at veterinary clinic and laboratory.

Taking history of patient

History taking or anamnesis is the process of taking information on animal patient from owners about its illness, onset of illness and feeding practice through careful questioning of the owner. In Veterinary practice, the disease is presented indirectly in the form of a complaint by the owner or the attendant. Thus, it is very necessary to have all the information from the owner. Most of the time, the owner or attendant fails to provide pertinent and adequate history and inaccurate history may lead to misdiagnosis. The clinician must substantiate these with rational question utilizing professional knowledge [1,5].

Without knowing history of animals it is difficult to diagnose disease problem of patient animals. Therefore, the history should be taken from the owners of the patient and recording the owner's complaint. Disease information should include the group(s) affected, the numbers of animal affected (morbidity) and the identities of the animals affected; the number of animals that have died (mortality) should be established. In order to get the accurate and complete history of patient the following things should be focused; patient data, present history, past history and environmental history. Patient data is essential to identify the patient and it includes: Owner's name, Owner's address: postal address, telephone, kebele, peasant association, species, breed, sex, age, name, ID No., body weight, description including color, marking, polledness, and other identification marks of patient [8,9].

Present history of patient

Present history of patient includes of recording the sequential events from the start of the patient chronic. Questions about physiological functions such as appetite, urination, defecation, rumination, respiration, sweating, milk production, gait, posture and also of the first symptoms shown by the animal should be asked. All these information deal with the current problem of the animal and the events associated with it. The points which going to be asked during that is as following [1,5,8].

Locations of the problems: The main attention at the complaint that a farmer has to say and from there clinician can tentatively say can be listed as following:

- * Digestive system involvement will be shown as absence of rumination, appetite, bloat or diarrhea
- * Respiratory system involvement will be indicated presence of nasal discharge, coughing, dyspnea.
- * Urinary system involvement will be manifested as frequent urination, passing red coloured or cloudy urine.
- * Musculo-skeletal and nervous system involvement will be manifested as lameness, inco-ordination, and paralysis.

Nature of illness: The clinician should be able to assess and find out the time of onset of disease, any change management practices and signs noticed by the farmers.

- * To assess to know the duration of disease whether it is peracute, acute, subacute or chronic
- * To know number of animal diseased and morbidity rate and mortality rate of animals
- * Determine whether any drug has been given for animals, before patient come to clinic for assurance
- * And the following question should be pointed:
- * When did the farmer notice the disorder? (time)
- * Did it occur suddenly/slowly? (acute /sub-acute / chronic nature)
- * What were the signs noticed? (anorexia/drop in milk yield/ others)
- * Are the animal fed / grazed in pasture / forest grazed? (getting information on management practices e.g. ketosis seen in stall fed animals, while babesiosis seen in forest grazed animals)
- * Is there any other animal affected with similar condition in the same herd / in other farmer's herd in the village (to find out if the disease is rapidly spreading)?
- * Ask if there has been any introduction of new animal to the herd / village (sick animal may have been bought from affected area and disease has started)
- * Is the affected animal vaccinated against food-and-mouth disease (FMD), anthrax, hemorrhagic septicemia (HS), Black quarter (BQ) (to find out if the animal is protected against common diseases).

Past history

Inquiring into the past history may help in arriving at a diagnosis. History of drenching a day or two earlier may cause aspiration pneumonia. History of past disease may be co-

related to the present illness. Past history will also give idea if such condition prevailed previously in the area.

- * Ask if such condition was reported previously too (reveal endemic nature of disease, or occurrence of a new disease)
- * Does this occur at certain period of time? (find out the seasonal occurrence of the disease)
- * Was the disease reported from other places in the locality? (area of spread / occurrence can be found out)
- * Has any animal recovered from such a sickness? (to aid in prognosis)
- * Is the disease restricted to certain age group / sex? (BQ is seen in animals between 1-3 years of age in both sexes 2, 4).

Environmental history

The environment in w*iosecurity and regional mineral deficiencies. Risk factors indoors may include ventilation, humidity, dust, stocking density, temperature, lighting, bedding, water availability, feeding facilities and fitments. Environment or surrounding of the animals may help in the diagnosis of disease. Animal grazing on pasture irrigated with sewerage water may suffer from nitrate poisoning. Parasitic diseases are more in animals, which are kept in marshy lands. Recent spraying of weedicide or insecticides may poison the animals. Environmental history can be divided into outdoor environment and indoor environment [9].

Outdoor environment history: It regard to the topography of land where animals are reared, vegetation, type of agriculture practiced in the locality, use of chemicals in agriculture (pesticide, weedicide) and system garbage disposal in the area. Animals that are grazed are likely to be infested by parasites and prone to vector borne diseases like babesiosis, trypanosomiasis, or animals that are grazed in the marshy area including paddy filed are likely to be infested by liver fluke etc.

Indoor environment history: It regards with the types of animal house. The following should be look assessed how is the house of animals is designed:

- * If there is proper ventilation,
- * In the rural area, traditionally animals are housed in the ground floor of the house where there hardly any ventilation and this will predispose the animals to respiratory diseases,
- * If the animals are stall fed check
- * If the animals are provided with enough drinking water,
- * If the floor is dry and clean, damp and dirty floor may lead to mastitis in milking animals,
- * If the bedding materials are used

- * If the materials are changed daily or topped daily to keep the animals dry and clean.
- * If the animals are housed separately see
- * If the height of the roof is at a required level,
- * If it is too low, in hot places animals may be subjected to heat stress in summer months,
- * If it is too high animals will be exposed to rain.

Restraint and approach to animals

Restraint is the term used to imply control of an animal and may be necessary for medical reasons and nonmedical procedures. Animals are often resisting to the clinical examination procedures. The animal must be restrained so that it can be examined carefully, safely and with confidence. The methods of restraint should be done, in order to able to carry out the examination safety and without danger to the clinician and assistants, the methods available may be classified as the following [6,9].

- **Physical restraint.**
- **Chemical restraint**
- **Verbal/Moral restraining**

Physical restraint: It is important to perform all the physical manipulations in a quiet and gentle manner in order to carry out the examination safely without causing danger to the clinician or his assistants and to avoid disturbing the patient. This manual is to give the advanced knowledge on how to restrain the domestic animals.

Restraints of the equine: Equipment used for restraint and handling should be effective without causing stress to the horse and should be designed for maximum safety of the handler and horse. A horse should be approached from the front and slightly to the left (near) side because they are accustomed to being handled on that side. They become nervous when handled from the right side 10. Any restraint method used to assist normal management or treatment of the horse should be the most mild and effective method available, and should be applied for the minimum amount of time necessary to carry out the task.

A halter and lead rope is the most common form of restraint. Generally, the safest knots are those that can be quickly untied even if the horse has pulled on it. When used by knowledgeable handlers, other acceptable forms of restraint include hobbles, twitches, lead chains, stocks and chutes 11. Tethering is a form of restraint that brings a high risk of injury to horses unless used correctly. Tethering does not refer to tie stalls or briefly tying a horse to a fixed object 11. The following lists are the methods how to restraint horse [8,10] (Figure 1.1):

- * *Applying a halter.*
- * *Rope twitch*
- * *The hand twitch*

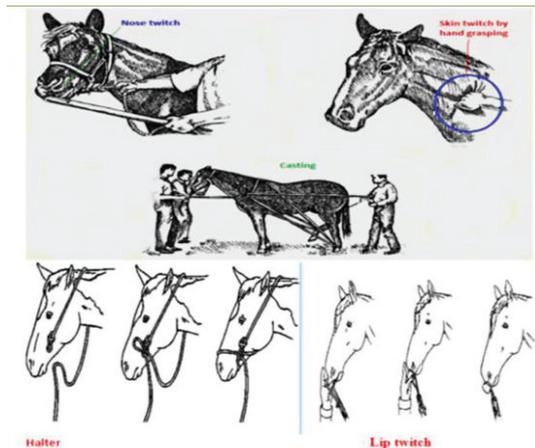


Figure 1.1: The methods for restraint of equine animals [12]. Halter: Used to restrain head of horse and donkey, it can be led or held for procedures such as injections. A halter can be made from a piece of sisal or cotton rope. A viod using nylon rope against the skin. The Lip twitch the twitch is useful to restrain a horse before a painful procedure, to examine it or gave it treatment. It is known that a twitch placed correctly on the nose cause the release of natural pain-killing substances in the horse's body. Two rope casting used to prevent kicking and it can be done safely without danger of being kicked.

- * *Neck skin grip:* To restrain the horse, grasp the loose skin of the neck.
- * *Twitch* is applied to the upper or lower lip or to the ear
- * *Nose twitch*
- * *Lifting the fore-leg and hind-leg* by unaided hands or with *Leg twitch*
- * A loop of strong cord or soft rope is applied to the appropriate part
- * *Two ropes one-person horse casting*
- * *Two ropes four people's horse casting*

Restraint of the cattle: Restraint of cattle depends on breed, age, sex and knowledge of animal behavior. For example, bulls are unpredictable and should be handled with care. Aggressive bulls for instance, will paw the ground with front feet, lower and shake the head. They may also make mowing sounds. Nervous cows will keep head and tail up and may have an anxious expression in their eyes [8,13]. The followings are the methods for restraint of cattle (Figure 1.2):

- * The nasal septum is gripped between the thumb and one finger or with 'bull-holder'
- * *Leg twitches* are also employed
- * *One rope locking two horns* on a post or tree
- * *One rope two-person cattle casting*
- * *Two ropes three-person cattle casting*
- * *Chest twitch:* Chest twitch is used for exceptionally restless cattle.

- * Procedure chest twitch: A rope is tied around the chest and held under tension by a strong pole twisted in a rope loop. However, chest twitch should be used only when there is no other method of restraint.
- * *Nose lead*: It restrains cattle by applying pressure to the nasal septum.
- * *Procedure of nose lead*: It is shaped like a pair of tongs with a large ball at the end of each arm, which fits against the nasal septum. Usually, it has a chain or rope at the end. Hold the lead out for the animal to sniff. As the animal raises its head to sniff, slip the nose lid into the nostrils and close quickly. Then tie the rope to the post for continuous restraint but remember it loses effect when the nasal septum becomes numb. Manually holding the nasal septum with index finger and thumb *nose grip* and slip in the nose lead. It is advisable to avoid using nose lead too frequently on the animal as it may become "head shy".
- * *Restraint using a crush*: A crush can be used to restrain many animals at a go. It can be constructed of posts or planks or steel tubing (Figure 1.2). However, the internal surface should be free of sharp edges or projections that can injure the animal. So, it should comprise of an assembly area with a funnel ending in a closed pen with the final run being just wide enough for one animal and sufficiently high enough to prevent it from jumping. Backward movement is prevented by a transverse bar inserted just behind the animal [13].

Restraint of sheep and goat: Sheep have an intense instinct to remain with the flock and as such, it is best to handle them as a flock initially before isolating the animal you want to perform certain procedures on. When disturbed, they will stamp their front feet and will use the head to attack. Hence, always work gently, calmly and with assurance around them. Sheep have very fragile bones that can easily be broken and heavily woolled sheep may become hyperthermised if chased around [13]. The following are the methods how to restraint and holding sheep and goat (Figure 1.3) [13].

Capturing a sheep: The sheep can be captured by driving the flock into a small pen or enclosure and then approach an individual animal slowly and swing your arm around the neck and front quarters and quickly wrap your other hand around the hindquarters or grasp the tail if present.

Shepherds crook: Hook a rear leg at the hock, quickly immobilizes sheep as above.

Halters: Can be used but remember sheep have a short nose and should be careful not to block the nostrils.

Restraint of goat: Goats do not tolerate rough treatment and will struggle violently if not properly handled. Therefore, restraint time should be as short as it is necessarily possible [18].

Walls and fences: Push the goat against the fence or wall with your legs and hip and leave your hands free for other

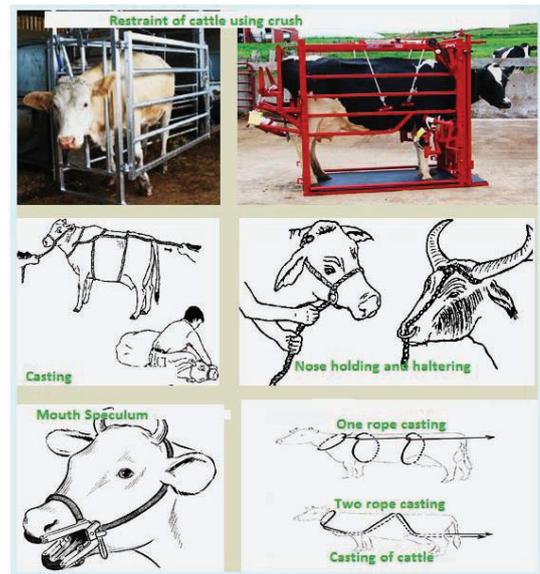


Figure 1.2: The methods of restraint of the cattle.



Figure 1.3: Restraint methods in sheep and Goat.

procedures. You can also push the hindquarters against a wall and then put your hand around the neck to keep it still for temperature taking or injection.

Restraint on lateral recumbence: The goat is placed parallel to your legs; the jaws are grasped with one hand while the inside rear leg is grasped with the other hand. Bring the leg forwards. The goat will be thrown off balance and fall on the ground.

Restraint of the Head: This is best when the goat is pushed in a corner and the body held against a wall. *Procedure:* Grasp the beard (if bearded) with one hand and encircle the neck with the other arm to stabilize the head.

Use of horns: This should only be for a short time as goats react violently when horns are held. Moreover, horns should not be used in very old goats as they break easily.

Cheek hold: Place one hand on either side of the cheeks

and wrap fingers around the mandibles to hold firmly. You can examine the eyes or take blood from the jugular.

Collars: Leather collar or neck chain can be used in dairy goats to lead or restrain them. They may be temporary or permanent (collars). Neck chains should be made of small, flat links, which don't catch easily as the goat rubs against a fence.

Restraint of Pet animals

Cats have always relied on speed, agility, caution, needle sharp teeth and dagger-like claws for survival. Therefore, they should be approached in a feeble manner. Restraint techniques of cat are as following [10,13]:

Leg restraint: Always place an index finger or middle finger between two legs. This provides a better grip to prevent escape.

Head – Mandible hold: The palm of the hand is placed under the cat's chin and the fingers are used to grasp mandible.

Scruff of the neck: Hold as much of the loose skin on the back of the cat's neck as possible. This prevents the cat from turning its head to bite.

Restraint with towel: Cover the head with a towel or cover the entire cat; then lift it up, isolate the needed part and uncover it. This is good for injection or examination.

Restraint bag: Feline restraint bag (cat bag) is normally used to restrict the movement of the cat and also to protect the handler from scratches.

Restraint with the adhesive tape: Apply adhesive tape around the legs starting with the hind legs then followed by the forelegs.

Gauze muzzle: They can be commercially available (leather muzzles) or home made using gauze.

Procedure of gauze muzzle: Make an over hand knot in the middle of the gauze to form a loop. Next, lower the loop over the cat's muzzle and tighten it on both ends of the gauze. Bring the gauze under the jaws, which are tied together under the mandible using an overhand knot with both ends brought behind the ears.

In restraint of dog the following devices prevent injury when used correctly and judiciously [10]: (Figure 1.4)

Muzzles: Commercial muzzles are made of leather, wire or fabric. There are also gauze or nylon rope muzzles.

Gauze or Rope Muzzles: For dogs with long noses, you do not need to pass the passive end between the eyes to secure the loop over the nose before tying the bow gauze after passing behind the ears.

Blanket and towels: These are used to remove unfriendly dogs from cages.

Restraint of poultry and birds

Free movement of the sternum is essential for respiration

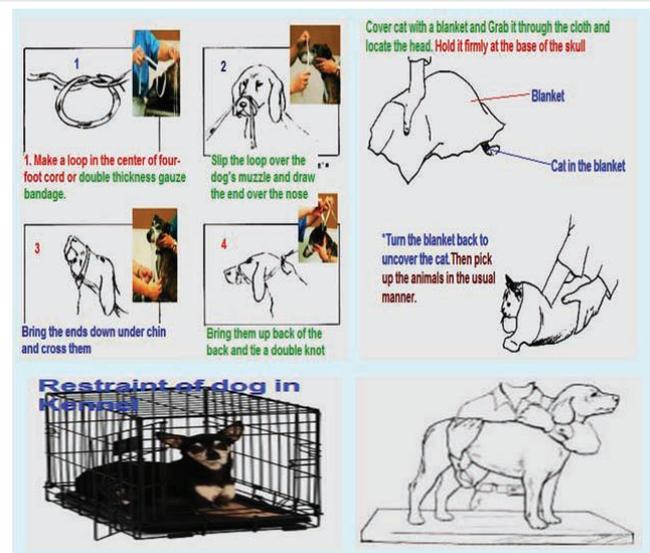


Figure 1.4: Restraint methods of Dog and cat.

in birds. They possess no diaphragm and their lungs do not expand and contract. They breathe through expansion and contraction of their air sacs facilitated by their intercostal muscles. Thus, any undue pressure on their sternum would restrict breathing. More than likely they had closed their hand around the chest inhibiting respiration. When handling a bird, fingers should never be closed around the chest, rather cupped in the hand to allow for sternal movement. During handling carefully monitor the bird for any signs of discomfort, stress or breathing difficulty. Due to struggling, the bird could contort and twist in such a way to constrict the air passages. Also during restraint, efforts to escape can lead to hyperthermia, especially if in a towel, so be alert if the bird begins to pant heavily [10].

There are various methods how to restraint poultry, the following lists are the common restraint methods during holding of poultry:

- * Use slow, steady movements with minimal noise
- * Catch individual birds by grasping both legs, just above the feet
- * Support the bird's body
- * Avoid too much pressure on thorax and compromising the ability to breathe
- * Carry the bird upright when possible
- * Place your arm over the bird's wings to minimize flapping
- * Extend the head between the index and middle finger,
- * Grasp the head with the thumb and index finger on either side of the head at the temporo-mandibular joint,
- * Using three fingers, place the thumb and middle finger just below the eyes and the index finger over the head, called the 'helmet grip,'

- * Crook the index finger behind the back of the head and gently place the thumb behind the lower mandible,
- * Gently circling the neck with the thumb and index finger as a tubular restraint collar would (Figure 1.5).

Chemical restraint

Chemical restraint may be defined as the use of drugs to bring about sedation or neuroleptosis, neuroleptanalgesia and anesthesia or short duration general anesthesia. This is a reversible process whose purpose is to produce convenient, safe, effective and inexpensive means of restraint so that clinical procedures may be carried out with minimum of stress, pain, discomfort, and toxic effects to the patient, the anesthetist or the clinician [13]. Drugs that is useful for this purpose includes: acepromazine, acetylpromazine, chlorpromazine, promazine and trimeprazine; members of this group can be used in most species of animals, butorphanol, hloral hydrat [8,13].

Verbal/moral restraint

The verbal methods of restraint is practiced by owner, the owner can restrain the animals by calling name of animals, feed provision and massaging of animals. When the horse is alert, the ears are flicked forwards and the horse is usually curious of one's approach. A nervous horse will continuously flick the ears back and forth especially when there is activity behind it while an angry or fearful horse will pull its ears backwards. This should however not be confused with laid-back ears where

a horse is concentrating on a difficult task such as calf roping or barrel racing. The tail always indicates the horse's attitude. A wringing or circling restraint of domestic, laboratory and wild animal's tail is an indication of nervousness. A tail held straight down indicates pain or sleeping, while a tail clamped tight indicates fear. Nevertheless, each horse is an individual and should be treated accordingly. Horses can be calmed by an even tone of voice and are most cooperative when handled quietly and decisively. They can also, be easily "bribed" with a handful of delicious foods like oats, carrots etc. Scratching behind ears, eye ridges and the neck will convince the horse you are friendly [5,10].

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Figure 1.5: Restraint methods how to holding domestic and wild birds.



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Research Article

Physical Examination

Physical examination is the methods of examination by means of applying general inspection, palpation, percussion and auscultation of animals to detect clinical signs of patient animals. General inspection is done some distance away from the animal; sometimes go round the animal or herd/flock, in order to get the general impression about the case¹. Attention should be compensated to the following items: behavior, appetite, defecation, urination, pasture, gait, body condition, body conformation, and lesions on outer surface of the body.

Palpation

Palpation is used to detect the presence of pain in a tissue by noting increased sensitivity and use fingers, palm, back of the hand, and fist, in order to get the information about the variation in size, shape, consistency and temperature of body parts and lesions, e.g., the superficial lymph nodes. The terms, which can be used to describe the consistency of parts during palpation, are [2,3].

- * **Resilient:** When a structure quickly resumes its normal shape after the application of pressure has ceased (e.g., Normal rumen)
- * **Doughy:** When pressure causes pit ting as in edema
- * **Firm:** When the resistance to pressure is similar to that of the normal liver (neoplasia/tumor)
- * **Hard:** When the structure possesses bone-like consistency (Actinomycotic lesion)
- * **Fluctuating:** When a wave-like movement is produced in a structure by the application of alternate pressure (hernia, hemorrhage/hematoma)
- * **Emphysematous:** When the structure is swollen and yields on pressure with the production of a crepitating or crackling sound (Table 1).

Percussion

Percussion is the methods of examination in which part of body to be examined is struck with sharp blow using fingertips to produce audible sound. Sound thus emitted will indicate the nature of the tissue / organ involved for example rumen when bloated will emit drum like sound. Some of the organs that can be examined by percussion are: gastro-intestinal tract, abdomen and thorax, frontal and nasal sinuses. The objectives of percussion are to obtain information about the condition of the surrounding tissues and, more particularly, the deeper lying parts. Percussion can examine the area of the subcutaneous emphysema, lungs, rumen and rump. Sounds produced from various structures can be described as following list [2,4,5].

- * Dull / flat: sound without resonance or echo, this type of sound can be heard on percussion of thick muscles or bone.
- * Full sound: sound heard is with resonance but not booming like drum. This type of sound is heard from tissues like lungs that contain air inside.
- * Tympanic sound: drum like sound can be heard, and this type of sound is heard from bloated rumen, abomasums and intestine.

Types of percussion

Immediate percussion: Using fingers or hammer directly strike the parts being examined.

Mediate percussion: Finger-finger percussion; Pleximeter-

Table 1: The structures that can be palpated and what they are palpated for.

Organs	Palpated For
Superficial Arteries	Pulse
Uterus	Pregnancy
Muscles	Pain/Swelling
Bone	Fracture/Growth
Stomach, Intestine	Pain
Esophagus	Obstruction
Rumen	Bloat, Impaction

hammer percussion. The quality of the sounds produced by percussion is classified as ⁴:

- **Resonant:** This is characteristic of the sound emitted by air containing organs, such as the lungs.
- **Tympanic:** The sound produced by striking a hollow organ containing gas under pressure, (tympanitic rumen or caecum).
- **Dull:** Sound emitted by a solid organ like the liver or heart.

Ballottement percussion: Tactile percussion or ballottement: is method in which palpation and percussion are combined together to feel structures that cannot be felt by either of these methods applied singly. This is normally used for pregnancy diagnosis in cows when the foetus cannot be palpated through per rectum. Here a firm–pushing stroke is applied on to the uterus and the hand after pushing is kept in contact with uterus so that the foetus will bound and strike on it. While firm pushing is done, this sets fluid in uterus into motion and foetus is made to bounce ². This modified percussion is used to detect late pregnancy in small ruminants, dogs and cats. And also, used for detection rebound of floated material shows pregnancy. Procedure: Apply a firm and interrupted push on the uterine region of the abdomen of small ruminants.

Fluid percussion: Used to detect fluid in the abdomen

Procedure: Apply a push on one side of the abdomen, percussion on the other side. The presence of wave–like fluid movement shows accumulation of fluid in the abdomen, ascites [6,7].

Auscultation

Word auscultation comes from ‘auscultona’ meaning ‘to listen’. This is a technique of listening to the sounds produced from organs in the abdominal and thoracic cavities. In olden days listening to these sounds were done with naked ears. This had certain limitations like the skin on animal being dirty and infested by parasites it was not healthy for the clinicians and was difficult to keep ears in contact on animal body due to constant movement. Therefore, an instrument was later developed for this purpose and this is called stethoscope [4,6] (Figure 1). The main objective of auscultation is that to listen the sounds produced by the functional activity of an organ located within a part of the body. This method used to examine the lung, trachea, heart and certain parts of the alimentary tract.

Types of auscultation

Direct auscultation: Spread a piece of cloth on the part to be examined using two hands to fix the cloth and keep your ears close to the body, then listen directly.

Indirect auscultation: Fix the probe of the stethoscope firmly on the part of the body to be examined and listen to the sounds produced by the functional activities of the body carefully.

Steps in auscultation: Place the ear piece into the ears, hold the chest piece and give a gentle tap on diaphragm, if no sound is heard adjust it by holding rubber tube with one hand and turning the chest piece with the other until there is ‘click’ sound. Tap again there should be amplified sound heard. Place the chest piece over the desired area and listen to the sound hear or lungs accordingly. Areas for listening to heart and lungs sounds are shown below, for rumen left flank region can be used [6] (Figure 1).

Succussion (shaking)

Succussion is the methods which used to determine the presence of fluid in the body cavities (thoracic and abdominal cavity). It is applied by shaking animals from side to side to set fluid into motions, and then the audible fluid sound is produced. Succussion method is only common in examination of small animals, but it is difficult in large animals [6].

Clinical Examination of the Patient

Physical examination can be carried out by taking vital sign such as; Temperature taking, Pulse taking, Respiration taking, Capillary Refill Time (CRT), Physical body condition, Normal demeanor, Abnormal demeanor [6,8].

Temperature taking

Temperature is the measures of how hot or cold the animal body is. Temperature can be measured by thermometer such as: digital and manual or mercury thermometer (Figure 2). On the basis of the ability to regulate body temperature animals are divided into two groups via homeotherms and poikilotherms. Homeotherms are those animals including man that can regulate their temperature in relation to the environmental temperature. Poikilotherms are those animals that are unable to regulate their body temperature in relation to the environmental temperature (Amphibians, Reptiles and fishes). Heat is generated in the body via the intracellular oxidation of food and the muscular activities. It is lost via the physical process of conduction, convection, and radiation and through the evaporation, respiration and excretion [2].

The body temperature is taken using a mercury or digital electronic thermometer placed carefully into the rectum. The

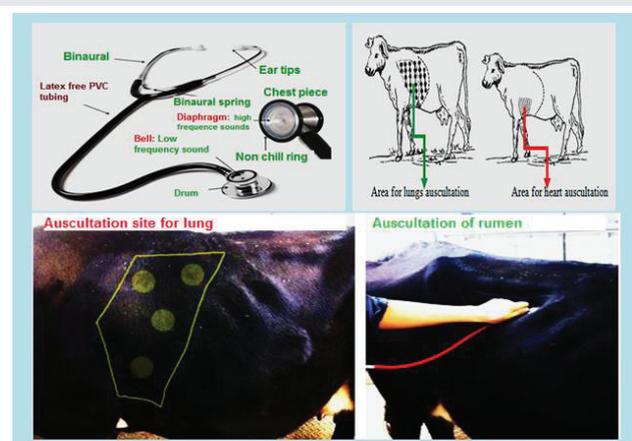


Figure 1: Part of stethoscope and site auscultation for lungs and heart.

thermometer should be lubricated before insertion and checked (in the case of a mercury thermometer) to ensure that the mercury column has been shaken down before use. It should be held whilst it is in the rectum. Sudden antiperistalsis movements in the rectum may pull the thermometer out of reach towards the colon. The thermometer is left in position for at least 30 seconds; the clinician should ensure the instrument is in contact with the rectal mucosa, especially if a lower than expected reading is obtained. The thermometer must be cleaned after removal from the patient. It must not be wiped clean on the patient's coat. If the animal's temperature is higher or lower than anticipated it should be checked again [2,6] (Figure 2).

Procedure: How to take temperature and how to recording temperature [9,10]:

- * The common sites for temperature taking are from rectum and vagina (approximately 0.5 degree centigrade higher in vagina).
- * The thermometer should be sterilized by disinfectant (antiseptics) before use.
- * It should be well shaken before recording of temperature to bring the mercury column below the lowest point likely to be observed in different species of animals. If the reading is not below 36°C, shake the mercury down to the bulb. Use flicking motions, taking care not to hit the thermometer on anything.
- * The bulb end of the thermometer should be lubricated with liquid paraffin or glycerin or soap especially in case of small pup and kitten.
- * Insert the thermometer in a rotational way and gentle manner. Care should be taken so that the bulb of the thermometer remains in contact with the rectal mucous membrane.
- * The thermometer should be kept in site for at least 3-5 minutes.

- * Pull out the thermometer, clean it and read the number.
- * Put a halter or head collar on the horse or donkey and have an assistant hold the head.
- * Read the value to define and explain a state of fever, hypothermia, and febrile or non-febrile animals.
- * The procedure for digital thermometer is different from that of the manual one, in digital thermometer just insertion of thermometer in to rectum of animals and after the time is reached the digital thermometer may give some sound. After that, the clinician should remove and interpret it for examination.

Method of recording temperature:

- * Keep the bulb of the thermometer immersed in the antiseptic solution for sterilization.
- * Bring down the column of the mercury before recording the temperature by shaking.
- * Lubricate the bulb with liquid paraffin or soap and water, when the thermometer is to be used in pup or kitten.
- * Insert the bulb of the thermometer into the rectum and tilt to one side so that the bulb of the thermometer touches the mucous membrane of the rectum.
- * Keep the thermometer in this position for one minute.
- * Take it out, wipe the faeces with cotton and read the temperature directly
- * Roll it until you can see a broad silver band of mercury (Figure 1.9).

Interpretation of thermometer: Thermometer reading will reveal if the temperature of animal being examined is normal (Table 2), above normal (fever) or below normal (subnormal). Based on this finding action taken will vary.

Fever: denotes the elevation of body temperature of animal above normal. It is a general reaction of animal and human

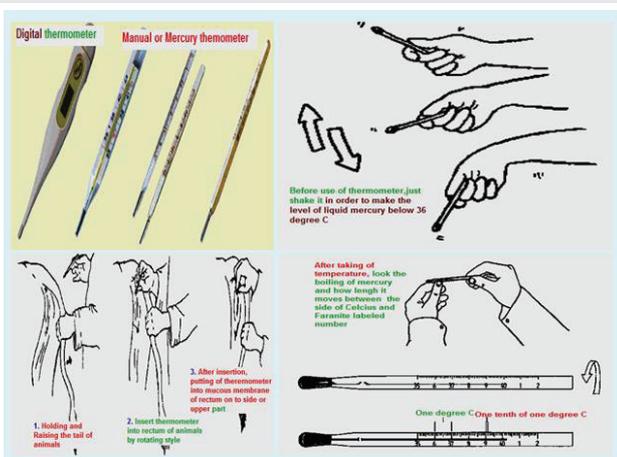


Figure 2: Types of theermometer and the procedure how to take rectal temperature in an animals. If the reading is not below 36oC, shake the mercury down to the bulb. Use flicking motions, taking care not to hit the themometer on anything. Take the themometer out of its case and hold it between th thumb and forefinger [12].

Table 2: Normal range rectal temperature of domestic animals.

Animal species	Temperature °C
Cattle/ Adult	37.8 –39.2
Calve	38.5 –39.8
Horse/Adult	37.2 - 38
Foal	37.5 -38.5
Sheep	38.9 - 40
Goat	38.6 –40.2
Pig/Adult	37.8- 38.9
Piglet	38.9 - 40
Small Dog	38.6 -39.2
Large Dog	37.5 –38.6
Cat	37.8 –39.2
Chicken	41.7

body to the action of infectious agents like bacteria, virus, parasites and exogenous substances like bacterial toxins. Sign of fevers are Animal will refuse to eat either completely or partially (anorexia), hair on the body might be seen standing up, dullness, and dry muzzle. Fever management: There are preparations to reduce temperature. Preparations like paracetamol, phenylbutazone is normally given to control fever (refer drug index for these preparations) in addition keeping animals in cool place [4].

Subnormal temperature / hypothermia: The temperature of animal drops below normal and this occurs when animals get exposed to extreme cold for example when a calf is exposed to heavy rain, when animal is in shock and a clinical condition like milk fever. Here the animal body is unable to regulate body temperature or the heat regulatory mechanism fails to generate heat to compensate the heat loss from the body.

Signs of hypothermia: Shivering, chattering of teeth, cold extremities and skin on touch, and reduced pulse and respiratory rates are observed.

Hypothermia management: Place the affected animal in warm place or provide shelter to protect from rain, rub extremities and apply liniments if available, provide warm porridge if animal has appetite, inject warm DNS / NS, inject calcium preparations in the case of milk fever the temperature will automatically rise.

Pulse taking

Pulse is defined as the regular expansion and contraction of the arterial wall caused by the flow of blood through it at every heartbeat. Pulse gives information with regard to the cardiovascular abnormalities.

It is influenced by exercise, excitement, annoyance, relative humidity, environmental temperature. Pulse can be adapted from the number of heart beats per minute by using stethoscope in less manageable animals. The rhythm of pulse should also be noticed while taking pulse. The pulse rate can rise rapidly in nervous animals or those which have undergone strenuous exercise. In such cases the pulse should be checked again after a period of rest lasting 5 to 10 minutes [4,6].

Procedures how to take pulse rate of domestic animals:

- ▶ Place the digits of fingers on the artery of animals. The anatomical location for arteries of domestic animal is detailed in table 3.
- ▶ Place the tip of the index / middle finger on the artery and applying gentle pressure until the pulse wave can be detected (Figure 3).
- ▶ Count the numbers of beats per minute, which mean count up to 15 minute and multiply by four, notice the quality and rhythm of pulse.
- ▶ The healthy animals may show the result as listed in table 4.

Note the pressure or pulsation of the arteries felt on the finger digits (Figure 3). It is useful to be able to find out how fast the heart is beating. For example, it can help you decide

Table 3: Site of pulse taking in domestic animals.

Animals	Site of pulse taking
Equine	External maxillary artery Transverse facial artery Median artery Great metatarsal artery
Cattle and pig	Middle coccygeal artery Facial artery Median artery
Sheep, Goat, Calf	Femoral artery.
Piglet, Dog and Cat	Femoral artery.

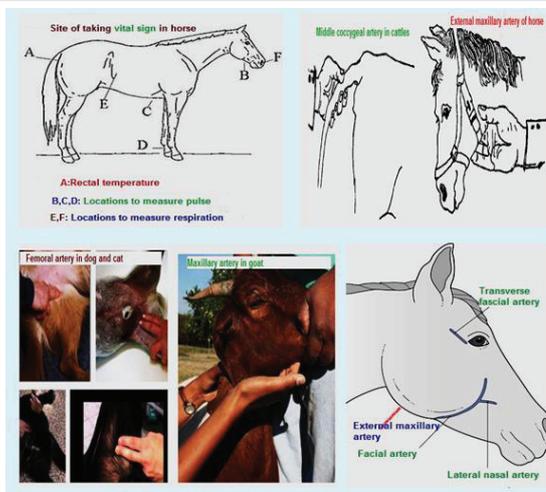


Figure 3: Anatomical location for pulse taking in domestic animals.

Table 4: Normal range of pulse rate in animals.

Animal species	Pulse rate/minute
Cattle	60-90
Young calves	100-120
Horse	28-42
Foal up to 1 year	70-80
Sheep	68 - 90
Goat	68 - 90
Pig	60 - 90
Dog	90 - 130
Cat	110 - 130
Chicken	200 - 400

whether colic is serious. An adult horse's heart beats more slowly than ours, especially when the horse is fit. It takes practice to find the pulse. There are several places where it can be felt. Using a watch with a second hand, count how many beats can be felt in a minute. Feel for it under the bone (mandible) at the side of the face [9]. Or feel for it behind the fetlock joint. Feel for it just above the hoof on the inside of the leg. It is useful to practice finding the pulse here because, if the horse has laminitis, this pulse will feel stronger. If you know what the pulse normally feels like here, it will help you recognize when it is different [10].

Factors influencing pulse

- * Species: Different species of animal have different pulse rate, which is number of rise and fall of arterial wall per minute.
- * Size: Higher in small than in large animals.
- * Age: Higher in young than adult animals.
- * Sex: Male slightly lower than female animal.
- * Parturition & Late stage of pregnancy: Relatively more pulse rate
- * Exercise: Increase pulse rate.
- * Ingestion of food: Cause momentary increase in frequency of pulse.
- * Posture: Pulse rate reduced about 10% when animal is recumbent than when standing [11,12].

Respiration taking

Respiratory movements can be observed at the right flank. Any change in the rate indicates respiratory involvement. Thoracic respiration is seen in animals suffering from acute peritonitis and abdominal respiration in pleurisy. Double expiratory movements are seen in emphysema in horses [3,4].

Types of respiration:

Costal respiration: In this type of respiration thoracic muscles are mainly involved and the movement of the rib cage is more prominent. It is seen in dogs and cats.

Abdominal respiration: This type of respiration is seen in ruminants such as; cattle, goat, sheep and yak. Here the abdominal muscles are involved and movement of the abdominal wall is noticed.

Costo- abdominal respiration: In this type of respiration muscles of both thorax and abdomen are involved so the movement of the ribs and the abdominal wall are noticed. The respiration rate is measured through counting of either contraction or expansion of the thorax and abdomen which can be observed during clinical examination. A method for respiration rate taking includes [6,13].

Inspection: Stand behind and to one side of the animal, and observe the movement of the thoracic and abdominal areas of the body.

Palpation: Put one hand in front of the nostril, feel the exchange of the gas; or put one hand on the lung area or the thorax and feel the respiratory movements.

Auscultation: Use stethoscope, listen to the respiration sound in the trachea or lung area.

Inspiratory or expiratory movements of the chest wall or flank can be counted. In cold weather, exhaled breaths can be counted. If the animal is restless the clinician should count the rate of breathing for a shorter period and use simple multiplication to calculate the respiratory rate in breaths/minute.

The calculation of breathing rate should be conducted by counting inspiration and expiration by looking flank movement or by placing stereoscope for 15 minute and multiply it by 4. After breathing rate taking is finished, the result should be as table 5 for normal animals. However if the abnormality is presented, the result may either greater or lower than normal breathing rate. Mouth breathing is abnormal in cattle and is usually an indication of very poor lung function or a failing circulation.

Visible mucous membrane

The mucous membrane in the eyes, mouth and vagina in the case of females can be examined to determine the health status of an animal. Examination of the mucous membrane should be done in natural light (sunlight) not in the lamplight. The abnormalities of color of mucous membrane are caused by different factors. Some of abnormalities which observed in mucous membranes can be classified to: pallor of the mucous membranes may indicate anaemia caused by direct blood loss or by haemolysis, a blue tinge may indicate cyanosis caused by insufficient oxygen in the blood, a yellow color is a sign of jaundice, the mucosae may be bright red (sometimes described as being 'injected mucous membranes') in febrile animals with septicaemia or viraemia, Bright red coloration of the conjunctiva is often seen, for example, in cases of bovine respiratory syncytial virus infection. A cherry-red coloration may be a feature of carbon monoxide poisoning. A greyish tinge in the mucosae may be seen in some cases of toxemia – such membranes are sometimes said to be 'dirty'. High levels of methaemoglobin, seen in cases of nitrate and/or nitrite poisoning, may cause the mucosae to be brown colored [4,6]. The normal color of different species of animal is listed in table 6.

The color of mucous membrane may change occurs in

Table 5: The respiratory rate of domestic animal per minute.

Species of animals	Respiration rate/minute
Cattle	25 - 30
Horse	8 - 16
Sheep	10 - 20
Goat	10 - 20
Pig	10 - 20
Dog	16 - 30
Cat	20 - 30
Chicken	15 - 30

various diseases as follow by the following [14]:

► **Anaemic mucous membrane.**

- ✓ Blood loss anaemia.
- ✓ Parasitic infestations leading to haemolysis.
- ✓ Tumours or leucosis.
- ✓ Iron deficiency anemia.
- ✓ Long-standing infectious diseases.
- ✓ Exposure to X-rays and some medications.

► **Congested mucous membrane.**

- ✓ High environmental temperatures and exercise.
- ✓ Any disease resulting in fever.
- ✓ Diseases of the heart, brain and its membranes.

✓ Hyperthermia

✓ Conjunctivitis

✓ Trauma

✓ Obstruction of jugular V

► **Yellowish or icteric mucous membrane.**

- ✓ Icterus of jaundice occurs due to increase of blood bilirubin concentration (blood parasites, leptospirosis, hepatitis, cholangitis, cholecystitis and cholangiohepatitis).
- ✓ Infectious anaemia and contagious pleuropneumonia of horses.

► **Chronic gastric dilatation.**

Disease like: Liver diseases, Fascioliasis, Hemolytic anemia, Hypophosphatemia.

- ✓ Cyanosed mucous membrane.
- ✓ Bluish discoloration of visible mucous membranes resulting from presence of reduced haemoglobin in blood capillaries.
- ✓ Carbon monoxide poisoning respiratory diseases
- ✓ Myocarditis, pericarditis.
- ✓ Plant and mineral intoxications.

Swelling of mucous membrane: Inflammation of mucous

membranes results in its swelling; in which case the mucous membranes may also be hot and tender (i.e. showing cardinal signs of inflammation). Marked swelling of conjunctival mucous membranes is characteristic of equine influenza. A slight degree of swelling is noticed in contagious pleuropneumonia of horse and cattle plague, anthrax and fowl diphtheria [14].

Capillary Refill Time (CRT)

Capillary refill time (CRT) is defined as “time required for return of color after application of blanching pressure to a distal capillary bed 15. This is taken by compressing the mucosa of the mouth or vulva to expel capillary blood, leaving a pale area, and recording how long it takes for the normal pink color to return. In healthy animals, the CRT should be less than 2 seconds. CRT of more than 5 seconds is abnormal, and between 2 and 5 seconds it may indicate a developing problem. An increase in CRT may indicate a poor or failing circulation causing reduced peripheral perfusion of the tissues by the blood [6,8].

Methods how to examine mucous membrane by capillary refill time as follow:

- ✓ This is taken by compressing the mucosa of the mouth or vulva to expel capillary blood, leaving a pale area
- ✓ Recording how long it takes for the normal pink color to return.
- ✓ In healthy animals, the CRT should be less than 2 seconds.
- ✓ A CRT of more than 5 seconds is abnormal, and between 2 and 5 seconds may indicate a developing problem.

Physical body condition

Body condition scoring is an important management practice used by producers as a tool to help optimize production, evaluate health, and assess nutritional status. Different scores can be given for individual animal and can further classified as normal, fatty, lean/thin, emaciation 6 (Figure 4).

Condition Score 1: Very thin: This animal’s skeletal structure is very prominent. Notice the deep depressions next to the spine, between the pelvis and rib cage, between the hooks and pins, and around the tail head.

Condition Score 2: Thin: The animal’s skeleton is still very apparent. The individual spinous processes are clearly visible, but there is a small amount of fat tissue over the spine, hooks, and pins.

Condition Score 3: Medium (Normal body condition): The animal appears smooth over the spine, ribs, and pelvis and the skeletal structure can be easily palpated. The hooks and pins are still discernible, with a moderate, rather deep depression between the pelvis and rib cage, hooks and pins, and around the tail-head.

Condition Score 4: Fat: There are no spinous processes detectable, and no depression in the loin area, which gives the top-line of the animal a flat, tabletop appearance. The ribs can no longer be felt, and the pelvis can only be felt with firm

Table 6: Normal color of mucous membrane of different animals.

Animal	Color of mucous membrane
Cattle, sheet and goat	Pale pink
Horse	Pale roseate
Pig	Reddish
Dog	Pale roseate
Cat	Pale pink

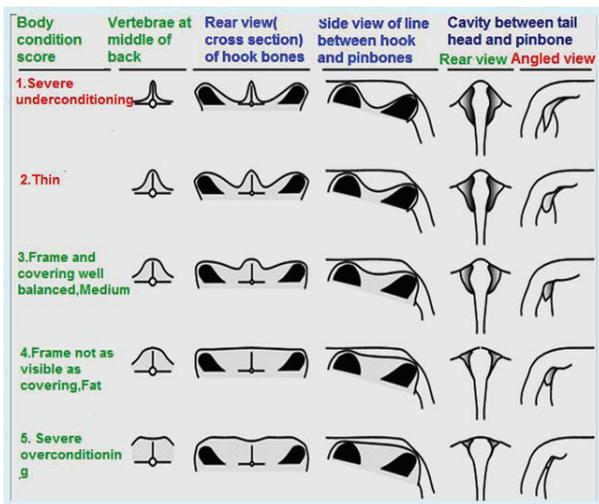


Figure 4: Body condition score of animals.

pressure. The hooks and pins have a rounded appearance due to areas of fat covering.

Condition Score 5: Very Fat: The animal appears rounded and smooth with a square-shaped appearance, because of the amount of fat filling in the loin. The skeletal structure is no longer visible, and can only be palpated with excessive pressure.

Normal demeanor: When, on being approached, an animal makes a normal response to external stimuli, such as movement and sound, the demeanor is said normal (bright). Normal reaction under these circumstances may consist of elevating the head and ears, turning towards and directing the attention at the source of stimuli, walking away and evincing signs of attack or flight [8].

Abnormal demeanor: Behavioral change/ response to external stimuli. The Abnormal demeanors in domestic animals are as follow list [6,8].

- ✓ Decreased response (depression): dull (apathetic); dummy state; comma.
- ✓ Excitation or increased response: apprehension (mildly anxious); restlessness; mania; frenzy.
- ✓ Posture: It denotes the anatomical configuration when they remain in stationary situation. How does it stand? How does it sit? How does it lie?

- ✓ Gait: It indicates about the locomotory process of an animal.
- ✓ Body conformation: shape and size of the different body regions relative to other regions.

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Research Article

Regional or systematic clinical examination

Clinical Examinations of the head and neck region

The clinical examination of head and neck can be undergone by further visual inspections and observations, before examination of head and neck the following question could be asked: [1]:

- * Movements of head and neck – normal or abnormal
- * Carriage of head – normal or tilted,
- * Can the animal see?
- * Can the animal hear?
- * Ocular or nasal discharge,
- * Salivation – normal or excessive,
- * Ability to prehend, masticate and swallow food
- * Mobility of the neck.

The perspective of this examination is to identify pale and discolored mucus membranes; assess problems of oral cavity and deranged appetite. The following points should be considered:

- * Visible mucous membrane
- * Eyelids, conjunctivae and eyes
- * Nasal regions and nasal mucous membrane
- * Prehension, mastication and deglutition
- * Salivation
- * Teeth eruption

Procedures

- * Visible mucous membrane examined by visual inspection to note the presence of lesions, discharge, glaucoma, nystagmus.
- * Examine the nose and nasal sinuses; lesions, discharges should be noted by percussion, palpation
- * Examine the mouth and appetite; oral lesions, salivation, feed intake should be noted. The rigidity of tetanus, the cunning leer or maniacal expression of rabies and acute lead poisoning are cases in point.

The symmetry and configuration of the bony structure should be examined. Doming of the forehead occurs in some cases of congenital hydrocephalus and in chondrodysplastic dwarfs, and in the latter there may be bilateral enlargement of the maxillae. Swelling of the maxillae and mandibles occurs in osteodystrophia fibrosa; in horses swelling of the facial bones is usually due to frontal sinusitis; in cattle enlargement of the maxilla or mandible is common in actinomycosis. Opisthotonos is an excitation phenomenon associated with tetanus, strychnin poisoning, acute lead poisoning, hypomagnesemic tetany, polioencephalomalacia and encephalitis ². Visible discharge should be noted; protrusion of the eyeball, as occurs in orbital lymphomatosis, and retraction of the bulb, as occurs commonly in dehydration, are important findings; spasm of the eyelids and excessive blinking usually indicate pain or peripheral nerve involvement; prolapse of the nictitating membrane usually characterizes central nervous system derangement, generally tetanus ². Excessive salivation or frothing at the mouth denotes painful conditions of the mouth or pharynx or is associated with tremor of the jaw muscles due to nervous involvement. Swellings below the jaw may be inflammatory, as in actinobacillosis and strangles, or edematous, as in acute anemia, protein starvation or congestive heart failure.

Examination of skin and appendages

The skin provides protection against minor physical injuries supports hair growth and offers some defence against microbial invasion. The condition of the skin is a reflection

of the general health of the animal, deteriorating in cases of ill health, ill thrift and debility. In some conditions, such as jaundice, the skin may provide through discolouration direct diagnostic evidence of a specific disease process. In other conditions, such as parasitism or severe mineral deficiency, a nonspecific general deterioration of skin health may occur causing a greater number of hairs than normal to enter the telogen or resting phase and a delay in their replacement, leaving the coat in poor condition with little hair [1].

Sebaceous secretions may be reduced, allowing the skin to become abnormally dry and inflexible and less able to perform its normal defence role in an already debilitated animal. In other cases, sebaceous secretion increases causing the skin to have either a greasy or a dry seborrhoeic, flaky Appearance [1]. Structures or parts associated with skin as its appendages are hoofs, hairs, horns, quills, claws, nails, sebaceous glands and sweat glands. Discrete skin lesions range in type from urticarial plaques to the circumscribed scabs of ringworm, pox and impetigo. Diffuse lesions include the obvious enlargements due to subcutaneous edema, hemorrhage and emphysema. Enlargements of lymph nodes and lymphatics are also evident when examining an animal from a distance [2,3].

The perspective point to assessing the condition of skin and coat to identify clinical signs of skin lesions such as:

- * Condition of the coat
- * Elasticity of the coat
- * Pruritus
- * Primary and secondary skin lesions
- * Dermatitis
- * Hyperkeratosis or parakeratosis
- * Presence of ectoparasites.

Discoloration of the skin may arise due to anaemia, cyanosis, jaundice, hyperaemia. In febrile conditions hair may be erect and in all chronic diseases with disturbances in nutrition, hair become rough, lusterless dry and coarse. Alopecia (Loss of hair) may occur due to diseases like ring worm, scabies, eczema, iodine and Vit A deficiencies, Procedure: Examine the skin and coat: grasp the skin of the upper part of the body and notice the elasticity, visual inspection of the condition of the coat and presence of skin lesions should be noted. The abnormalities which occurred from skin due to disease and others are summarized in table 1. The disease of skin can be divided into two lesions, primary and secondary. As it is described in table 1 the following is the most common caused by diseases:

Primary lesion

- * Macule: cause by early stage of pox –Tick bites.
- * Vesicle is caused by: Vesicular stomatitis or Foot and mouth disease
- * Pustules: caused by pastular dermatitis

- * Wheals: cause by urticarial, allergic conditions
- * Papulae: caused by eczema, pox

Secondary skin lesion

- * *Dandruff or scales* or pityriasis cause by: Mange, Vit. A deficiency, Ringworm.
- * *Crust or scab* caused by: Pox, Dermatitis.
- * *Erosion or ulcers* caused by: Foot and mouth disease, Ulcerative dermatitis, Burns, uncreative lymphangite, epizootic lymphangite.

Examinations of the thoracic cavity

Examination of the thoracic cavity guides to show the regional anatomy of the lungs and the heart, and perform physical examination of the lung and the heart area. Regional anatomy of the lungs –locate the lung area. The lung is located on the external surface of the thoracic region by forming an imaginary triangle by using the points at the angle of the scapula, olicranun process and the second intercostals space from the last [1,4].

Physical examination of the thorax (lung area)

- Inspection –note respiratory movements
- Palpation –check the presence of pain by applying pressure
- Percussion –notice resonant sound
- Auscultation –note bronchial sounds (trachea and anterior part of the lungs) and alveolar sounds

Palpation

Chest palpation can be useful to identify thoracic pain which may be caused by rib fractures and pleuritis. Gentle pressure should be applied to the thorax using the palm of the hand and the animal observed for a pain response. The entire thorax should be explored in a systematic manner to identify focal areas of pain. In addition to pain, subcutaneous emphysema may be detected as a spongy sensation which may be accompanied by crackling noises. This clinical sign is sometimes seen in outbreaks of respiratory syncytial virus and is caused by rupture of emphysematous bullae in the lungs [1].

Table 1: The pathological lesion of skin in domestic animals and its causative agents.

Primary skin lesions	Description	Causative agents	Secondary skin lesions	Description
Spot	Circumscribed red area of skin which is not elevated or swollen	Flea bites	Erosion	Destructive separation of the epidermis (contagious pustular dermatitis of sheep and goat).
Papule	Firm elevation of skin surface caused by cellular infiltration	Pox	Ulcer	Circumscribed shallow destructive area of tissue with or without elevated edges (equine ulcerative lymphangitis).
Vesicle	Elevation of superficial layer of skin with accumulation of serous fluid beneath it.	FMD, vascular stomatitis, pox	Crust	A firm mass consisting of dried inflammatory exudate or blood.
Blister	A large vesicle	?	Dandruff	Secondary to ectoparasites
Pustule	A pus-filled vesicle	?	?	

Auscultation

During auscultation, the stethoscope should be moved systematically to cover the whole of thoracic lung fields with the aim of identifying any abnormal sounds present, their location and their occurrence in relation to the respiratory cycle. The location of an abnormal sound is deduced from the position of maximal intensity. Particular attention should be given to the apical lobe if bacterial pneumonia is suspected or the diaphragmatic lobe if lungworm is suspected [1,2].

Abnormal sound and its causative which is heard from auscultation of lung are listed as (www.bu.edu.eg):

- ✓ Moist rales: pulmonary edema, bronchopneumonia, chronic bronchitis and pulmonary hemorrhage.
- ✓ Dry rales: Spasm in bronchial muscle, bronchitis, early stage of pneumonia
- ✓ Crepitate rales: it is rise from; Bronchiolitis, interstitial pneumonia.
- ✓ Emphysematous sound: pulmonary emphysema, pneumothorax.
- ✓ Frictional sound: pleurisy, pericarditis.
- ✓ Absence (dull) sound: Tumor, TB, consolidation of lung, lung collapse.
- ✓ Grunting sound: It is forced expiration associated with closed epiglottis and usually associated with painful conditions either in thoracic or in the abdominal cavity.

Pulmonary grunting sound are like: pleurisy, traumatic pericarditis, sever pneumonia and pulmonary emphysema.

Abdominal grunting sounds are like: Sever pain in Vaginitis, Urethritis, Urolithiasis, Cystitis, Nephritis, Pyometria, Peritonitis, Traumatic reticuloperitonitis, Bloat and Impaction

Abnormalities associated with respiratory system:

Epistaxis: It is bleeding from nostril of animals; it is caused by some disease like: Trauma, Pulmonary hemorrhage, Rhinitis, Anthrax and High blood pressure

Sneezing: It is caused by irritation or some response occur in mucous membrane of nose. It can occurred when rhinitis is occurred, aspiration pneumonia and in halation of irritant smoke.

Nasal discharge: When serous or some discharge is coming out of nostril in some cases. The discharge can be Serous (in early stage of diseases or mucoid in late stage or after secondary bacterial infection). The discharge may contain gas bubbles, Copious (acute diseases or scanty in chronic form of disease). Tinged with blood -With bad odor (infection or in gangrenous pneumonia). Unilateral (in unilateral affection of upper respiratory tract) or bilateral in affection of lower respiratory tract. The causes of nasal discharge may be: Bacterial pneumonia, chronic bronchitis, IBR (Infectious

Bovine Rhinotracheatitis), Gangrenous pneumonia, Rhinitis Pyogenic infection of Paranasal sinuses.

Dyspnea: difficulty to breath, it is characterized by mouth breath, dilated nostrils, pumped anus, abnormal respiratory rhythm, extension of head and neck, cyanosis of mucous membrane. The major causes of dyspnea are like: Stenosis of upper respiratory tract, Bronchopneumonia, Bronchitis, Impaction, Pulmonary odema and congestion, Traumatic pericarditis

Coughing: Coughing may occurred by irritation around larynx and respiratory tract. It is cause by Bronchitis, Parasitic pneumonia, interstitial pneumonia (viral pneumonia), Pericarditis, Pneumonia (bacterial pneumonia), and Pleurisy.

Regional anatomy of the heart

The heart is suspended by great vessels and located on the left median mediastinum of ventral thorax. The left side of the heart apex reaches the chest wall. The percussion examination in all animals is the same in cranial and dorsal border.

Cranial limit: Cranial limit is the caudal border of the triceps, from the caudal angle of the scapula to the olecranon of the radius bone in standing animals.

Dorsal border: Dorsal border is on the line, which runs from caudal angle of the scapula to the tuber coxae of os coxae bone.

Basal border: Basal border is from olecranon of the radius to the last intercostal space at the dorsal border. The basal border line is straight in cattle but is concave in horse and dog.

In case of ox, the basal border of the lungs runs in almost straight line from the sterna end of the 6th rib to 11th intercostals space at dorsal border. In horse, the basal border of the lungs runs in a strong curve from the sterna end of the 6th rib through the middle of the 11th or 12th intercostals space and onto the vertebral end of the 16th intercostals space. In dog, the basal border of the lungs runs in a strong curve from the sterna end of the 6th rib to the 11th intercostals space.

Topographic location of the heart

Ox: The heart extends from 3rd to the 5th or 6th rib. Five seventh of the heart lies to the left of the median plane and it is in contact with the left thoracic wall between the 4th and 5th intercostals spaces. Heart auscultation is restricted to a small area near the olecranon.

Horse: three fifth of the heart lies to the left of the median plane and in direct contact with the left thoracic wall in the region of 4th and 5th rib, auscultation and percussion can be done on the left side between the 3rd and 5th intercostals space.

Dog: about fourth seventh of the heart lies on the left side of the midline. It occupies the thoracic space between the 3rd and the 7th rib and between the sternum and half the height of thoracic cavity.

For clinical observation, it is necessary to pull forward the fore limb, by doing so the heart is felt in the lower third of

the chest between 4th and 6th ribs, especially the 5th intercostals space. And also anatomy location of heart is summarized in Table 2.

After locating the heart the following should be noted through physical examination (palpation, percussion, auscultation) the following would be examined [1,5,6] (Table 2).

- * Heart rate
- * Abnormal variation in heart rate
- * Heart sounds
- * Normal heart sounds (dub-lab)
- * Adventitious heart sounds (murmurs)
- * Pericardial frictional sounds
- * Venous pulsation (jugular pulsation)

Anatomical locations of valves⁵

- ✓ Mitral valves: Examined on the left side in the 5th intercostals space, 4 inches above the sternal extremity of 5th rib.
- ✓ Tricuspid valves: Present on the right side in the 3rd intercostals space and 3 inches above the sternal extremity of the 4th rib.
- ✓ Aortic semi-lunar valve: Present on the left side in the 4th intercostals space level with the shoulder point [5].

Examinations Digestive System

The clinical examination of digestive system is to check the normal motility and rate of abdominal normality in domestic animals⁹. The examination of digestive system of animals may start from mouth into rectum. The examination which applied over mouth, esophagus can show the manner of regurgitation, mastication, prehension and etc. Regurgitation in ruminat or other animals may rise from different abnormality like: Megaoesophegus, Oesophegitis, Pharyngeal paralysis, obstruction or pharyngitis and Hypomagnesaemia. The abnormality of prehension which mean when animals fail to take food by their mouth is caused by the following diseases:

- * Tetanus
- * Trauma in the jaw or mandible

- * Stomatitis
- * Glossitis
- * Foreign body in the mouth
- * Actinobacillosis
- * Actinomycosis
- * Foot and mouth disease

Pica is the most common abnormal behavior of animals, it is usually due to nutritional deficiencies and it may be manifested in the form of eating of abnormal or unusual diet of large ruminants, excessive licking or urine drinking. This abnormality may rise from:

- ✓ Rabies
- ✓ Actinomycosis
- ✓ Water deprivation
- ✓ Salt deficiency
- ✓ Lack of roughage
- ✓ Hypophosphatemia
- ✓ Ketosis (acetonemia)

Excessive salivation results either from excessive production of saliva, dysphagia or as a response of painful condition in the oral cavity. It is caused by:

- ✓ Hypomagnesaemia
- ✓ Esophageal obstruction
- ✓ Stomatitis
- ✓ Lead poisoning
- ✓ Foot and mouth diseases
- ✓ Pharyngeal paralysis or pharyngitis
- ✓ Calf diphtheria
- ✓ Esophageal obstruction
- ✓ Paralysis or esophegitis.

Swallowing or deglutition means that transportation of masticated food from the oral cavity to the stomach through the pharynx and esophagus. However, when animals fail to swallow clinically it is called as dysphagia, dysphagia can be caused by esophageal and pharyngeal disease (obstruction, inflammation and paralysis).

The examination of rumen, reticulum and omasum

Examination of rumen can be performed by inspection, palpation, percussion and auscultation; stomach tube can be used as well. In bloat case, the left side would be bulged, and

Table 2: Anatomical location heart in equines and ruminants [5].

Area	Equines	Ruminants
Base	From 2 nd to 6 th intercostal space	From 3 rd to 6 th rib
Apex	Half an inch from the last sternal	One inch from the diaphragm segment
Posterior border	Opposite to the 6 th rib	Opposite to the 5 th rib
Left surface	Composed of left ventricle and extends from 3 rd to 6 th rib	Extends from 3 rd to 4 th rib
Right surface	Extends from 3 rd to 4 th rib	Not examined

the motility would be decreased. The reticulum is a pouch-like structure in the forward area of the body cavity. It is the most cranially situated compartment of the stomach apposite to 6–8th intercostal spaces on the left side of the abdomen. It is known as honeycomb for it arranged in network resembling honeycomb. A small fold of tissue lies between the reticulum and the rumen, but the two are not actually separate compartments.

The reticulum lies close to the heart (<https://www.extension.umn.edu>). The reticulum is examined for the presence of foreign object lodge in honeycomb appearance tissue and the method is by backgrasp, below pole lifting, up and down hill leading, hand palpation. The examination of omasum and abomasums is done by exploratory puncture. Abomasum has 'U' shaped structure laying on the abdominal floor on the right side opposite to 7–9th ribs or intercostal spaces. Abomasum can produce sound like "tinkling or high pitched metallic sound" once per 15 minutes and can be stimulated by tactile percussion over the abdomen. Abomasal movement may be absent in case of abomasal displacement (right or left), Impaction, abomasal ulcers. The examination of the displacement of abomasums is done by inspection, palpation, and auscultation.

This disorder of stomach can be detected by taking stomach contents sample. Insert the stomach tube or nasal tube after cleaning, disinfecting and lubrication or trocarisation.

Palpation of rumen: When touching rumen with fist or hand, in normal animal it gives resilient palpation. However in abnormal case it produces the following:

- ✓ *Doughy palpation: impaction*
- ✓ *Emphysematous palpation: tempany*
- ✓ *Painful palpation: peritonitis, traumatic reticulitis*

Auscultation of rumen: It is the procedure by which checking the sound which produced from rumen by stethoscope. Normally "Gurgling or Booming" sound in the normal rate of 2–5 / 2 minutes, the sound occurred due to the movement of the fluid and food particles in the rumen. The abnormal auscultation of rumen reveals that, the sound which varies from normal it is in case of:

- ✓ Increased ruminal sound: Early stage of diarrhoea, early stage of tempany vagal indigestion and Esophageal obstruction.
- ✓ Decreased ruminal sound: Ketosis (acetonemia), Febrile condition, Vagal indigestion Mild impaction, Abomasal displacement, Simple indigestion and Traumatic reticulitis
- ✓ Absent of ruminal sound: Acute impaction, Severe tempany, Abomasal impaction, Diaphragmatic hernia, late stage of vagal indigestion, Ruminitis and Sugar beet arsenic or organophosphorous poisoning.

Ruminal atony and lack of rumination: It is a condition which characterized by lack of ruminal movement and lack of rumination together with decrease of food intake and decreases the amount of faecal matter output. It is caused by the following abnormality (<http://www.bu.edu.eg>):

- ✓ Acidosis or alkalosis,
- ✓ Vagal indigestion
- ✓ Abdominal pain
- ✓ Sudden change in the diet,
- ✓ Ruminal tempany and impaction
- ✓ Traumatic reticulitis
- ✓ Prolonged oral use of sulphonamide and antibiotics.

Causes of distended or increased sized abdomen:-

In examination of abdomen of animals, abnormal shaped abdomen in large ruminants may be either by increased sized (distended) or reduction in the size of the abdomen. This abnormality can be caused by:

- ✓ **Distention of abdomen or increased sized abdomen:** Abomasal displacement, Tempany, Ascitis or liver diseases, Impaction, Urine retention, Pyometria, Normal after heavy meal or in the late stage of pregnancy.
- ✓ **Decreased sized abdomen:** Pyelonephritis, Severe diarrhea, TB and liver abscess, Starvation and malnutrition or deficiencies

Rectal examination of internal abdominal structures

The internal abdomen like intestine and some organs can be examined in large animals through rectum. The method and procedure for rectal palpation is by making cut and smooth the nail; wear shoulder long glove; lubricate; cone shape of the fingers; insert in rotating way; notice: the hand cannot open, or even grasp organs inside. It's necessary or possible to use tranquilizer to reduce the sensitivity of the rectum in horse. In bloat case, the pressure in the abdomen would be very high, so it would be difficult to insert the hand inside.

The pathological condition occurring in the ruminant and other domestic animals ruminant are listed as following [1]:

- ✓ **Reticulum**– actinobacillosis/actinomycosis infection of the oesophageal groove, neoplasia of the oesophageal groove, reticular abscess, reticuloperitonitis.
- ✓ **Rumen**– secondary free gas bloat, frothy bloat, ruminal acidosis, cold water ruminal atony, neoplasia of rumen, rumen collapse syndrome, rumen foreign body, rumen impaction, vagal indigestion.
- ✓ **Omasum**– omasal impaction.
- ✓ **Abomasum**– right abomasal dilatation and torsion,

abomasal rupture, left abomasal displacement, abomasal impaction (dietary), abomasal ulceration, abomasal neoplasia.

- ✓ *Small intestine* – Johne's disease, salmonellosis, winter dysentery, bovine virus diarrhoea, gut tie (intestinal strangulation), foreign body intestinal obstruction, intussusception, neoplasia of small intestine, prolapse of the intestines through the mesentery, torsion of the root of the mesentery, small intestine rupture post calving, spasmodic colic.
- ✓ *Large intestine* – caecal dilatation and torsion.
- ✓ *Rectum* – rectal perforation, rectal prolapsed
- ✓ *Abdomen/peritoneum* – ascites, uroperitonium, fat necrosis, focal or diffuse peritonitis, acute pneumoperitonium.
- ✓ *Liver* – abscessation, hepatitis, cholangitis, Fasciolosis

Auscultation of the rumen help in recognizing the rate, quality and rhythm of the normal ruminal movements. The ruminal movements arise from the churning action of the organ. The rate of ruminal movement in health animals is 2-5 in cattle, 3-6 in sheep, 2-4 movements every two minutes in goats. It decreases in cases of rumen atony; diseases of reticulum, omasum and abomasum; impaction and late stage of tympany; also in severe feverish conditions and in traumatic reticuloperitonitis. Increased rate is seen in early stage of digestive disorders such as tympany and the form of vagus indigestion with hypermotility [1].

Absence of rumen movements is occurred by the following causes [1,5]:

- ✓ Severe dilation of the rumen with gases (tympany) or with food (impaction).
- ✓ Toxic conditions.
- ✓ The quality could be described as strong in healthy animals, weak in cases of ruminal atony and very strong as in early stages of digestive disorders such as tympany and vagus indigestion with hypermotility.

Constipation is the abnormality in movement of faeces in intestine of animals; it is occurred when there is reduction in the movement of alimentary tract resulting in passage of small hard amount of faecal matter. It may also associate with tensmus or straining (sign of pain when there are problems in the pelvic cavity, alimentary tract or urogenital organs. The major causes of constipation are like:

- ✓ Fever
- ✓ Tetanus
- ✓ Septicemic conditions
- ✓ Zinc poisoning

- ✓ Ruminal atony
- ✓ Traumatic reticulitis
- ✓ Lack of water Indigestion
- ✓ Ketosis (acetonemia)
- ✓ Abomasal impaction
- ✓ Hepatitis
- ✓ Abomasal displacement (right)
- ✓ Ruminal impaction
- ✓ Tapeworm infestation

Colic is an abnormal or the sign of disease of intestinal, it is common in horse and also ruminant animals. Signs of colic in large ruminants in the form of restlessness, kicking of the abdomen, or rising and laying down frequently are similar to that of horses but in horse are clearer than that in large ruminants. It is cause by:

- ✓ Peritonitis
- ✓ Traumatic reticulitis
- ✓ Abomasitis
- ✓ Omasal or abomasal impaction
- ✓ Ruminal tempny
- ✓ Abomasal displacement
- ✓ Impaction (lactic acidosis)
- ✓ Hepatitis
- ✓ Cystitis
- ✓ Traumatic pericarditis
- ✓ Urolithiasis
- ✓ Obstruction of intestine of horse
- ✓ Parasitic obstruction of intestine

Diarrhea: when faeces of animals become water due to some disease, it is most common problem in large ruminants and there are many factors that affecting in the type of diarrhea according to age of the animal, physical conditions of the animal, feeding system, aim of breeding, infectious agents (virus, bacteria, protozoaas well as physical causes of diarrhea .

The major causes of diarrhea in young calf are as:

- ✓ Coccidiosis
- ✓ Colibacillosis
- ✓ Salmonellosis

- ✓ Sodium chloride poisoning
- ✓ Copper and vitamin A deficiency
- ✓ Lead, mercury and nitrate poisoning
- ✓ Dietary scour due to bad feeding management
- ✓ Rotavirus
- ✓ Parvovirus
- ✓ Enterovirus

Causes of diarrhea in adult and growing animals:

- ✓ Lactic acidosis (ruminal impaction)
- ✓ Fasciolosis
- ✓ Salmonellosis
- ✓ Coccidiosis
- ✓ Antibiotic induced diarrhea
- ✓ Aflatoxicosis
- ✓ Parasitic gastroenteritis
- ✓ Ecoli
- ✓ John's disease (no blood, no mucous membrane, only dark watery diarrhea)

Examination of the urogenital system

In the horse and cow only the left kidney is accessible for palpation from the rectum. The right kidney lies further forward and cannot be reached by the hand. In the horse, the left kidney extends back to about four inches behind the last rib and its inner border is separated from the median line by about the same distance. In ruminants, it is loosely suspended below the lateral processes of the first lumbar vertebrae. Sometimes it may be shifted over to the right side. In dogs the kidneys lie in the lumbar region.

The manifestations of the urinary tract diseases include abnormal constituents and appearance of urine, changes in the volume of daily urine flow and frequency, pain and dysuria and uremia⁶. Examination of urogenital examination is important to identify the regional anatomy, undertake clinical and physical examination of urinary system and assess urinary abnormalities, perform clinical examination of female and male reproductive organs as well as the mammary glands and teats. Identify the anatomic structure of the kidney on live animal: the left kidney is located ventral of the transverse process of the 3rd to 5th lumbar vertebrae. Normally left kidney its position varies according to the degree of fullness of the rumen as when the rumen is fully distended, the left kidney present ventral to the transverse process of 5 and 6th lumbar vertebrae. While when the rumen not distended, the left kidney lies ventral to transverse process of 1, 2 or 3rd lumbar vertebrae. Normally

right kidney present ventral to transverse process of the 2, 3rd lumbar vertebrae. The kidney can be examined by inspection, external palpation, rectal palpation, and urinalysis. The examination of the urinary bladder is performed by stimulating the sense of urination from the lower part between the two hind legs on small animals or rectal palpation on large animals. Attention should be paid to the paralysis of the bladder and retention of urine and rupture. During palpation the size of kidney may increase due to neoplasm, hydronephrosis, acute nephritis and pyelonephritis, But the size is decreased due to dehydration and advanced chronic interstitial nephrosis⁶.

- * Take the sample of urine examine through physical methods
- * Fresh urine is collected with test tube after stimulating urination by palpating the perineal region or by inserting catheter
- * Clinical examination of the mammary glands and teat- inspection and palpation to detect the presence of swelling and lesions on the teats/decrease in size and shape, any discharge, temperature of the udder, consistency, and pain reactions are performed.
- * Clinical examination of milk samples: after collecting the milk samples in clean test tubes one can apply different physical and laboratory examinations
- * For the gross examination of the milk, the change of the color, odor, viscosity and flakes in the milk should be identified.

External palpation of the kidneys in the horse is not performed due to the considerable thickness and rigidity of the abdominal wall. Kidneys can be palpated through the abdominal wall in the majority of small and medium sized dogs. In sheep, goat and pigs, external palpation of the kidneys is of little value. In the cat, kidneys are large and pendulous therefore easily palpable. Identification is aided by recognition of the hilus on the attached border. In cattle, rectal palpation may help in examination of the kidneys¹.

Abnormality which associated with urinary system are as:

Frequency of urination: Frequency may increase due to: cystitis, calculi (incomplete obstruction), increase the fluid intake especially in winter, injection of diuretic. However, frequency of urination can be decreased in case of dehydration, urethral obstruction, and diarrhea.

Anuria and dysuria: Anuria is complete absence of urination which may be associated with painful urination (dysuria), while stranguria means that dripping of urine as in case of cystitis, urethral calculi, urethritis, spinal cord trauma and hemorrhage into urinary tract.

Haematuria: Presence of blood in the urine due to blood parasite, hypophosphatemia, pyelonephritis, systitis, trauma in the kidney or urethra, urolithiasis, and inappropriate use of urethral catheterization.

Pyuria: Presence of pus in the urine due to inflammation lesion in the pyelonephritis, abscesses in kidney and urethra.

Examination of reproductive system of animals

Clinical examination of male genital organs, visual inspection and palpation are employed to examine the testes, prepuce and the penis after withdrawing from the prepuce. The abnormality which observed from male genital are like orchitis, cryptorchidism, scrotum hernia, obstruction of urethra by calculi, phimosis, paraphimosis, inflammation of the prepuce, testes and penis are noted^{1, 2, 7}. Clinical examination of female genital organs, visual inspection and palpation of the vaginal region, use of vaginal speculum to examine the inside of vagina and intra-rectal examination of the cervix, uterus and ovaries would be performed [1,2,4].

The procedures of rectal palpation:

- First of all restraining of animals(cow): by approaching from rear and tail is grasped firmly about midway of its length, the animal may jump from side to side as the hand is inserted into the rectum. The veterinarian or technician must be move readily from one side to other with the cow.
- Preparation of safety: the arm must be covered with plastic sleeve with lubrication. Hand is inserted by forming the thumb and fingers into a cone tip and with a slight rotary motion, forcing into rectum. When hand is inserted, there may wave of peristaltic contraction will encountered from rectal wall.
- If the animal show defecation, the clinician or technician should have to expel it from rectum, then the floor of rectum is depressed with the hand which has been inserted half way to the elbow.
- The genital tract should be lie just below and wholly on the floor of the pelvis. The whole part of genital tract may be contracted and lying in the caudal part of pelvic canal, genital tract s position may vary forward until uterus is hanging over the brim of the pelvis into the abdominal cavity, but with only the vagina and cervix in the pelvic canal.
- The abnormality which is present in diseased animals are like:
 - Pyometra
 - Tumor and abscesses in the retroperitoneal tissues around the vulva and vagina.
 - Cervix will appear a firm, rope like structure, cervix may feeling like aturkey neck which mean long.
 - A pedundulous uterus, if empty may pulled up into pelvic canal.
 - Cervix ordinarily lies along the middle of the pelvis, directly under rectum and above bladder. But, if bladder

is full with urine, the remain part of cervix is present in pelvic canal, reproductive tract. Particularly, cervix, may lie over on the right side of the pelvis and wedge in between the bladder and wall of the pelvis.

- The cervix ordinarily lies along the midline of the pelvis, directly under the rectum and above the bladder. But if the bladder is full and remains in the pelvic canal, the reproductive tract, particularly the cervix, may lie over on the right side of the pelvis and be wedged in between the bladder and the wall of the pelvis.

Examination for pregnancy involves palpation of the uterine horns to determine size differences and then later the presence of fetal membranes, cotyledons and increased blood flow in the uterine artery. The palpation of ovary is started from the position where the hand is placed over the coiled uterine horns. The ovaries are palpated to detect the presence and size or degree of developments of follicles and corpora lutea. The ovary, when picked up under the edge of the broad ligament is turned up and backward on the top of the broad ligament for examination. It is held in a transverse position with the free edge of the ovary toward the examiner and the mesovarial edge away from the examiner. The attached end of the ovary (end to which the bursa is attached) is lateral. The determination of size and location of ovarian structures requires measurement with the finger tips and the consistent orientation of the ovary with respect to the body wall; also the identification of the structure as being in certain defined regions of the ovary. Finger widths constitute a rough unit of measurement. There are about nine regions of the ovary; they are upper surface, lower surface and greater curvature along free margin. Each of these has right, left and middle regions.

Examinations of the nervous and musculoskeletal system

An animal can response to change in the environment and complete linking of the different in the body that this response is called coordination. There are two types of coordination system; they are like nervous and endocrine system. The nervous system also classified into central and peripheral nervous system. The interaction of nervous and muscular system aids the animals to stand, walk and coordinating with its environment. The examination of nervous and musculoskeletal system is to detect the clinical signs of various problem associate with these systems.

Observation the behavior of the animal and checking the responses of the animals while applying different stimuli:

- Examine the brain by corneal reflex, the pupil reflex.
- Examine the spinal cord of the neck and thorax by withers reflex.
- Examine the spinal cord of the back part by applying hoof stimuli and anus stimuli.
- Move the animal to and from or uncomfortable position to examine the locomotors.

- Examine the joints long bones and different muscles: arthritis, dislocation of the joints, rickets or osteocalcin, muscular atrophy, central or peripheral origin paralysis [1,2].

Examination of skeletal muscle of an animal

The musculoskeletal system is composed of the bones of the skeleton, joints, ligaments, muscles and tendons. In addition to the nervous system, the musculoskeletal system is important for the maintenance of posture and for locomotion. The aim of the clinical examination is to identify the site and the cause of the lameness. Identification of the affected limb, the site of the lesion within the limb and assessment of the severity of the lesion, are primary aims of the initial part of the examination. Localization to a foot or an upper limb problem may be possible by observation. Abnormalities of posture and gait, weight bearing and gross swellings, wounds and deformities should be noted. Posture means the anatomical configuration of animals when they remain standing. And sitting in other words, it means how the animal stands and sits. Postural abnormalities can be of great help in making a diagnosis (Table 3).

Gait: Gait is denotes the locomotor processes of the animals. A locomotor disturbance of the animal is judged by the movements (Walking, running, trotting, circling etc.). There are certain diseases, which interfere with the process of locomotion (Table 4). The gaits of the dog are commonly used patterns of locomotion that can be divided into two main groups: symmetric and asymmetric. With symmetric gaits such as the walk, trot, and pace, the movement of the limbs on one side of the dog's body repeats the motion of the limbs on the opposite side with the intervals between foot falls being nearly evenly spaced. With asymmetric gaits such as the gallop, the limb movements of one side do not repeat those of the other and the intervals between foot falls are unevenly spaced. When considering gaits, one full cycle is referred to as a stride [8].

Examination of superficial lymphnodes of animals

Examination of superficial lymphnode istocompare each paired node for size and consistency with the contralateral node. Grossly enlarged lymph nodes may have been seen during observation of the patient before it is handled. Observation and palpation is possible when the animal is restrained. Normal characteristic of lymphnode can be identified as ther following:

- * Size: lymphnode vary greatly in large animals, but it is larger in young animals than adult.
- * Consistency: firm on palpation
- * Surface: lobulated in large lymphnode and smooth surface
- * Temperature: it takes the normal skin temperature
- * Pain: painless on palpation
- * Skin: movable freely over surface of examined lymph node

Table 3: Abnormal posture in domestic animals.

Animals	Site of pulse taking
Equine	External maxillary artery Transverse facial artery Median artery Great metatarsal artery
Cattle and pig	Middle coccygeal artery Facial artery Median artery
Sheep, Goat, Calf	Femoral artery.
Piglet, Dog and Cat	Femoral artery.

Table 4: Abnormality of gait in domestic animals.

Gait	Disease
Lameness and disinclination to move	Laminitis, foot rot
Stiff gait	Arthritis
Walking in circles / head pressing	Gid (Coenuruses)/otitis/ listeriosis
Dragging of the hind leg	Subluxation of medial patellar ligament

- * Movement: mobile in relation to the neighboring tissues

Anatomical Location of lymphnodes (LN):

Lymph nodes are round or bean-shaped structures that are widely distributed throughout the body. Lymph node are imbedded in connective tissue or fat, which concentrated in cervical, axillary, and inguinal regions of neck, armpits, and groin, respectively. They are typically less than 1/2 inch in length, depending on the size of the animal. The lymph nodes filter the lymph before returning it to the veins [1,9].

Submandibular lymph nodes: situated and are palpable on the medial aspect of the 'angle of the jaw' where the horizontal and vertical rami of the mandible meet. (Normal size is 1.5 to 2 cm)

Prescapular lymph nodes: It lies subcutaneously and underneath the cutaneous muscle just anterior to the shoulder joint (Normal size 1 cm × 3.5 cm)

Axillary lymph nodes: Found on each side of the chest in the armpit area. Normally only palpable in young calves without heavy muscling (Normal size 1.5 cm)

Popliteal lymph nodes: These nodes are found surrounded by dense muscle tissue immediately behind the stifle. It found on each rear leg on the opposite side of the knee. They are relatively superficial and easy to feel (normal size 1-1.5 cm)

Inguinal lymph nodes: These are usually palpable as a small group of fairly mobile and firm structures adjacent to the inguinal canal. (Normal size 0.5 cm)

Supramammary lymph nodes: These are normally readily palpated on the caudal aspect of the udder just above the upper limit of the mammary glandular tissue, normal size 2.5 cm [4,10] (Figure 1).

Abnormalities of lymph node in large animals may be enlarged and inflamed by disease like: Blood parasites, Actinobacillosis, 3 days fever [ephemeral fever], caseous lymphadenitis (corynebacterium) or edematous skin disease and epizootic lymphangitis, mastitis.

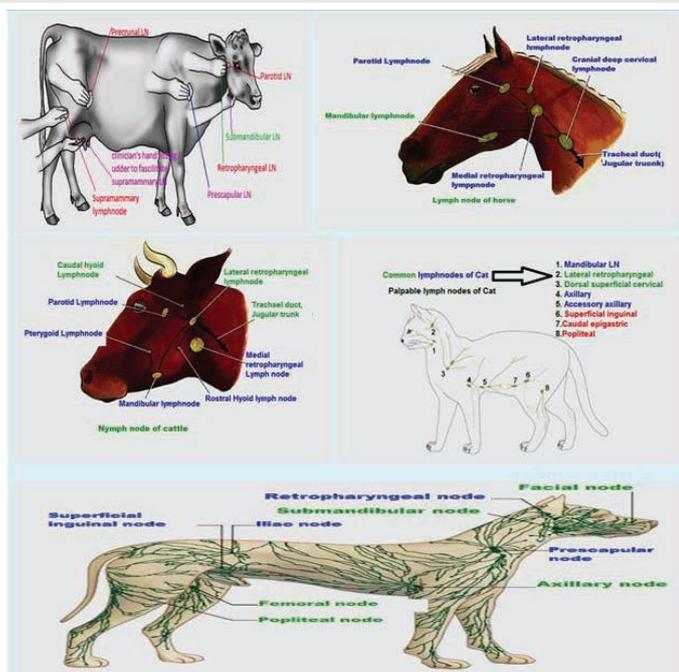


Figure 1: Anatomical location of animals node.

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Research Article

Preparation and administration of medicaments body weight of animals and drug dosage

The body condition of a livestock is generally assessed by visual observation. The references to an animal being too thin, in good flesh, or obese all relate to the animal's weight. Therefore, the weight of an animal can be used as a measurement tool to determine its well-being, or the presence of problems which may threaten the health of the horse. The weight of small animals and poultry may be carried out by balance by automatic balance machine several methods can be used to determine the body weight of livestock in an approximate scale. Because of the variety of dosage forms in veterinary medicine and the diversity of animal and bird species treated, drug or dosage delivery sometimes requires the development of specific devices to ensure fast, safe, effective and low cost efficient treatment. The domestic animals body weight estimation equation as following [1].

*Equation of equine body weight estimation:

$$\text{Weight (kg)} = \frac{(\text{girth measurement in cm})^2 \times (\text{length measurement in cm})}{11,900}$$

*Equation of Bovine body weight estimation:

$$\text{Live weight in lbs.} = \frac{\text{Length} \times \text{Girth square}}{300}$$

Ibs = pound. 1 Ibs = 0.454 Kg; 1 Inches = 2.54 cm.

Formulations of Drug Dosage Forms of Animals are listed as following².

Oral dosage forms: Refers to administration of drug through the mouth. The most commonly used preparations are solid oral dosage forms such as tablets, capsules, granules, powder, paste and boluses.

Parenteral dosage forms: The most common parenteral dosage forms are stable aqueous solutions and subcutaneous implants.

External dosage forms:

- ✓ Ointment- semisolid preparation for external application.

- ✓ Cream- a viscous semisolid, consisting of oil in water emulsion or water in oil emulsion.
- ✓ Dusting powder e.g., popular antibacterial agent applied on animal wounds.
- ✓ Lotion- an aqueous solution or suspension for local application.
- ✓ Spray-a drug applied in liquid form by pressure. **forms:** gaseous and volatile liquid anaesthetic agent (drugs), given by inhalation, e.g., Halothane.

Inhalation dosage forms: gaseous and volatile liquid anaesthetic agent (drugs), given by inhalation, e.g., Halothane.

The Common Veterinary Drugs

Anthelmintic drugs [3,4]

Anthelmintics are drugs that are used to treat infections with parasitic worms. This includes both flat worms (flukes and tapeworms) and round worms (nematodes, round worms, whipworms, hookworms, pinworms, threadworms, and filarial worms). Anthelmintic can be classified into the following based on their mode of action: Nicotin agonist, acetyl cholinesterase inhibitor, GABA agonist, GluCl potentiator, calcium permeability increase, B-tubulin binding, proton ionophores, inhibitor of malate metabolism, inhibitor of phosphoglycerate kinase and mutase, and inhibitor of arachidonic acid. Base on spectrum of action, anthelmintics can be classified as: broad spectrum (kill a wide variety of worms) or narrow spectrum (kill one or two varieties).

Benzimidazole (albendazole, cabendazole, fenbendazole, flubendazole, mebendazole, oxfendazole, oxibendazole, parbendazole and triclabendazole). The benzimidazole bind to a specific building block called beta tubulin and prevent its incorporation into certain cellular structures called microtubules, which are essential for energy metabolism. Then parasite will die or paralyzed from action of benzimidazole

drugs. It is broad spectrum in treatment of all nematode, cestode and adult form of fasciola. Triclabendazole is narrow spectrum for fasciolosis, and also broad spectrum for fasciola species, which means it can kill all stages of fasciola (larva and adult stage). However, it is not recommended for pregnant animals due to its teratogenic effect.

Imidazothiazoles (tetramisole, levamisole): it is broad spectrum against nematodes. Levamisole is selectively as a cholinergic agonist at synaptic and extra synaptic nicotinic acetylcholine receptors on nematode muscle cells. This causes spastic paralysis of susceptible nematode by selecting gating acetylcholine receptors on nerve muscle. Levamisole is a nematocidal compound effective against lung and GI tract nematodes and annelid (leech) but not effective against cestode and trematode parasites, and it is not ovicidal. Levamisole has been shown to have immune modulatory effects, that is; it enhances immune responsiveness by restoring the number of T lymphocytes to normal when they are depleted. It is not used in horse because of its limited efficacy against many equine parasites.

Tetrahydropyrimidines (pyrantel, morantel and oxantel): These drugs act selectively as agonist at synaptic and post synaptic nicotinic acetylcholine receptors on nematode muscle cells and produce contraction and spastic paralysis. Pyrantel and morantel are 100 times more potent than acetylcholine, although slower in initiating contraction.

Organophosphate Compounds (Haloxon, coumaphos, dichlorvos, crufomate and naphthalophos): Organophosphate inhibits many enzymes, especially acetyl cholinesterase, by phosphorylating their esterification site. This blocks cholinergic nerve transmission in the parasite, which results in spastic paralysis. Originally, they were used extensively as insecticides, then as ectoparasiticides. Dichlorvos is used as an anthelmintic in horses, pigs, dogs, and cats; trichlorfon in horses and dogs; and coumaphos, crufomate, haloxon, and naftalofos in ruminants. Haloxon is probably the safest organophosphate anthelmintics for use in ruminants.

Piperazine (Diethylenediamine): The drug has good efficacy profiles against ascarid and nodular worm infections of all species of domestic animals, moderate for pinworm infections. Piperazine acts by blocking transmission by hyperpolarizing nerve membranes at the neuromuscular junction leading to parasite immobilization by flaccid paralysis and consequent removal from predilection site and death. Piperazine is a selective agonist of gamma-amino butyric acid (GABA) receptor resulting in opening of chloride channels and hyperpolarization of the membrane of the muscle cells of the nematode parasites. Mature worms are more susceptible to the action of piperazine than the younger stages.

Bunamide: It is effective against the common tapeworm species and it is most effective when given after fasting. It is formulated as tablets (hydrochloride salt) or suspension (hydroxynaftoate salt) for oral administration in companion animals and ruminants, respectively. It acts by disrupting the tapeworm's tegument and reduces glucose intake, as a result, the subtegumental tissues are exposed and the worm destroyed by the host's digestive enzymes.

Praziquatel: its mode of action is by increasing the cestode's cell membrane permeability; this disintegrates the worm's outer tissue covering.

Macrocyclic Lactones (abamectin, ivermectin, doramectin, eprinomectin, and selamectin): The macrolides act through selective toxic effects on insects, acarines, and nematodes. However, they do not possess efficacy against cestode and trematode parasites due to both trematode and cestode they haven't receptor for this ivermectin or lack of GABA receptor. The macrocyclic lactones induce reduction in motor activity and paralysis in both arthropods and nematodes. The parasitic effects are mediated through GABA and/or glutamate-gated chloride channels (GluCl), collectively known as ligand-gated chloride channels. The endectocides cause paralysis and death of both arthropod and nematode parasites due to their paralytic effects on the pharyngeal pump which affects nutrient ingestion, and on the parasite somatic musculature limiting its ability to remain at the site of predilection in the host.

Anti-microbial and anti fungal [5-7]

Antimicrobial therapy is based on the selective toxicity of a drug for invading organisms, i.e. ability to kill or inhibit an invading microorganism without harming the cells of the host. Antimicrobial can be classified into different based on their action, they are like:

Inhibition of cell wall synthesis: penicillins, cephalosporins.

Impairment of cell membrane function: Polymyxin, tyrocidin, and the polyene antifungal agents, nystatin and amphotericin B that bind to cell-wall sterols.

Reversible inhibition of protein synthesis: Affect the function of 30s or 50s ribosomal subunits, and are bacteriostatic drugs which include chloramphenicol, tetracyclines, macrolides (erythromycin) and clindamycin.

Alteration of protein synthesis: Bind to the 30s ribosomal subunit and affect cell membrane permeability which eventually leads to cell death, eg aminoglycosides (eg; streptomycin, gentamicin).

Inhibition of nucleic acid function or synthesis: Rifamycins (rifampin) which inhibit DNA-dependent RNA polymerase, and quinolones (oxolinic acid).

Interference with microbial metabolism: Sulphonamides, trimethoprim.

Inhibition of viral enzymes: These agents block the viral enzymes that are essential to DNA synthesis, thus halting viral replication, eg nucleic acid analogues (zidovudine, acyclovir, and vidarabine).

The most common bacteriocidal (kill bacteria) antimicrobials are like: Agents that alter microbial cell wall or membrane permeability are generally bactericidal. These include the penicillins, cephalosporins, aminoglycosides and polymyxins. Essentially bacteriostatic agents inhibit bacterial protein synthesis, chloramphenicol, macrolides and tetracyclines.

The importance of combination of two or more antimicrobial drugs useful in veterinary medicine, the importances of combining are:

- * To overcome infection
- * To avoid rapid emergence of resistant mutants, especially in prolonged therapy with drugs such as streptomycin which tend to induce rapid bacterial resistance
- * To prevent inactivation of the antimicrobial agent by bacterial enzymes, eg, the use of co-amoxiclav, a combination of amoxicillin and clavulanic acid, an α -lactamase inhibitor
- * To achieve a synergistic effect, as is exemplified by cotrimazine, a combination of sulphadiazine and trimethoprim
- * To reduce the severity or incidence of adverse reactions where the organisms are fully sensitive to each drug.

Common veterinary antimicrobials [5-8]

Penicillins (procaine penicillin, penicillin G): It is betalactames Penicillins which interfere with the synthesis of the bacterial cell wall peptidoglycan (the major constituent of G+ve bacteria cell wall). After attachment to binding sites on bacteria, they inhibit the transpeptidase enzyme involved in cross linking of the peptidoglycan chain, the 3rd and final stage of bacterial cell wall synthesis. It is narrow against G+ve aerobes and anaerobes bacteria.

Cephalosporins: It is beta lactamase group, which have certain therapeutic advantages over penicillins which include their relative resistance to β -lactamase, their broad-spectrum of activity, their ability to reach the CNS, and less likelihood to cause allergic reactions, hence they are suitable for use in rabbits, guinea pigs, and reptiles. They inhibit the β - lactam-binding proteins involved in bacterial cell wall peptidoglycan synthesis, hence are bactericidal. Cephalosporin have four generation, they are like:

1. **First-generation cephalosporins:** cefadroxil, cefalexin, cefazolin, cephacetril, cephradine and cephexazole
2. **Second-generation cephalosporins:** cefaclor, cefamandole, cefmetazole, cefonicid, cefprozil, and loracarbef. Cephamycin; cefotetan and cefoxitin.
3. **Third-generation cephalosporins:** cefdinir, cefixime, cefoperazone, cefotaxime, ceftizoxime, and ceftriaxone.
4. **Fourth-generation cephalosporins:** Examples are cefepime and ceftipime. They have a wide antibacterial spectrum (*Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, and *Pseudomonas*). Cefepime is highly resistant to β -lactamases.

Aminoglycosides (streptomycin, dihydrostreptomycin, neomycin, tobramycin, kanamycin, gentamicin, amikacin, netilmicin): They bind to the 30S ribosome and inhibit the rate of bacterial protein synthesis and the functionality of mRNA translation, resulting in the synthesis of abnormal proteins.

Aminoglycosides alter cell membrane permeability causing nonspecific membrane toxicity. Their effect is bactericidal and is enhanced by agents that interfere with cell wall synthesis (eg, β -lactam antibiotics). They are effective against many aerobic G-ve and some G+ve organisms; *Leptospira* spp are also affected.

Tetracycline (Tetracycline, oxytetracycline, demeclocycline, doxycycline, methacycline, and minocycline): Tetracyclines are bacteriostatic; they inhibit microbial protein synthesis by binding to 30S ribosome and block the attachment of aminoacyl tRNA to the mRNA-ribosome complex. As a result, they block the addition of amino acids to the growing peptide chain. It is broad spectrum against G+ve and G-ve bacteria, spirochaetes, rickettsiae (*Anaplasma*, *Cowdria*, and *Ehrlichia* species), mycoplasmae, chlamydiae, amoebae, and some protozoa (*Theileria* and *Babesia* species).

Strangles in horses; actinomycosis and actinobacillosis; anthrax; pasteurellosis; clostridial diseases; respiratory and urinary tract infections in dogs and cats; psittacosis in birds, rickettsial diseases; bovine anaplasmosis, caprine heartwater, canine ehrlichiosis, mycoplasma infections of poultry including borreliosis, coryza and erysipelas all respond to tetracycline therapy.

Chloramphenicol: Chloramphenicol binds with the 50 s ribosomal subunit to inhibit peptide bond formation and protein synthesis in the bacterial or disease causing organism. It is a broad-spectrum bacteriostatic agent active against many gram-positive and gram-negative bacteria, *Rickettsia*, *Mycoplasmas*, and *Chlamydia*. It has an excellent therapeutic activity against *Salmonella*.

Macrolide (oleandomycin, tylosin, carbomycin, spiramycin, tiamulin, tilmicin): It inhibits bacterial protein synthesis by binding to the 50s ribosome, preventing translocation of amino acids to the growing peptide. It is effective against gram positive organisms such as *staphylococci*, *mycoplasma*, *spirochaetes*, and certain mycobacteria are sensitive to the group.

Lincosamide: They are effective against gram-positive cocci, anaerobes, and Toxoplasma and mycoplasma species.

Sulphonamides: It is broad spectrum antimicrobial drugs which inhibit bacteria and protozoa (coccidiosis). Long acting (sulphamethoxyypyridazine, sulphamethoxine, sulphadoxine). Enteric or gut-activesulphonamides (Phthalylsulphathiazole, Succinyl sulphathiazole, Sulpha-bromethazine, Sulphaquinoxaline, Sulsalazine, Sulphacetamide). Being impermeable to folic acid, many bacteria must rely on their ability to synthesise folate from PABA. Pteridine and glutamate in contrast the mammalian in cells cannot synthesize folic acid and must obtain preformed folate as a vitamin in their diet. The sulphonamides are structurally similar to PABA, the sulphonamides competitively inhibit dihydropteroate synthetase, the enzyme that catalyses the incorporation of PABA into dihydrofolic acid.

The folic acid is required for pure and D.N.A synthesis which without it bacteria growth is inhibited. Sulphonamides have a broad-spectrum of activity against both gram-positive and gram negative bacteria, and some protozoa (coccidia, Neospora, Toxoplasma, rickettsiae).

Antifungal Drugs

Fungal infections (mycoses) are classified into two types: topical (superficial), which affects the skin and mucous membranes, and systemic which affect areas as the blood, lungs, or C.N.S. The most common disease of fungus in domestic animals are rised from the strain of fungus like: *Blastomycosis*, *Cryptococcosis*, *Histoplasmosis (epizootic lymphangitis)* and *Coccidioidomycosis*. The most common drug available for treatment of fungal infections are like:

- ✓ Superficial agents (griseofulvin) Polyene macrolides (amphotericin B, nystatin)
- ✓ Imidazole derivatives (ketaconazole (the prototype), miconazole, clotrimazole, fluconazole, itraconazole, enilconazole, and terconazole)
- ✓ Antimetabolites (5-flucytosine)

Antiprotozal Drugs

- ✓ **Anticoccidiosis:** Sulphonamides, Quinazolines, Quinolones, Symmetrical triazinones and Thiamine antagonists
- ✓ **Antitypanosomiasis:** Diamidines (Diminazene acetate, Phenamidine, Stilbamidine and Pentamidine), Phenanthridines (phenidium, dimidium, homidium and isometamidium), Quinapyramine Compounds (quinapyramine chloride, quinapyramine sulphate and suramin), Organic arsenicals (melarsomin, or melarsoprol)
- ✓ **Antiprotozoal drugs (Amicarbalide isethionate, buparvaquone, halfuginone, menoctone, parvaquone tetracycline, imidocarb, quinuronium sulphate).** It can inhibit anaplasma like: anaplasmosis, babesiosis, coccidiosis, theileriosis, ehrlichiosis, and hepatozoonosis and in avian spirochaetosis.

Analgesia and Anesthesia Drugs

The most common drugs in surgery are used in preoperative, during surgery and post-surgery. General anesthesia is preceded by premedication with one or more drugs. The importance of premedication drugs are

- ✓ Reduce the amount of anaesthetic required (injectable and inhalant), so reducing side effects (and costs!)
- ✓ Relax muscles
- ✓ Provide analgesia
- ✓ Provide sedation
- ✓ Suppress vomit reflexes
- ✓ May help calm a stressed animal
- ✓ May help make the animal easier to handle
- ✓ Improve recovery (fewer tremors and less vocalising)

Common premedication drugs

Opioid drugs (Oxymorphone, Fentanyl, Morphine, and Butorphanol): Opioid produce their effect by binding three different receptors [μ (μ), κ (κ), and δ (δ)] as either agonists, partial agonists or antagonists. The location of these receptors varies, but in general, resides within the brain and spinal cord. Provide potent analgesia; concurrent administration can lower the dose of inhalant or barbiturate general anesthetic for surgery; mechanism mediated by receptor binding in the brain and spinal cord; long history of use in research; reversible with Naloxone.

Alpha-2 agonists: It is used for their sedative and analgesic properties in a variety of species. Produces analgesia of short duration; can be combined with Ketamine to produce adequate surgical anesthesia in many species; effects can be reversed with a subcutaneous α_2 antagonist injections such as Atipamezole.

Xylazine: it has potent sedative effect, but cardiovascular depression can be profound. The side effect of this drug is like: vomiting, diuresis, GIT motility depression, platelet aggregation.

Acepromazine maleate (Phenothiazine): It has tranquilliser action which makes calm aggressive and apprehensive animals. The IV, IM, SC or oral dose may be used in the dog or cat. Doses: dogs: 0.01-0.1 mg/kg IV, intramuscularly (IM), subcutaneously (SC), 1-3mg/kg oral; cats: 0.05-1.0mg/kg IV, IM, SC, 1-3mg/kg oral.

Atropine: It is parasympatholytic, blocking acetylcholine (ACh). It can increase the occurrence of cardiac dysrhythmias. The main uses of atropine now are to facilitate ophthalmic examination by dilating the pupil (mydriasis) or to treat or prevent certain types of bradycardia or bradyarrhythmias. Atropine is used to decrease salivary and airway secretions during anaesthetics. Doses: dogs/cats: 0.02 mg/kg IV, 0.045 mg/kg IM or SC

Diazepam: It has a sedative, tranquilliser, anticonvulsant, and skeletal muscle relaxant and appetite stimulant actions in cats. When used alone, diazepam occasionally results in excitation rather than sedation, so it is most often used in combination with another sedative, usually ketamine. Dose: dogs/cats: 0.1-0.5mg/kg IV

Medetomidine: It is alpha 2-adrenergic agonist, Good skeletal muscle relaxation; sedation and analgesia are induced by administration. Sedation is reversed by administration of atipamezole. Doses: dogs: 0.01-0.05 mg /kg IV, IM, SC; cats: 0.05-0.15 mg/kg IV, IM, SC

Nonsteroid anti-inflammatory drugs (meloxicam, carprofen, flunixin and ketoprofen). It has about 13 different classes of drugs which share inhibitory activity of the cyclooxygenase (COX) enzyme. The COX enzyme facilitates the production of Prostaglandin G₂ (PGG₂) which then follows a variety of enzymatic processes in the production of several compounds that are involved in normal physiological processes and production of Prostaglandin E₂ (PGE₂). PGE₂ specifically play a role in the perception of pain in the periphery and within the central nervous system. Thus, blockade of PGE₂ by

COX inhibition is effective in control of discomfort at the site of insult and within the central nervous system. Two forms of the COX enzyme have been well characterized (COX-1 and COX-2). As a result, COX inhibitors are often referenced as non-selective COX inhibitors or selective COX-2 inhibitors. This distinction has been made because inhibition of COX-2 is believed to be the predominant method of NSAID function to provide analgesia and anti-inflammatory action even though this “consensus” is still under debate. Over the past 10 years, several NSAIDs have emerged for veterinary use that are COX-2 selective, such as Carprofen and Meloxicam which can be administered once every 12–24 hours in most species. Newer drugs (Carprofen, Meloxicam) include a long duration of analgesic activity; newer drugs demonstrate analgesic quality that rivals some opioids; not a DEA controlled substance; there are multi route administration methods for several NSAIDs; relative safety when administered at prescribed dosages

Alpha-2 antagonists (yohimbine): it is used as reversal agents for α_2 agonists. Administration at the end of a procedure where the anesthetic combination included Xylazine or Medetomidine, α_2 antagonist will aid in reducing anesthesia time and prompting anesthetic recovery.

General anesthesia

Ketamine: Ketamine is associated with increased muscle rigidity and excessive salivation. Ketamine may cause increased heart rate, cardiac output, and blood pressure. Ketamine is used in combination with other injectable agents such as α_2 agonists or benzodiazepines to reduce or eliminate many of the less desirable side effects if used alone. Doses: dogs: 11–22 mg/kg IM, IV, SC; cats: 1.0–33mg/kg IM, SC, IV. Ketamine + Diazepam: Both drugs can be mixed in a single syringe prior to administration. Advantages include limited cardiovascular effects including minimal hypotension as compared to Ketamine/Xylazine combinations. Ketamine + xylazine + acepromazine: increases the depth of anesthesia and substantially prolongs the duration of anesthesia as well as recovery time. The combination will produce less cardiovascular depression than xylazine-ketamine. Dose Diazepam 0.1–0.4 mg/kg and ketamine 5 mg/kg given IV.

Barbiturates: Sodium Pentobarbital, Methohexital, Thiopental. Barbiturates function as GABA agonists and are considered to be good anesthetic agents but provide unreliable sedation at low dosages and inadequate analgesic effect at any dose. Pentobarbital, the most commonly used drug of this class, is considered a long acting anesthetic. Methohexital and Thiopental are considered short and ultra-short acting anesthetics and were more commonly used as induction agents in large animal species. Rapid anesthetic onset; provides a prolonged duration of surgical anesthesia; decades of use has characterized many research side effects; Pentobarbital is the active drug in manufactured euthanasia solutions.

Local anesthesia

Local anesthesia block nerve conduction by inhibiting influx of sodium ions through ion-selective sodium channels in nerve membrane leading to impairment of the generation of action potential. The sodium channel itself is a specific receptor for local anesthetic molecules. Local anesthesia can be classified

into topical or surface anesthesia and regional anesthesia. Topical anesthesia's are like 2% or 4% lignocaine HCl or other can be used for the relief of pain in abrasions or eczematous areas. Infiltration anesthesia applies to nerve ending of animals at the actual site of the operation to block nerve.

Linear infiltration: it is done on the line of the incision or just parallel to the line of the incision. The amount to be infiltrated is 1 ml/cm in small animals and 2 ml/cm in case of large animals. The anaesthetic is to be infiltrated into the subcutaneous area as well as into the muscles. The technique can be used for any type of the surgical operation like rumenotomy, cystotomy.

Inverted 'L' or 'T' or inverted 'V' block: 2% Lignocaine HCl is injected into the tissues bordering the dorsocaudal aspect of the last rib and ventrolateral aspect of the lumbar transverse processes. Inverted 'V' block technique is primarily used for teat surgery or management of accidental wounds of the limbs.

Field Block and Ring Block: It is the technique by which a wall of the local anaesthetic is formed around the site of operation. The volume used will depend on the size of the animal and the area to be blocked.

The common drugs which used in local anesthesia are listed as following:

Lidocaine: It has rapid onset of action, Duration of action is variable (depending on uptake) but will be around 1 hour without epinephrine, and 2 hours with epinephrine.

Bupivacaine: It has prolonged duration of action (8 hrs) when combined with epinephrine. It is therefore used whenever long action is required (post-op analgesia; prolonged surgery etc)

Mepivacaine: it is widely used in horse as it causes very little swelling and edema in the area of injection, it lacks vasodilatory action. Onset of action is faster and reliability of block greater than with prilocaine.

Pirocaine: It has slower onset of action, and spreads less well compared to lidocaine. The unique ability of prilocaine to cause dose dependent methemoglobinemia limits its clinical usefulness. The main use is in the horse as it causes less swelling but great accuracy is needed when doing specific nerve blocks.

Routes of Drug Administration

Medications are administered to cattle by injection for a variety of reasons. For example, vaccines (biological) are injected to prevent disease, and antibiotics or anthelmintic may be injected to treat bacterial or parasitic infections, respectively. Other medications may be given by injection for supportive medical care, to relieve pain or to enhance production. Injection may be the best, or the only, route of administration for a particular medication [8].

The routes of drug administration in domestic animals are summarized as following form (**Figure 1-3**) [8,12]:

Oral administration: There are large numbers of pharmaceutical preparations available for oral administration. Solid dosage forms (powders, tablet, capsules, pills, etc.) and liquid dosage forms (syrups, emulsion, mixture, drench, electrolytes, etc.)

Parenteral administration: (IV, IM, SC, Id, epidural, subconjunctival): It refers to a drug administration by injection directly in to the tissue fluid or blood without having to cross the intestinal mucosa (Table 1).

Intravenous route (IV): Gives swift, effective and highly predictable blood concentration and allows rapid modification of dose and is used for emergency treatment. Jugular vein is the common route of drug administration in large animals (horse, cattle, sheep and goat) usually given through jugular vein, in pig-ear veins, in the dog and cat-cephalic vein and recurrent tarsal vein (Figure 1).

Intramuscular (IM) route: Absorption occurs either haematogenous or via lymphatic and is usually fairly rapid except for long acting preparation.

Subcutaneous (SC) route: Preferred when slow and continuous absorption of drug is required. The injected drug disperses through the loose connective tissues. They dissolve in tissue fluid before it can enter either capillaries or lymphatic.

Intradermal route (ID): Used for testing hypersensitivity test and for vaccination.

Epidural route: Refers to deposition of drug up on or outside the dura matter. E.g. Introduction of local anesthetics between the first and second coccygeal vertebra and lumbosacral to eliminate straining.

Subconjunctival: Disposition of a pharmaceutical preparation beneath the conjunctiva.

Topical or local application: It refers to external application

Recommended needle size for domestic animals	Inch (") x diameter (gauge)		
	Subcutaneous (SC)	Intramuscular (IM)	Intravenous (IV)
Dog & Cat	1/2" x 22 ga	1" x 22 ga	
Horse	1" x 18 ga	1 1/2" x 18 ga	1 1/2" x 18 ga
Calf	1/2" x 20 ga	1" x 20 ga	1" x 20 ga
Cow	1" x 16 ga	1" x 16 ga	1 1/2" x 16 ga
Calf	1/2" x 16 ga	1" x 16 ga	1" x 18 ga
Goat	1/2" x 22 ga	1" x 18 ga	
Sheep	1/2" x 18 ga	1" x 18 ga	1" x 18 ga
Hog	1/2" x 18 ga	1" x 18 ga	2" x 18 ga
Sow	1" x 18 ga	1" x 18 ga	4" x 18 ga
Pig	1/2" x 20 ga	1" x 18 ga	1" - 1 1/2" x 20 ga
Poultry	5/8" x 25 ga	1/2" x 22 ga	

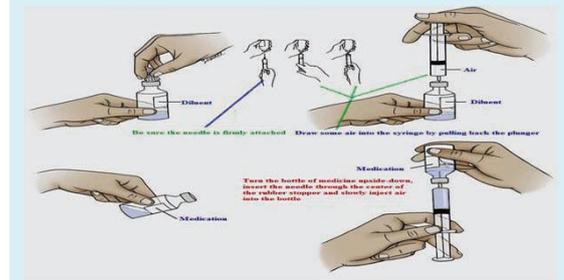


Figure 2: Types of needles and methods how to drawing out of medication bottle.

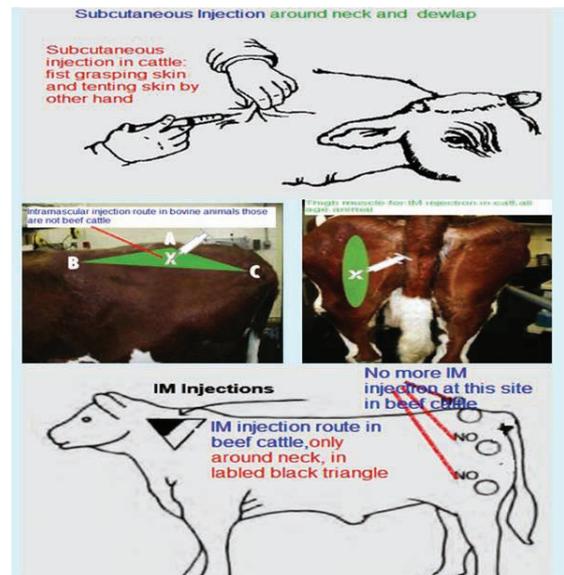


Figure 3: Parenteral route of drug administration in bovine animals.

of drug to the body surface for localized action at accessible site, such as skin, eyes, body orifices, body cavity.

Drug dose calculation

If drugs are used incorrectly, disease organisms can build up resistance making drugs ineffective. This usually happens from under dosing or overdosing drugs. It is best to calculate the correct dosage based upon the weight of each animal determined by using a scale or estimated by using heart girth measurement [1,10]. Dose is the quantity of the drug to be administered at one time and expressed in mg/kg or IU/kg.

General Equation for calculating and converting of the concentration of drugs.

*Dose required (Equals drug volume in ml or cc) = Animal weight (kg) x dosage (mg/kg) divide by Concentration of drug (mg/ml).

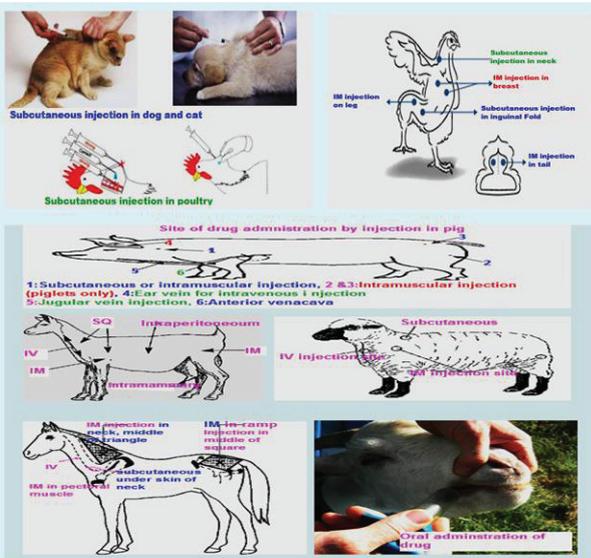


Figure 1: Route of drug administration site in domestic animals, cat, dog, poultry, pig, shoat and horse.

Table 1: Site of parenteral injection in domestic animals.

Animals species	Intramuscular injection site	Intravenous injection site	Subcutaneous injection site
Horse, Buffalo	*Rump is avoided in horse it may cause some sensitivity like swelling, heat and pain. So that it is difficult to drain abscess. Therefore neck muscle is the best site for Equine animals. Thigh also possible.	Jugular vein	Horses have very little loose skin in which to give an SQ injection compared to other domestic species. Under the skin at the side of the neck (due to horse have skin in the fatty or loose connective tissue layer which is less sensitive to drug.
Cattle, yak	Rump, thigh (semimembranosus and semitendinosus)	Jugular vein	Under the skin at the side of the neck, Dewlap.
Sheep, Goat	Thigh	Jugular vein	Behind the elbow or just over a rib. Under the skin on the inside of the hind leg.
Dog, cat	Thigh	Cephalic, Saphenous veins	Under Skin over the middle of the back or just behind the shoulders.
Chicken	Chest(breast muscle) , Pectoral muscle	Wing vein	Under skin, after parting the feathers on the breast. Under skin at back of neck in small chicks.
Pig	Thigh	Ear vein	Under skin inside the thigh for small pig and Under skin at the behind of the ear

*The unit conversion, 1 ml (millilitre) = 1 cc (cubic centimeter); 1 kg (Kilogram) = 2.2 lbs (pound); 1 cm = 0.394 inch and 1 inch = 2.54 cm; 1 foot = 0.3048 m and 1 m = 0.033

*Convert the concentration of a solution expressed in percent into mg/ ml: Multiply percent figure by 10 = given % x 10 mg/ml.

*Percent solution = Weight in gram x 100 divide by the volume of solution in mls.

*Weight in grams = Volume of solution in mls x percent solution divide by 100.

Example: If one cow is presented to clinic, The Clinician Undergoes Clinical Examination and He Prescribed the Oxtetracycline 10%. The cow weighs 350 kg and requires a daily injection of 10% drug in solution at the 10 mg / kg dose rate. Calculate the volume of the dose to be administered for Cow.

*** Solution:**

- 10% solution = 10 x 10 mg/ml = 100 mg in 1ml.
- Dosage rate = 10 mg/kg
- Dosage rate x body weight = 10 mg/kg x 350 kg = 3500mg
- The volume of drug required = 3500mg/100mg/ml = 35 mls/ day
- Vets often use Latin abbreviations to denote how often the dog should receive the antibiotic during the day:
- SID: Once a day dosing
- BID: Twice a day dosing
- TID: Three times a day dosing
- QID: Four times a day dosing

- Stat: Immediate at that day

Method and procedure how to load a syringe during taking drug (Figure 2) [11]:

- Be sure the needle is firmly attached.
- Draw some air into the syringe by pulling back the plunger.
- Turn the bottle of medicine upside-down, insert the needle through the centre of the rubber stopper and slowly inject air into the bottle.
- Now draw the medicine into the syringe.
- Keep the bottle above the syringe so any air bubbles in the syringe go to the top.
- Push the plunger carefully so the air comes out.
- Now see if the right amount is in the syringe (the top of the plunger should be on the line for the proper dose).

Withdraw more medicine or squirt some back into the bottle until the right amount is in the syringe.

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Research Article

Veterinary laboratory guidance

The Veterinary Diagnostic Laboratory has the great role in diagnosing medical testing for infectious agents, toxins, and other causes of disease in animal diagnostic samples which submitted by veterinary practitioners serving animal owners, public health officials, wildlife management, and scientists with research projects. This Veterinary Diagnostic Laboratory guidance focused on the discipline of special veterinary faculty like pathology, clinical pathology, microbiology, virology, immunology, parasitology, and serology.

Principle of biosafety in laboratory

The principle gives some awareness for the lab worker and student, how to operate in laboratory and what is the prohibition act in laboratory and etc. The main safety in laboratory is listed as following [1-3]:

- ✓ Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas.
- ✓ Safety glasses, face shields (visors) or other protective devices must be worn when is necessary to protect the eyes and face from splashes, impacting objects and sources of artificial ultraviolet radiation.
- ✓ It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in canteens, coffee rooms, offices, libraries, staff rooms and toilets.
- ✓ Open-toed footwear must not be worn in laboratories.
- ✓ Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in laboratories.
- ✓ Smoking and/or application of cosmetics shall not take place in the laboratory; Pipetting shall not be done by mouth.

- ✓ Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited in the laboratory working areas.
- ✓ Appropriate gloves must be worn for all procedures that may involve direct or accidental contact with blood, body fluids and other potentially infectious materials or infected animals. After use, gloves should be removed aseptically and hands must then be washed.
- ✓ Storing human foods or drinks anywhere in the laboratory working areas is prohibited.
- ✓ Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing.
- ✓ The laboratory should be kept neat, clean and free of materials that are not pertinent to the work.
- ✓ Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day.
- ✓ All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse.
- ✓ The laboratory should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period;
- ✓ Personal protective equipment such as long-sleeved lab coats or gowns, closed-toe footwear, disposable gloves, masks, safety glasses, face shields, and oronasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory.
- ✓ The laboratory door should be closed when work is in progress and ventilation should be provided by

extracting air from the room (Where biosafety cabinets are used, care shall be taken to balance ventilation systems).

- ✓ Emergency response plans should be developed to deal with the biohazard of spills. Some of the items addressed in the plans should include having effective disinfectant available for cleaning spills, removal of and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops.
- ✓ Used laboratory glassware and other contaminated material shall be stored safely. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise decontaminated before disposal. Re usable material shall be decontaminated by appropriate means.
- ✓ No infectious material shall be discarded down laboratory sinks or any other drain.
- ✓ Any accidents or incidents shall be recorded and reported to the Safety Officer.
- ✓ Some common terms used in veterinary clinical diagnostic and laboratory practice are listed below [1,4]:
- ◆ **Diagnosis** is an art of precisely knowing the cause of a particular disease. (Dia =thorough; gnosis = knowledge). The diagnosis is based on accurate history, careful examination of animal, collection of material for laboratory examination and correlation and interpretation of findings.
- ◆ **Snap diagnosis:** This is to give an opinion about the cause of disease by merely looking at the animal. It is often erroneous unless the animal is showing pathognomonic symptoms.
- ◆ **Tentative diagnosis:** The tentative diagnosis is based on clinical symptoms and physical examination. It is to give an approximate cause of disease.
- ◆ **Symptomatic diagnosis:** The symptomatic diagnosis is based on a few important symptoms without knowing the cause of disease.
- ◆ **Confirmatory diagnosis:** It is based on clinical, physical and laboratory findings. It includes the exact cause of disease and determination of which one of several diseases may be producing the symptoms.
- ◆ **Differential diagnosis:** This is the process of exclusion for differentiating among diseases having similar symptoms or closely related diseases, e.g. diabetes mellitus and diabetes insipidus.
- ◆ **Test therapy diagnosis:** The diagnosis of disease depends on the response of the animal to a particular drug or medicine.

- ◆ **Clinical diagnosis:** This diagnosis is based on the inspection of animal by looking the clinical symptoms.
- ◆ **Physical diagnosis:** This diagnosis is based on examination of animal by physical methods like palpation, percussion and auscultation.
- ◆ **Laboratory diagnosis:** The laboratory diagnosis is based on laboratory findings like examination of clinical samples such as serum, feces, blood etc.
- ◆ **Symptoms:** Any subjective evidence of disease of an animal characterized by a change in patient's condition indicative of some bodily or mental state as told by the owner.
- ◆ **Signs:** An indication of the existence of something, any objective evidence of disease, which is perceptible to the veterinarian. These are based on the veterinarians' observations.
- ◆ **General symptoms:** The general symptoms include symptoms involving whole body like fever/rise in temperature.
- ◆ **Systemic symptoms:** These symptoms are an indication of involvement of a system of body like vomiting, diarrhoea indicating the involvement of digestive system; respiratory symptoms, nasal discharge indicate the affections of lungs and respiratory passage.
- ◆ **Pathognomonic symptoms:** These symptoms are specific for a particular disease and based on that one can diagnose the disease with confirmation, e.g. rusty brown nasal discharge in equine infectious anemia.
- ◆ **Lesions:** Lesion is the pathological alterations in structure or function and can be detectable either by naked eyes or microscopically.

The veterinary diagnostic laboratory guidance is the best guidance of the clinician and veterinarian who work over research and laboratory. Therefore, the most common examination undertaken during practice is as listed below:

- ✓ Examination of blood
- ✓ Examination of feces
- ✓ Examination of urine
- ✓ Examination of cerebrospinal fluid
- ✓ Examination milk
- ✓ Examination skin scraping
- ✓ Antimicrobial sensitivity test
- ✓ Serological and immunological
- ✓ Molecular biology examination
- ✓ Postmortem/necropsy examination

Examination of Blood in Domestic Animals

General scientific fact about blood

Blood is a bodily fluid in animals that delivers necessary substances such as; nutrients and oxygen to the cells and transports metabolic waste products away from those same cells. Blood which is a vital special circulatory tissue is composed of cells suspended in a fluid intercellular substance (plasma) with the major function of maintaining homeostasis [5]. The components of blood include plasma (the liquid portion, which contains water, proteins, salts, lipids, and glucose), red blood cells and white blood cells, and cell fragments called platelets [6].

Blood plays an important role in regulating body systems and maintaining homeostasis. It performs many functions within the body including:

- Supplying oxygen to tissues (bound to hemoglobin, which is carried in red cells)
- Supplying nutrients such as glucose, amino acids, and fatty acids either dissolved in the blood or bound to plasma proteins (e.g., blood lipids)
- Removing waste such as carbon dioxide, urea, and lactic acid
- Immunological functions, including circulation of white blood cells and detection of foreign material by antibodies
- Coagulation, which is one part of the body's self-repair mechanism (blood clotting by the platelets after an open wound in order to stop bleeding)
- Messenger functions, including the transport of hormones and the signaling of tissue damage
- Regulating body pH
- Regulating core body temperature
- Hydraulic functions, including the regulation of the colloidal osmotic pressure of blood.

Medical terms related to blood often begin with hemo- or hemato- (also spelled haemo- and haemato-), which is coined from the Greek word α (haima) for "blood". In terms of anatomy and histology, blood is considered a specialized form of connective tissue, given its origin in the bones. Blood cells produced at different stages of development differ in morphology and function. Thus, primitive (fetal) cells fabricated early in gestation have markedly different properties from adult counterparts produced during late gestation and in postnatal life (Table 2). Primitive erythrocytes (RBCs) are formed in the yolk sac, whereas definitive RBCs are produced by the liver and later spleen and bone marrow. Primitive RBCs are nucleated in circulation until approximately day 12.5 (E12.5) of gestation, after which nuclei gradually become condensed before being shed between E14.5 to E16.5 [7].

Both primitive and definitive RBCs have basophilic cytoplasm when first produced due to abundant rough endoplasmic reticulum [8]. Haematogenesis is the process of differentiation of blood from bone marrow; it is regulated by erythropoietin (EPO) hormone secreted from kidney. Secreted molecules also are important regulators of hematopoietic development during gestation. Erythropoietin (EPO) sustains both primitive and definitive erythropoiesis by stimulating proliferation and differentiation of immature primitive and definitive RBCs [9]. Different cell lineages occupy specific locations: granulocytes, lymphocytes, and macrophages are concentrated near the endosteum and arterioles, and megakaryocytes and erythroid cells are located near venous sinuses [10]. Hematopoietic cells are derived from a common pluripotent stem cell which gives rise to lymphoid and myeloid progenitor cells. Lymphoid progenitor cells generate lymphocyte progeny, whereas the myeloid progenitor cells generate erythroid cells, megakaryocytes, basophils, eosinophils, and a common granulocyte macrophage cell that produces neutrophils and macrophages (Figure 3) [11].

Stages of erythropoiesis include rubriblasts, prorubricytes, rubricytes, metarubricytes, reticulocytes, and mature RBCs [12]. As erythroid precursors mature, the cells become smaller, the nuclear to cytoplasmic ratio decreases, the cytoplasm becomes less basophilic and more polychromatophilic, and the nuclear chromatin becomes condensed. In mammals, the nucleus is extruded before maturation to a mature RBC [12]. Red blood cells, or erythrocytes (erythro- = "red"; -cyte = "cell"), specialized cells that circulate through the body delivering oxygen to other cells, are formed from stem cells in the bone marrow. In mammals, red blood cells are small, biconcave cells that, at maturity, do not contain a nucleus or mitochondria; they are only 7–8 μm in size. In birds and non-avian reptiles, red blood cells contain a nucleus.

The red coloring of blood comes from the iron-containing protein hemoglobin. The principal job of this protein is to carry oxygen, but it transports carbon dioxide as well. Hemoglobin is packed into red blood cells at a rate of about 250 million molecules of hemoglobin per cell. Each hemoglobin molecule binds four oxygen molecules so that each red blood cell carries one billion molecules of oxygen. There are approximately 25 trillion red blood cells in the five liters of blood in the human body and also for domestic animals is detailed in table 1, which could carry up to 25 sextillion (25×10^{21}) molecules of oxygen at any time. In mammals, the lack of organelles in erythrocytes leaves more room for the hemoglobin molecules. The lack of mitochondria also prevents use of the oxygen for metabolic respiration. Only mammals have anucleated red blood cells; however, some mammals (camels, for instance) have nucleated red blood cells [13].

The advantage of nucleated red blood cells is that these cells can undergo mitosis. Anucleated red blood cells metabolize anaerobically (without oxygen), making use of a primitive metabolic pathway to produce ATP and increase the efficiency of oxygen transport. Not all organisms use hemoglobin as the method of oxygen transport. Invertebrates that utilize

hemolymph rather than blood use different pigments containing copper or iron to bind to the oxygen. Hemocyanin, a blue-green, copper-containing protein is found in mollusks, crustaceans, and some of the arthropods. Chlorocruorin, a green-colored, iron-containing pigment, is found in four families of polychaete tubeworms. Hemerythrin, a red, iron-containing protein, is found in some polychaete worms and annelids [13].

Leukocytes or white blood cells (from the Greek word *leuco*, white) differ considerably from erythrocytes in that they are nucleated and are capable of independent movement to exit blood vessels. Leukocytes may be classified as either granulocytes or agranulocytes based on the presence or absence of cytoplasmic granules that stain with common blood stains, such as Wright's stain. These stains contain an acid dye, eosin, which is red, and a basic dye, methylene blue, which is bluish [14].

Granulocytes are named according to the color of the stained granules (i.e., neutrophils, which have granules that stain indifferently; eosinophils; and basophils). The differentiations of white blood cell in domestic animals are detailed in figure 2. The nuclei of granulocytes appear in many shapes and forms, leading to the name polymorphonuclear leukocytes (from the Greek poly, many; morpho, form). However, the term is commonly used to indicate neutrophils, because they are normally the most prevalent granulocyte. Monocytes and lymphocytes are the two types of agranulocytes (Figure 1) [14].

Blood platelets, also called thrombocytes, are fragments of megakaryocytes, large cells formed and residing in the bone marrow. Thrombocytes are the smallest of the formed elements in the blood. They are surrounded by a plasma membrane and contain some organelles, but not nuclei. The appearance of platelets in a stained smear may be considerably different from

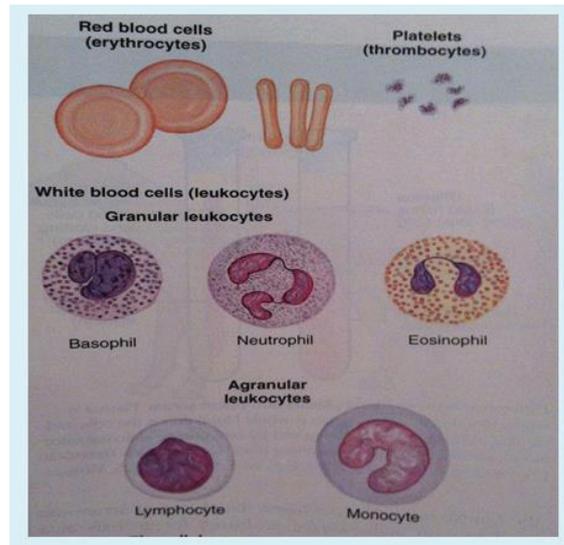


Figure 1: Normal blood cells (white, red and platelet) of animals.

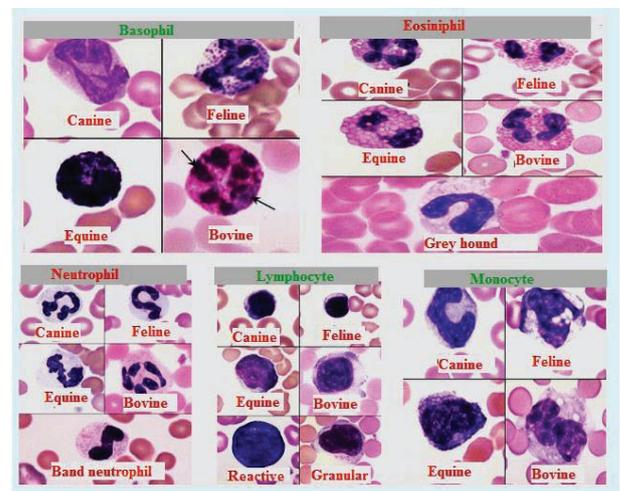


Figure 2: Leukocytes of domestic animals with their comparison [15].

Table 1: The numbers per microliter of blood cell and platelets in domestic animal [14].

Blood elements	Horse	Cow	Dog	Chicken
Erythrocytes	8-11 x 10 ⁶	6-8 x 10 ⁶	6-8 x 10 ⁶	2.5-3 x 10 ⁶
Total leukocytes	8-11 x 10 ³	7-10 x 10 ³	9-12 x 10 ³	20-30 x 10 ³
Neutrophils	4-7 x 10 ³	2-3.5 x 10 ³	6-8.5 x 10 ³	5-10 x 10 ³
Lymphocytes	2.5-4 x 10 ³	4.5-6.5 x 10 ³	2-3.5 x 10 ³	11-18 x 10 ³
Eosinophils	200-500	150-500	200-500	600-2,000
Monocytes	400-500	350-500	450-600	2-3 x 10 ³
Basophils	<100	<100	<100	200-900
Platelets	150-450 x 10 ³	300-500 x 10 ³	300-500 x 10 ³	25-40 x 10 ³

Table 2: The site of hematopoiesis/generation of RBC in domestic animals.

Age of animals	Site of hematopoiesis
Embryo	Yock sack then liver
3 rd to 7 th month*	Spleen
4 th and 5 th months*	Marrow cavity (granulocytes and platelets.)
7 th months*	Marrow cavity (erythrocytes.)
Birth	Mostly bone marrow; spleen and liver when needed.
Birth to maturity	Numbers of active sites in bone marrow decreases but retain ability for hematopoiesis.
Adult	Bone marrow of skull, ribs, sternum, vertebral column, pelvis, proximal ends of femurs.

their actual appearance in circulating blood, where they are oval disks. In smears they may appear as circular disks, star-shaped fragments, or clumps of irregular shape. Substances released by platelets and lodged on their surface membranes stimulate clotting and help cause local constriction of the injured blood vessel [15].

Blood cell collection

Equipments and reagents required for hematological blood examination.

- Hematocrit centrifuge
- Compound microscope
- Sahlis instrument
- Capillary tube
- Hematocrit reader
- Distilled water

- Haemocytometer pipette
- Haemocytometer counting chamber
- WBC diluting fluid
- RBC diluting fluid
- Slides cover slip, special cover slip
- ESR stand, ESR tube
- Filter paper
- blood lancets
- lead pencils
- marker
- Giemsa stain, Wright's stain
- Vacutainer tubes(Figure 4)
- Vacutainer needles
- syringes
- needle holder
- Gloves
- Blood samples
- Staining racks and others

The blood is collected from animals through puncture of vein using syringe and needle. In laboratory animals or poultry, however, it is collected directly from the heart [17-24]. The sites of blood collection in different animal species are described in figure 5 and table 3.

Anatomical Location of the Veins of Domestic Animals

Jugular vein

The jugular vein is situated in the jugular groove, over

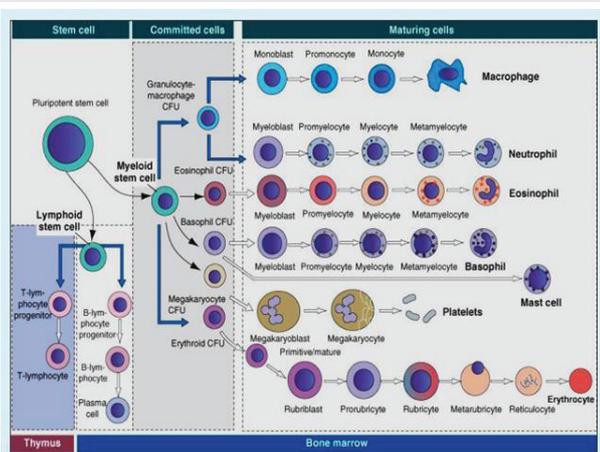


Figure 3: Mode of blood cell formation or haemopoiesis [16].

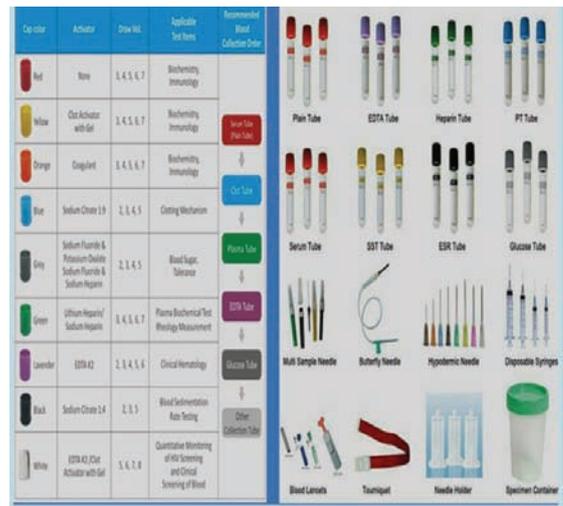


Figure 4: Equipment of blood collection in domestic animals.

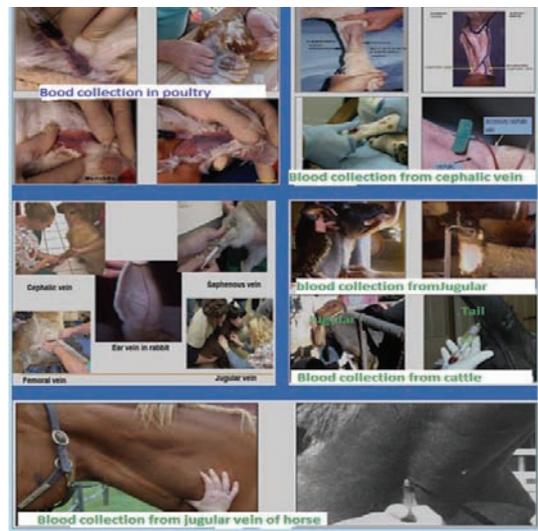


Figure 5: Anatomical site of blood collection in domestic animals.

Table 3: Common site of blood collection in domestic animals.

Animals species	Site of blood collection
Cattle	Jugular vein, ear vein
Horse	Jugular vein
Camel	Jugular vein
Sheep	Jugular vein
Goat	Jugular vein
Pig	Ear vein, anterior venacava
Dog and Cat	Cephalic vein, Recurrent tarsal vein
Poultry	Wing vein, Heart, Small bird from: Brachial, jugular, and femoral veins.

the trachea and carotid artery on both sides of the neck [25]. Before collection of blood, the hairs of the area are removed with the help of scissors and the area is shaved with razer. The antiseptics like spirit is applied over the area of vein puncture and the animal is properly restrained. The vein is raised by applying pressure at ventral point of puncture. The vein can

be felt by finger tapping. Blood is collected by using a 16 gauge needle in large animals and 18 gauge needles in small animals. The needle is inserted in the vein by force in an angle; blood can be collected directly in the collection tube or by applying syringe over needle to suck the blood. After collection of blood, the pressure is released and needle is removed. Apply antiseptics at the site of vein puncture. In casted animals, the neck should be turned upwards as it helps in distention of vein for puncture [26]. The right jugular is the largest and by far the most preferred site for avian blood collection. It is easily visualized by applying a small amount of alcohol to the featherless tract on the right side of the neck. The vein should be occluded with a thumb or forefinger at the level of the thoracic inlet prior to venipuncture [27].

Ear vein

The ear vein technique is a simple, quick and reliable method of taking blood samples from cattle for determination of the haematocrit values of blood [28]. Small amounts of blood (1–2 ml) can be collected from ear vein [29,30]. The area should be cleaned and shaved properly at the dorsal side of the ear. Antiseptics should be applied over the area. The vein is raised by applying pressure of thumb and index finger of left hand and blood is collected using a 20 or 22-gauge needle with syringe. After collection of blood, the pressure is released and the vein puncture site is rubbed with cotton dipped in spirit. Ear blood was more sensitive than jugular blood for the detection of parasitacmia [31].

Cephalic vein

The cephalic vein is situated in the anterior surface of the forelegs, from where hairs should be removed and the area is shaved [26]. For proper disinfection, 70% alcohol spirit is applied over the area. Use a tourniquet around the elbow to raise the vein. Vein is felt by fingertip and a needle of 20–22 gauge is inserted along with syringe. The needle should be in the right side of the vein and a click like sound appears when the needle penetrates the vein. Collect blood in desired quantity and remove the tourniquet, needle and syringe. Apply antiseptic at the site of puncture.

Recurrent tarsal vein

This vein is situated on the lateral side of the hock joint where it takes a round [26]. The pressure should be applied over the shfle joint. The area must be cleaned, shaved and properly disinfected. The blood is collected with a 20 gauge needle with syringe. After collection of blood, pressure is released and needle is removed. The antiseptics are applied over the vein puncture area.

Poultry

In poultry, small amount of blood (1–2 ml) can be collected from wing vein [32]. However, if more blood is required, it should be collected directly from the heart. The wings are extended and the area should be cleaned. Feather or fluffs are removed from the site and then the vein is raised by applying pressure at the root of wing. The needle (20 gauges) is inserted

in the vein and blood is collected slowly. Remove the pressure, before withdrawal of needle and apply proper disinfectant vein puncture site. For the heart puncture, the bird is placed on its back with extended neck and wings against the chest [33].

The needle (20 gauges) along with syringe is applied from dorsal side in the groove of neck and the needle is directed forward in slightly raised position. When it punctures the heart, blood comes out in the syringe. Collect the desired quantity of blood and remove the needle. Apply proper disinfectant on the area of needle puncture. The other method for collection of blood from poultry is from jugular vein. For Blood collection from jugular vein of birds, first the neck is cleaned and fluffs are removed and the area is properly disinfected.

The head of the bird is held at its base between fore and middle fingers and then with the help of the thumb the jugular vein is so placed that it rests on the ridge or the solid background of cervical vertebrae. The needles are inserted into the vein and collect the blood in syringe. After collection of blood, remove the needle and syringe and apply disinfectant at the site of vein puncture. After the collection of blood from animal, the needle is removed from syringe and the blood is slowly transferred to the collection tube in order to avoid the breakage of erythrocytes and formation of hemolysis 23.

Interpretation of Examined Blood

The wet film of blood is examined for the demonstration of movements of microfilaria or hypanosomes. For this a drop of fresh or anticoagulant added blood is placed over a clean and dry slide. It is covered with a cover slip and examined under microscope. The parasites are seen moving in between the erythrocytes. The trypanosomes are recognized by their swirling movements. These parasites can also be demonstrated by lysis of blood using saponin.

Interpretation of red blood cell and white blood cell

Increased total erythrocyte count (erythrocytosis) is reported in following conditions. Dehydration, hemoconcentration, exercise, occlusion of vein for a longer period, brucellosis, campylobacteriosis, leptospirosis, rinderpest, pasteurellosis, acute poisoning, chronic heart disease, pulmonary fibrosis, shock, vomiting, diarrhoea, hypoxia [34–37].

Decreased total erythrocytic count (erythropenia) occurs in following conditions: Anemia, anaplasmosis, babesiosis, leptospirosis, copper, lead and phenothiazine poisoning, equine infectious anemia, defective blood formation, leukemia, hemorrhage, aflatoxicosis, coccidiosis, fasciolosi [38,39].

The total leucocyte count is performed by hemocytometer using a WBC diluting pipette. The blood is sucked in pipette up to 0.5 mark and then WBC diluting fluid, as in case of total erythrocyte count, upto 11 marks. Remove the rubber tubing, keeping the pipette in horizontal position. Mix the contents of pipette by rotating the pipette between the palms and discard the first few drops. Fill the space in between coverslip and counting chamber and wait for 1–2 min for settling of cells. Put the counting chamber under microscope and count

the cells in four large corner squares of the ruled area under low power. Calculate the total leucocytes per cubic mm of blood by multiplying 50 with the count. The white blood cell (WBC, leukocyte) count of birds is obtained using manual techniques because the presence of nucleated erythrocytes and thrombocytes interfere with the counting of white blood cells using electronic cell counters [40,41].

For total leucocyte count in poultry, the diluting pipette of erythrocytes is used because of high leucocytic count. Like total erythrocytic count, fill the RBC diluting pipette upto mark 0.5 and fill it with "WBC diluting fluid-poultry" upto mark 101. Remove the rubber tubing and mix the contents, discard a few drops of contents and fill the counting chamber as described earlier. After 1-2 min, count the cells under low power/high power in four corner primary squares of the counting chamber and calculate the number of cells by multiplying 5000 to get total leucocyte count per cumm of blood [40].

Interpretation of white blood cell

Leucopenia is the decrease in total leucocyte count and is observed in following conditions: Canine distemper, infectious canine hepatitis, feline panleukopenia, mucosal disease, hog cholera, swine influenza, chronic intoxication of lead, bismuth, arsenic, mercury, pesticides, prolonged antibiotic therapy, rinderpest, bovine malignant catarrh, extreme debility and loss of resistance, exposure of X-rays, salmonellosis, toxoplasmosis, PPR, brucellosis, chlamydiosis, leishmaniosis, tuberculosis, blue tongue, lymphosarcoma, hepatic cirrhosis, mastitis, copper deficiency, aflatoxicosis, BHV-1 infection, bracken fern poisoning [37,42].

Leucocytosis is the increase in number of leucocytes and is observed in following disease conditions. Pasteurellosis, leptospirosis, salmonellosis, local infection of *streptococci*, *staphylococci*, and *coynebacterium*, uremia, diabetes, gout, malignancy, viral infections, rabies, histoplasmosis, tuberculosis, strangles, blood protozoan infections, colibacillosis, chronic enteritis, bovine immunodeficiency syndrome, acidosis, burn, gangrene [37,42,43].

Following are some factors which affect the total leucocytic counts:

- ◆ **Age:** TLC high at birth in pups, calves TLC low at birth in piglets
- ◆ **Species:** Bovines have more lymphocytic count while canines have more neutrophilic count.
- ◆ **Excitement:** Excitement causes an increase in TLC.
- ◆ **Pregnancy:** During pregnancy total leucocyte count increases.
- ◆ **Estrus:** During estrus TLC increases.
- ◆ **Digestion:** In dogs and pigs after eating food, there is increase in total leucocyte count and neutrophilic count.

Differential leucocyte count and interpretation

For differential leucocyte count, a thin blood smear is prepared on glass slide. A drop of fresh or anticoagulant mixed blood is placed on one corner/end of slide and spread as smear with the help of another slide using its thin edge at an angle of 45°. Smear should be uniform and thin and it should not have any air bubble. Dry the smear in air and mark the identification number in the thick portion of the smear. Thereafter fix the smear in methanol for 5 min and dry in air. Stain the smear with Giemsa stain diluted to 1:10 in distilled water for 30 min. Wash the slides, dry in air and examine under oil immersion of the microscope. Count at least 200 cells by battlement/zigzag method. Cells counted are neutrophils, lymphocytes, eosinophils, monocytes and basophils.

The interpretation is as the following listed [44-47].

Lymphocytosis: Increased number of lymphocytes is observed in viral infections, tuberculosis, brucellosis, hypothyroidism, following vaccination, Leukemia, Adrenocortico insufficiency etc.

Lymphopenia: Decrease number of lymphocytes is observed in canine distemper, infectious canine hepatitis, corticosteroid therapy, hypothyroidism, BHV-1 infection, Q-fever, foot and mouth disease, mucosal disease etc.

Neutrophilia: Increased number of neutrophils is observed in septicemic diseases, uremia, gout, coronary thrombosis, pyogenic infections, acute inflammation, cancer, arthritis, pyometra, post-surgical operation, coronary thrombosis, pregnancy, calf diphtheria, rheumatic fever.

Neutrophilia (Shift to left): Increased number of neutrophils with immature cells is observed in leptospirosis, traumatic reticulopericarditis (TRP), metritis, canine distemper, glanders, endocarditis, synovitis etc.

Neutropenia: Decrease number of neutrophils is observed in pasteurellosis, bovine viral diarrhoea, and infectious canine hepatitis etc.

Eosinophilia: Increase number of eosinophils is observed in allergy, parasitic infection, skin disease, anaphylactic reaction, convalescence, eosinophilic myositis and granulocytic eosinophilic leukemia.

Basophilia: Inverse number of basophils is observed in Pox infections, sinus-tissue, splenectomy, cirrhosis, Hodgkin's disease, introduction of foreign protein.

Monocytosis: Increase in number of monocytes is observed in tuberculosis, brucellosis, trypanosomiasis, convalescence, monocytic leukemia, carbonate and chloride poisoning, Systemic fungal diseases (histoplasmosis, blastomycosis, cryptococcosis, coccidioidomycosis, aspergillosis),

Hyperhemoglobinemia: Increase in hemoglobin is observed in polycythemia, dehydration, leptospirosis, equine influenza, acid indigestion.

Hypohemoglobinemia: Decreased in hemoglobin content is observed in anemia, theileriosis, strangles, anaplasmosis, Degnala disease, fasciolosis.

Erythrocytic sedimentation rate

The erythrocyte sedimentation rate (ESR) is measured by using Westergren pipette [48,49]. Suck the anticoagulant mixed blood in Westergren pipette upto mark '0' and fix it in stand in vertical position. Leave this for one hour at room temperature. After one hr record the reading on graduated pipette, it is the mm fall of erythrocytes per hour. Care should be taken that there should not be any air bubble in the pipette. Pipette should be filled exactly to '0' mark. ESR should be performed with one hour of blood collection.

The values of ESR are increased in tuberculosis, carcinoma, nephritis, gout, heavy metal toxicity, rheumatoid arthritis, pleurisy, canine distemper, pyometra, pericarditis, peritonitis, leptospirosis, filariasis, pneumonia, fracture, trypanosomiasis, equine infectious anemia and parasitic diseases. Decreased erythrocyte sedimentation rate has been recorded in hemolytic jaundice, sickle cell anemia and in brucellosis [49-51].

Increased hematocrit value: Dehydration (Increased total protein and/or albumin, Increased sodium and chloride, Increased BUN and creatinine, increased urine specific gravity), ketosis, leptospirosis, rotavirus infection, influenza, rinderpest, Renal: cysts, tumors, hydronephrosis, Erythropoietin-secreting tumors (paraneoplastic), High altitude, Hyperthyroidism, Chronic lung disease (Increased reticulocyte count and nucleated red blood cells, Decreased PO₂ on arterial blood gas, low SpO₂ on pulse oximeter, Pulmonary pathology on thoracic radiographs), Chronic cardiac disease [36,52,53].

Decreased hematocrit value: Anemia, parasitic infection, muscular dystrophy, gestation, lactation, theileriosis, strangles, anaplasmosis, blue tongue, endocarditis, hemolysis (Immune-mediated (IMHA), Infectious: hemotropic mycoplasma, rickettsial diseases, babesiosis, cytauxzoonosis, heartworm, Zinc toxicity) and Bone marrow disease/myelophthisis (e.g. lymphoproliferative, myeloproliferative disorders, metastatic neoplasia, myelofibrosis) 35,53.

Blood smear

Principle of smear preparation

A small drop of blood is placed near the frosted end of a clean glass slide. A second slide is used as a spreader. The blood is streaked in a thin film over the slide. The slide is allowed to air-dry and is then stained. EDTA anticoagulated blood is preferred. Blood smears can also be made from fingerstick blood directly onto a slide. Three methods may be used to make blood smears [54, 55]:

- The cover glass smear,
- The wedge smear and
- The spun smear. The spun smear requires an automatic

slide spinner. For the purpose of lab exercise, we will use the wedge smear (Figure 6).

Procedure how to make blood smear [56-59] (Figure 7).

- Using the stopper piercer, place a drop of blood, about 2 mm in diameter approximately inch from the frosted area of the slide.
- Place the slide on a flat surface, and hold the narrow side of the nonfrosted edge between your left thumb and forefinger.
- With your right hand, place the smooth clean edge of a second (spreader) slide on the specimen slide, just in front of the blood drop.
- Hold the spreader slide at a 30 angle, and draw it back against the drop of blood.
- Allow the blood to spread almost to the edges of the slide.
- Push the spread forward with one light, smooth, and fluid motion. A thin film of blood in the shape of a bullet with a feathered edge will remain on the slide.
- Label the frosted edge with patient name, ID number and date.

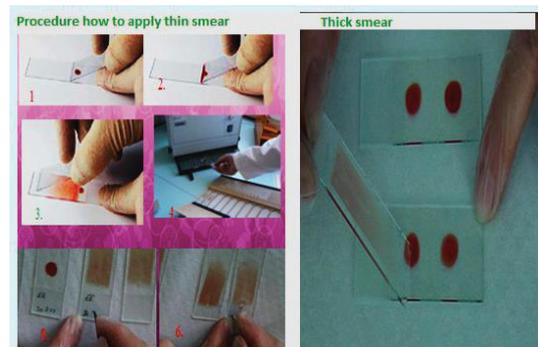


Figure 6: Thin smear and thick smear.

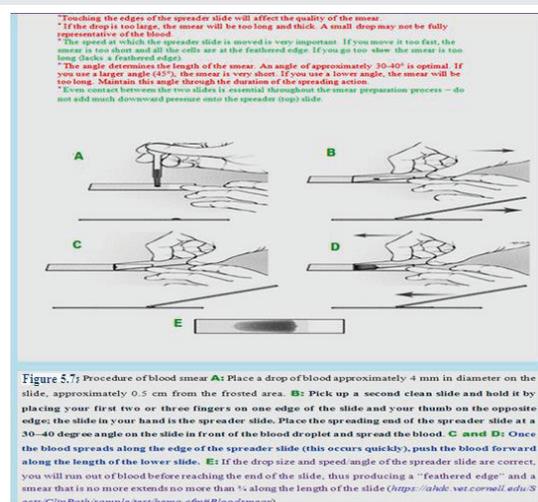


Figure 7:

- Allow the blood film to air-dry completely before staining. (Do not blow to dry. The moisture from your breath will cause RBC artifact).

The good blood smear should have the following:

A dense body; this should take up about 2/3 of the entire smear and should blend smoothly into the monolayer area. A well developed feathered edge. This edge should have a fine, feathery appearance; if there is a thick line of blood where the slide stopped, it's an indication of a poorly made smear. A monolayer area just behind the feathered edge. This region should be noticeably thinner than the body, but should blend in with the body of the smear. Often this area is only about 1/2 cm wide. Prior to staining, if the slide is held up to the light, there is a rainbow effect seen just behind the feathered edge on wellmade smears.

- ◆ A good blood film preparation will be thick at the drop end and thin at the opposite end.
- ◆ As soon as the drop of blood is placed on the glass slide, the smear should be made without delay. Any delay results in an abnormal distribution of the white blood cells, with many of the large white cells accumulating at the thin edge of the smear.
- ◆ The blood smear should occupy the central portion of the slide and should not touch the edges.
- ◆ The thickness of the spread when pulling the smear is determined by the 1) angle of the spreader slide (the greater the angle, the thicker and shorter the smear), 2) size of the blood drop and 3) speed of spreading.
- If the hematocrit is increased, the angle of the spreader slide should be decreased.
- If the hematocrit is decreased, the angle of the spreader slide should be increased.

Common causes of a poor blood smear:

- Drop of blood too large or too small.
- Spreader slide pushed across the slide in a jerky manner.
- Failure to keep the entire edge of the spreader slide against the slide while making the smear.
- Failure to keep the spreader slide at a 30 angle with the slide.
- Failure to push the spreader slide completely across the slide.
- Biologic causes of a poor smear:
 - ✓ Cold agglutinin - RBCs will clump together. Warm the blood at 37 oC for 5 minutes, and then remake the smear.
 - ✓ Lipemia - holes will appear in the smear. There is nothing you can do to correct this.

- ✓ Rouleaux - RBC's will form into stacks resembling coins. There is nothing you can do to correct this.

The WBCs are unevenly distributed and RBC distortion is seen at the edges. Smaller WBCs such as lymphocytes tend to reside in the middle of the feathered edge. Large cells such as monocytes, immature cells and abnormal cells can be found in the outer limits of this area. Spun smears produce the most uniform distribution of blood cells.

Blood staining

Wright's Stain

The Wright's stain is a Romanowsky stain. A Romanowsky stain is any stain combination consisting of eosin Y or eosin B with methylene blue and/or any of its oxidations products. Such stains produce the typical purple coloration of leukocyte nuclei and neutrophilic granules as well as the numerous blues and pinks found in other cell types. Methyl alcohol is used as both a solvent and fixative in this procedure [60-64].

Procedure:

- Attach a clothes pin (or use forceps) to the thick edge of the blood smear.
- Place the slide in the Coplin jar with Wright's stain. Allow to stand 5-10 seconds.
- Raise the slide out of the stain and allow the majority of the stain to run off the slide.
- Place the slide in the first jar containing deionized water. Allow to stand 10-20 seconds.
- Remove the slide carefully and dip several times in the second jar containing deionized water to rinse off the excess stain.
- Wipe off excess fluid from the back of the slide. Place the slide upright on a paper towel with the feathered edge up and allow to air dry.
- When completely dry, examine the smear with the microscope (lower power 10x) as follows:
 - **Determine the overall staining quality of the blood smear:**
 - Stain should not be too dark or too pale.
 - There should be no stain precipitate present on smear.
 - RBCs should be appropriate color of reddish pink.
 - Lymphocytes have dark purple nuclei with varying shades of blue cytoplasm
 - Neutrophils have dark purple nuclei with reddish, granular cytoplasm.
 - Monocytes have a lighter purple nucleus with a gray-blue cytoplasm.

- Eosinophils have bright red/orange granules
- Basophils have dark purple nuclei and granules
- Scan the edges and center of the slide to be sure there are no clumps of RBCs, WBCs or platelets. The interpretation result of blood cell is illustrated in table 4.
- In the blood there is some hemoparasite are observed like dirofilaria immitus, parafilaria and some protozoal (Figure 8).

Packed Cell Volume

The examination of the blood film for the qualitative assessment of the blood should include blood smear examination for digression from normal of the cell size, shape, distribution, haemoglobin concentration, colour, and intracellular inclusions 55, 65. Analysis of the collected blood samples was divided into the quantitative and qualitative quantification of the red cell, white cells and platelet parameters. The packed cell volume (PCV) was carried out using the microhaematocrit method while the haemoglobin concentration was carried out using the cyanmethaemoglobin method (Figure 9) [66]. The erythrocyte count was estimated by using the haematocytometer method while the erythrocyte indices (mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) were calculated using the methods described by [67].

Procedure of Microhematocrit [68]:

- * Fill the capillary tube two-thirds to three quarters full with well-mixed, oxalated venous blood or fingertip blood. (For fingertip blood use heparinized tubes, and invert several times to mix.)
- * Wipe the excess blood from the outside of the tube. Use plain(anticoagulant free)
- * Microhematocrit tubes with anticoagulant blood.
- * Push sealing clay into one end of each microhematocrit tube. Seal one end of the tube with clay.

Table 4: The morphological color of blood under staining.

Blood cell	Cytoplasm	Nucleus(chromatin)
Erythrocytes	Yellow to pinkish	Purple
Lymphocytes	Blue	Purplish red
Monocytes	Light blue	Purple
Eosinophil	Yellow to brownish red rods	Light purple
Basophils	Dark purple granules	
Neutrophils	Yellow to brownish red rods	Light purple
Thrombocytes	Grey-blue	Purple

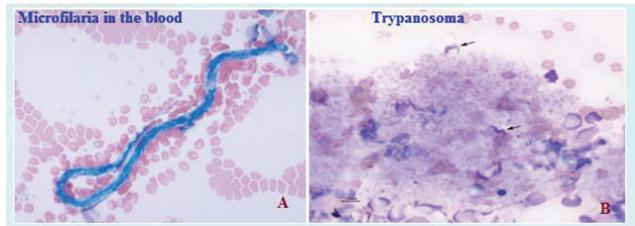


Figure 8: The presence of infectious agent in blood. A: Presence of *Dirofilaria immitis* (heartworm) or *Dipetalonema-Acanthocheilonema reconditum*. B: Trypanosomes (arrows) are caught up within this platelet clump in an anemic dairy coe (<http://www.eclinpath.com/d>).

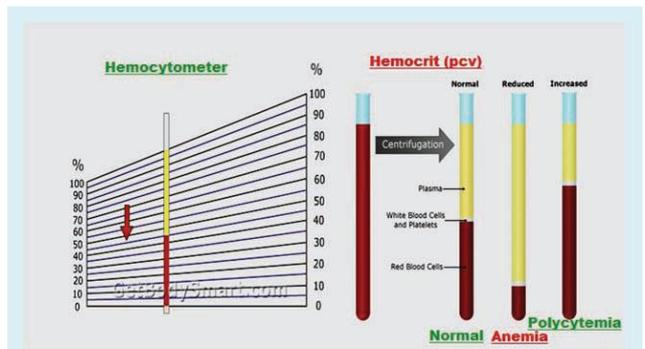


Figure 9: Hemocrit centrifuge result or PCV and Hemocytometer.

- * Place the filled tube in the microhematocrit centrifuge, with the plugged end away from the center of the centrifuge.
- * Centrifuge at a preset speed of 10,000 to 12,000 rpm for 5 minutes. If the hematocrit exceeds 50 percent, centrifuge for an additional 3 minutes.
- * Place the tube in the microhematocrit reader. Read the hematocrit by hematocrit tube reader.

Hematocrit Value

Hematocrit is the percentage of blood volume made up by red blood cells and it is known as packed cell volume (PCV). It can be determined by macro and micro methods. Macromethods are used by Wintrobe tube. With the help of a long needle and syringe, fill the Wintrobe tube with blood upto mark 100. Centrifuge the tubes at 3000 rpm for exactly 30 min. Record the reading of packed cell volume in percent, i.e. mass of erythrocytes settled down in tube. Micromethods is the method use microcapillaries of 1.0 mm diameter and 7-8 cm length are used. The capillaries are filled with blood by capillary action from collection vial. Seal one end of capillary with plasticin and centrifuge in microcentrifuge for 5 min. Remove the capillaries and put them on reader scale to calculate hematocrit value [69].

Packed Cell Volume (PCV) which is also known as haematocrit (Ht or Hct) or erythrocyte volume fraction (EVF) is the percentage (%) of red blood cells in blood [69]. Packed Cell Volume is involved in the transport of oxygen and absorbed nutrients. Increased Packed Cell Volume shows a better transportation and thus results in an increased

primary and secondary polycythemia. Haemoglobin is the iron containing oxygen-transport metalloprotein in the red blood cells of all vertebrates with the exception of the fish family, channichthyidae as well as tissues of invertebrates [70]. Haemoglobin has the physiological function of transporting oxygen to tissues of the animal for oxidation of ingested food so as to release energy for the other body functions as well as transport carbon dioxide out of the body of animals [71,72]. Packed Cell Volume (PCV) reading indicated either an increase in number of Red Blood Cells (RBCs) or reduction in circulating plasma volume.

Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration indicate blood level conditions. A low level is an indication of anaemia [73]. The haematocrit or packed cell volume (PCV) is considered an integral part of an animal's complete blood count result, along with haemoglobin concentration, white blood cell count and platelet count [74,75]. In mammals, haematocrit is independent of body size [75]. The Packed Cell Volume (PCV) can be determined by centrifuging heparinized blood in a capillary tube (also known as microhaematocrit tube) at 10,000 RPM for 5 minutes [76]. This separates the blood into layers. The volume of Packed Red Blood Cells divided by the total volume of the blood sample gives the Packed Cell Volume (PCV). Because a tube is used, this can be measured by measuring the lengths of the layer. According to Wikihow (2013c), the Packed Cell Volume (PCV) of animals can be determined to know their anaemia state; the haematocrit is used to screen animal to determine the extent of anaemia. A low haematocrit combined with other blood abnormal blood tests, confirms the diagnosis. The haematocrit is decreased in a variety of common conditions including liver and kidney diseases, malnutrition, vitamin B12 and folic acid deficiencies, iron deficiency, pregnancy among others [76]. A low haematocrit with a low MCV with a high RDW suggests a chronic-iron-deficient anaemia resulting in abnormal haemoglobin synthesis during erythropoiesis [75].

An elevated haematocrit is most often associated with dehydration, which is a decreased amount of water in the tissues, diarrhea etc. These conditions reduce the volume of plasma causing a relative increase in RBCs which concentrates the RBCs, called hemoconcentration [77,78]. Kopp and Hetesa (2000) and Chineke et al. (2006) documented that high PCV haematocrit reading indicated either an increase in the number of circulating RBC or reduction in circulating plasma volume. An elevated haematocrit may also be caused by an absolute increase in blood cells, called polycythemia. This may be secondary to a decrease amount of oxygen, called hypoxia or a result of proliferation of blood forming cells in the bone marrow (Polycythemia vera) [76].

Mean Corpuscular Volume or "Mean Cell Volume" (MCV), is a measure of the average red blood cell volume that is reported as part of a standard complete blood count. The MCV is calculated by dividing the total volume of packed red blood cells (also known as haematocrit) by the total number of red blood cells. The resulting number is then multiplied by 10. The red blood cells get packed together when they are spun around at high speeds in a centrifuge [79,80].

Haemoglobin deficiency can be caused either by decreased amount of haemoglobin molecules, as in anaemia, or by decreased ability of each molecule to bind oxygen at the same partial pressure of oxygen [81]. In any case, haemoglobin deficiency decreases blood oxygen carrying capacity. Haemoglobin deficiency is, in general, strictly distinguished from hypoxemia, defined as decreased partial pressure of oxygen in the blood [81]. Other common causes of low haemoglobin include loss of blood, nutritional deficiency, and bone marrow problems among others. High haemoglobin levels may be caused by exposure to high altitudes, dehydrations, and tumours, among others [82]. The ability of each haemoglobin molecule to carry oxygen is normally modified by altered blood PH or CO_2 , causing an altered oxygen-haemoglobin dissociation curve. However, it can also be pathologically altered in, example carbon monoxide poisoning. If the concentration is below normal, this is called anaemia. Decreased haemoglobin, with or without an absolute decrease of red blood cells, leads to symptoms of anaemia [81]. The Mean Corpuscular Haemoglobin or "Mean Cell Haemoglobin" (MCH) is the average mass of haemoglobin per red blood cell in a sample of blood [83-85]. Mean Corpuscular Haemoglobin Concentration (MCHC) is a measure of the concentration of haemoglobin in a given volume of packed red blood cells. MCHC is very significant in the diagnosis of anaemia and also serve as a useful index of the capacity of the bone marrow to produce red blood cells. It is reported as part of a standard complete blood count. It is calculated by dividing the haemoglobin by the haematocrit [86].

White Blood Cell Count is a test to determine the number of WBCs (Figure 10). Five [87,88] and diverse types of leucocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as hematopoietic stem cell. An increase in the number of leucocytes very the upper limits is called leucocytosis and a decrease below the lower limit is called leucopenia. A high white blood cell indicate another problem, such as, infection, stress, inflammation, trauma, allergy, or certain diseases, for this reason, a high white blood cell count requires further investigation [89]. High white blood cell count could be caused by infection, immune system disorders, stress, anaemia, bone marrow tumour, infectious diseases inflammatory disease, severe physical stress, tissue damage (for example, burns) among others [89-92]. A low white blood cell count, or leucopenia, is a decrease in disease

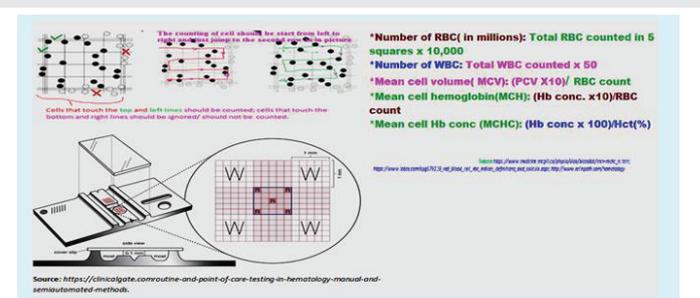


Figure 10: Hemocytometer and calculation of total blood count. The common formulae which used to calculate the total number of blood cell hemoglobin.

fighting cells (leucocyte) circulating in animal's body [92,93]. A low number of WBCs may be due to bone marrow deficiency or failure (for example, due to infection, tumour or abnormal scarring), disease of the liver or spleen, radiation therapy or exposure [92-94].

Component of blood smear

Blood smear have several areas while diagnosing under microscope, they are like feathered edge, the monolayer, the body and base of the smear (Figure 11). Among the layer of blood smear monolayer is the best area where the cells are examined in detail and differentiated counted is conducted, due to it dries quickly and cells are well spread (not overlapping) and not disrupted. Red blood cells are separated or barely touching, with little overlapping. In any species but the horse, rouleaux, consisting of stacks of 3 or more red cells, in this area are an abnormality suggestive of increased globulin concentration, which is common in animals with inflammatory disorders [68,95,96].

The animals which have very anemic and polycythemic state, it is difficult to find an area where the RBC are not

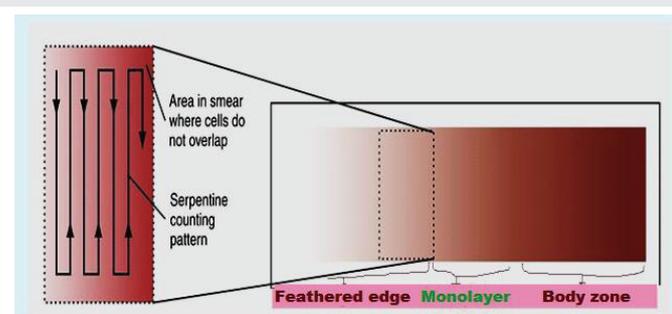


Figure 11: The layer of blood smear.

Table 5: The change of blood cell from examination (<http://www.eclinpath.com/hematology>).

Blood cell	Shape	Size	Color	Inclusion	Pattern	Infectious Agent
RBC	*Acanthocyte *Echinocyte *Eccentricocytes *Keratocytes *Schistocyte *Spherocytes *Ovalocytes *Target Cells *Stomatocyte	*Macrocyte *Microcytes *Anisocytosis (Variation In Size)	*Hypochromasia (Too Little Hemoglobin), *Polychromasia (Immature Cells containing RNA or Reticulocytes).	*Siderocytes (Iron), *Basophils *Stippling (RNA), *Howell-Jolly Bodies (Retained Nuclei), *Heinz Bodies (Oxidized Hemoglobin).	*Agglutination (Noted As Present Or Absent), *Rouleau Formation	*Babesia, *Anaplasma Spp.
Blood cell	Toxic Change	Reactive Lymphocyte	Smudge Cell	Abnormal Cell	Infectious Agent	
WBC	*Cytoplasmic Basophilia, *Döhle Bodies, *Cytoplasmic Vacuolation, *Toxic Granulation, *Immature Chromatin.	Indicating Antigenic Stimulation	A Few Smudged Cells Will Not Impact The Count But Moderate to Many Will Smudged Cells Are More Frequently Seen In "Aged" Or Stored Samples.	*Presence of Leukemia *Blasts *Dysplastic Changes In Leukocytes, Iron In Leukocytes, *Leukocytes, *Histocytes, *Mast Cells.	*Anaplasma Spp.	Platelets: Size And Degree of Granularity Is Assessed, Along With The Presence of Anaplasma Sp.

touching or overlapping. So that the clinician should have to search from feathered edge. The feathered edge of blood smear is the best for detection of platelet clump and some hemoparasite like microflaria, the smear should be examined under lower power microscope. In feathered zone, the RBC is completely flattened and lack of central pallor, mimicking spherocytes [96,97].

In body zone of blood smear the thick area of a smear dries too slowly for leukocytes to spread out. This area is too thick, however the smear should be examined under low power microscope to detect or identify low number of potentially cells (blast in leukemia), microfilaria, trypanosomes and certain RBC change like hypochromasia and agglutinates (Table 5) [68,97].

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Research Article

Examination of feces

Fecal examination is indicated in diarrhoeic diseases, constipation, anorexia, jaundice, hepatitis, lungworm infection, anemia, weakness, low milk production and in dysentery. Examination of feces is mainly carried out for the detection of parasites, their ova/larva/oocyst, blood and other foreign material. The fecal examination for diagnosis of parasitic infections is probably the most common laboratory procedure performed in a veterinarian clinic. Sometimes cultural examination of feces is performed for isolation of bacteria or virus in order to establish the etiology of diarrhea/dysentery. The bacterial or viral antigens are also demonstrated in feces using immunological methods like immunofluorescence, enzyme linked immunosorbent assay and dot immunobinding assay. Parasitic worm eggs from the respiratory system may be coughed into the throat and swallowed and appear in the feces. Parasitic forms seen under the microscope have characteristic morphologic features that with a little practice can be diagnostic for a particular parasite. Fecal examination can reveal the presence of parasites in many parts of the animal body. This clinic will concentrate on parasites inhabiting the digestive system. Fecal examination should be done on fresh samples. If fecal samples are used after being in the environment for hours or days accurate reading of parasite indicators cannot be guaranteed. Also, free-living nematodes rapidly invade a fecal sample on the ground and can confuse diagnosis. Several grams of feces should be collected immediately after observing defecation [1].

Collection of fecal sample in domestic animals

When collecting fecal samples, first make certain that the feces is from the animal in question. Secondly, secure a fresh sample that is free from rocks, soil, bedding, and other foreign materials. Place the fecal sample in a plastic vial, glass jar, waxed cup, or plastic bag. If the examination does not follow closely after collection, preserve the sample in a refrigerator [2,3]. In large animals, one should wear gloves and lubricate

the hands with water and collect feces from rectum. If feces is not present in rectum, stimulate the mucosa of rectum with finger, the animal will defecate within few minutes [4].

Collect 5-10 gm feces in a clear, dry glass container. If the feces are to be collected for cultural examination, it should be collected in sterilized glass vials [5]. In small animals like calves, sheep, goat and dogs, the feces is collected by using index finger. In poultry, the cloaca1 swabs are collected for examination. If the processing of a fecal specimen must be delayed, it may be: Refrigerated (but not frozen) for several days (not recommended for samples with live larvae that you intend to examine using the Baermann technique) and Fixed, 10% formalin (5% formalin-saline is better for protozoal cysts). Add fixative to feces at a ratio 3:1 (v:v) and mix well. Do not fix samples intended for use with the Baermann technique the larvae may destroy by the formalin [6].

The procedure how to take feces:

- * The operator places an obstetrical sleeve on one arm
- * The arm is formed into a cone and the animal's tail held to one side with the opposite gloved hand.
- * Gentle pressure is applied to the anal sphincter until penetration into the rectum is obtained.
- * A fecal aliquot of sufficient size for the intended laboratory procedure is scooped with the sleeved hand and removed from the animal.
- * The fecal sample is placed in a separate container or the obstetrical sleeve is inverted off the arm such that the fecal sample is trapped inside.
- * Small calves, sheep, goats, and swine: restrain manually. Gently pass a gloved, lubricated finger through the anus and massage the rectal wall to stimulate rectal evacuation.
- * If feces are not produced, collect feces with finger.

Techniques of fecal examination in veterinary laboratory

After fecal sample is collected from domestic animals, it would present to laboratory for examination. The methods

for examination of collected fecal sample are direct smear, flotation, and gross examinations.

Direct fecal smear examination

The direct smear method involves mixing a very small amount of feces with a water or saline solution. Place the mixture on a slide, overlay it with a cover glass, and examine the entire smear under a low power microscope. Observe the mixture for worm eggs and larvae and protozoa trophozoites and cysts. The use of a small amount of feces and the presence of fecal debris lower the reliability of this examination method [7,8].

The fecal sample can be examined by qualitative (flotation and sedimentation) or quantitative methods (Mack master method) if the direct smear method interpreted negative result.

Flotation examination

Examination by the flotation method is more complex and requires more time, but is usually more accurate than the direct smear [8]. The feces is mixed with either saturated sugar solution, saturated salt solution, saturated sodium nitrate solution, 41% magnesium sulfate solution or 33% zinc sulfate solution. The flotation method uses the principle that most fecal particles fall to the bottom of the tube or vial. Parasite eggs and cysts rise to the top of the salt or sugar solution, which is the result of a weight difference between feces, parasite eggs, and cysts within the solution. In pure water, parasite eggs and cysts settle to the bottom rather than float; whereas in the salt or sugar solution, they float due to the higher density of the solution [9].

Instrument and some reagent required:

- Plastic containers or two beakers
- Saturated sugar,
- Saturated salt,
- Saturated sodium nitrate,
- Magnesium sulfate a
- Zinc sulfate solutions.
- Gauze or tea strainer, double layer of cheesecloth
- Measuring cylinder
- Centerfuge
- Stirring rod
- Mortar and pistle
- Test tube
- Test tube rack
- Pipettes

- Balance
- Teaspoon
- Microscope
- Slide and cover slide

The procedure how to process the fecal flotation is listed as following steps [9,10].

The flotation method involves the use of a flotation solution that has a specific gravity, greater than 1.2, such as a salt or sugar solution (Figure 6.1)

- Simple flotation, about one gram of feces is taken and grinded and mixed with 42 ml of saline water
- Then filter it through a fine sieve or muslin cloth or gauze in to test tube or cylinder until it form meniscus (up to top of tube).
- A clean glass slide or cover slide is placed on the mouth of test tube or cylinder. Then left it for 10- 15 minute at room temperature without disturbance, then remove coverslide/ slide and examine under 10 x of microscope.
- In centrifugation flotation, the first step as simple flotation is similar, except this method use centrifugation. Mixed the contents and centrifuge at 1500 rpm for 5 minute, the tube is taken out and placed without disturbance. Transfer the small amount of superficial contents of the tube on a clean and dry glass slide.
- Place the cover slip on a slide and examine it under a microscope and the parasite ova may be observed under microscope as Figure 6.2.



Figure 1: The step of fecal flotation. A. Mix 2 to 5g of feces with approximately 10ml of flotation solution in a disposable cup until the consistency is uniform. B. Strain the mixture. C. Pour the strained mixture into a 15-ml centrifuge tube. D. Fill the tube with flotation solution to form a slight positive meniscus; do not overfill the tube. E. Place a coverslip on top of tube. F. Put the tube in the centrifuge, make sure the centrifuge is balanced, and spin at 1,200 rpm for 5 minutes. G. Remove the tube and let it stand for 10 minutes. H. Lift the coverslip directly upward and place on a glass microscope slide and examine the entire area under the coverslip at 10X magnification.

- The unfiltered material left on sieve or muslin cloth is examined for tapeworm segment.

Sedimentation method

The sedimentation technique is qualitative methods for detection of trematode egg in feces of domestic animals. The majority of trematode eggs are too large and heavy to float reliably in the flotation fluids normally used for nematode eggs. They do however sink rapidly to the bottom of a faecal/water suspension and this is the basis of the faecal sedimentation technique.

Instrument and some reagent required:

- Plastic containers or two beakers
- Gauze or tea strainer, double layer of cheesecloth
- Measuring cylinder
- Stirring rod
- Mortar and pestle
- Test tube
- Centrifuge
- Test tube rack
- Methylene blue
- Pipettes
- Balance
- Teaspoon
- Microscope
- Slide and cover slide

Procedures:

- Weigh or measure 3 g of faeces and grinded it, then transfer it into cylinder [1].

- Pour 40-50 ml of tap water into cylinder1.
- Mix faeces and water thoroughly.
- Filter the suspension through a tea strainer or double-layer of cheese cloth into cylinder [2].
- Pour the filtered material into a test tube. And allow to sediment for 5 minutes
- Remove the supernatant with a pipette very carefully.
- Re-suspend the sediment in 5ml of water.
- Allow to sediment for 5 minutes.
- Discard the supernatant carefully.
- The eggs of liver flukes are heavy and do not float on water and settle in sediment.
- Stain the sediment by adding one drop of methylene blue
- The dyes stain the faecal particles a deep blue or green leaving the trematode eggs unstained. The egg of trematode is revealed in figure
- Transfer a small drop of the stained sediment to a microscope slide using a pipette.
- Cover droplet with a coverslip and examine under a microscope at 10 x 10 magnifications.

McMaster egg counting technique

McMaster is the quantitative methods for determining the number of nematode eggs per gram of feces in order to estimate the worm burden in an animal. Advantage of this technique is quick as the eggs are floated free of debris before counting.

Equipment required:

- * Beakers or plastic containers
- * Balance
- * Tea strainer or cheesecloth
- * Measuring cylinder
- * Stirring device (fork)
- * Pasteur pipettes and rubber teats
- * Flotation fluid
- * McMaster counting chamber (Figure 3)
- * Microscope

Procedure of McMaster techniques [8,13-15]:

- * Weigh out 2 gm of feces and transfer in to container 1
- * Add 60 ml of saturated salt solution into container 1

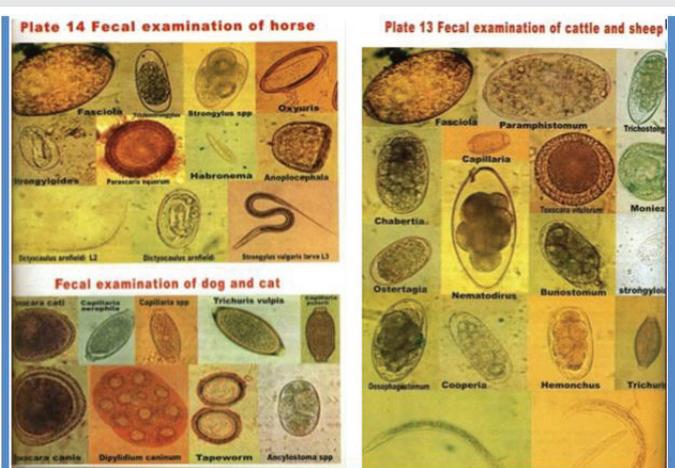


Figure 2: The larvae eggs and larvae of different species of helminths in domestic animals.

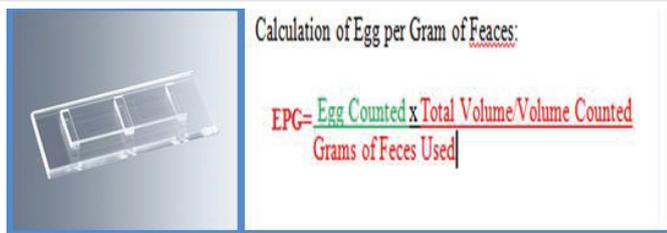


Figure 3: Macmaster two chamber and equation for egg per gram of faeces.

- * Mix feces with saturated salt solution by stirring device
- * Filter fecal from container 1 into container 2 or cylinder by gauze or sieve
- * Take a sub- sample with a Pasteur pipette from container 2
- * Fill both side of the McMaster counting chamber with the sub sample
- * Allow the counting chamber to stand for 3-5 minutes
- * Examine the sub sample of filtrate under a microscope at 10x 10 magnification
- * Count all eggs and coccidian oocytes within the engraved area of both chambers
- * Focus first on the etched lines of the grid, then go down a tiny bit, the egg will be floating just below the top of the chamber.
- * The calculation of egg from chambers is: Multiply the total number of eggs in the 2 chambers by 100= eggs per gram (EPG) or multiply the total by 50. This gives the EPG (egg per gram of feces) of faeces. (Example: 50 eggs seen in chamber 1 and 100 eggs seen in chamber 2 = $(50 + 100) \times 50 = 25,000$ epg.)

Baermann apparatus technique

In 1917, while working in Java, the Dutch physician Dr. Baermann developed a simple method for isolating nematodes from soil [16]. Nowadays, the veterinarians and nurses are using this method for the extraction of live larval stages of nematode parasites from feces. The Baermann technique is used to isolate lungworm larvae from faecal samples and infective larvae from faecal cultures. It is based on the active migration of larvae from faeces suspended in water and their subsequent collection and identification [17].

Berman technique makes use of two characteristics of parasitic larval nematode behavior like; the warmer it is, the more active the larva (37 to 40 °C is as warm as you want to get), and, in addition, some larvae are thermotactic and will move towards the warmer water under the filter paper. And the other characteristic is like most parasitic larval nematodes are poor swimmers. Therefore, the following events take place when the sieve is placed in the water: The larvae will be moving around in a random fashion and within any given time interval

some of them will migrate through the tissue and fall into the water. Because they can't swim they sink to the bottom and over time a number accumulate there. The more active the larvae are (i.e. the warmer the water) the greater the number of larvae that accumulate at the bottom in a given time interval. The longer you wait, the more larvae will fall to the bottom of the dish, but with time, the fecal sample breaks down and begins to pass through the tissue leading to an accumulation of sediment along with the larvae [8,18].

Equipment required:

- * Funnel
- * Funnel stand
- * Rubber or plastic tubing
- * Rubber bands
- * Clamp or spring clip
- * Cheesecloth
- * Simple thin stick
- * Strainer
- * Microscope
- * Test tube
- * Pasteur pipette
- * Small petridish

The procedure of baermann apparatus technique [18] (Figure 4):

- * First of all, construct the funnel with its standing; fit a short piece of tubing which is closed at one end with a clamp or string clip to the stem of a funnel
- * Support the funnel by a stand
- * Weigh the fecal culture/ faeces about 5- 10 gm and place on a piece of double layer cheesecloth
- * Form the cheesecloth around the faeces as a pouch
- * Close the pouch with a rubber band
- * Fix a supporting stick under the rubber band
- * Place the pouch containing faecal culture material or faeces in the funnel and trim the surplus cheesecloth off
- * Fill the funnel with lukewarm water and covering the faecal material
- * Leave the apparatus in place for 24 hours, during time larvae actively move out of faeces and ultimately collect by gravitation in the stem of the funnel.
- * Draw 10-15 ml of fluid from the stem of the funnel into a test tube or petri dish.



Figure 4: The procedure of Baermann apparatus technique for diagnosis of larvae of nematode like lung worm. A. Place a sieve in a custard dish or other similar container, B & C. Spread about 10 grams of feces on a piece of tissue paper and place it into the sieve, D & E. Place warm water in the custard dish until it just covers the feces, taking care not to disrupt the feces. Allow to sit for at least one hour, f. Lift off sieve, G. I and K. Using a Pasteur pipette, remove a drop of the sediment at the bottom of the tube and place it on a microscope slide for examination.

- * Leave the tube to stand for 30 minutes. Remove the supernatant with a Pasteur pipette.
- * Transfer a small aliquot of the remaining fluid using a Pasteur pipette to a microslide, add a drop of iodine and cover with a coverslip. For positive samples a transfer of larvae to a microslide for identification at 10 x 10 magnifications may be required. It is important to differentiate between *Muellerius capillaris*, *Dictyocaulus spp*s and other species as the treatment is different.

Examination of feces for blood

Take 2–3 ml of saturated solution of benzidine in glacial acid in to small test tube, and then add 2–3 ml of hydrogen peroxide. The feces 2 gm is mixed with distilled water and fecal suspension is poured in the benzidine solution drop by drop. If blood is present in faeces, it would generate blue colour.

Preparation of fecal culture

Many nematode eggs are alike and species such as *Haemonchus*, *Mecistocirrus*, *Ostertagia*, *Trichstrongylus*, *Cooperia*, *Bunostomum*, and *Oesophagostomum* cannot be clearly differentiated from the eggs in faecal samples. For these parasites, differentiation can be achieved by the use of faecal cultures. They provide a suitable environment for the hatching and development of helminth eggs into the infective stage (L₃).

Equipment required for fecal culture:

- Fork
- Spoon depressor

- Spatula
- Water
- Jars
- Containers
- Charcoal (It should be added with equal amount with feces and used to make moist feces to become damp.)

Procedure of fecal culture:

- Break up collected faeces finely using a mortar and pestle or stirring device
- Faeces should be moist and crumbly
- If faeces are too dry, add water
- If faeces are too wet, add charcoal until the correct consistency is obtained
- Transfer the mixture to jar or other containers
- Leave the culture at room temperature for 14–21 days, at this time all larvae should have reached the infected stage
- If an incubator is available at laboratory, the culture should be placed at 27 °C and left for 7–10 days.
- Add water to culture regularly, every 1–2 days
- Larvae are identified using Baermann technique

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Research Article

Examination of urine

Urinalysis usually is performed as a screening technique for acquiring a large amount of information about several body systems. It is studying the kidneys related to the detection of abnormal component in urine that may be of renal origins and relation of urine concentration ability to kidney function. Analysis of urine is conducted through collection of samples. Urine is normally yellow in all domestic animals. The yellow color is derived from bilirubin excreted into the intestine and reabsorbed into the portal circulation as urobilinogen [1].

Urine collection

Methods of urine collection are the most important [2]. The urine can be collected by gentle tickling of the perineum around the vulva with a piece of straw or the fingers may encourage a cow or heifer to urinate. In male animals similar handling of the prepuce may be followed by urination. The Cattle very often will urinate during examinations, so have a suitable container ready. Continuous stroking of the skin just below the vulva of cows will usually induce urination. Once collected, the urine sample should be inspected, smelled and its contents tested [3]. Urine can be collected by catheter methods, in cow and heifers plastic catheter 0.5cm in diameter and 40 cm long may be passed into the bladder. The methods how catheter is inserted into bladder for collection of blood, first disinfection of vagina and placed gloved forefinger into suburethral diverticulum and insert catheter over the finger into urethra. Then urine may flow freely from the bladder into sterile syringe via catheter [3].

Horses both sexes urinate only while resting and cease feeding for the time and cows urinate similarly to mares, male cattle on the other hand urinate not only while feeding but also while walking, old dogs and pigs male void the urine in the interrupted jerky stream 1. Since bulls and steers cannot be catheterized, longer observation may be needed to obtain a sample. A collecting urinal may be strapped on male swine,

cattle, sheep, or goats to obtain a sample. Catheters can be used on both male and female horses. In male horses, manual pressure on the bladder via the rectum will sometimes induce urination. Close observation will enable collection from dogs and cats, but catheterization can be used successfully. Other collection methods from dogs and cats include applying pressure on the bladder and using a collection cage [4,5].

Storage of Sampled Urine

Urine sample can be examined immediately after collection. However, the sample is expected to be delayed, the urine sample should be kept at 4°C without adding any preservative. For long preservative toluene can be added in urine to form a layer over urine, this suit for chemical examination. One drop of 40% formalin can be used as preservative in urine; however it may give false reaction for sugar examination [6].

Physical examination of urine

The terms important in urination problem [1,7]:

- ✓ Polyuria refers to increase urine output
- ✓ Oliguria refers decrease in urine output
- ✓ Anuria is condition in which no output of urine.
- ✓ Dysuria is difficult or painful micturition.
- ✓ Stranguria is slow, dropwise, painful discharge of the urine caused by spasm of the urethra and bladder

Physical examination of urine includes assessment of color, clarity (transparency or turbidity), and specific gravity. Normal urine color, which varies from colorless or pale yellow to dark yellow, is associated with the presence of yellow pigments called urochromes, the end products of hemoglobin [7,8]. Cloudy urine usually is associated with presence of red blood cells, white blood cells, epithelial Cells, crystals, casts, bacteria, lipid, mucus, or semen within the urine sample. Clear or slightly cloudy urine should not obviate microscopic examination of the urine sediment as abnormal findings are still possible [9].

Myoglobin is dark red colour heme containing protein, stored in muscle. Change in permeability of myolemma

causes myoglobin leak in plasma, which is cleared by kidney swiftly [10]. Myoglobin is released in the blood plasma as a result of damage to muscle tissue, because of its small size and lack of binding to haptoglobin, it is rapidly removed from blood by the kidney and excrete into urine. Hemoglobin, released when the red blood cell becomes hemolysis, rapidly and tightly bound to haptoglobin [11]. Generally, the urine can be examined by physical like examination of urine color, odour, consistence/turbidity, foaming and specific gravity [1,3].

Examination of urine color and turbidity

The color of urine may be observed at the time of collection by keeping it in glass tube. The color of normal urine of domestic animals is water yellow to amber [12]. However, in certain disease conditions the color of urine alters as table below. Urine may be light to dark yellow and pale pink in color in bovines suffering from urolithiasis. Freshly voided urine from the healthy animals is usually clear, except in horse where it usually thick and cloudy due to the presence of calcium carbonates crystals and mucous [13].

Cloudy Urine is not Necessary Indication of Pathology in Horse [14]. Interestingly, in bovine obstructive urolithiasis urine may still be transparent and clear [15,16]. The variation in the colour of urine of the affected animals on day zero probably could be due to the variation in the concentration of urine, accumulation of sediments and haemorrhage. Dirty yellow coloured urine might be due to presence of sedulous materials in the urinary bladder. Brownish urine is indicative of mixing of blood in the urine, which could be due to haematuria or nephritis. Reddish colouration of urine is indicative of haematuria, which could be due to injury by calculi or inadvertent haemorrhage while performing surgery [17] (Table 1).

Normally the urine of equines is turbid and cloudy due to calcium carbonate and mucous while the urine of other animals is clear and transparent. Yellowish turbidity can be observed in pyogenic infections of kidneys. Kidney damage may be responsible for hematuria, albuminuria and proteinuria, which gives turbid urine. Cloudiness in urine is due to presence of leucocytes, erythrocytes, epithelial cells, bacteria, mucous, fat

and/or crystals. Of these, leucocytes give a white cloud while the red turbidity is seen due to erythrocytes and epithelial cells [2].

Bacteria form dorm cloudiness in urine and fat forms cloud. On addition of chloroform or ether in the urine sample urine becomes transparent. On centrifugation, the fat comes at the top of urine while other turbid material settles down [2].

Odour of urine

The normal odor of urine is uremic, but some disease may change the odor of urine. Some of the example like, ketosis may produce sweetish or fruity odor of urine, pyogenic infection of kidney produce fetid odour. The abnormality sample may give the odour of ammonia, acetone or fetid odor [3]. Normal urine has a slight odor of ammonia; however, the odor depends on urine concentration. Some species, such as cats and goats, have pungent urine odor because of urine composition. Bacterial infection may result in a strong odor due to pyuria; a strong ammonia odor may occur if the bacteria produce urease [20].

Specific gravity of urine

Specific gravity determined by number of particles per unity of solvent. In time of ingestion of large amount of water specific gravity of urine as low as 1.001 is normal due to excess water for hemostasis. However, in the time of dehydration specific gravity of urine is increased due to marked conservation of water is expected. Specific gravity (SG) which is directly proportional to urine osmolality, measures solute concentration and urine density, or the ability of the kidney to concentrate or dilute the urine over that of plasma. Specific gravity in health varies with the state of hydration and fluid intake [15,17]. The range of specific gravity of urine in normal cattle is 1.025–1.045 with an average of 1.035 and in the obstructive urolithiasis it ranges from 1.008 to 1.025.

Under normal conditions, urine SG ranges between 1.015 and 1.040 in healthy dogs and between 1.036 and 1.060 in healthy cats [1,21]. An animal that is dehydrated or has other causes of prerenal azotemia will have hypersthenuric urine with an SG >1.025–1.040 (depending on species). Dilute urine in a dehydrated or azotemic animal is abnormal and could be caused by renal failure, hypo- or hyperadrenocorticism, hypercalcemia, diabetes mellitus, hyperthyroidism, and diuretic therapy. In cases of diabetes insipidus, values <1.010 can be expected (Table 2).

Glucosuria increases the SG despite increased urine volume [20]. The specific gravity increases in acute interstitial nephritis, cystitis, diabetes mellitus and dehydration and it decreases in chronic interstitial nephritis, pyometra and diabetes insipidus (Table 2).

Foaming

On shaking, the normal urine produces white foams but in case of proteinuria, the amount of foam is in excess which remains for a longer duration. In icteric animals, the colour of foam may become green or yellow brown. The foams are red to brown in colour in case of hemoglobinuria.

Table 1: The abnormal color of urine with its cause (www.veterinaryworld.org/Vol1.6/sept2013/11.pdf).

Abnormality of urine	The color change of urine
Hematuria	Red colour urine, cloudy.
Hemoglobinuria	Brown to red colour.
Icterus	Yellow brown colour to greenish yellow
Pyogenic infections of Kidney	Yellowish green colour.
Azoturia in Horse	Brown to black colour of urine
Phenothiazine treatment	Red colour urine
Methylene blue treatment	Blue to greenish urine
Acriflavin treatment	Green coloured urine
Diabetes melintensis	Pale coloured urine
Diabetes insipidus	Pale coloured urine
Increased water up take	Pale coloured urine
Chronic interstitial Nephritis	Pale coloured urine
Pyometra	Pale coloured urine

Chemical examination of urine

Urine PH

The reaction of urine is determined by using pH strips or pH meter. Under normal conditions the urine of ruminants and horse is alkaline while in canines and feline it is acidic [22]. The pH of alkaline urine is 7.4–8.4, while that of acidic urine is 6–7. Urine becomes acidic during starvation, fever, treatment of ammonium chloride, sodium chloride, calcium chloride and sodium acid phosphate. The alkaline urine occurs in cystitis and due to treatment of acetate, bicarbonate; citrate and nitrates of sodium or potassium [22]. Urine pH will affect crystalluria because some crystals, such as struvite, form in alkaline urine, whereas other crystals, such as cystine, form in acidic urine [20,22].

Urine pH is a measurement of the kidneys ability to conserve hydrogen ions, thus it provides a rough but useful estimate of the body's acid-base status. However, urine pH does not necessarily reflect the body's pH, as it is highly influenced by diet, recent feeding, bacterial infection, storage time, metabolic and respiratory alkalosis, and urinary retention [23]. High protein diets, such as those consumed by carnivores produce neutral to acidic urine.

Herbivores tend to produce alkaline urine. Any animal may produce alkaline urine immediately after eating due to buffering that occurs in response to gastric acids. Alkaline nature of the urine is frequently linked to urinary tract infections. The bacteria break down urea and forms ammonia contributing towards the alkalinity of urine. Obstruction and renal tubular disease may also create alkaline urine. Acidic urine is commonly observed in animals with diabetes mellitus, especially if the animal is ketoacidotic. Excess or deficient dietary protein may lead to acidosis, as can Fanconi syndrome and metabolic acidosis [24]. The release of ammonia due to the breakdown of urea in the retained urine renders it alkaline. Struvite and calcium apatite uroliths are mostly found in urine with alkaline pH, while cystine stones are formed at the acidic pH. However, pH is variable in the formation of urate, silicate and calcium oxalate stones [25–27].

Urine Glucose

The urine sugar or glucose is measured by the following method using Benedict's reagent. Take 0.5 ml of urine in a test tube and add 5.0 ml of Benedict's reagent and mix them thoroughly (Figure 1,4). Place this tube in a boiling water bath or flame for 5 min. Remove the tube from heating and keep on test tube stand for a few minutes and note the change of colour.

A urine dipstick test is a basic diagnostic tool used to determine pathological changes in patient's urine in standard urinalysis. A standard urine test strip may comprise up to 10 different chemical pads or reagent which react (change color) when immersed in and then remove from urine sample. The analyzing urine test is test for presence of proteins, glucose, ketones, haemoglobin, bilirubin, urobilinogen, and nitrite and leucocytes as well as testing Ph and specific gravity or test for infection by different pathogens (Figure 2,3).

Normally there should not be any glucose content in urine. Glycosuria occurs due to hyperglycemia and in diabetes mellitus, acute or chronic pancreatitis, hyperthyroidism, hyperadrenalism, hyperpituitarism, increased intracranial pressure, enterotoxemia. Certain drugs like streptomycin, chlortetracyclin, penicillin, tetracyclin and chloramphenicol may also lead to glycosuria [28]. False-negative results can occur with high urinary concentrations of ascorbic acid (vitamin C) or with formaldehyde (a metabolite of the urinary antiseptic, methenamine, which may be used for prevention of bacterial urinary tract infections). False-positive results may occur if the sample is contaminated with hydrogen peroxide, chlorine, or hypochlorite (bleach) [22].

Glucosuria in combination with hyperglycaemia reflects a tubular resorption defect in which the renal tubules fail to reabsorb glucose from the glomerular filtrate [29]. Nonpathologic glucosuria is associated with eating (postprandial), excitement

Table 2: The clinical identification of urinalysis [18].

Parameter	Clinical pathological condition
High specific gravity (>1.035)	Nephrotic syndrome, dehydration, acute glomerulonephritis, heart failure, liver failure.
Low specific gravity (<1.005)	Diabetes insipidus, nephrogenic diabetes insipidus, acute tubular necrosis.
Protein in urine	Renal disease, fever, congestive heart failure (CHF), hypertension, tumors.
Glucose in urine	Sugar, levels are obviously important in diagnosing diabetes.
Ketone bodies in urine	Ketonuria occurs in diabetes mellitus and starvation.
Bilirubin	Liver damage or disease.
Blood	Hemoglobin or myoglobin. Kidney damage, infection, kidney or bladder stones, kidney.
Cancer	Blood disorders, among other conditions.
Red blood cells (RBC's) in the urine	Haematuria
White blood cells (WBC's) in the urine	Infection, urinary tract infections (UTI)
Crystals	Hypercalcemia

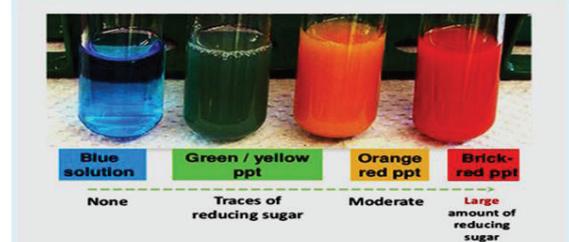


Figure 1: Benedictic test for glucose. Positive Benedict's Test: Formation of a reddish precipitate within three minutes. Reducing sugars present (Glucose). Negative Benedict's Test: No color change (Remains Blue). Reducing sugars absent (sucrose). <https://microbiologyinfo.com/benedicts-test-principle-composition-preparation-procedure-and-result-interpretation/>

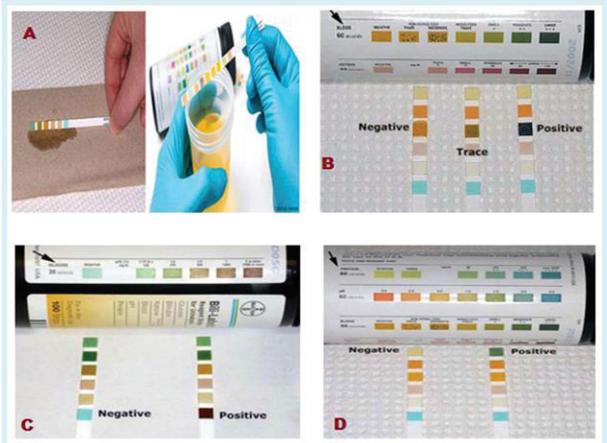


Figure 2: Urine dip stick test. A : Removal of excess urine horizontally will prevent the mixing of chemicals from different reagent pads. B : Traces heraturia and marked hematuria or hemoglobinuria with Bayer reagent strips. C: Marked glucosuria with Bayer reagent strips. D : Marked proteinuria with Bayer reagent strips (http://vetbook.org/cat/index.php?title=Urine_dip-stick_analysis).

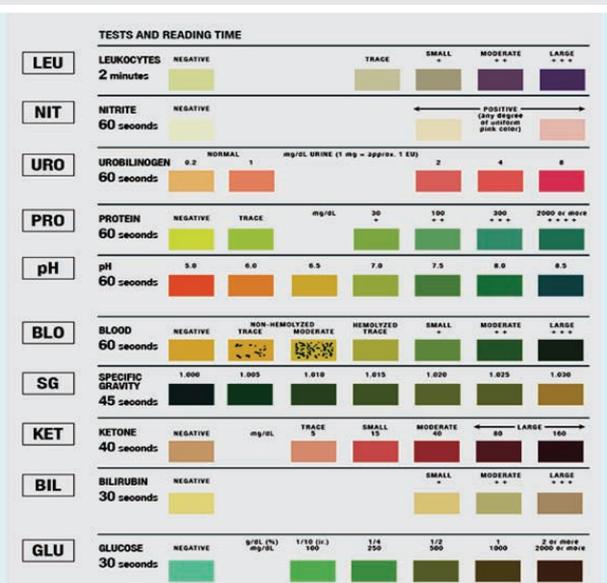


Figure 3: The reading and testing time of urine by dipstick testing method.

and stress (especially in cats and horses). Pathologic glucosuria is associated with diabetes mellitus, acute renal failure, and urinary obstruction in cats and milk fever in cattle. Numerous factors can decrease urine glucose values. These include refrigeration, ascorbic acid (vitamin C), salicylates, penicillin and presence of bacteria³⁰.

Protein

Urine protein levels are most conveniently determined with a dipstick. Normally, there is little to no protein present in urine. The presence of protein in urine is called proteinuria^[21]. Proteinuria may result from glomerulonephropathy, tubular transport defects, inflammation or infection within the urinary tract. Increased protein level in the urine might be due to acute nephritis or inflammatory exudation resulting from pyelitis, urethritis, cystitis and urolithiasis ^[31].

The glomerulus does not typically filter larger plasmaproteins, such as albumin and globulins, but it freely filters smaller proteins, which are reabsorbed in the proximal tubules of the kidneys unless there are significantly increased amounts of these proteins, or impairment of renal tubule reabsorption is present. The main protein in urine is albumin which comes under certain disease conditions. Robert's reagent test compositions are like; Nitric acid 1 part and saturated MgSO₄ 5 parts.

The procedure of Robert reagent test ^[32]:

Take 2 ml urine in the test tube, and then overlay it with 2 ml of Robert's reagent

Allow the urine to run slowly down along the wall of test tube. If albumin is present in the urine, a white ring will appear at the inter junction of two fluids which should be graded as follow: Very heavy ring (Highly positive), Heavy ring (Positive), A wide ring (Moderately positive), A narrow ring (Mild positive) and No ring (Negative).

Proteinuria may occur under some physiological conditions like excessive muscular exertion, stress, more protein in diet and convulsions which may disappear after removal of the factor. In disease conditions, proteinuria may occur in nephritis, acute interstitial nephritis, pyelonephritis and nephrosis. Phenol, arsenic, lead mercury, sulfonamides, phosphorus, turpentine and ether may cause proteinuria. Proteinuria may also occur in cystitis, prostatitis, pyelitis, urethritis, ureteritis, and urolithiasis and due to mixing of vaginal and prepuccial discharges in the urine ^[16]. The microscopic examination of urine is of great clinical importance. The important structures to identify include crystals, erythrocytes, leukocytes, casts and bacteria ^[33].

Ketone body test

Ketone bodies present in the urine react with the sodium nitroprusside which decomposed into sodium ferricyanide, ferric hydroxide and sodium nitrate. Then, these compounds in alkaline medium form complex with ketone bodies and produce purple colour. This colour is indication of positive test. Composition of Ross Reagent like Sodium nitroprusside 1 part and ammonium sulphate 100 parts ^[32].

The procedure:

- Place a half inch layer of Ross reagent and add 5 ml of urine.
- Shake the 2 components
- Add 1-2 ml of ammonium hydroxide.
- Wait for five minutes. Development of purple color ring at junction is the indication of presence of ketone bodies.
- **Results:** Slight purple, Moderate purple, Dark purple and Dark purple to black color ring

Bilirubinuria

The testing of bilirubin in urine is carried out by Gmelin test. The bile pigments are oxidized by acids and produced colored derivatives [32].

Procedure:

- Take 2 ml of nitric acid and 2 ml of urine in test tube.
- Presence of green to violet colour ring at the junction of two fluids is indication of presence of bilirubin in urine.
- **Result:** Bilirubinuria is seen in hepatocellular diseases like ICH (infectious canine hepatitis), leptospirosis, neoplasia; obstruction of bile duct; jaundice; and toxicides.

Crystalluria examination in urine

Crystalluria is a frequent finding during the routine examination of urine sediments. In most instances the precipitation of crystals of calcium oxalate, monosodium urate crystals, triple phosphate, calcium phosphate and amorphous phosphates or urates is caused by transient super saturation of urine, ingestion of specific foods and also associated with pathological conditions such as urolithiasis, acute uric acid nephropathy, and ethylene glycol poisoning, and hyperesinophilic syndrome and due to some sulfa drug like sulphadiazine [34].

Cellular evaluation of the sediments

Evaluation of the cellular components in the urine sediment is complicated by the fact that cells may originate from several areas such as the vascular system, interstitial tissue, urothelium or the genital tract (Table 3). The presence of increased number of white blood cells are evident in cystitis and pyelonephritis. Pyuria indicates a purulent process at some point in the urinary tract especially urethritis or cystitis [23].

Test for haemoglobinuria, myoglobinuria and hematuria

The urine sediment is examined for the presence of white

Table 4: Benedict test for glucose in blood.

Color change of test	The degree of sugar
Blue (-)	No sugar
Blue to green sediment (+)	mild sugar
Yellow with heavy sediment (++)	moderate sugar
Orange with heavy sediment (++++)	Highly positive for sugar

blood cells, red blood cells, epithelial cells, crystals, bacteria, fat spermatozoa and fungi. High numbers of calcium carbonate crystals are expected in urine of equine. Squamous epithelial cells from the genital tract or distal urethra are often present in urine samples. Transitional epithelial cells originating from the more proximal urinary tract also observed.

Large clumps of transitional epithelial cells or cellular atypia should show the possibility of urinary tract neoplasia (transitional cell carcinoma). Blood reagent pad detects hematuria, hemoglobinuria and myoglobinuria. Intact erythrocyte produces a speckled color change, whereas hemoglobin and myoglobin produce a uniform color change [35]. Presence of partial hemolysis of erythrocyte in urine sample can indicate the combination of hemoglobinuria and myoglobinuria. If hemoglobinuria is due to hemoglobinemia rather than lysis of erythrocytes with in urinary tract, clear red discoloration of the serum or plasma should be observed. Dark red urine not accompanied by hemoglobinemia or intact red cells on urine sediment examination is consistent with myoglobinuria. Myoglobin is a very small molecule with low renal threshold and discoloration of the plasma is not expected when there is muscle break down and release of myoglobin. There will be increase of muscle origin enzymes like creatine kinase and aspartate aminotransferase with in myoglobinuria [35]. In sedimentation of urine is urine discoloration is due to hematuria, there will be numerous red blood cells and increased turbidity. However, if urine remain transparent when color change is due to hemoglobinuria [36,37]. Myoglobinuria and hemoglobinuria may cause a tea-colored appearance of the urine, and although both cause positive results on the urine dipstick for blood, myoglobinuria may be differentiated from hemoglobinuria by performing a series of simple tests. Myoglobinuria is brown, and often only a few RBCs are present in the urine. Hematuria produces a reddish sediment in spun urine samples. Red or brown urine with a negative dipstick result for blood indicates a dye in the urine. Hemoglobin produces a reddish or brown coloration in the spun serum, whereas myoglobin does not discolor the serum. CK levels are markedly elevated in myoglobinuria. Results of radioimmunoassay for the specific measurement of serum or urine myoglobin can be delayed by several days and are not useful in immediate diagnosis and treatment [38].

The myoglobinuria may be clinically detectable as a red-brown or chocolate-brown discoloration of the urine. This discoloration can be differentiated from that caused by hemoglobin by spectrographic examination or with the use of orthotoluidine paper strips. Urine becomes dark when myoglobin levels exceed 40 mg/dL of urine [39]. Hemolysis indicates some intravascular hemolysis and myoglobinuria is indicated by muscle pathology [40].

Table 3: Types and characteristic of urinary cast [19].

Casts in urine	Source of cast	Causes of presence of cast in urine
Protein	Mucoprotein	Normal health, fever, exercise, diuretics, renal disease
Granular	Degenerating cellular casts	Glomerular disease, tubular disease, pyelonephritis, viral infections or aggregated proteins
Waxy	Final stages of granular cast	Advanced renal failure or other conditions with dilated tubules with diminished flow degeneration
Fatty	Lipid-containing renal	Nephrotic syndrome tubular cells
RBC's	Red blood cell	Glomerulonephritis, Tubulointerstitial nephritis, acute tubular injury/necrosis
WBC	White blood cells	Pyelonephritis, glomerulonephritis, Tubulointerstitial nephritis
Epithelial cell	Renal tubular cells	Epithelial cell Renal tubular cell

The pattern of increase in muscle enzymes and myoglobin concentrations in horses with RER suggested that the high plasma AST and CK activities commonly observed at rest in symptom-free Standard bred horses are probably a result of repeated subclinical episodes of rhabdomyolysis after exercise, rather than leakage due to abnormal sarcolemmal permeability [41]. Myoglobinuria is a common finding in adult horses with acute paralytic myoglobinuria but is not a common finding in acute nutritional muscular dystrophy in young farm animals, except perhaps in yearling cattle with acute muscular dystrophy.

White muscle disease is associated to vitamin/selenium deficiency but it is exacerbated by other factors such as exercise, environment (climatic conditions may be involved), nutrition and some toxicants and also myoglobinuria may cause by necrosis of skeletal muscle in horse like azoturia (paralytic myoglobinuria, Monday morning disease, sacral paralysis). It is a disease of horses associated to strenuous exercise after a long resting period and a diet rich in carbohydrates [42,43].

Hematuria

Hematuria is the presence of intact blood in the urine which appears as gross blood clots passed at the beginning (caused by urethral damage), after exercise in horse (caused by cystic calculi), or at the end of urination (caused by vesicle damage) or as more uniformed discoloration of the urine throughout the urination without clots. If the blood is equally distributed in the urine, it could be caused by kidney damage. At the centrifugation or sedimentation of urine the RBC is sedimented and the upper part become clear no red colouration [40,45] (Figure 4). The most causative agent of hematuria are classified as prerenal causes (trauma to kidney, septicemia and purpura hemorrhagica in horse), Renal causes (acute glomerulonephritis, pyelonephritis, tubular damage due to sulphonamide toxicity, embolism and renal infarction), Post renal causes (urolithiasis, urethritis, cystitis and enzootic hematuria in cattle, tumor in urinary bladder [35,44].

Hemoglobinuria

Hemoglobinuria is the presence of hemoglobin in the urine which caused by hemolysis of RBC. The true haemoglobinuria is manifested by deep red discoloration of the urine caused by lysis effect of *Bacillary hemoglobinuria*, *babesiosis*, copper intoxication, water intoxication on RBC [46]. False hemoglobinuria is occurs with cases of hematuria, when RBC are destroyed and liberate their contents of hemoglobin into urine.

Generally, to differentiate hemoglobinuria from myoglobinuria and hematuria (Figure 5,6), which all have a positive blood test on a urine dipstick, evaluate the color of the supernatant after centrifugation of the urine; hematuria will have a clear supernatant, whereas hemoglobinuria and myoglobinuria will not. To differentiate hemoglobinuria from myoglobinuria, evaluate the plasma color; hemoglobinuria will have a pink to red plasma color, whereas myoglobinuria will not [47]. If necessary, ammonium sulfate precipitation or urine protein electrophoresis can be used to differentiate



Figure 4: Centrifugation result of hematuria.

(http://www.youpetsbestfriend.com/your_pets_bes_friend/2008/07/bladder-blockage.html).



Rapid test to distinguish hematuria from hemoglobinuria. The onset of red urine during or shortly after a blood transfusion may represent hemoglobinuria (indicating an acute hemolytic reaction) or hematuria (indicating bleeding in the lower urinary tract). If freshly collected urine from a patient with hematuria is centrifuged, red blood cells settle at the bottom of the tube, leaving a clear yellow urine supernatant. If the red color is due to hemoglobinuria, the urine sample remains clear red after centrifugation.

Figure 5: The urine test which distinguish hematuria form hemoglobinuria.

(<https://emedicine.medscape.com-artical/206885-ocerview>).



Figure 6: The differentiation of hemoglobinuria from hematuris.

Red-brown discoloration of the urine suggests hemoglobinuria, hematuria, or myoglobinuria; however, red discoloration of the plasma (right) indicates that hemoglobinuria is present and this produced the hemeoglobinuria (<http://vetbook.org/wiki/cat/index.php?title=/Hemoglobinuria>).

hemoglobinuria from myoglobinuria [42]. Ammonium sulfate precipitation test, 5 ml of urine is mixed well with 2.8 mg of ammonium sulfate and centrifuged. Hemoglobin precipitates, myoglobin does not and if the supernatant remains dark after centrifugation, suspect myoglobinuria [48].

Myoglobinuria

Myoglobinuria is the presence of myoglobin in the urine. The myoglobinuria may be clinically detectable as a red-brown or chocolate-brown discoloration of the urine. Myoglobinuria

is caused by massive muscle necrosis (rhabdomyolysis) [49]. Myoglobinuria caused by acute myositis (e.g., toxoplasmosis), Compartment syndrome, Crush injury, Extreme exercise (rhabdomyolysis), and Tornequet syndrome prolonged Seizures. Myoglobinuria is usually the result of rhabdomyolysis or muscle destruction. Any process that interferes with the storage or use of energy by muscle cells can lead to myoglobinuria.

The release of myoglobin from muscle cells is often associated with an increase in levels of creatine kinase (CK), aldolase, lactate dehydrogenase (LDH), serum glutamic-pyruvic transaminase (SGPT), and other enzymes. When excreted into the urine, myoglobin, a monomer containing a heme molecule similar to hemoglobin, can precipitate, causing tubular obstruction and acute kidney injury

Myoglobin is a very small molecule with low renal threshold and discoloration of the plasma is not expected when there is muscle break down and release of myoglobin. There will be increase of muscle origin enzymes like creatine kinase and aspartate aminotransferase with in myoglobinuria. This enzyme can measure the urine discoloration is due to myoglobin [35]. Hemolysis indicates some intravascular hemolysis and myoglobinuria is indicated by muscle pathology [40]. The pattern of increase in muscle enzymes and myoglobin concentrations in the horses with RER suggested that the high plasma AST and CK activities commonly observed at rest in symptom-free Standardbred horses are probably a result of repeated subclinical episodes of rhabdomyolysis after exercise, rather than leakage due to abnormal sarcolemmal permeability [41,42].

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The myoglobinuria may be clinically detectable as a red-brown or chocolate-brown discoloration of the urine. This discoloration can be differentiated from that caused by hemoglobin by spectrographic examination or with the use of orthotoluidine paper strips. Urine becomes dark when myoglobin levels exceed 40 mg/dL of urine [42]. As the cell breaks down, large quantities of Potassium, aldolase, phosphate, myoglobin, CK, lactate dehydrogenase (LDH), aspartate transferase (AST) and urate leak into the circulation [50]. When more than 100g of muscle tissue is degraded the plasma's myoglobin binding capacity is overwhelmed and free myoglobin causes renal morbidity by several mechanisms [51].

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Research Article

Skin scraping

Skin scrapings are part of the basic database for all skin diseases. There are two types of skin scrapings, superficial and deep. Superficial scrapings do not cause capillary bleeding and provide information from the surface of the epidermis. Deep skin scrapings collect material from within the hair follicle; capillary bleeding indicates that the sampling was deep enough. Skin scrapings are used primarily to determine the presence or absence of mites. Skin scrapings are best performed using a skin-scraping spatula, which is a thin metal weighing spatula commonly found in pharmacy or chemical supply catalogs. These spatulas are reusable and do not cause injury [1]. Skin scrapings help diagnose fungal infections and scabies. For fungal infection, scale is taken from the border of the lesion and placed onto a microscope slide. Then a drop of 10 to 20% potassium hydroxide is added. Hyphae, budding yeast, or both confirm the diagnosis of tinea or candidiasis. For scabiesscrapings are taken from suspected burrows and placed directly under a coverslip with mineral oil; findings of mites, feces, or eggs confirm the diagnosis [1,2]. The skin scraping can be classified into two, superficial and deep scraping.

Superficial skin scraping

Superficial scraping used to identify surface mites and multiple scrapings should be taken to increase the likelihood of ectoparasites (*Cheyletiella*, *Sarcoptes*, *Psoroptes*, *Otodectes*, and *Demodex* mites, fleas and lice) detection [2,3].

The procedure of superficial scraping [3-6]:

- Remove the hair coat in the area by gentle clipping and after clipping the hairs the are has to be sterilized with antiseptics (chlorhexidine, soap)
- A few drops of liquid paraffin can be applied and spread over the skin scraping site, then scraping with a blunt scalpel blade

- Holding the blade perpendicular to the skin or slightly deviated away from the direction of scraping, the blade is gently passed over large areas of the skin in a sweeping pattern.
- Don't be apply 10% KOH directly to skin
- Take the scabed hair and skin content into petri dish
- Emulsion of scant material(superficial epidermis) is spread over a microscope slide or petri dish
- Add 2 -3 drops of 10% KOH into scabed sample and mix it
- It is warmed for about 3 minutes
- Covered with a cover slide and the slide is examine under microscope at 10x low power, to see adult, larvae, nymph and egg of parasite

Deep skin scraping

Deep skin scraping is important in the diagnosis of burrowing and deep follicular mites such as *Sarcoptes scabiei* and *Demodex* sp. The technique is repeated until capillary blood oozes out and multiple sites should be scraped to maximize detection of ectoparasites.

Procedure [2,3,7]:

- The hair should be clipped with scalpel blade. Holding the blade perpendicular to skin and blade is gently passed over a small area of lesional skin in a sweeping pattern. The skin should be pinched firmly between the thumb and forefinger to help extrude the parasite from deeper epidermal layer.
- Place the scalpel blade with material removed into petri dish or other container
- The collected material is mounted onto glass slide in liquid paraffin or potassium hydroxide.
- Cover aslide should be applied into sample collected on slide and examine it under low power objective of microscope

- Scan the parasite entire of slide and mite structure will observe as figure 1.

Examination of fungus from scraping

Fungal examination of skin scrapings is indicated when fungal infection of skin is suspected. The site of collection of scrapings should be the most active lesion at skin. Since some fungal infections spread towards the periphery, it is advisable to collect the scrapings from centre as well as from periphery of the lesion. When dermatophytes fungi affect skin and hair of the animal it will produce white greyish circular lesion accompanied with crust, scale, hyperkeratosis and alopecia. Those lesions normally found in the face, neck, chest, leg and body of the animal [8]. Before collection of the scrapings, skin is swabbed with 95% alcohol or spirit to remove any saprophytic organism. The scalpel should be moistened with mineral oil and the skin scrapped deeply to include hairs.

The skin scrapings are collected in sterilized petridishes containing 10% potassium hydroxide or sodium hydroxide. Fungi are usually isolated on Sabouraud dextrose agar which inhibits growth of most bacteria. The addition of chloramphenicol and cycloheximide increases sensitivity by inhibiting some of the fast growing contaminating bacteria and other microorganism [10]. Hair plucking can be collected and inoculated using sterile forceps into one of two media like sabouraud's agar or dermatophyte test medium. The culture should be kept at room temperature for 7-10 days and checked for appearance of colony of white colony and red coloration of the medium, which indicates presence of dermatophytes. However, if no growth after 3 weeks, the sample can be negative of ring worm [3].

Sabouraud's dextrose agar (SDA) and isolated selective media containing cycloheximide, penicillin and streptomycin were used in most diagnostic laboratories until point-of-care fungal culture medium was developed. In the late 1960s Dermatophyte Test Medium (DTM) was developed for field evaluation by paramedical personnel for skin infections in military forces operating in the tropics [11]. Dermatophyte Test Medium is a nutrient growth medium with antibiotics to suppress bacterial and contaminant fungal overgrowth and a colour indicator to aid in the early recognition of possible dermatophyte species. The colour change in the medium from yellow to red is the result of a pH change triggered by fungal growth. The first published article concluded that colour change alone was diagnostic of a dermatophyte, but numerous studies shortly thereafter documented a wide range of contaminants that also cause a red colour change in the medium [12,13].

The Wood's lamp has UV light, which is directed on the intact skin or on the scrapings collected in petridishes (Figure 2). If the fungus *Microsporum* is present, it gives yellow-green fluorescence while no fluorescence is observed in negative or trichophyton fungal infection of skin. The Wood's lamp is a point-of-care diagnostic tool, with which a test can be performed in clinic. It is an ultraviolet lamp that was invented in 1903 by Robert W. Wood as a light filter used in communications during World War I.

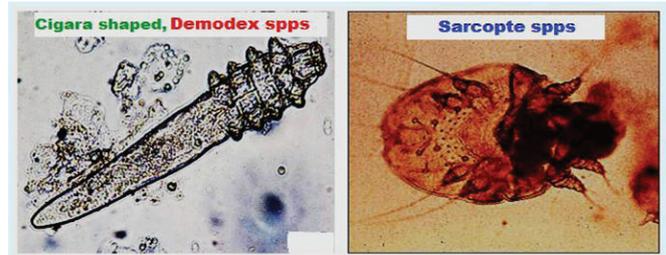


Figure 1: The structure of mite which obtained from scraping of skin (<http://orsofia.com/30206/bogdan-the-mengy-pup>).



Figure 2: Wood's lamp for diagnosis of fungal disease in domestic animals.

The original glass filter material has been replaced by newer materials (e.g. barium-sodium-silicate glass incorporating 9% nickel oxide) that coat the inside of glass tubes. The Wood's lamp glass is deep violet blue and is opaque to all visible light rays except the longest red and shortest violet wavelengths. It is transparent in the violet/ultraviolet band between 320 and 400 nm with a peak at 365 nm and a broad range of infrared and the longest, least visible red wavelengths [14]. Fluorescence occurs when light of shorter wavelengths initially emitted by the lamp, is absorbed and radiation of longer wavelengths is emitted [14]. Thus, it excludes most of the burning and tanning shorter rays (<320 nm) and the visible rays longer than 400 nm). A Wood's lamp is often mistakenly referred to as a "black light" but these are distinctly different things [14].

A black light is composed of a clear glass that filters medium- and short-wave ultraviolet light (UV) and emits a large amount of blue visible light along with long-wave UV light. An example of a black light is the black light bulbs in bug catchers. It is hard to see fluorescence due to the large amount of visible light. Many microbial organisms produce phosphors as a result of their growth on skin and/or hairs and this can aid in detection and/or confirmation of infection. With the exception of *T. schoenleinii*, dermatophytes that produce fluorescence are members of the *Microsporum* genus. The primary dermatophyte of veterinary importance that produces fluorescence is *M. canis*. Clinical reports of *M. gypsum* or *M. persicolor* dermatophytosis in dogs and cats note a lack of fluorescence on infected hairs [15-18].

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Research Article

Milk examination

Milk production potential is a function of the number of mammary epithelial cells in the gland, as well as the secretory activity of those cells [1,2]. Therefore, improved lactation performance can be achieved under conditions that enhance mammary cell proliferation (or decrease apoptosis), biochemical and structural differentiation of mammary epithelium, and synthesis and secretion of milk components [1].

Milk sample can be collected first wash the udder and the teats thoroughly and dry with a paper towel. Swab the teats with 70 per cent alcohol. The first couple of squirts should be discarded unless used for a field test and, about 10 ml milk squirted into a sterile 30 ml Macartney bottle or sterile 30 ml plastic universal container held nearly horizontally. Avoid touching the mouth of the bottle with the teat and code the samples numerically. The samples must be submitted chilled using either crushed ice or cooling bricks in an insulated container².

Milks must be refrigerated if transport is slightly delayed, or frozen if transport is delayed more than 2-3 days. Clinical mastitis episodes associated with Gram-negative bacteria frequently cause a profound leukopenia, neutropenia, lymphopenia and monocytopenia as a result of the endotoxemia and an increased packed cell volume². After sample is collected, the sample can be examined by agglutination test for mastitis and brucellosis.

Milk ring test

The milk ring test is a type of agglutination test that detects brucella antibodies in milk samples and is recommended as a screening test for bovine brucellosis by OIE [3]. It consists of *Brucella abortus* cells stained with hematoxylin, which are added to a sample of 1 to 2 ml of milk and incubated at 37°C for one hour³. If antibodies for *Brucella abortus* are present in the milk

the antigen agglutinate with the cells, and float to the surface where it forms a sharp blue or purple colored cream layer. The milk column underneath will remain white. The milk ring test (MRT) and ELISAs are available for detecting antibodies to *Brucella* infection in milk and the MRT test is cheap, simple and requires no specialised equipment to perform. It detects anti *Brucella* IgM and IgA bound to milk fat globules. When tests for detecting *Brucella* antibodies in milk and serum are considered, the principal methods for detecting infected herds and for diagnosing brucellosis in individual animals are the serological tests which are mainly used for diagnosis of brucellosis [4].

MRT antigen is prepared from concentrated, killed *B. abortus* strain 99 or 1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000g for 10 minutes at 4°C, followed by resuspension in haematoxylin-staining solution⁵. The milk ring test indicates the presence of agglutinins (antibodies) in the milk, by addition of stained bacteria to the milk sample followed by incubation for 30-60 minutes. The agglutinins present in milk of cow or sheep will clump the *Brucella* the stained agglutinin antigen complex rises the fat globules. This causes the cream layers at the top to bottom depolycoloured [5]

Procedure MRT

The milk ring test should be performed by the following procedure [6-9]:

- Collect milk from an animal, sample is identified by date of collection and refrigerated
- Gentle mix the milk in sample tube to ensure distribution of cream
- Place 1 ml of milk in a plastic agglutination tube to give a column of milk about 2 cm high
- Add 1 drop of 0 milk ring test antigen or *B. abortus* suspension by pipette into test tube.
- Gentle mix thoroughly the contents within 1 minutes of adding antigens and avoiding frothing and incubation the mixture at 37 °C for 39-40 minutes and then examine

- The interpretation should be observed as: Positive test result: cream layer is deeply coloured and beneath cream layer is white, Negative test result: cream layer is white, milk beneath is white coloured as indicated at figure 1.

Mastitis test

Mastitis is a disease of many mammalian species. At least, 137 infectious causes of bovine mastitis are known to date and in large animals the commonest pathogens are *staphylococcus aureus*, *streptococcus agalactiae*, other *streptococcus* species and *Coliform* as reported by [10]. *Staphylococcus* sp. is the main aetiological agents of clinical and subclinical mastitis in cows while, *S. aureus* and *Escherichia coli* are most commonly isolated pathogen from the clinical mastitis, coagulase negative *Staphylococci* (CNS) are the most frequently isolated pathogens from the subclinical cows mastitis [11,12].

The major bacteria can be split into two categories, those that are cow associated (or contagious), and those which are environmental in origin [12]. The cow-associated bacteria are *Staph. Aureus* and *Strep. Agalactiae* while the main environmental bacteria are *Strep. Uberis*, *Strep. Dysgalactiae* and coliforms [13]. The most important major pathogens involved in bovine mastitis worldwide are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella* spp [13]. Mastitis can be tested by somatic cell count and california mastitis test.

California mastitis test

The California mastitis test (CMT) is a simple cow-side indicator of the somatic cell count of milk. It operates by disrupting the cell membrane of any cells present in the milk sample, allowing the DNA in those cells to react with the test reagent, forming a gel [14] (Figure 2).

Procedure of California Mastitis Test [15]:

- Collection of milk samples from each quarter of udders in the CMT paddle. The paddle has four shallow cups marked in different to identify the individual quarter from which milk sample was obtain.
- Take about 2 ml or 1 teaspoon of milk from each quarter
- Add an equal amount of CMT solution to each cup in the paddle

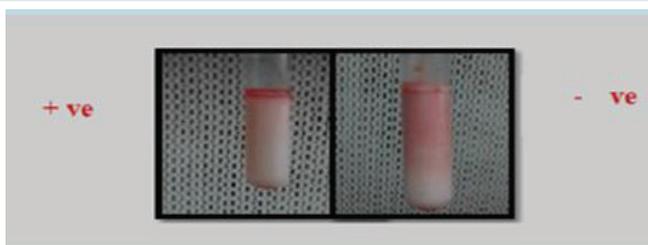


Figure 1: Milk ring test to detect the presence of brucella spp in milk sample [7].

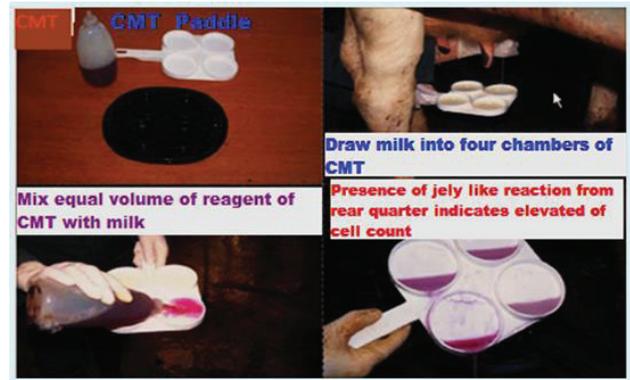


Figure 2: California mastitis test [16].

- Rotate the CMT Paddle in a circular motion to mix the milk contents do not mix more than 10 seconds.
- The result is observed after about 20 seconds. The result is more gel formation

Somatic cell count (SCC)

Somatic cells which found in milk are composed of white blood cell and sloughed epithelial cells. Cell white blood cell found in normal milk from uninfected glands include neutrophils, macrophages, lymphocytes and epithelial cells [16,17]. SCCs are a measure of the number of cells present in the milk. In healthy udder the scc is made up predominantly of epithelial cells. In the inflammation of the udder, white blood cells enter the udder to combat the infection and the scc rises. Numerical scc are measure using the automatic fossomatic methods, which is used to determine sccs in bulk milk and individual cow samples. sccs can either be performed by bulk milk presented monthly, three monthly and annual average or on an individual cows [17]. The macrophages have an important role in providing surveillance in the uninfected gland. When bacteria invade and colonize the mammary gland, the macrophages respond by initiating the inflammatory response that attracts polymorphonuclear cells (PMNs) into the milk to engulf and destroy the bacteria. The largest factor that influences the SCC of milk is mastitis. The SCC of a cow that is not infected with mastitis is usually less than 200,000 cells/ml and many cows maintain SCC values < 100,000 cells/ml [18].

Physical examination of milk

The milk can be examined physical by observing the consistency, color and odor or given milk samples, it is summarized as following [19–21]:

Color of Milk

- ✓ Normal: white in buffalo and whitish yellow in cow
- ✓ Yellow: ratio containing carotene, colostrum, some breed like Jersey cow

Abnormal:

- ✓ Blood stained: Highly produced animal's pathological discoloration

- ✓ Red: Dicumarol toxicity, Leptospirosis, Staphylococcosis, Bacterium erythrogene
- ✓ Green: Corynebacterium
- ✓ Yellow: most of bacterial Mastitis, Pseudomonas xantha.

Odor

- ✓ Normal milk: milky odor

Abnormal:

- ✓ Fetid (putrefied) odor: Gangrenous mastitis, Dry cow mastitis
- ✓ Fecal odor: Coliform mastitis
- ✓ Rancid odor: Milking in bad ventilated place or leaving milk for 2 days in environment
- ✓ Antiseptic odor: Excessive dipping of teat
- ✓ Acetone odor: Ketosis

Consistency

- ✓ Normally: milk consistency

Abnormally: Watery milk (decrease consistency): Streptococcal mastitis

- ✓ Increased consistency: Corynebacterial mastitis

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Research Article

Microbiology examination

Veterinary microbiology laboratory examination is required to establish the clinical samples, etiology and line treatment through antibacterial sensitivity testing. Veterinarian should have to go for antimicrobial sensitive test before treatment of patient with antibacterial drugs, without testing of antimicrobial sensitive, the bacteria or microorganism may develop resistance to that drugs. In microbiology laboratory examinations, the clinical samples can be examined in different ways, the ways are by direct examination and culturing on media and isolation of organism.

Direct examination in microbiology examination

The collected samples can be directly examined are tissues, organ swabs (nasal, fecal, vaginal, and prepuccial and conjunctival), urine, blood and exudate [1]. The sample can be collected for bacterial culture or direct examination from different organ and postmortem as it is detailed in table 1.

Staining and its principle in Microbiology diagnosis

Direct examination of the collected sample can be conducted by smearing of tissue or organ on clean and dry glass slide. Thin smear are prepared from blood, exudate and swabs, air dried and fixed on flame of Bunsen burner for a few seconds. A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so they are much more visible. Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image [3]. The stain can be classified into simple and differential staining [4]. Simple staining are used to stain negatively charged particles, usually the bacterial cell wall components and nucleic acid carrying negative charge which strongly attract the basic stain with the positively charges chromogen.

The morphology and arrangement of the bacteria are visualized by simple staining. The basic stains commonly used are methylene blue, crystal violet and carbol fuchsin.

Whereas, differential staining uses three reagents like primary stain, decolourizer and counter stain. The primary stain imparts colour to all the cells, the decolourizer is used to establish a colour contrast and counter stains the cells that are decolourised [5]. Staining are adding the color to bacterial cell and used to determine bacterial morphology and to distinguish bacteria from different species by differential staining characteristics. Before staining all slides are fixed by heat, methyle alcohol and formalin (Figure 3). Fixation is important to make permeable staining to bacterial cell by killing vegetative bacteria and protoplasmic shrinkage [1].

Gram staining technique

Gram staining is used in bacteriological to differentiate Gram negative (stain red to pink) from Gram positive (stain purple to blue) bacteria. Cell wall, aging or death may cause Gram positive bacteria to appear Gram negative (Figure 1) [6]. The cell walls of the two groups are morphologically and chemically quite different. One explanation for the differential staining reaction emphasizes the higher lipid content of the cell walls in Gram-negative bacteria. During the decolorization

Table 1: Sample collection for bacterial isolation from sick animals and carcass [2].

Predominant clinical sign or post mortem lesions	Bacteria	Samples
Septicemia, toxemia, sudden death, hemorrhages, subcutaneous oedema, gas gangrene.	Bacillus anthracis, Clostridium Pasteurella, E. coli, Salmonella, Erysipelothrix, Staphylococcus	Blood, body fluids, parenchymatous organs, bone marrow, lymphnode
Enteritis, dysentery	E. coli, Salmonella, Mycobacterium paratuberculosis, Clostridium, Campylobacter, Brachyspira hyodysenterica	Tied off portions of affected intestine, mesenteric lymphnodes
Pneumonia, coughing	Pasteurella, Mannheimia, Bordetella, Histophilus, Mycoplasma	Affected lung, transtracheal aspirates, nasal swabs, mediastinal lymph nodes.
Nervous signs	Cl.botulinum, Cl.Tetani, L.monocytogenes, Acetobacterium pyrogenes, E. coli	Serum, contents of the rumen and large intestine, wound swab or tissue, brain, liver, spleen, CSF
Abscess, granuloma, arthritis	Staphylococcus, Streptococcus, Corynebacterium, Actinomyces, Actinobacillus, Brucella, Pseudomonas, Erysipelothrix, Fusobacterium, Mycobacterium, Nocardia, Mycoplasma	Pus from edge of lesion, swab, intact abscess, joint aspirate.
Mastitis	As for abscess, also Leptospira, Enterobacteria, yeasts, Mycobacteria	Milk, supramammary lymph nodes
Skin lesions	As for abscesses, also Dermatophilus congolensis and dermatophytes	Fresh scabs, skin biopsy, skin scraping
Abortion, infertility	Brucella, Leptospira, Campylobacter, Listeria, Chlamydia, Salmonella, Coxiella	Foetus, foetal membranes, foetal stomach, vaginal swabs, blood from dam

step, alcohol may extract the lipids, increasing the porosity or permeability of the cell walls. Thus, the crystal violet-iodine complex is easily lost.

The Gram-positive bacteria, however, do not have lipid-rich cell walls. Their cell walls become dehydrated during the alcohol treatment, decreasing the porosity so that the crystal violet-iodine complex is retained [7]. Gram-positive organisms are able to retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. Gram positive cell walls typically lack the outer membrane found in Gram-negative bacteria [8].

The cell walls of gram positive bacteria are thicker with less lipids substance than gram negative bacteria (Figure 2). The solvent dissolves the lipids, which combined with thinner cell walls, washes out or decolorizes the stain. Counter stain (safranin) is added to exaggerate the contrast with gram negative cells. However, in gram positive bacteria, when washed with solvent, the cell pores close becoming less permeable and are able to retain the stain and cell become purple. The crystal violet is primary stain, which stains everthings in the smear purple blue. Gram's iodine acts as a mordant that causes the crysatal violet to penetrate and adhere to the gram positive cell [8].

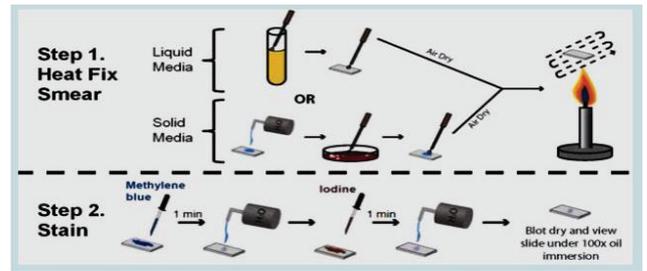


Figure 3: General staining techniques in veterinary microbiology (<http://microbeonline.com>).

The acetone alcohol mixture acts as the decolorizer that washes the stain away from everything in the smear except the gram positive organisms. The safranin is the counter stain that stains everything in the smear that has been decolorized in gram negative organisms [9]. The method of Gram stain is named after its inventor, the Danish scientist Hans Christian Gram 1853–1938, who developed the technique while working with Carl Friedländer in the morgue of the city hospital in Berlin in 1884. Gram devised his technique not for the purpose of distinguishing one type of bacterium from another but to make bacteria more visible in stained sections of lung tissue [10].

The following are procedure of gram staining (Figure 1,3) [2,11,12]:

First of all, presenting all material required for staining, like staining reagent, bacterial sample, Bunsen burner, slide, cover slide, water, pipette, loop, forceps.

- ✓ First sterile the loop and take specimen of bacteria or swabs in to slide
- ✓ Fixation of slide with the specimen by passing over heat (flame) several time us in forceps.
- ✓ Flood the fixed smear with crstal violet solution and allow remaining for 60 seconds.
- ✓ Rinse off the crstal violet with distilled or tap water
- ✓ Flood the slide with iodine solution and allow remain for 60 seconds.
- ✓ Rinse off the iodine solution with distilled or tap water
- ✓ Flood the slide with decolorize for 5 seconds
- ✓ Rinse off the decolorizer with distilled or tap water
- ✓ Flood the slide with safranin and allow to remains for 30 seconds.
- ✓ Rinse off the Safranin with distilled or tap water.
- ✓ Dry the slide on absorbent paper and place in an upright position.
- ✓ Put stained slide under microscope at 100 x objectives and observe the color change of bacterial specimen, and the gram positive (Stain deep violet to blue) and negative bacteria (stain pink to red) will be observed.

Gram Staining Procedure		Gram Positive Cell Wall		Gram Negative Cell Wall	
Process of test	Appearance of Cells	Effect of Step	Effect on Cell Wall	Effect of Step	Effect on Cell Wall
Step 1: Begin with heat fixed cells		Step 1: Cell wall remains clear.		Step 1: Cell wall remains clear.	
Step 2: Flood slide with crystal violet dye for 1 min.		Step 2: Peptidoglycan cell wall is flooded with crystal violet and appears purple.		Step 2: Cell wall is stained purple from the crystal violet dye.	
Step 3: Add iodine solution for 1 min.		Step 3: A crystal violet-iodine complex is formed within the peptidoglycan cell wall trapping the purple stain.		Step 3: A crystal violet-iodine complex is formed but does not adhere to the cell wall due to the thin layer of peptidoglycan.	
Step 4: Wash slide with alcohol for 20sec.		Step 4: The crystal violet-iodine complex is trapped with the peptidoglycan cell wall and doesn't wash out.		Step 4: The crystal violet-iodine structure is washed out of the thin peptidoglycan layer.	
Step 5: Counter stain with safranin.		Step 5: As the peptidoglycan cell wall remains stained purple the red safranin has no effect.		Step 5: The red safranin stains the washed gram negative cells.	

Figure 1: The procedure of gram staining (http://www.wikiwand.com/en/Gram_stain).

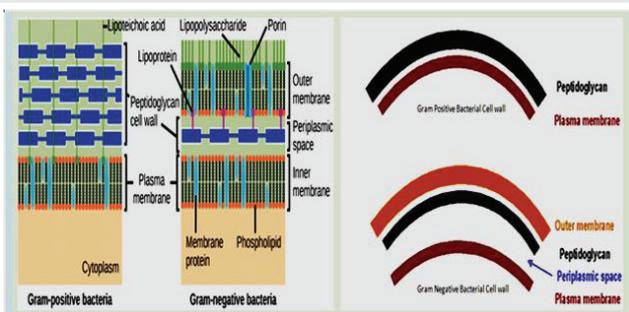


Figure 2: Structure of gram positive and negative bacteria (<https://bio.libretexts.org>).

Acid fast staining (The Ziehl–Neelsen stain)

The outer layer of the mycobacterial cell wall consists mainly of long-chain fatty acids called mycolic acids [13]. These mycolic acids comprise approximately 40% of the dry weight of the bacterium and are partially responsible for its acid fastness and the relative impermeability of the cell wall, including impermeability to antibacterial agents [14,15].

The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolorization by both acid and alcohol and hence they are referred as acid fast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast [16]. Zhiel neeslen is used to detect mycobacteria, chlymdia, nocardia and brucella spp [17]. Once stain penetrated and combine with the mycolic acid, and the cells resist decolorization even when a dilute acid alcohol solution is applied. Therefore organism said to be acid resistance or acid fast [18].

Preparation of reagent before staining [11]:

- ✓ **Ziehl neelsen carbolfuchsin:** Dissolve 3 g basic fuchsin in 100 ml 95% ethyl alcohol. Prepare a 5% phenol solution by dissolving 5g phenol in 100 ml distilled water. Prepared the Ziel Neelsen carbolfuchsin by mixing 10 ml alcoholic basic and 90 ml 5% phenol and allowing the mixture to stand for 24 hr. Filter the solution prior to use.
- ✓ **Acid alcohol:** Mix 2 ml concentrated hydrochloric acid & 98 ml 95% ethyl alcohol.
- ✓ **Methylene blue:** Prepare solution of methylene blue by adding 1.5 g powdered methylene blue to 100 ml 95% ethyl alcohol. Slowly add the alcohol to dissolve the powder. Add 30 ml saturated alcoholic solution for methylene blue to 100ml distilled water and 0.1 ml 10% potassium hydroxide. Filter and dilute it 1: 20 with distilled water to prepare the final methylene blue counterstain.

Staining Procedure

The acid fast staining can be conducted as the following procedure for bacteria which have mycolic acid. The procedure is started by applying the primary dye (carbolfuchsin or malachite green) and putting the smear to heat. The importance of heat is to enhance penetration and retention of the dye into the cells of bacteria. The general use of the reagents are like; Acid alcohol is decolorize removes the red stain from bacteria that are non-acid fast. Acid fast organism retains the red colour since carbolfuchsin is more soluble in the cell wall waxes than acidic alcohol. But, in non-acid fast bacteria cell wall lack of wax components and carbolfuchsin remove rapidly and cell become colorless [11,17,19]:

- ✓ Allow the film to air dry and then gently heat and fix it.

- ✓ Add additional stain (carbol fuchsin) and heated stain remain on slide for 2–5 minute
- ✓ Wash off the stain with clean water
- ✓ Cover the smear with 3% acid alcohol or 20% sulfuric acid for 2–5 minutes, until the smear is sufficiently decolorized (pale pink).
- ✓ Add a bit more decolorizer for very thick slides or to those continue to red dye
- ✓ Wash well with water
- ✓ Cover the stain with malachite green stain or methylene for 1–2 minutes
- ✓ Wash off stain with clean water
- ✓ Wipe the back of the slide clean, and place it in a draining rack for smear to air dry
- ✓ Examine the smear microscopically, using 100x oil immersion and identify bacterial whether acid fast or non-acid fast.
- ✓ Interpretation: Acid Fast Bacilli: Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded. Cells: Green (malachite green) or Blue (methylene blue). Background material: Green (malachite green) or Blue (methylene blue). The color is depend on the reagent which used in procedure as table 2.

Spore staining

Bacterial spores are bacterial mechanism that is intentionally set in an attempt to secure themselves to the adverse effects of the external environment (Figure 4). Spore is extremely resistance to harsh environment and disinfectants [20,21]. Endospore staining is the type of staining to recognize

Table 2: The interpretation of acid fast staining of bacteria (<http://microbeonline.com>).

Reagent	Acid Fast	Non-Acid Fast
Carbol Fuchsin with heat	Red (Hot Pink)	Red (Hot Pink)
Acid Alcohol	Red	Colorless
Methylene Blue/Malachite Green	Red	Blue/Green

the presence spore in bacterial vegetative cells and can penetrated wall thickness of spore bacteria. Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell (Figure 5). Spore shape may also be of diagnostic use. Spores may be spherical or elliptical [22]. There are two major pathogenic spore forming genera, Bacillus and Clostridium [23].

Procedure of spore staining (Figure 6) [24,25]:

- ✓ Prepare a smear of the acid fast species of bacteria and make it air dry and heat fix.
- ✓ Put a beaker of water on the hot plate and boil until

steam is coming up from the water. Then turn the hot plate down so that the water is barely boiling.

- ✓ Place the wire stain rack over the beaker which now has steam coming up from the boiled water.
- ✓ Cut a small piece of paper towel and place it on top of the smear on the slide. The towel will keep the dye from evaporating too quickly, thereby giving more contact time between the dye and the bacterial walls.
- ✓ Flood the smear with the primary dye, malachite green or carbonfuchsin, and leave for 5 minutes. Keep the paper towel moist with the malachite green. Do not let the dye dry on the towel.
- ✓ Remove and discard the small paper towel piece.
- ✓ Wash really well with water and move the slide and wire rack from the boiling
- ✓ Water to the regular stain tray to finish up the last step in the procedure.
- ✓ Place the smear in the stain jar or flood the smear with the counterstain dye, safranin, and leave for 30 seconds-60 seconds.

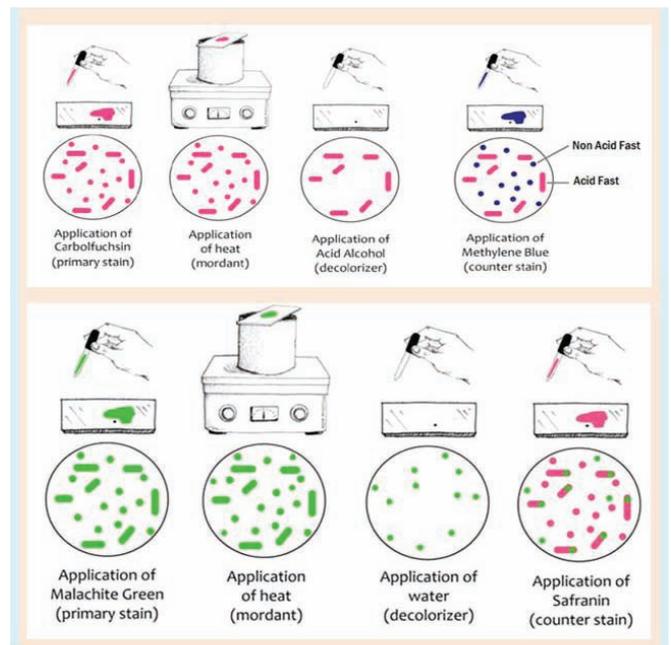


Figure 6: Procedure of spore staining. <http://laboratoryinfo.com> and <http://microbiologyinfo.com>.

- ✓ Wash well with water. Blot dry with bibulous paper.
- ✓ Examine the slide under microscope for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink which picked up form safranin dye. The spore can be one terminal (*Cl. Tetani*), central (*Bacillus anthracis*) and subterminal (*Cl.perfringe*) location.

Capsule staining

The capsule stain is a type of differential stain which selectively stains bacterial capsules. A capsule is a substance that is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Capsules can be polysaccharide, polypeptide, or glycoprotein. Capsules are associated with virulence in several microorganisms, including *Streptococcus pneumoniae* and *Neisseria meningitidis*, because capsules provide a mechanism for these pathogens to evade the host immune system. Because of their structure and composition, heat and water will dislodge capsules from bacteria during laboratory procedures. In the capsule staining procedure, the primary stain is crystal violet, and all parts of the cell take up the purple crystal violet stain. There is no mordant in the capsule staining procedure. A 20% copper sulfate solution serves a dual role as both the decolorizing agent and counter stain. It decolorizes the capsule by washing out the crystal violet, but will not decolorize the cell. As the copper sulfate decolorizes the capsule, it also counter stains the capsule. Thus, the capsule appears as a faint blue halo around a purple cell [26].

Procedure of capsule staining [27]:

- ✓ Obtain a clean glass slide. Choose one of the above broth cultures, and agitate your broth culture to disperse the bacteria.

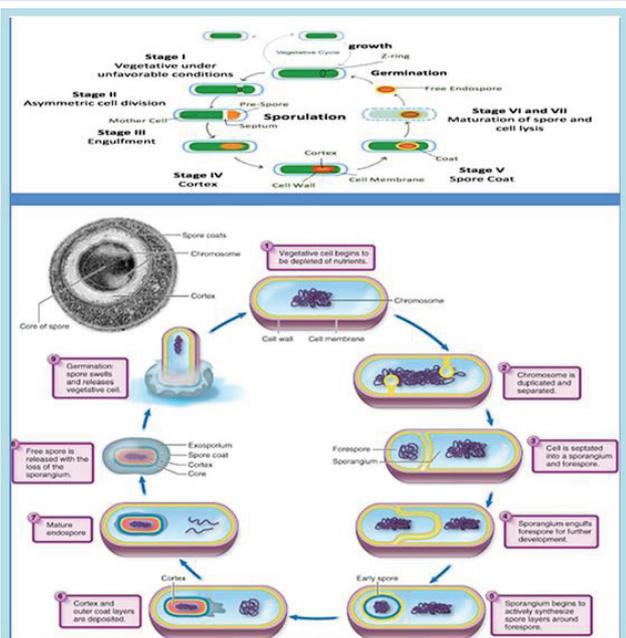


Figure 4: Sporulation of bacteria while change from unfavorable condition. [http://commons.wikimdia.org/wiki-File :Sporulation_in_Bacillus_subtilis.jpg](http://commons.wikimdia.org/wiki-File:_Sporulation_in_Bacillus_subtilis.jpg), http://online.science.psu.edu/micrb106_wd/node/6057.

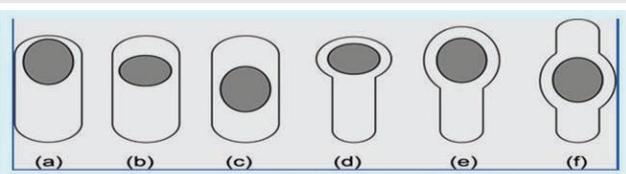


Figure 5: Morphology of endospores : Terminal(a,d,e); Subterminal (b) ; Central(c,f) ; circular(b,d), ellipsoid(a,c,e,f).<http://ttkamop.elte.hu>.

- ✓ Prepare a smear using 2–3 loopfuls of the broth culture. Allow the smear to air-dry, but do not heat fix this slide! Heat will cause the capsule to dislodge.
- ✓ Cover the smear with crystal violet, and stain the smear for 2 minutes.
- ✓ Tilt the slide and rinse with 20% copper sulfate solution. Do not rinse with water! Water will remove the capsule from the cell.
- ✓ Let the slide air dry for a few minutes. Do not blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.
- ✓ Observe the slide under oil immersion, and Look for purple cells surrounded by a clear or faint blue halo on a purple background. (The halo is the capsule.) You may need to decrease the amount of light in order to make the capsule easier to see.
- ✓ Clean your microscope with lens cleaner, removing all oil from lenses and the capsule will be observed as figure 7.

Cultural methods

The purposes of culturing media in veterinary clinical samples are to cultivate the organism and to obtain the discrete colonies for isolation of organism in the pure culture. The cultural media which used in veterinary laboratory are classified in to four groups, like simple, enriched, selective and differential or biochemical media [26,28].

Simple media: It is also called general media or universal media. It is a media which used for bacteriological examinations of the clinical specimens and support the microorganisms that do not require special nutrients. Example: nutrient broth, nutrient agar.

Enriched media: The cultured media that enriched with whole blood, serum, vitamins, special extraction which support for growth of fastidious organisms. Example: Blood agar, serumagar, chocolate agar.

Selective media: It allows the growth of certain types of organisms and prevents or slows down growth of bacteria other than pathogens for which media are intended. Example: MacConkey agar, Salmonella shigella agar.

Biochemical or differential media: It is the media that used to identification of one bacterial species from other. The media which indicator substances are added to differentiate bacteria. Example: TCBS agar differentiates sucrose fermenter to non-sucrose fermenter. The routine bacteriological examinations are carried out by using blood agar or tryptose soya agar plates/ MacConkey agar plates. Example: mannitol salt agar, DNase agar, blood agar, MacConkey agar and eosin-methylene blue agar.

MacConkey Agar is both selective and differential [29–31]. MacConkey agar is a selective culture medium, which

is used to grow gram negative bacteria and to identify those which ferment lactose and also differential, meaning that this medium differentiates or distinguishes between groups of bacteria on the basis of a color change reaction. MacConkey's contains two additives that make it differential; neutral red (a PH indicator) and lactose (a disaccharide) [29–31].

MacConkey Agar media contains crystal violet and bile salts, which inhibit most gram-positive organisms and select for gram-negative organisms (Figure 8). It also contains the substrate lactose and the pH indicator neutral red, which allow differentiation among gram-negative bacteria based on their ability to ferment lactose. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

Lactose fermenter bacteria are like *Citrobacter* spp, *Klebsiellasp*, *Escherichia coli*, and *Serratia* spp. and lactose fermenter are like *Proteus* spp, *Shigella* spp, *Yersinia* spp, *Salmonella* spp, *Edwardsiella* spp, *Hafnia* spp, *Morganella* spp, *Providencia* spp and Gram positive bacteria are nor growth on mackonkey [32,33]. Growth on MacConkey agar indicates the organism is resistant to crystal violet and bile salts, and is likely to be gram-negative. Enteric bacteria that have the ability to ferment lactose can be detected using the carbohydrate lactose, and the pH indicator neutral red. Growth which is a pinkish-red color indicates the organism has the ability to ferment lactose [33].

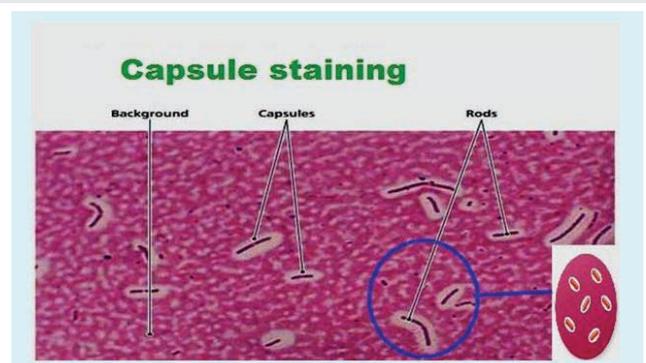


Figure 7: Capsule staining. Capsule staining is negative staining techniques due to its stain only bacterial cell and background, but capsule stay in unstained around bacterial cell [27].

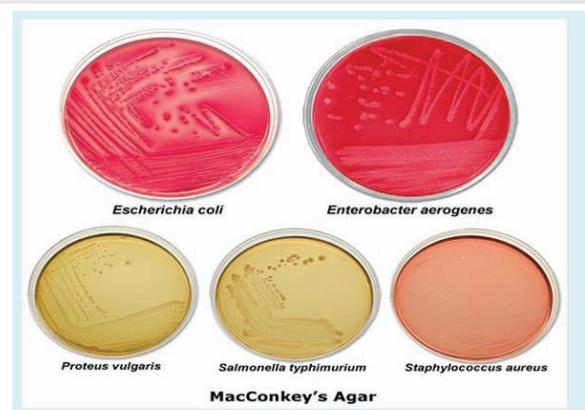


Figure 8: Macconkey Agar : (<http://chrysacsiovi.tk>).

Eosin Methylene Blue (EMB) agar is both selective and differential. Eosin methylene blue is selective for gram negative bacteria, the dyes eosin and methylene blue found in medium which inhibit growth of gram positive bacteria [33,34]. It also contains lactose, allowing differentiation between organisms which ferment lactose and produce acid end-products, and organisms that do not ferment lactose. Small amounts of acid production result in a pink colored growth (e.g. *Enterobacter aerogenes*) while large amounts of acid cause the acid to precipitate on the colony, resulting in a characteristic greenish, metallic sheen (e.g. *E. coli*). Organisms which do not ferment lactose will be colorless, taking on the color of the medium [34].

Blue agar indicates the organism can grow in the presence of the dyes eosin and methylene blue and is likely a gram-negative. Growth which is a pink color indicates the organism can ferment lactose to form weak acid end-products, and growth which exhibits a green metallic sheen indicates the organism can ferment lactose to form strong acid end-products [35] (Figure 9).

Mannitol salt agar (7.5% NaCl) is a medium selective for staphylococci and differential with respect to mannitol fermentation. It contains a high concentration (about 7.5%–10%) of salt (NaCl), making it selective for Gram-positive bacteria (*Staphylococcus* and *Micrococcaceae*) since this level of salt is inhibitory to most other bacteria. *Staphylococcus aureus* produces yellow colonies with yellow zones, whereas other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium. If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow [36].

Fermentation of mannitol is only seen in the pathogenic species of *Staphylococcus* and is signaled by the production of acidic products leading phenol red in the media to change from a neutral red–orange to bright yellow. Non-pathogenic staphylococci produce small colonies surrounded by red or pink–purple zones due to the production of basic by products due to metabolism^{36,37}. A change in the color of the agar from pink to yellow indicates the organism has the ability to ferment mannitol. Yellow coloration can usually be seen around the sides of the bacterial growth [38].

Blood Plates: All organisms could growth on the blood plates, but they are not selective. Blood agar is a rich medium that has been supplemented with fresh 5–10% blood. The hemolytic response can be dependent upon the type of blood. Sheep blood is commonly used, but some organisms require rabbit or bovine blood [39,40]. Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells.

The degree of the hemolysis is an especially useful tool for differentiation among Gram-positive cocci. The three types of hemolysis are (Figure 10) [39,39]:

- **Beta hemolysis:** When the bacteria cause complete lysis of red blood cells and hemoglobin, this results in complete clearing of the blood around colonies.

- **Alpha hemolysis:** when bacteria causes partial lysis of red blood cells and hemoglobin, which results in a greenish–grey or greenish–yellow discoloration of the blood around the colonies.
- **Gamma hemolysis:** when the bacteria couldn't causes the hemolysis of the red blood cells and hemoglobin. Then no any change in the medium.

Culture transfer techniques

Various media types used in microbiology labs include agar slants, agar deeps, agar plates, and broths. An agar slant is a solid media in a test tube with a slanted surface on which to culture the microorganism. These are typically inoculated by streaking the surface of the slant with a sterile loop. An agar deep is a solid media in a test tube which does not have a slanted surface. These are typically inoculated by stabbing the media with a sterile needle. An agar plate is a solid media which is contained in a Petri plate, providing an optimal surface on which to culture microorganisms. Like the agar slants, these are inoculated by streaking the surface with a sterile loop. Broth tubes are a liquid medium which can be inoculated by a sterile loop, needle, or pipette [26].



Figure 9: The eosin methylene blue. Quadrant 1 : Growth on the plate indicates the organism, *Escherichia coli*, is not inhibited by eosin and methylene blue and is gram-negative bacterium. The green metallic sheen indicates *E. coli* is able to ferment lactose to produce strong acid end-products. Quadrant 2 : Growth on the plate indicates the organism, *Pseudomonas aeruginosa*, is not inhibited by eosin and methylene blue and is a gram-negative bacterium. The absence of color in the bacterial growth indicates *P. aeruginosa* is unable to ferment lactose. Quadrant 3 : Growth on the plate indicates the organism, *Enterobacter aerogenes*, is not inhibited by eosin and methylene blue and is a gram-negative bacterium. The pink color of the bacterial growth indicates *E. aerogenes* is able to ferment lactose to produce weak acid end-products. Quadrant 4 : Absence of growth indicates the organism, *Staphylococcus aureus*, is inhibited by eosin and methylene blue and is a gram positive bacterium.

(<http://iws2.collin.edu/dcairn/CCCCC%20Micro/embagar.htm>).



Figure 10: Types of hemolysis on blood agar.

Procedure

- ✓ Label each tube appropriately.
- ✓ Using a sterile needle, obtain a small amount of culture from the broth tube containing
- ✓ Use the needle to inoculate a nutrient agar deep tube by stabbing the needle into the agar deep.
- ✓ Using a sterile loop, obtain a loop full of culture from the broth tube containing cultured bacteria. Use the loop to inoculate a nutrient agar slant tube by streaking the agar slant with a zig-zag motion.
- ✓ Using a sterile loop, obtain a loop full of culture from the slant of cultured bacteria
- ✓ Use the loop to inoculate a nutrient broth tube by gently swishing the loop around in the liquid broth.
- ✓ Make sure all caps are loose, but secure.
- ✓ Incubate at 30 °C for 48 hours.

Isolation of pure colony

A colony forms on a plate when a single microbe is inoculated onto the surface of the plate and reproduces until there are enough cells to form a visible colony. Since a colony theoretically forms from a single cell, a colony should then represent a pure culture. One way to obtain single, isolated colonies is using the quadrant streak method [41]. The quadrant streak plate method allows sequential dilution of the original microbial material over the entire surface of a fresh plate (Figure 11). After the original sample is diluted by streaking it over successive quadrants, the number of organisms will be decreased. Usually by the third or fourth quadrant only a few organisms are transferred, and these produce single, discrete colonies.

Procedure [41-143]:

- Label different nutrient agar plates.
- Divide the agar plate into 4 quadrants.
- Place a loopful of culture onto the plate in Quadrant 1 with a sterile loop and streak the loop very gently using a back and forth motion.
- Sterilize loop. Go back to the edge of Quadrant 1 and extend the streaks into Quadrant 2, going back into Quadrant 1 twice.
- Sterilize loop. Go back to the edge of Quadrant 2 and extend the streaks into Quadrant 3, going back into Quadrant 2 twice.
- Sterilize loop. Go back to the edge of Quadrant 3 and extend the streaks into Quadrant 4, going back into Quadrant 3 twice. Be careful NOT to go back into Quadrant 1!

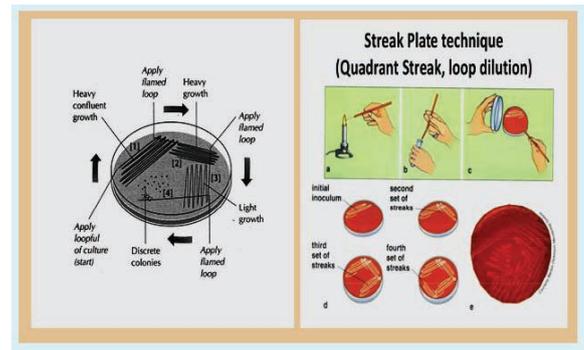


Figure 11: Four streaking techniques in isolation and inoculating method of bacteria.

- Tape plate closed on both sides. Make sure the plate is labeled with your name, date, and the organism(s), and incubate upside down (to prevent condensation from getting on to agar) at 30 °C.

Serial dilution

The purpose of serial dilution in veterinary microbiology laboratory is to quantifying the number of bacteria in a broth culture and also necessary to quantify the number of living bacteria in a particular sample. A Pure culture may be obtained by serially diluting the sample with sterile water to the point of extinction in number of cells. This method is used to isolate the organisms, if it is present in large number in the mixture [42]. The liquid culture needs to be diluted, often 1-million-fold, before it can be plated. When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make serial dilutions. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture.

Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data. To estimate the number of bacterial in the original culture, the number of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a 10^{-6} dilution was plated, and 123 colonies were counted following incubation. The total dilution plated would be 10^{-7} (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be: $(123) \times 1/10^{-7} = 1.23 \times 10^9$ CFU/ml. Note that the results are expressed as “colony forming units (CFU)” per ml [42,43] (Figure 12).

Calculate the number of bacteria (colony forming units) per milliliter or grams of sample by dividing number of colonies by dilution factor multiplied by amount of specimen added to agar plate, the equation can be:

$$C = \frac{N}{S * D}$$

$$C = \text{concentration} \left(\frac{\text{CFU}}{\text{mL}} \right)$$

$N = \text{Numbers of colonies}$

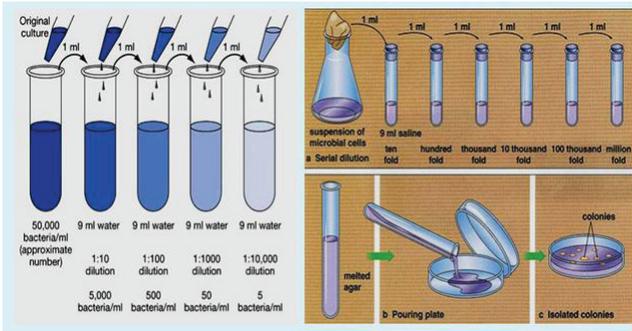


Figure 12: Serial dilution methods in bacterial isolation.

$D =$ Dillutions blank factor

$S =$ Volume transferred to plate

Procedure how to dilute [43]:

- ✓ Prepare serial dilutions of the broth culture as shown below. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer 0.1 ml of the final three dilutions ($10^{-5}, 10^{-6}, 10^{-7}$) to each of three nutrient agar plates, and label the plates.
- ✓ Position the beaker of alcohol containing the glass spreader away from the flame. Remove the spreader and very carefully pass it over the flame just once (lab instructor will demonstrate). This will ignite the excess alcohol on the spreader and effectively sterilize it.
- ✓ Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.
- ✓ Repeat the flaming and spreading for each of the remaining two plates.
- ✓ Invert the three plates and incubate at room temperature until the next lab period.

The effective on growth of bacteria

Bacterial growth is depending on the temperature may affect bacterial enzymes, membrane fluidity. The enzymes are less active at low temperatures due to low kinetic energy and increasingly as temperature increase. However, when temperature becomes too high, enzymes become denatured and will cease its functions. At low temperatures, the lipids in a cell membrane can pack too tightly and solidify this prohibiting membrane from proper functions. When temperatures become excessively high, the lipid bilayer can become too fluid and lose its integrity. Bacteria can be divided into four depending on ability to survive in temperature environment; all prokaryotes can be classified into four general groups depending on their temperature requirements [44].

- ✓ Psychrophile: Those bacteria grows at ranging from -5 to 20 °C

- ✓ Mesophile: Those bacteria grow at ranging from 20 to 45 °C. Example, *Staphylococcus aureus* (45°C) and *Staphylococcus epidermidis* (40°C).
- ✓ Thermophile: Those bacteria grows at ranging from 45 to 80 °C
- ✓ Hyperthermophile: Those bacteria grows at ranging from 80 to 105 °C
- ✓ Bacterial growth is also dependent on the presence of oxygen in the environment, as different bacteria have different oxygen requirements depending on the types of enzymes they possess. The major bacterial oxygen classes are aerobes, microaerophiles, obligate anaerobes, aerotolerant anaerobes, and facultative anaerobes [44,45] (Figure 13).

- Aerobes require atmospheric O₂ (20%), and use O₂ as the final electron acceptor in the electron transport system.
- Microaerophiles require O₂ at below atmospheric concentrations, typically 2-10%. Microaerophiles have a limited ability to neutralize toxic oxygen, so excess O₂ will kill the bacteria. However, microaerophiles do use O₂ as final electron acceptor in the electron transport system. Example: *Campylobacter jejuni*, *Helicobacter pylori*.
- Obligate Anaerobes cannot survive in the presence of any oxygen. Obligate anaerobes lack the enzymes necessary to break down the toxic by-products of oxygen. Examples are like: *Clostridium*, *Bacteroides*.
- Aero tolerant: Anaerobes grow equally well in the presence or absence of oxygen. They do possess enzymes necessary to neutralize toxic oxygen by-products, but

	a. Obligate Aerobes	b. Facultative Anaerobes	c. Obligate Anaerobes	d. Aerotolerant Anaerobes	e. Micro-aerophiles
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concentration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase [SOD] allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

Figure 13: Classification of bacteria depending on their environment (requirement of oxygen).

they never use O₂ as a final electron acceptor. Example: *Enterococcus faecalis*, some *Lactobacillus*

- Facultative Anaerobes are also able to live either in the presence or absence of oxygen, but they prefer oxygen so they can carry out aerobic respiration with O₂ as final electron acceptor to maximize ATP yields. These organisms can use other electron acceptors if O₂ is not available, such as fumarate and nitrate. They can also utilize fermentative metabolism in the absence of oxygen. Example: All *Enterobacteriaceae* (*E.coli*), some *Bacillus*, *Staphylococcus aureus*.
- Obligate aerobic bacteria: They have respiratory enzymes and lack the capacity for fermentations. Example: *Pseudomonas*, some *Bacillus*, *Mycobacterium tuberculosis*
- Capnophilic bacteria require increased concentration of carbon dioxide (5% to 10%) and approximately 15% oxygen. This condition can be achieved by a candle jar (3% carbon dioxide) or carbon dioxide incubator, jar or bags. (*Haemophilus influenzae*, *Neisseria gonorrhoeae*).

Cultivation of bacteria on anaerobic condition

Specialized methods are necessary to culture organisms anaerobically. One such method is the use of fluid thioglycollate broth, which is a reducing medium. It contains sodium thioglycollate, which reacts with molecular oxygen keeping free oxygen levels low. The sodium thioglycollate in the broth creates a redox potential in the tube, with higher levels of oxygen at the top of the tube, and a complete absence of oxygen at the bottom of the tube. Fluid thioglycollate broth also typically contains a redox potential indicator such as resazurin, which produces a pink coloration in an oxidized environment.

A second method used to culture organisms anaerobically is the use of a GasPak Jar and candle method [45] (Figure 14). This is a specialized culture vessel in which an anaerobic environment is generated after inoculated media are sealed into the chamber. Anaerobic conditions are created by adding water to a gas generator envelope that is placed in the jar just before sealing. There are two chemical tablets in the envelope, sodium borohydride and sodium bicarbonate. Water reacts with these chemicals, producing hydrogen gas from the sodium borohydride and carbon dioxide from the sodium bicarbonate. The hydrogen gas combines with free oxygen in the chamber to produce water, thus removing all free oxygen from the chamber. This reaction is catalyzed by the element palladium, which is attached to the underside of the lid of the jar. The carbon dioxide replaces the removed oxygen, creating a completely anaerobic environment [45].

Biochemical test

There are two levels of biochemical tests. The Primary Identification test in which, once a pure culture is obtained, the results from a few comparatively simple tests can often identify the bacterium to a generic level. The Secondary Identification of Bacteria occurs once the bacterium has been identified to a

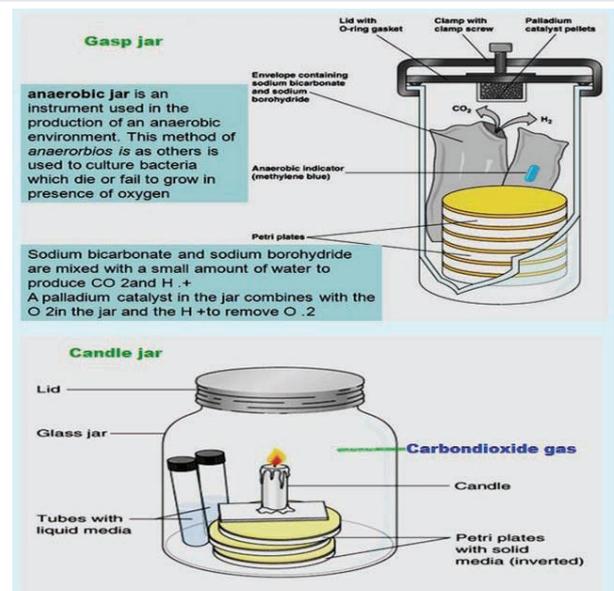


Figure 14: Creating anaerobic environment in culturing of bacteria by using jar and candle jar.

generic level, some of the tests can be carried out to identify the species. Secondary biochemical tests are: Fermentation of carbohydrates, Citrate utilization, Decarboxylation of amino acids, gelatin liquefaction, hydrogen sulfide test, indole test, Methyl red test, nitrate reduction test, ONPG test, Urease test and Voges Proskauer test [46]. The bacteria generally classified based on their biochemical test and staining and morphological structure is detailed in figure 15.

Catalase test

Catalase is the enzyme which present in most cytochrome containing aerobic and facultative anaerobic bacteria used to break hydrogen peroxide into oxygen (O₂) and water (H₂O). When the hydrogen peroxide is added to bacterial sample, it produces some bubble. When bubble is formed it shows, that bacteria is catalase positive [47] (Figure 16).

- ✓ Catalase positive:
- ✓ *Staphylococcus aureus*,
- ✓ *Micrococcus*,
- ✓ *Bacillus*,
- ✓ *Listeria monocytogenes*,
- ✓ *Enterobacteriaceae*,
- ✓ *Gonococcus* & *Meningococcus*,
- ✓ *Vibrio cholerae*,
- ✓ *Pseudo/Aero/Plesiomonas*,
- ✓ Catalase negative:
- ✓ *Streptococcus* spp
- ✓ *Clostridium*

Triple Sugar Iron Agars test

Triple Sugar Iron Agars (TSI) is used to determine if bacteria can ferment glucose and/or lactose and if they can produce hydrogen sulfide or other gases (Figure 17) [48]. This method used to differentiate various *Enterobacteriaceae*, including *Salmonella* and *Shigella*, which are intestinal pathogens. TSI contains three sugars: glucose, lactose and sucrose. Lactose and sucrose occur in 10 times the concentration of glucose (1.0% versus 0.1%). Ferrous sulfate, phenol red (a pH indicator that is yellow below pH 6.8 and red above it), and nutrient agar are also present. The tube is inoculated by stabbing into the agar butt (bottom of the tube) with an inoculating wire and then streaking the slant in a wavy pattern. Results are read at 18 to 24 hours of incubation [38,49,50].

Procedure:

Preparation of media: Add 3.0 gram of Beef extract, 3 gram of yeast extract, 15 gram of peptone, 5 grams of protease peptone, 10.0 grams of lactose, 10.0 gram of saccharose, 1.0 gram of glucose, 0.2 gram of ferrous sulphate, 5.0 gram of

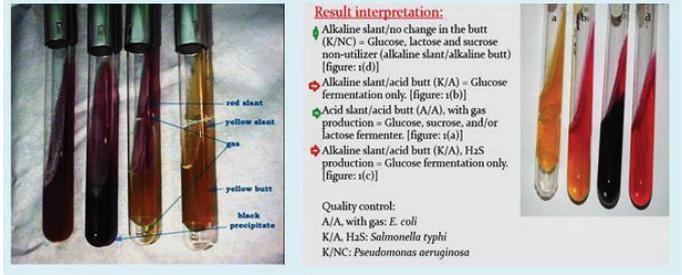


Figure 17: Triple sugar test and its interpretation.

sodium chloride, 0.3 gram of sodium thiosulphate, 0.024 gram of phenol red and 12 gram of agar and make the mixture up to 1000ml with distilled water. The Peptone mixture and the Beef and Yeast extracts provide the nutrients essential for growth. Sodium chloride maintains the osmotic balance of the medium. The Bacteriological agar is the solidifying agent.

The procedures are performed as below [51]:

- Sterilize the inoculating needle in the blue flame of the bunsen burner till red hot and then allowed to cool.
- From the rack, take the Trypticase soy broth tube containing the 24–48 hour culture, remove the cap and flame the neck of the tube.
- Using aseptic technique, take the culture of the organism from the TSB (tryptic soy broth) tube with the needle.
- Again flame the neck of the tube and replace the tube in the test tube rack.
- Take a sterile TSI slant tube from the rack, remove the cap and flame the neck of the tube.
- Stab the needle containing the pure culture into the medium, upto the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant.
- Again flame the neck of the TSI tube, cap it and place it in the test tube rack.
- Incubate at 37°C for 18 to 24 hours.

Interpretation:

- A yellow slant on TSI indicates the organism ferments sucrose and/or lactose.
- A yellow butt shows that the organism fermented glucose.
- Black precipitate in the butt indicates hydrogen sulfide production (*Salmonella typhi*).
- Production of gases other than hydrogen sulfide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube.

Urease test

Urease test is used to differentiate urease positive proteus

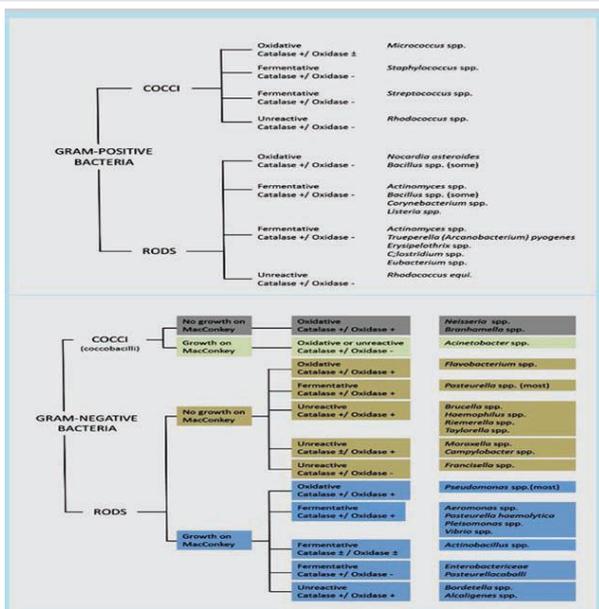


Figure 15: Classification of bacteria depending on their morphological biochemistry (www.afrivip.org).

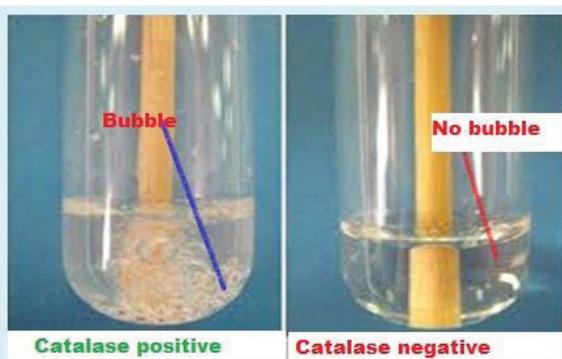


Figure 16: Catalase test and its interpretation.

species from other members of enterobacteriaceae. Some species of bacteria possess the enzyme urease and able to hydrolyze urea with the release of ammonia and carbon dioxide. Urea broth is a yellow orange color. The enzyme urease will be used to hydrolyze urea to make ammonia. If ammonia is made, the broth turns a bright pink color and it is positive and in negative result no color change. The result of interpretation positive test is magenta to pink colour (Figure 18) [38,52].

Motility test

Major bacteria are motile by flagella. Motility can be temperature dependent and some bacteria tend to be motile at ambient temperature but not at 37 °C. Semisolid motility media are important with Terazolium salts can be added to these media aid in detection of motility. Before autoclaving the motility medium, 0.05 g of 2, 3, 5-triphenylterazolium chloride is added per litre of medium. Terazolium is colorless, but a bacterium is grown the dye is incorporated into bacterial cells where it is reduced to an insoluble red pigment, formazan. The red colour forms only in the area of medium where the bacterium is growing. Motility media are prepared in test tube, two test tubes of the medium are stab-inoculated using a straight wire. One tube is incubated at room temperature and the other at 37°C. The tubes are examined for motility after 24 and 48 hrs. Motile bacteria migrate through semisolid medium which become turbidity. If TTC has been incorporated into the medium, the motility is demonstrated by a red colour throughout the agar. The growth of non-motile bacteria is confined to the stab line [53] (Figure 19).

Coagulase Test

The coagulase test usually relates with pathogenicity, some staphylococci can be negative to the slide coagulase test, but positive to the tube test.

Slide coagulase test: Heavy loopful of the staphylococci culture is emulsified in a drop of water on microscope slide. A loop of rabbit plasma is added and mixed well with the bacterial suspension. The slide is gently rocked and a positive reaction is indicated by clumping within 10 seconds (Figure 20).

Tube coagulase test: 0.5 ml of rabbit plasma is placed in a small test tube. A suspension (0.01) of an overnight broth culture is added to the rabbit plasma test tube. The tube rotated gently to mix the contents and then incubated at 35–37°C, preferably in a water bath. Alternatively one to three large well isolated colonies can be transferred into 0.05 ml of rabbit plasma in a tube and incubated at 35–37°C. A positive with degree of clotting of the plasma can occur in 2–4 hours. Many weak coagulase positive strains will coagulate at the plasma only after overnight incubation [17,38] (Figure 20).

Citrate utilization test

Citrate utilization test is commonly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests, that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products [54].

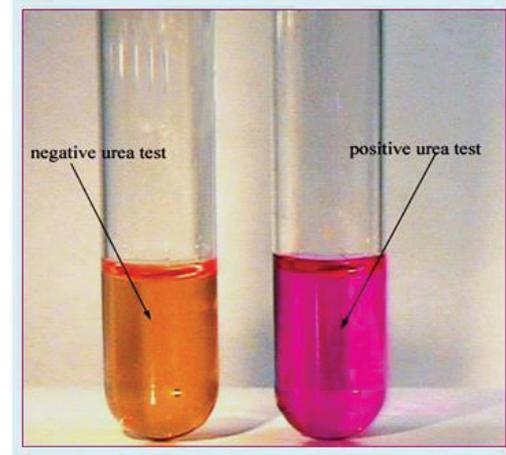


Figure 18: Urease test and its interpretation.

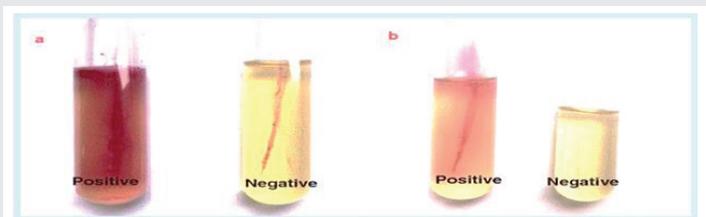


Figure 19: Motility test medium tubes containing TTC inoculated with *Escherichia coli* (a) and *Bacillus cereus* (b). Positive before treatment negative after treatment with prodigiosin [53].

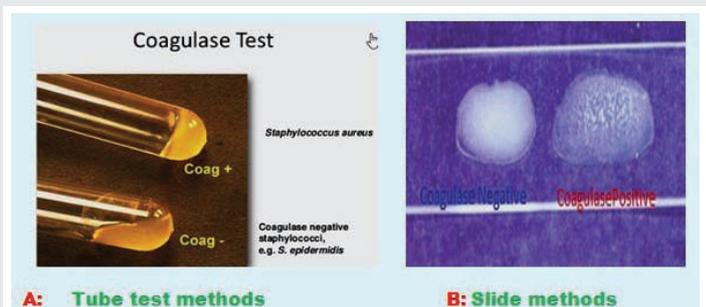


Figure 20: Coagulase test by two methods.

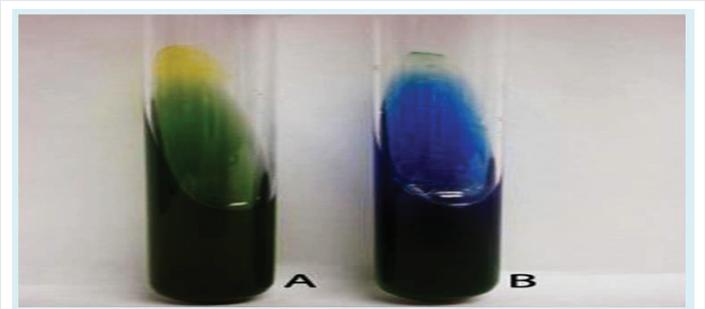


Figure 21: Citrate test to differentiate enterobacteriaceae spp. A : Citrate negative : No growth and no color change will be visible, the medium remain the deep forest green color of uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant. B : Citrate positive : Growth of bacteria will be visible on the slant and medium will be an intense prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.

Procedure of citrate utilization test:

- Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
- Incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
- Observe the development of blue color; denoting alkalinization (Figure 21).

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Research Article

Serological and immunological test

Immunological and serological methods can detect the antibodies specific to a particular infection in serum or antigen in different body tissues or excretions or secretions. The animal diseases have been diagnosed by immunological and serological test since very long. The antibodies are produced in animal body as a result of body defense responses against the causative agents. However, detection of antibodies in serum is not sufficient to conclude diagnosis of animal's disease. The presence of antigen in body tissues or fluids directly correlates with the presence of incrimination agent in the system. It is, therefore, advisable that paired sera should be tested before arriving at any diagnosis. The detection of antigen is considered more specific for diagnosis. Here, many serological and immunological tests are described and the readers may use any of these according to their suitability and availability of materials. Serology is the science which study about serum and other body fluids. In laboratory term, serology is termed to the diagnostic identification of antibodies in the serum [1].

Serologic testing for infectious agents or for an antibody response demonstrating exposure to an infectious agent is one of the most common ways immunologically based tests are used in clinical medicine. Immunologic testing is also used to evaluate the immune system itself in cases of suspected hypersensitivity or autoimmunity, to estimate resistance to a particular disease, or to document an immunodeficiency state. Immunologic principles are used in diagnostic tests not intended to evaluate either infection or immunity. Serologic tests can be classified as tests of primary antigen-antibody interaction, secondary tests of interaction, or tertiary tests of interaction [2].

Agglutination test

Agglutination is the clumping of particulate antigens, such as bacterial cells, in presence of an immune serum or antibody or complements. For agglutination, the antigen should be in

particulate form of suspension and antigen-antibody reacting sites must be present on the surface of the particles. The interaction between antibody and a particulate antigen results in visible clumping called agglutination. Agglutination reactions are more sensitive than immune precipitation [3].

The application of agglutination is to:

- ✓ In diagnosis of bacterial diseases such as brucellosis, salmonellosis, yersiniosis.
- ✓ Blood typing prior to transfusion.
- ✓ Diagnosis of rickettsia1 diseases like Q-fever.
- ✓ Diagnosis of viral diseases like influenza.

Various types of agglutination tests commonly used in the diagnosis of animal diseases are mentioned below Agglutination test commonly used in the diagnosis of domestic animals at veterinary laboratory can be classified into slide agglutination test, Tube agglutination test, capillary tube agglutination test and others [3-5].

Slide methods of agglutination

This method is quick, simple and requires smaller quantity of reagents. In this test, take a clean, dry glass slide and place a drop of antigen suspension over the middle area of the slide. Add one drop of test serum and mix properly with the help of a clean glass rod or toothpick. If the homologous antibodies are present, clumps of bacterial cells will occur through agitation. The clumping can be seen by naked eyes or by using a light microscope. A saline-antigen control should also be used for comparison of the results. The test is mostly used for identification of salmonella organisms or pollorum disease in poultry [5].

Tube agglutination test

The tube agglutination test is used for the diagnosis and screening of herds for brucellosis in animals [6,7]. For diagnosis of brucellosis, a 0.5% carbol saline is used in cattle and buf-faloes and 5.0% carbol saline for sheep and goats. To perform

this test two fold serum dilutions are made in carbol saline as 1:5, 1:10, 1:20, 1:40..,and so on. An equal amount of brucella antigen is added into tubes and kept at 37 °c in incubator for 24 hrs. If agglutination occurs, the clumps of antigen and antibody complexes will settle down leaving the clean supernatant. In case of no agglutination, the turbid suspension remains same. The standard tube agglutination is useful in diagnosis of those diseases in which immune carriers are present. There are some sets up to which the test is considered to be negative, e.g. brucellosis positive reactions at 1:20 and 1:40 dilutions are interpreted as negative and doubtful respectively [6,7].

The titer should be expressed as the highest dilution of antiserum which gives visible agglutination and expressed in IU (International Units). In the brucellosis it is considered 40 IU positive in sheep and goats and 80 IU in man and cattle. To avoid discrepancies in the results, the paired sera should be tested and an increase in fourfold titer of agglutination should be considered as a result of recent infection [8].

ELISA Test in veterinary laboratory practice

ELISA (Enzyme linked immune sorbent assay) is an antigen antibody reaction. In 1971, ELISA was introduced by Peter Perlmann and Eva Engvall at Stockholm University in Sweden. It is a common laboratory technique which is usually used to measure the concentration of antibodies or antigens in blood. A number of enzymes have been used for ELISA such as alkaline phosphatase, horse radish peroxidase and beta galactosidase. Specific substrate such as ortho-phenyldiaminedihydrochloride (for peroxidase), paranitrophenyl phosphate (for alkaline phosphatase) are used which are hydrolyzed by above enzymes to give colored end product. Antibodies or antigens present in serum are captured by corresponding antigen or antibody coated on to the solid surface⁹.

Enzyme linked immune sorbent assay methods can be classified in to three based on the binding structure between antibody and antigens such as: indirect, direct and sandwich method [9]. ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. A positive control serum and a negative control serum would be included among the 96 samples being tested in 96 well as it described (Figure 1) [10]. ELISA is the abbreviation of enzyme-linked immunosorbent assay. It is a useful and powerful method in estimating mg/ml to µg/ml ordered materials in the solution, such as serum, urine, sperm and culture supernatant [11].

Direct ELISA

For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme. Direct ELISAs involve attachment of the antigen to the solid phase, followed by an enzyme-labeled antibody [9,10]. Direct ELISA test is considered to be the simplest types of ELISA the antigen is adsorbed to a plastic plate, then an excess of another protein is added to block all the other binding sites. After excess enzyme- antibody complex is washed off, enzyme antibody bound to antigen left [12].

Procedure (Figure 2):

- A buffered solution of the antigen to be tested for is added to each well of a microtiterplate; where it is given time to adhere to the plastic through charge interactions.
- A solution of nonreacting protein, such as bovine serum albumin or casein, is added to well (usually 96-well plates) in order to cover any plastic surface in the well which remains uncoated by the antigen.
- The primary antibody with an attached (conjugated) enzyme is added, which binds specifically to the test antigen coating the well.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The higher the concentration of the primary antibody presents in the serum, the stronger the color change. Often, a spectrometer is used to give quantitative values for color strength.

Indirect ELISA

Procedure (Figure 3):

- * Antigen is coated in microtiter well
- * Coat the micro titer plate wells with antigen.
- * Block all unbound sites to prevent false positive results.
- * Add sample containing antibody (e.g. rabbit monoclonal antibody) to the wells and incubate the plate at 37°C.

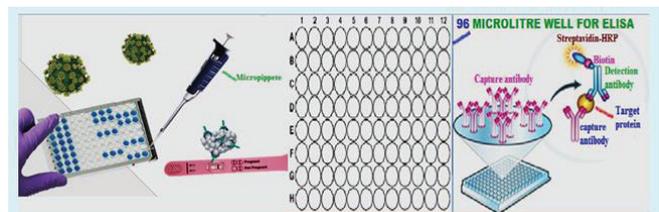


Figure 1: The microliters well for ELISA.

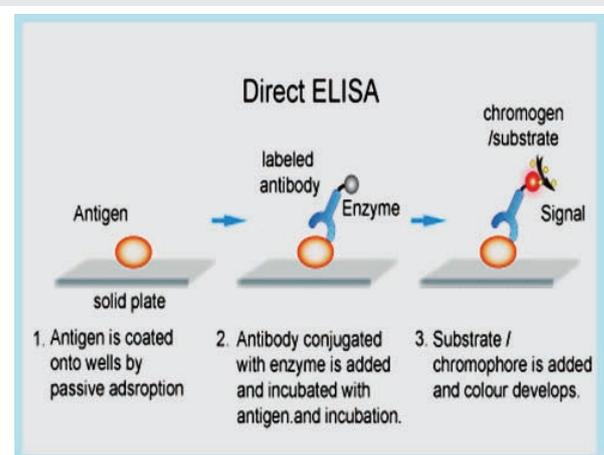


Figure 2: Direct ELISA and its procedure.

- * Wash the plate, so that unbound antibody is removed.
- * Add secondary antibody conjugated to an enzyme (e.g. anti- mouse IgG).
- * Wash the plate, so that unbound enzyme-linked antibodies are removed.
- * Add substrate which is converted by the enzyme to produce a colored product.
- * Reaction of a substrate with the enzyme to produce a colored product

Sandwich Method

In methods of sandwich, antibody is coated in microtiter.

Procedure (Figure 4):

- Prepare a surface to which a known quantity of antibody is bound.
- Add the antigen-containing sample to the plate and incubate the plate at 37°C.
- Wash the plate, so that unbound antigen is removed.
- Add the enzyme-linked antibodies which are also specific to the antigen and then incubate at 37°C.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme to produce a colored product.
- Reaction of a substrate with the enzyme to produce a colored product

Competitive methods

The antigen-antibody mixture is then added to the microtitre well which is coated with antigen.

Procedure (Figure 5):

- Antibody is incubated with sample containing antigen.

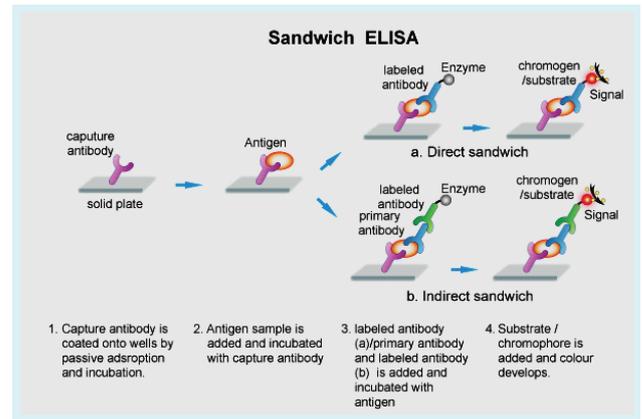


Figure 4: Procedure of sandwich ELISA techniques.

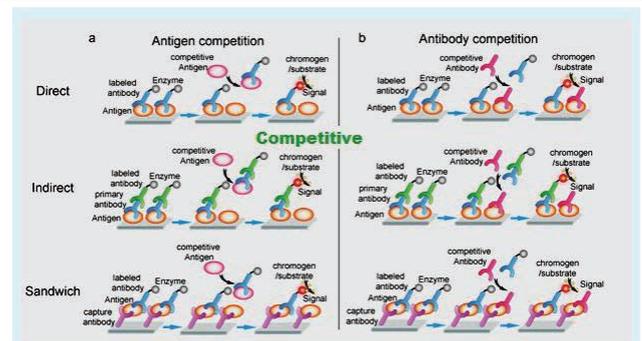


Figure 5: Competitive ELISA procedure.

- Antigen-antibody complex are added to the microtitre well which are pre-coated with the antigen.
- Wash the plate to remove unbound antibody.
- Enzyme linked secondary antibody which is specific to the primary antibody is added.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme into a fluorescent signal.

Allergic test: Histologically, the area which has been injected with allergen is heavily infiltrated with mononuclear cells comprising mainly of macrophages and lymphocytes. The cell involved in allergic reactions are T-lymphocytes, macrophage, regulatory B-cell and basophiles [13,14]. Allergic reaction test is being used to diagnosis chronic disease like tuberculosis, Para tuberculosis and glander. The allergens are prepared by killing the causative organisms of disease and filtering them from culture media. These allergens are named after their principal organisms like tuberculin for *Mycobacterium tuberculosis*. Special purified protein derivative (PPD) is used now a days for eliciting delayed type of hypersensitivity reactions and detection of reactors. Single intradermal (SID) and comparative intradermal (CID) tests are recommended for routine screening of animals to detect the reactor of tuberculosis and Johne's disease.

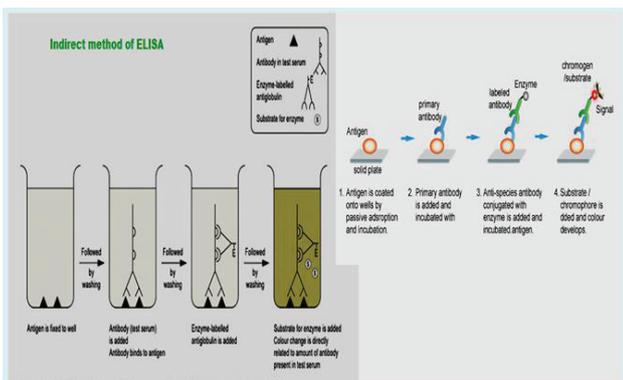


Figure 3: Indirect ELISA procedure [8].

Single Intradermal (SID) Test: Single intradermal (SID) test: This test is applied by the intradermal injection of 0.1ml of bovine tuberculin PPD into a skin fold at the base of the tail or into the cervical fold and the subsequent detection of swelling as a result of delayed hypersensitivity. The reaction is read between 48 and 96 hours after injection with a preference for 48 – 72 hours for maximum sensitivity and at 96 hours for maximum specificity. The positive reaction constitutes a diffuse swelling at the site of injection¹⁵. The main disadvantage of the SID test is its lack of specificity and the number of no visible lesion reactors (NVLs) which occur.

Mammalian tuberculin is not sufficiently specific to differentiate between reactions due to infection with *M. bovis* and infection with *M. avium*, *M. tuberculosis* and *M. paratuberculosis* including vaccination or *Nocardia farcinicus*. The maximum permissible of NVL reactors is 10% and when this rate is exceeded, tests other than the SID test should be used [16]. The other disadvantages of SID test include failure to detect cases of minimal sensitivity, in old cows and in cows which have recently calved; as well as in early infection, in some cattle in an unresponsive state, referred to as energy which is developed due to antigen excess or immunosuppression which in-turn caused by nonspecific factors such as malnutrition and stress [17].

Tuberculin Test: Tuberculin was invented by Koch in 1890, and was first used experimentally in treating tuberculosis in man. Numerous experiments showed this to be the case and since 1891 the use of tuberculin as the diagnostic agents for tuberculosis of cattle. Tuberculin is the sterilized and filtered glycerin extract of cultures of tubercle bacilli. It contains the cooked products of the growth of these bacilli, but not the bacilli themselves [18]. Tuberculin testing is done for the diagnosis of tuberculosis in animals and birds. In bovines, the tuberculin is injected in the skin of neck; in pigs, in ear and in birds, in wattle.

Tuberculin skin test: The tuberculin test based on a delayed type hypersensitivity to *mycobacterial* tuberculo-protein, is the standard ante-mortem test in cattle [17]. It is convenient, cost effective method for assessing cell mediated responses to a variety of antigens and it is “gold standard” for diagnostic screening for detection of new or asymptomatic *M. tuberculosis* complex infection [19]. The reaction in cattle is usually detectable 30–50 days after infection [20]. The tuberculin is prepared from cultures of tuberculosis or *M. bovis* grown on synthetic media [21]. The tuberculin test is usually performed between the mid necks, but the test can also be performed in the caudal fold of the tail.

The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold of the tail [22]. Bovine tuberculin is more potent and specific and the potency of tuberculins must be estimated by biological methods, based on comparison with standard tuberculins and potency is expressed in the international unit (IU) [23]. In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose

at least 2000 IU in cattle. In cattle with diminished allergic sensitivity, a higher dose of bovine tuberculin is needed and the volume of each injection dose must not exceed 0.2ml [24]. Cell mediated hypersensitivity, acquired through infection can be demonstrated systematically by fever or ophthalmically by conjunctivitis, or dermally by local swelling, when tuberculin test or its purified protein derivative (PPD) is given by the subcutaneous, conjunctival or intradermal route, respectively [25].

Procedure [26]:

- In single intradermal test, 0.1 ml of tuberculin or PPD is injected intradermally in the middle portion of neck of bovines, a site preferred due to maximum sensitivity.
- Prior to tuberculin injection, the skin should be cleaned, shaved and thickness of skin fold measured using a Vernier calliper.
- The reaction can be read after 72 hours of the injection; the positive reaction is characterized by a hot, red and painful swelling with an increase of 4 mm or more in skin fold. However, some diagnostic consider that skin thickness should be double to declare a positive.

Johnin test

Johnin purified protein derivative (PPD) procured from IVRI, Izatnagar was used for the test. One square inch area in mid neck of each goat was shaved and mopped with medispirit. Skin thickness prior to injection was measured using a sliding vernier calipers and 0.1 ml of Johnin PPD was injected intradermally. A pea sized bubble was formed at the site of injection. After 72 h skin thickness was measured and an increase in skin thickness of 4 mm or above from initial reading was taken as positive [27]. An intradermal injection was given to each animal on the left side of the neck at the crossing point of a line between the ear and the shoulder joint and a line between the jaw and the shoulder crest [27].

Procedure of Johnin test:

- * First restraint animals and clean and shave the area of cervical region
- * Inject 0.1 ml of Johnin in intradermal route into cervical shaved area
- * If tuberculin and Johnin test done at the same time, just inject Johnin in opposite side of neck
- * An increase of 5 mm or more in thickness of skin fold after 72 hours is considered positive for Johnin's disease.
- * This test is also not able to detect cases of minimal sensitivity such as may occur in the early or late stages of disease, in old cows and in cow which have recently calved.

Double Intradermal Test (DID): The double intradermal test is performed in same manner as in single intradermal test, the only difference is that a second injection of tuberculin or

Johnin is given 7 days after the first injection at the same site and the skin thickness is measured 24 hrs after the last injection. In positive reactor, there should be an increase of 5 mm or more in skin thickness. Double intradermal test is useful to detect those animals which are poorly sensitized for any reason.

Comparative Test: Comparative intra-dermal (CID) test: In this test, two sites on the mid neck, 10–12cm apart, are shaved and the thickness is measured in millimeters with caliper before the injection of tuberculin [28]. In the CID test, 0.1ml of avian PPD and 0.1ml of bovine PPD are injected intradermally into separate clipped sites on the side of the neck. Care must be taken in placing the injection as varied from place to place in the skin. After 72 hours the thickness of the skin at the sites is measured again [29].

When the change in skin thickness is greater at the avian PPD injection site, the result is considered negative for BTB. When the change in skin thickness increased at both sites, the difference between the two changes is considered. Thus, if the increased in the skin thickness at the injection site for the bovine (B) is greater than the increase in the 1+–skin thickness at the injection site at the avian (A) and $(B - A)$, is less than 1mm, between 1 and 4 mm, or a 4 mm and above, the result is classified as negative, doubtful, or positive for bovine tuberculosis, respectively and the animal with the evidence of infection is termed as reactor [16].

The comparative test is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other *mycobacteria*. This sensitization can be attributed to the antigenic cross reactivity among *mycobacterial* species and related genera [30].

Short thermal test: Intradermal tuberculin (4ml) is injected subcutaneously into the neck of cattle which have a rectal temperature of not more than 39°C (102°F) at the time of injection and for 2 hours later. If the temperature at 4, 6 and 8 hours after injection rises above 40°C (104°F), the animal is classed as a positive reactor. The temperature peak is usually at 6 – 8 hours and is generally over 41 °C (105.8°F) [21].

Stormont test: Stormont test is a more sensitive test than short thermal test of tuberculosis in cattle. This test relies on the increased sensitivity of the test site, which occurs after a single injection [31]. The test is performed similarly to the SID test in the neck with a further injection at the same site 7 days later. An increase in the thickness of 5mm or more, 24 hours after this second injection, is a positive result. The loss of sensitivity is probably due to the general immunological hypo-reactivity that occurs associated with parturition [32].

Mallein Test: The mallein is a bacteria free filtrate from a fluid cultivated of *Pseudomonas mallei*, the cause of glanders in equine. The mallein test is routinely test to screen the equines for glanders and is considered quite specific. As the glanders is a communicable and notifiable disease, the test has its significance in detecting positive reactors in eradication programme of disease.

Procedure of mallein test:

- ✓ Inject 0.2 ml of mallein intradermally into skin of lower eyelid using tuberculin syringe
- ✓ After 48 hours the positive reaction like edema of eyelid within blepharospasim and severe purulent conjunctivitis will be marked.

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Research Article

Antimicrobial susceptible test

Anti-microbial susceptibility test is useful to guide the clinician in the selection of antimicrobial agents to which clinical condition being treated will respond. There are three principal methods of antimicrobial susceptibility testing like disc diffusion, broth dilution and agar dilution¹.

Disc diffusion test

It is performed by impregnating of antimicrobial agents onto an agar plate seeded with the bacterium to be stored. The antimicrobial agents diffuse into agar creating a zone saturated with the agent, in which organisms susceptible to that agent will not growth. The edges of zone are the point of minimum inhibition concentration [1].

Reagents for the disk diffusion test: Mueller Hinton agar medium is considered to be the best for routine susceptibility testing of non fastidious bacteria for the following reasons:

- ✓ It shows acceptable batch-to-batch reproducibility for susceptibility testing.
- ✓ It is low in sulphonamide, trimethoprim, and tetracycline inhibitors.
- ✓ It gives satisfactory growth of most nonfastidious pathogens.
- ✓ A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Müeller-Hinton agar preparation includes the following steps.

- * Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions.

- * Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath.
- * Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.
- * The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C).
- * Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar.
- * A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

Procedure for performing the disc diffusion test

The growth method is performed as follows:

- * At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth.
- * The broth culture is incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours)
- * The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. To perform this step properly, either a photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

- * Direct Colony Suspension Method: As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from 18- to 24-hour agar plate (a nonselective medium, such as blood agar, should be used).
- * The suspension is adjusted to match the 0.5 McFarland turbidity standards, using saline and a vortex mixer. This approach is the recommended method for testing the fastidious organisms, *Haemophilus* spp., *N. gonorrhoeae*, and streptococci, and for testing staphylococci for potential methicillin or oxacillin resistance.

Inoculation of Test Plates

- * Adjust Turbidity Of Inoculum: Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
- * Inoculate the swab over dried mellerian hintan agar by streaking: The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.
- * Impregnated disks: The lid may be left a jar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Reading plates and interpreting results

The interpretation is listed as following [1,2]:

Susceptibility is high probability of success following treatment, whereas resistance is low probability of success following resisatnce. After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc.

Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate (Figure 1). The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light. If blood was added to the agar base (as with streptococci), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed. If the test organism is a *Staphylococcus*

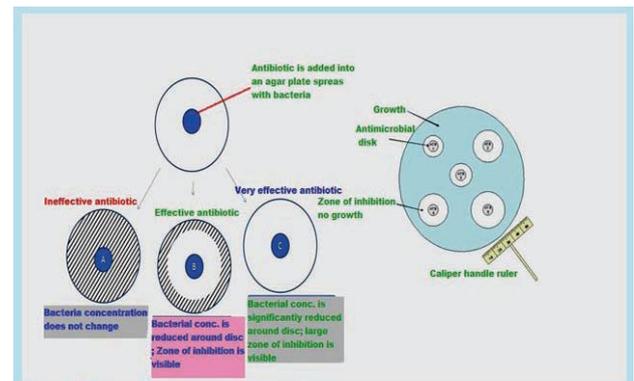


Figure 1: Agar diffusion and antimicrobial test.

or *Enterococcus* spp., 24 hours of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 hours. Transmitted light (plate held up to light) is used to examine the oxacillin and vancomycin zones for light growth of methicillin- or vancomycin- resistant colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin or vancomycin resistance.

The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored. When using blood-supplemented medium for testing streptococci, the zone of growth inhibition should be measured, not the zone of inhibition of hemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter.

Dilution methods

Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in log² serial dilutions (two fold). Minimum Inhibitory Concentration (MIC) Diffusion tests widely used to determine the susceptibility of organisms isolated from clinical specimens have their limitations; when equivocal results are obtained or in prolonged serious infection e.g. bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise (Figure 2). Also the terms 'Susceptible' and 'Resistant' can have a realistic interpretation. Thus when in doubt, the way to a precise assessment is to determine the MIC of the antibiotic to the organisms concerned. There are two methods of testing for MIC are Broth dilution method and Agar dilution method.

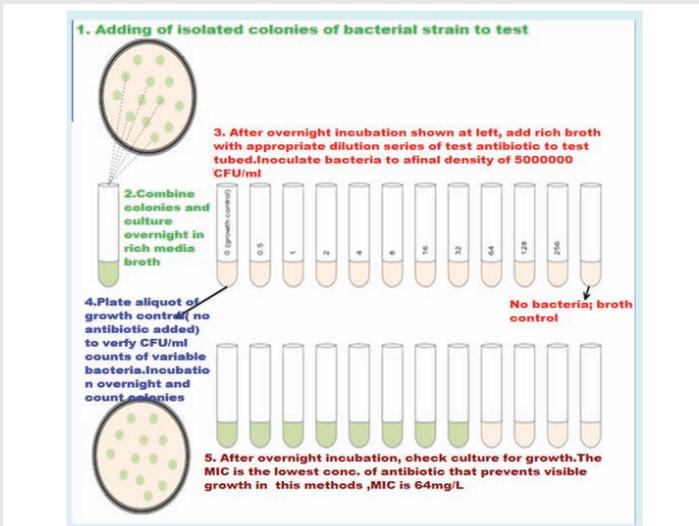


Figure 2: Broth dilution methods. The MIC is the lowest concentration of antibiotic that lacks turbidity.

Broth dilution method

The Broth Dilution method is a simple procedure for testing a small number of isolates, even single isolate. The broth dilution method is based on serial dilutions of an antimicrobial agent in tubes, or microtitre plates, to which a standard concentration of the test bacterium is added [3]. Results are read as a MIC in mg/mL, that is, the lowest concentration of antibiotic that completely inhibits the growth of the bacterium; this is the first tube showing no discernible growth. If the contents of the tubes are cultured onto agar plates then the plate with no growth represents the tube with the minimal bactericidal concentration (MBC) of antimicrobial and may

be different to the tube indicating the MIC. The tubes are incubated and examined for turbidity. A turbid sample is an indication of bacterial growth, whereas a clear sample is an indication of inhibition of bacterial growth. The MIC is the lowest concentration of the antibiotic being tested that inhibits the growth of the bacteria, resulting in a sample that lacks turbidity [4].

The agar dilution method

The agar dilution method is similar to the broth dilution method in that the antimicrobial agent is serially diluted and the dilutions added to the agar medium. The test bacterium is spot-inoculated at a standardized concentration on to the agar surface. Agar plates are prepared with antimicrobial concentrations that encompass the quality control range and the breakpoints. Agar plates containing antimicrobial concentrations must be used within a week of preparation, and therefore, the method requires more media preparation than the disk diffusion method and is more likely to be used by laboratories with a high sample throughput [4].

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Research Article

Veterinary molecular laboratory examination

Molecular biological techniques are the best dimensions to diagnosis the disease process and its causative agents. The molecular biology technique is performed with sophisticated instruments or equipment's required specialized trained personnel and undertaken in routine diagnostic laboratories. The importance of using molecular biology techniques is to diagnosis many bacterial and viral diseases genomes by giving the standard for protein resolution, nucleic acid resolution, and blotting techniques. The following techniques are used in veterinary molecular biology laboratory

Polyacrylamide gel electrophoresis

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'-methylenebisacrylamide and the high molecular weight complex compounds are allowed to pass through gel matrix of the polymer of acrylamide by applying electric current (Figure 1). This method has high resolution capacity which can be further enhanced by addition of sodium dodecyl sulfate (SDS) which allows the resolution on the basis of molecular weight of the compound [1].

The Required Reagents: In the polyacrylamide gel electrophoresis, one requires 0.2 M sodium phosphate buffer (pH 7.2), acrylamide-bisacrylamide ratio (22.2:0.6), ammonium persulfate (15 mg/ml distilled water), TEMED (N, N, N', N'-Tetra methylethylene diamine), bromopheno1 blue (50 mg/100 ml distilled water) and staining solution, 50% Glycerol, 1X TBE, 0.5% xylene cyanol, Ethidium bromide, 10 microgram/ ml.

Procedure of Gel Electrophoresis [2]:

- Prepare an appropriate gel volume that is more than sufficient to fill the chamber formed between the gel plates. Mix gentle stirring the 44 ml water, 5% gel mix and 50 ml of 10% acrylamide solution for 100ml chamber.

- Before adding gel mix into chamber, add 1 ml of 10% ammonium persulfate and 75 micro litre of temed.
- Mix gently using a 50 ml syringe without a needle and dispense mixture into the chamber using the syringe and fill the space entirely. Promptly insert slot former into the top of the chamber and clamp tightly in place and allow gel to polymerize 15 min to 1 hr.
- After polymerization, remove bottom spacer and slot former, and mount gel in electrophoresis apparatus and fill upper and lower tanks with 1x TBE buffer, submerging upper slots.
- Remove any trapped bubbles from the space under the gel in the lower tank.
- The water layer is then removed and the tubes are placed in gel apparatus. In 40 ml solution of protein (sample, which may be bacteria/ virus/ rickettsia/chlamydia/ myoplasma in purified form), add 10 ml of tracking dye (bromophenol blue 0.05%) by using micropipette to each gel. Also use positive control to compare the results.

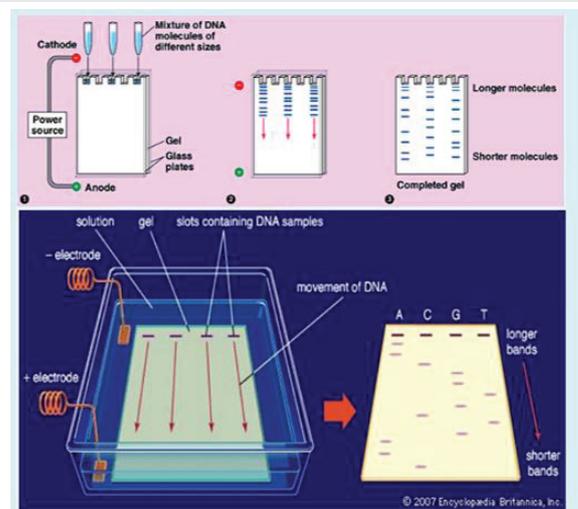


Figure 1: Structure of Gel Electrophoresis.

- Run the gel by applying a voltage of 2- 10 V per centimeter of gel and run the gel long enough to resolve the sample fragments of interest. As a rough guide, the bromophenol blue dye front comigrates with DNA fragments of about 50 bp, whereas the xylene cyanol dye front runs with 400 bp fragment in a 5% gel.
- Turn off power supply and remove the gel from apparatus.
- Separate the two plates with gel and adhering to one of them and the resolved DNA can now be detected by autoradiography if radiolabeled or ethidium bromide staining.
- Autoradiography may be performed on the wet gel by covering it with clear plastic wrap and applying it to XAR-5 film in a cassette.
- Gel can be transferred to whatman 3MM paper and covered with clear plastic wrap. This gel can be autographed or dried on a gel dryer and the autoradiographed.
- Ethidium bromide staining can be performed by carefully layering 50-100 ml of an ethidium bromide solution, 10 microgram/ ml, onto the gel while it is still adhering to one of the plates. Allow ethidium bromide solution to diffuse into gel and stain DNA for 15 minute.
- Gently rinse excess stain from the gelsurface with water and cover the gel with clear plastic wrap and visualize DNA by UV transillumination [plastic wrap directly against UV source, because glass absorbs UV light.
- Gel may now be photographed and identified fragments can now be cut from gel with a new razor blade and recovered by electroelution.

Nucleic acid hybridization

The nucleic acid hybridization is the process wherein two DNA or RNA single chains from different biological sources, make the double catenary configuration, based on nucleotide complementarity and of contingent sequence homology of the two sources, resulting DNA-DNA, RNA-RNA or DNA-RNA hybrids (Figure 2) [3]. After electrophoresis, the cells are plated on an agar solidified medium and blotted with a disc of nitrocellulose paper. The paper later treated to lyse cell and dissociate DNA to be hybridized with the probe and finally to be auto radiographed [4]. For this the nitrocellulose paper is applied on both sides of the gels to get duplicate blots of the gel.

The nucleic acid used to detect the presence of specific nucleic acid in the immobilized sample is radioactively labeled is known as probe. If complementarity exists between the probe and the immobilized nucleic acid, hybridization will occur and this can be measured by a scintillation counting or autoradiography and it is known as dot-blot hybridization [5,6]. When hybridization takes place on a solid carrier is named blotting and is divided in 3 categories: Southern

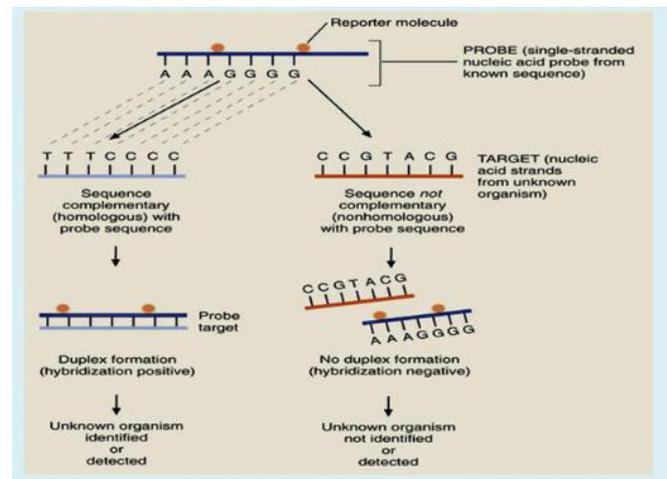


Figure 2: Principles of nucleic acid hybridization. Identification of an unknown organism is established by positive hybridization (duplex formation) between a nucleic acid strand from the known sequence (probe) and a target nucleic acid strand from the organism to be identified. Failure to hybridize lack of homology between the probe and target nucleic acid. (<http://basicmedicalkey.com>).

blotting whereby DNA molecules are identified using DNA or RNA probes; Northern blotting whereby RNA molecules are identified using RNA or DNA probes; Western blotting whereby protein sequences are identified using specific antibodies [3]. After overnight blotting, the nitrocellulose membranes are slightly rinsed in distilled water and heated at 80°C for 2 hours.

The membranes are then kept at room temperature till hybridization. For hybridization, specific probes are used in order to make a confirmatory diagnosis. The probes are purified, characterized, nucleic acid sequence, which can be specific for a given species of organisms [7,8]. The hybridization probe is a fragment of DNA or RNA of variable length (usually 100–1000 bases long) which can be radioactively labeled. It can then be used in DNA or RNA samples to detect the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe. The probe thereby hybridizes to single-stranded nucleic acid (DNA or RNA) whose base sequence allows probe target base pairing due to complementarity between the probe and target (Figure 3,4) [8].

There are two ways how to synthesize probes like nick translation and random primers.

Probe synthesis

Probes can be synthesized by using randomized primers and nick translation.

Probe synthesis by “nick-translation”

The DNA molecule double stranded is subject to DNase I action that is a nonspecific site endonuclease and that takes to pieces randomly, phosphodiesteric bonds, in the presence of Mg^{2+} ions, generating single chain breaks “nicks”; as a result of exonuclease 5-3' action of a DNA polymerase I from *E. coli* nicks are enlarged simultaneously with exonuclease 5-3' action of DNA polymerase I, this shows its 5'-3' polymerization activity using marked dNTPs and thus a translation of the single chain

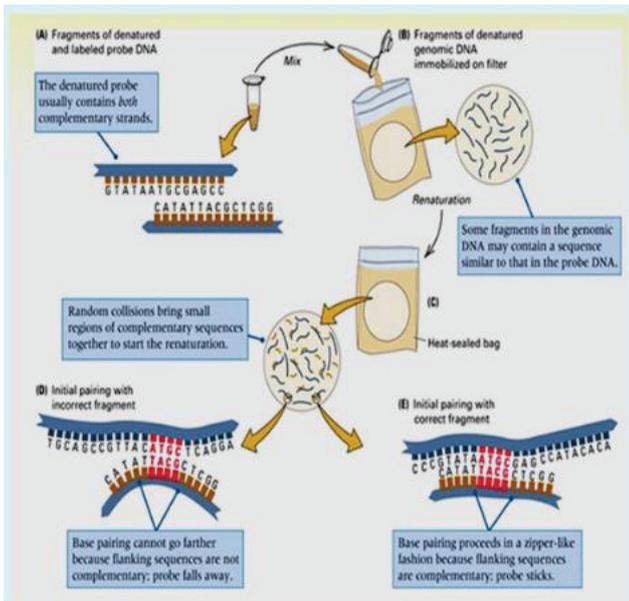


Figure 3: Nucleic acid hybridization process.

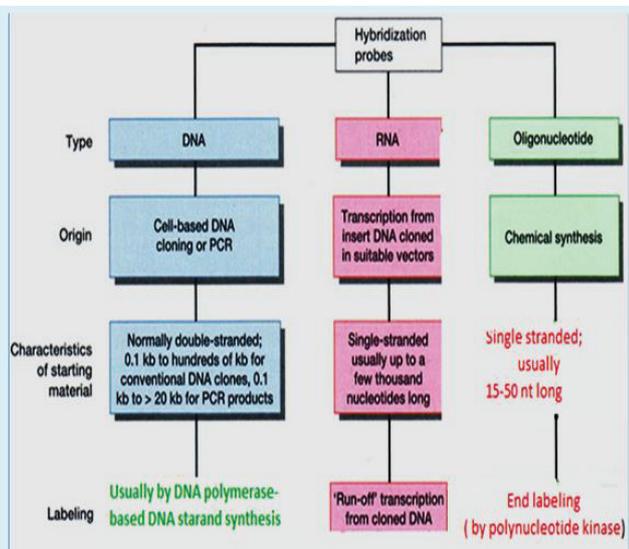


Figure 4: Hybridization of DNA and RNA.

break is carried out (nick-translation). DNA double stranded sequences thus obtained and marked uniformly are subject to thermal denaturation by breaking the hydrogen bridges and obtaining single chain probes.

Probe synthesis using randomized primers.

Randomized primers are heterogenous sequence oligonucleotides that can hybridize in many sites of the matrix chain. DNA is isolated from the salmon sperma or calfe timus and is acted over it with DN-ase I so as a high population of single chain DNA oligonucleotide sequences are obtained having dimensions of 6-12 nucleotides. Automaticoligonucleotide synthesizer having all the 4 types of dNTPs in the reaction medium. Thermic denaturation of the matrix and production of DNA single chains, Attaching of randomized primers in various sites of single chains (at random).

Using 3 types of simple nucleotides and one marked radioactively, in the persence of DNA polymerase I and Klenow-DNA pol I(it has no exonuclease 5'-3' activity), completion of holes among primers, by copying the matrix information, By heat denaturationradioactively marked probes are obtained.

The probes are of two types they are detailed as below (Figure 5) [3].

Radio Labeled Probes: Mostly used radioactive labels are phosphorous 32 (³²P), sulphur 35 (³⁵S) and tritium (³H) which are detected by process of autoradiography. These probes are used for identification and detection of the probes after hybridization .

Biotin Labeled Probes: Biotin labeled probes are non-radioactive like enzyme reactions (peroxidase, alkaline phosphate) and luminescence (Adamantyl Phosphate derives,Lumi-Phos). Instead of radioactive material, the probe is labelled with biotin which is detected by avidin-peroxidase conjugate by using an enzyme substrate like diaminobenzidine tetrahydrochloride. The colour developed by the substrate at tlie reaction site is visible by naked eye; and the blotted nitrocellulose sheets can be stored for long period. Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support, generally nylon or nitrocellulose membranes [10].

Types of blotting

The electrophoresis of nucleic acid and protein blotting and its detection by using probes is classified into three types^{11, 12} (Figure 6).

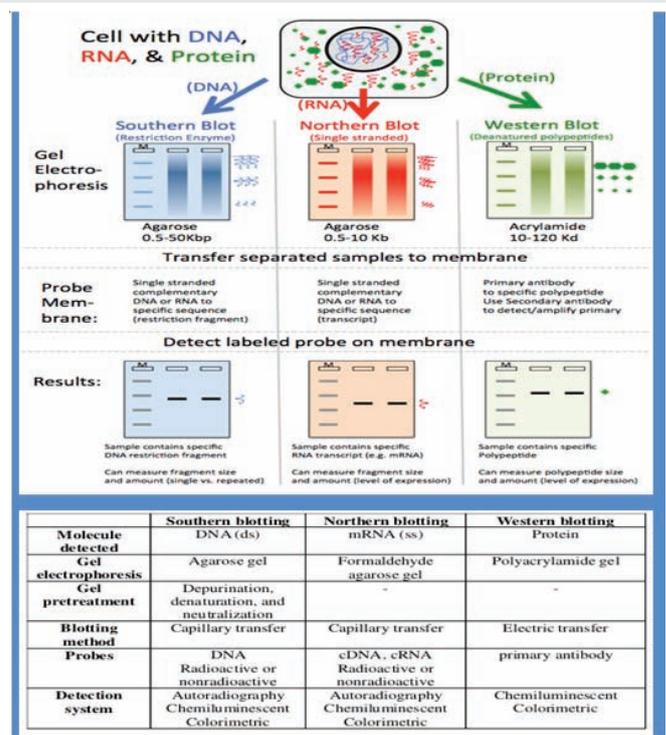


Figure 5: Comparison of southern, northern and western blotting techniques.

Southern blotting: It is a method used for detection of a specific DNA sequence in DNA samples. DNA is separated by electrophoresis, then transferred to nitrocellulose paper and denature into single strands that can be hybridized with a specific probe¹³ (Figure 7).

Procedure [7,14,15]:

- * High molecular weight DNA strands are cut into smaller fragments by restriction endonucleases.
- * The DNA fragments are separated by size by agarose gel electrophoresis and then transferred to a nitrocellulose membrane which is placed on the top of the gel.
- * In Southern blotting, before transfer, DNA is usually denatured with alkali for denaturation of the double stranded DNA.
- * The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe, and

destroys any residual RNA that may still be present in the DNA.

- * After transfer of the DNA fragments to the nitrocellulose membrane which is done by capillary action or may be by electrotransfer, vacuum transfer or centrifugation, the membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe (a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined).
- * The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe or by development of color on the membrane if a chromogenic detection method is used.

Northern blotting

The north blot is a techniques used in molecular biology research to study gene expression by detection of RNA (isolated mRNA) in a sample¹⁶. When the RNA is electrophoresed and blotted on nitrocellulose paper and is detected by nucleic acid probe. The northern blot technique is used to study gene expression by detection of RNA (or isolated mRNA) in a sample. It is used to identification and qualification of RNA expressed in cells. The RNA population is separated by size during gel electrophoresis followed by transfer of the RNA onto a membrane (nitrocellulose or nylon)¹⁷.

Procedure [7,18]:

The procedure includes the following steps RNA isolation, Probe generation, denaturing, hybridization and washing and detection (Figure 8):

- The nucleic acid molecules (RNA samples) are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane but for RNA in Northern blotting, alkali denaturation is not necessary and would in any case hydrolyze the molecules.
- A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them.
- The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat.
- After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that

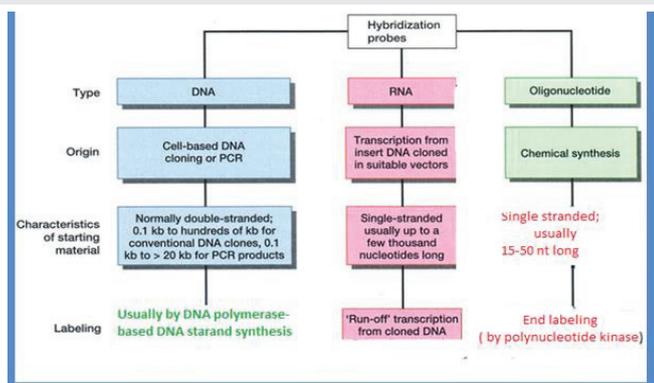


Figure 6: Hybridization of DNA and RNA probes.

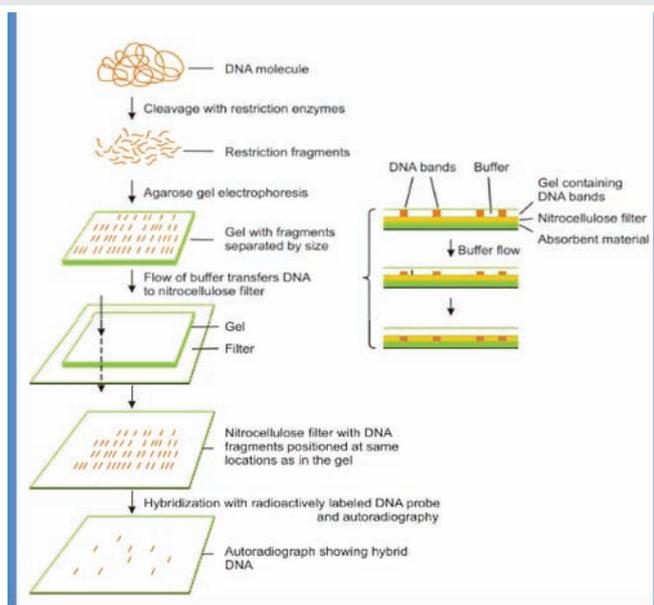


Figure 7: Procedure of southern blotting in separation of DNA.

can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition.

- The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film.

Western blotting

Western blotting does not involve nucleic acid hybridization. The western blot (alternatively, immunoblot) is used to detect specific proteins in a given sample of tissue homogenate or extract. Proteins are separated on a gel, transferred to a membrane and detected by antibodies. When protein is electrophoresed and is detected by using monoclonal or polyclonal antibodies and enzyme conjugates and substrate and by producing a coloured visible reaction; it is known as western blotting or immunoblotting [19].

Procedure (Figure 9):

The principle of the Western blotting is based around a few broad steps [20]:

- The extraction of cellular proteins from a complex mixture of intracellular and extracellular proteins (from tissue, cells, etc.).
- Quantification of protein concentration and electrophoretic separation of proteins within a gel matrix; transfer to a membrane with a high affinity for proteins;
- Blocking the membrane to reduce non-specific binding;

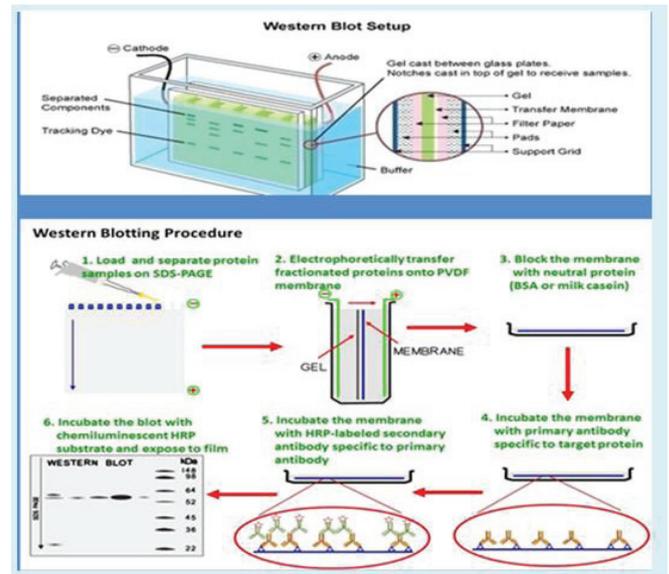


Figure 9: Procedure and technique of western blotting.

- Antigen detection by antibodies specific for the protein(s) of interest;
- Incubation with a secondary antibody linked to a label (e.g., chemiluminescent or fluorescent);
- Development and detection of the signal, which is theoretically proportional to the degree of antigen/antibody binding (Figure 10).
- Quantification of the resulting bands using densitometry

Polymerase Chain Reaction (PCR)

The PCR is a widely used technique in molecular biology because of its high sensitivity, specificity and user-friendly nature. In 1971, the idea by Kjell Kleppe [21] was described to replicate short DNA fragments by nucleotide primers *in vitro*, but the invention of the complete assay is credited to Kary Mullis [22]. By the development of related disciplines (enzymology, oligosynthesis, electromagnetism, etc.), there is a large variety of PCR technologies available nowadays [23]. At the present time, the PCR is based on a thermostable DNA polymerase, which amplifies a specific region of the target DNA initiated by short, 15–30 bp long oligonucleotides (primers), following the principle of Watson–Crick base pairing.

PCR is a highly sensitivity procedure for detection of infectious agents in host tissues and vectors. Pcr can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. However, PCR do not differentiate viable and nonviable organisms or incomplete pieces of genomic DNA. PCR may use to diagnosis chronic persistence infection like retroviruses bovine leukaemia virus, caprine arthritis/ encephalitis virus [24].

The material which required in processing of PCR are listed below [25,26]:

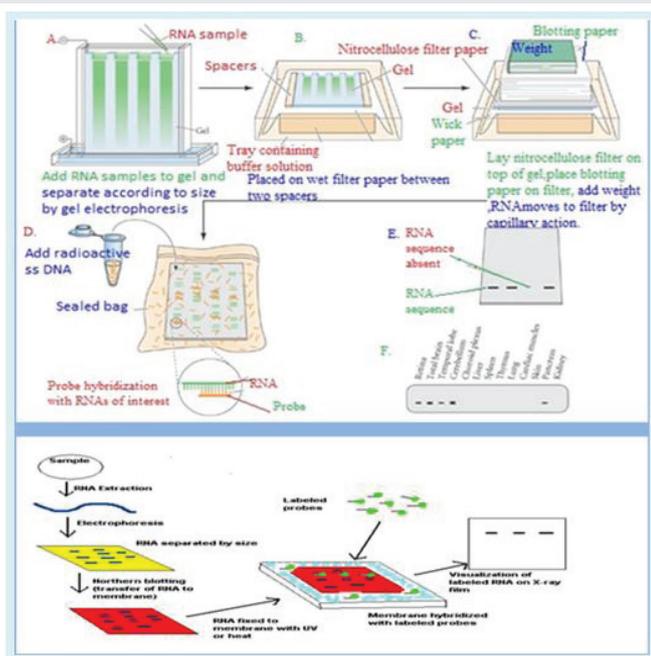


Figure 8: Northern blotting for RNA identification.

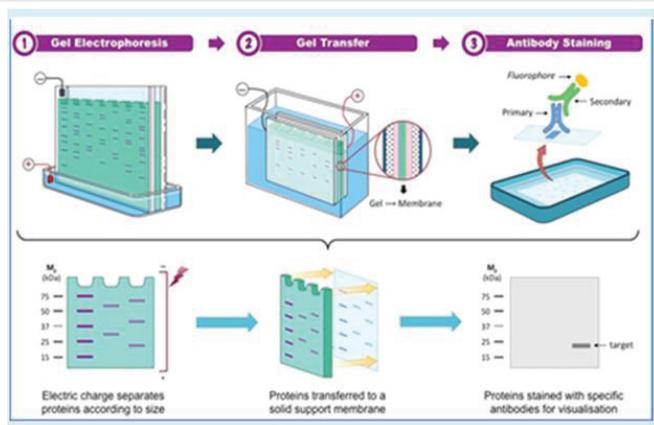


Figure 10: Gelelectrophoresis procedure in identification of antibodies.

- 1.5 ml microcentrifuge tubes
- 0.2 ml PCR tubes (16 tubes)
- Microcentrifuge tube rack
- Empty tip boxes for holding PCR tubes
- The streak plates of your starch degraders
- Inoculating needle
- Micropipettors and tips
- Purified soil DNA
- Nuclease-free H₂O
- 10X PCR buffer
- 25 mM MgCl₂
- Deoxynucleotides (1.25 mM dNTPs each)
- Forward primer (designated 11F)
- Reverse primer (designated 1492R)
- *Taq* polymerase (*Taq* polym-erase)
- Positive control

The PCR reaction requires the following components [27]:

DNA Template: The double stranded DNA (dsDNA) of interest, separated from the sample.

DNA polymerase: Usually a thermostable *Taq* polymerase that does not rapidly denature at high temperatures (98°) and can function at a temperature optimum of about 70°C.

Oligonucleotide primers: Short pieces of single stranded DNA (often 20–30 base pairs) which are complementary to the 3' ends of the sense and anti-sense strands of the target sequence.

Deoxynucleotide triphosphates: Single units of the bases

A, T, G, and C (dATP, dTTP, dGTP, dCTP) provide the energy for polymerization and the building blocks for DNA synthesis.

Buffer system: Includes magnesium and potassium to provide the optimal conditions for DNA denaturation and renaturation; also important for polymerase activity, stability and fidelity.

All the PCR components are mixed together and are taken through series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine.

Denaturation: This step involves heating the reaction mixture to 94°C for 15–30 seconds. During this, the double stranded DNA is denatured to single strands due to breakage in weak hydrogen bonds.

Annealing: The reaction temperature is rapidly lowered to 54–60°C for 20–40 seconds. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

Elongation: Also known as extension, this step usually occurs at 72–80°C (most commonly 72°C) to raise the reaction temperatures so *Taq* polymerase extends the primers, synthesizing new strands of DNA. In this step, the polymerase enzyme sequentially adds bases to the 3' end of each primer, extending the DNA sequence in the 5' to 3' direction. Under optimal conditions, DNA polymerase will add about 1,000 bp (base pair) /minute.

Final elongation: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

Final hold: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

The Requirements in processing of PCR in veterinary molecular laboratory like: [26,28–33]:

Thermostable DNA polymerase: The use of thermostable DNA polymerases in PCR has made it simple because single addition at the beginning is sufficient for the entire amplification process without any further requirement. These enzymes are obtained from thermophilic bacteria that have capacity to replicate at higher temperatures. Such enzymes are also produced in *E. coli* through recombinant DNA technology and at present both natural and recombinant enzymes are commercially available. These include *Taq* polymerase, AmpliTaq, Vent, *pfu* DNA polymerase, *Tth* DNA polymerase, *UITma* etc.

Deoxyribonucleic acid Triphosphates: It is used in 200 pM concentration which is enough to synthesize 12.5 pg DNA. Each batch of dNTP should be checked for pH and pH 7.0 should be adjusted using 1N NaOH.

Primer: Primer is oligonucleotide sequence with 17-30 nucleotides having 50% GC content 5' end primer is used for PCR product modification like addition of restriction site but 3' end should be intact without any inter or intra-primer complementarity which may lead to primer dimer formation in PCR.

PCR primers are specific short string of ss DNA known as oligomers [Various kinds of primers are as under:

Random primers: These are random hexamers of four nucleotides A, T, G and C and are used for c DNA synthesis. Universal primers-Primer sequences flanking the cloning sites of plasmids are termed as universal primers.

Degenerate primers: Every possible combination of nucleotides that code for a given amino acid sequence is called degenerate primers.

Specific primers: Primers designed to amplify a specific target DNA of defined length.

News fed Primers: Primers used to amplify a segment interval to the previously amplified PCR product.

Buffers and Magnesium Chloride: The most commonly used buffers (lox) include 100 mM Tris hydrochloride, 500 mM potassium chloride and 15 mM magnesium chloride. It also contains 0.1% (w/v) gelatin and has pH 8.5 at room temperature. Magnesium is a required cofactor for thermostable DNA polymerases, and magnesium concentration is a crucial factor that can affect amplification success. It is used with taq or Ampli Taq DNA polymerase. However, buffer should be used as per the recommendatlom of supplies and it may vary with DNA polymerase. Magnesium chloride is very critical in PCR and is responsible for formations of soluble complex with d NTPs which is essential for incorporation. It also stimulates polymerase activity, increases Tm of ds DNA and promotes primer annealing.

Low concentration of magnesium chloride results in low yield while excess concentration may lead to accumulation of non-specific products. The EDTA used for dissolving DNA should not have more than 0.1 mM concentration otherwise it will affect magnesium ion concentration.

Template DNA: Sample is prepared by lysing the cells through boiling in hypotonic solution. Care should be taken to avoid contamination of template DNA with PCR inhibitors which include heparin, protease-K porphyrin, ionic and nonionic detergents and phenol.

Procedure of PCR how to perform in veterinary disease diagnosis (Figure 11) [34,35]:

- Prepare the reaction mixture in PCR tube as following
 - ✓ Nuclease-free water - 59.0 μ l
 - ✓ 10 x PCR buffer - 5.0 μ l
 - ✓ dNTPs (2mM each) -1.0 μ l

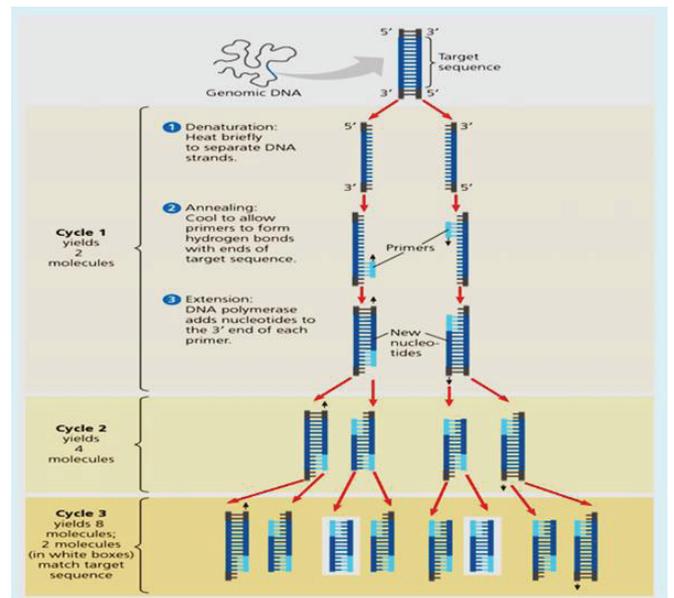


Figure 11: The procedure of PCR.

- ✓ Primer I(100 p moles) - 1.0 μ l
- ✓ Primer I1 (100 p moles) - 1.0 μ l
- ✓ Template DNA - 2.0 μ l
- Mix well and centrifuge at 5000 rpm for 15 sec and add 1 μ l enzyme Taq polymerase (2.54/ML).
- Mix well and again centrifuge at 5000 rpm for 15 sec.
- Heat the PCR mixture at 94°C for 4 min for denaturation.
- Again heat the PCR mixture at 94°C for 1 min.
- Incubate the mixture at 55°C for 1 min.
- Incubate the PCR mixture at 72°C for 3 min.
- Repeat the process from step 5 to 7 for 54 cycles, however, the number of cycles depends on denaturation temperature, duration and amount of template DNA. At higher denaturation temperature, enzyme activity is lost and more number of cycles would not help. If the template concentration is low, the number of cycles can be increased.
- After 54 cycles, incubate the mixture at 72°C for 5 min. This is the final extension step to ensure all PCR products are blunt ended.
- Cool the product in ice. It should be immediately chilled and should not be left at room temperature as the residual enzyme activity may lead to formation of non-specific product.
- For amplification of RNA by PCR, complementary DNA (cDNA) is synthesized first using reverse transcriptase and then cDNA is amplified in PCR as given above

- Confirmation of PCR product is the ultimate result of PCR standardization.

The PCR amplified product is confirmed through either of the following methods [36-39]:

- Agarose gel electrophoresis: It can confirm the size of product when run in 1% agarose with molecular weight markers and dye.
- Restriction enzyme analysis: The product can be fragmented using restriction enzyme and then run on agarose gel for expected fragments.
- Nested primer PCR: In this, amplify a region of PCR product using nested primers that flank the region internal to the product and confirm the specific PCR product.
- Nucleotide sequencing: Sequence the PCR product for confirmation.
- Southern blotting: Transfer the PCR product on nitrocellulose after electrophoresis and carry out hybridization using DNA probe.

Interpretation of PCR

Positive PCR: When comparing with other pathogen isolation PCR usually generates more positive results because it requires much less pathogen. Positive PCR can be obtained in cases in which viral culture and isolation test results are negative due to PCR detects pathogen DNA rather than active pathogen reproduction and also it can identify asymptomatic animals that harbor nonviable organisms⁴⁰. Animals that harbor a viable pathogen can be identified if the initial screening PCR is followed by culture or viral isolation. False-positive PCR results can occur through the undetected inclusion of contaminating DNA within the test reaction⁴¹:

- ✓ Excessive PCR cycling resulting in amplification of DNA similar to the target DNA
- ✓ low specificity of the PCR primers (primers that amplify similar nonpathogen DNA)
- ✓ Carryover contamination in post-PCR analysis using conventional PCR techniques. Contamination can be monitored through inclusion of negative control reactions in which the test protocol is identical, but target DNA is not included.

The development of a new PCR test should include primer testing using target DNA with similar sequences (e.g., primers for equine herpesvirus should be tested against those for equine herpesvirus 4) to ensure that the primers have good specificity to the target DNA [42]. The exponential amplification of pathogen genetic material using PCR generally leads to an increase in positive results compared with most other conventional methods. In addition to the basic PCR technique, other advances in PCR technology (nested PCR, real-time PCR) have been used to increase the specificity and sensitivity of PCR-based diagnostic testing [43].

Negative PCR: Negative PCR results from infected animals which occur when samples from infected animals do not contain target DNA or RNA. Negative PCR results can be isolated from cerebrospinal fluid samples that lack of bacterial infection or from serum of infected animals that have passed short lived viremic phase of infection with encephalitis virus [44]. False negative PCR results can occur when samples contain appropriate target DNA, no DNA are amplified but pathogen DNA are present due to handling or laboratory errors (poor DNA extraction from samples, DNA degradation, poor primer performance, poor reaction optimization, transcription errors and presence of contaminating inhibitory substances in the samples [45-47]).

Polymerase chain reaction- enzyme linked immunosorbent assay technique

Polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) is an immunodetection method that can quantify PCR product directly after immobilization of biotinylated DNA on a microplate. It is used to detect bacteria, fungi, viruses and other pathogenic organisms [48,49]. It can detect nucleic acid of microorganism instead of protein (figure 12), it is more sensitive method compared to conventional PCR method, with shorter analytical time and lower detection limit [48]. PCR-ELISA is a more accurate diagnostic test than other molecular and serological tests [50].

PCR-ELISA methods can be undergone in three steps [48,51] and also indicated in figure 12:

- * Amplification: The gene of microorganism is amplified through PCR in the presence of digoxigenin-11-dUTP (DIG-dUTP). DIG-labelled PCR products will then bind to specific oligonucleotide probes, labelled with biotin at their 5' end.

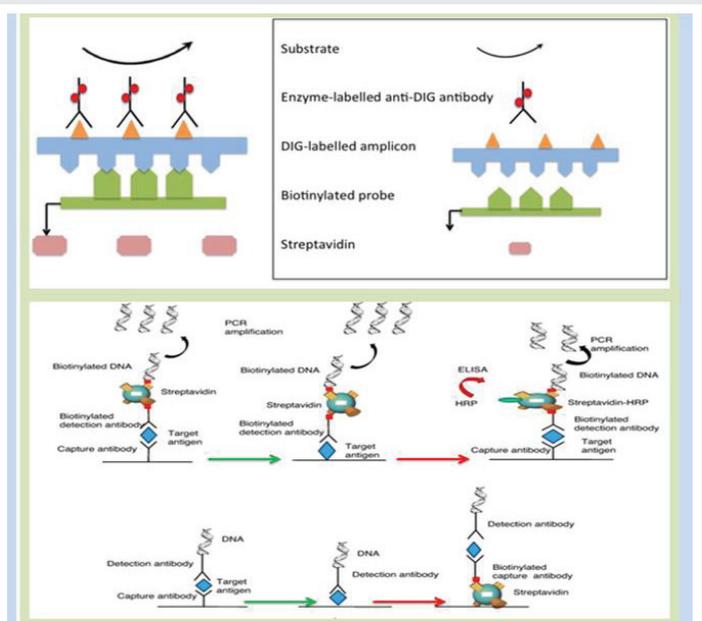


Figure 12: The methods of immuno-PCR or PCR-ELISA technique in detection of microbial genome instead of the protein (antigen).

- * Immobilization: Immobilization of interested gene of isolated gene into microplate. Presence of streptavidin coated on plates and biotin on the 5' end of the formed hybrid can viable the procedure. Affinity of avidin-biotin interaction forms avidin-biotin complexes; this allows only PCR products to bind with specific gene interest to microplate. Other uninterested product could be washed out.
- * Detection: the formed complexes can't be detected by naked eyes, therefore it can be detected by using an anti-DIG-peroxidase conjugate through substrate 2,2'-azino-di-3-ethylbenzothiazoline sulfonate. Form this conjugation blue-green color reaction will be developed, this is visible and also measure by spectrophotometer.
- * Fluorescein probe can also detect the complexes formed in PCR-ELISA detection, using antifluorescein antibodies conjugated to horseradish peroxidase to detect the hybridized fluorescein labeled oligonucleotide probe.

PCR-ELISA technique is used to detect parasite, fungi and bacteria from sample of diseased animals [52-55].

- * The first step for detection of microorganism is that collection of sample from diseased animals, fecal sample, blood and etc.
- * Then DNA of the agents can be isolated from sample
- * Identification of primer used for amplification: the primer used for amplification for *Trypanosma spp*s are listed as following :
- * Prime for *Trypanosome vivax* : ILO1264 (CAG CTC GGC GAA GGC CAC TTG GCT GGG) and biotinylated ILO1265 (biotin-TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG), the fluorescein-tagged oligonucleotide for hybridizing with the PCR products obtained is designated violo-3, and has the sequence: (FL-CA GAG CAG TCT CGG CGC GCC CCA TGT TC-FL). The biotinylated oligonucleotide designated violo-4 (biotin-CT GGA GGT GAA CAT GGG GCG CGC CGA GAC TGC TCT G) is used as a positive control when detecting by ELISA the PCR product of *T. vivax* DNA amplification ⁵⁵.
- * *Primer for T. brucei*: the pair of primers designated bruolo-1 (biotin-AA GAA CCA TTT ATT AGC TTT GTT GC) and bruolo-2 (CGA ATG AAT ATT AAA CAA TGC GCA G). The fluorescein-tagged oligonucleotide primer designated bruolo-3 (5-FL-CAA TGT GTG CAA TAT TAA TTA CAA GTG TG-FL-3) is hybridized with the PCR products obtained. The primer, bruolo-4 (biotin-TAT TTA ATG TTG CAC ACT TGT AAT TAA TAT TGC AC ACA TT), is used as a positive control when detecting by ELISA the PCR products of *T. brucei* DNA amplification, because it is complementary to the fluorescein-tagged oligonucleotide primer ⁵⁵.
- * The following steps are undergone to detect the PCR product by ELISA:

- ✓ The ELISA is performed in microtitre plates coated with streptavidin diluted in carbonate coating buffer to detect the PCR product.
- ✓ The plates were shaken briefly after addition of the coating buffer, incubated at room temperature for 2 h, and then stored at 4 °C for up to 1 week after removal of the buffer.
- ✓ Wells of the microtitre plates is washed six times with Tris-buffered saline
- ✓ The PCR products is analyzed by ELISA diluted 1:10 with hybridization buffer and 100 µl of each sample is dispensed into each well and incubated at 37°C for 1hr to facilitate the binding of the biotinylated PCR products to the streptavidin coating in the wells of the plate.
- ✓ The PCR product binded to streptavidin is denatured by adding NaOH in each well and incubating for 2 min at room temperature.
- ✓ The unbound PCR product is removed by washing the plates six times with TBS/Tween-20.
- ✓ Fluorescein-labeled oligonucleotide probe (at 1 ng/ml) in hybridization buffer is added into each well and the plate incubated at 55 °C for 2 hr.
- ✓ The fluorescein-labeled probe is revealed by introducing into each well of anti-fluorescein antibody conjugated to alkaline phosphatase, diluted in TBS/1% BSA.
- ✓ The reaction proceeded at 37 °C for 30 min.
- ✓ An amplifier (alcohol dehydrogenase plus diapherase) is added at well and the assays transferred to 37°C for 6 min.
- ✓ The action is terminated within a 6 min interval, by the addition of sodium chloride into each well.
- ✓ The optical densities of the reactions in each of the wells can be determined using an ELISA microtitre plate reader
- ✓ From interpretation, the conjugation of PCR product and substrate of ELISA produces blue green color reaction which can be measure by spectrophotometer.

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Research Article

Postmortem examination in veterinary practice

Necropsy is the examination of animals after death and it helps in diagnosis of diseases. Necropsy includes systemic examination of dead animals, recording pathological lesions and interpretation approach to diagnosis of disease. The knowledge of histopathology, microbiology, immunology and toxicology is required for confirmation. The necropsy examination is an integral part of disease investigation; therefore veterinarian should have to know the techniques of postmortem examination, recording of lesions and collection of proper material for laboratory.

The reference for all postmortem examination [1-8]:

Equipment needed for postmortem

The equipment and disinfectant reagent which required during postmortem operation are listed below:

- Gloves
- Boots
- Coveralls
- Protective glasses
- Boning knife
- Steel - for sharpening
- Scissors
- Forceps
- Pruning shears - AKA rib cutters
- Wire cutters
- Plastic wide mouth containers

- 10% buffered formalin (from a veterinarian)
- Sealable bag (Zip-lock bags)
- Permanent marker
- Needles
- Syringes

Disinfectant reagents

Phenolics: General disinfectant. These are active against most bacteria except for spore forming bacteria, such as Anthrax and Clostridium. Some viruses may be sensitive to these compounds.

Alkalis: Examples: Lye, Lime, and Sodium Carbonate. These act against most bacteria as well as spore forming bacteria (i.e. Anthrax, Clostridium) as well as some viruses.

Hypochlorites: Examples: Sodium hypochlorite and Chlorinated lime. These chemicals have a wide antibacterial spectrum, but have little activity against spore forming bacteria and Mycobacterium (eg. the causative agent of Johne's disease). They are active against viruses and protozoa as well. The activity of the chemical is greatly reduced by organic material and high pH (Alkali environments).

Chloramine: Active against most bacteria including spore forming bacteria and Mycobacterium.

Quaternary Ammonium: Active against most bacteria, except Mycobacterium. Will also act against some viruses. Activity is greatly reduced by the presence of organic matter.

Chlorhexidine: Active against most bacteria and fungi, but not against spore forming bacteria or viruses. Activity greatly reduced by the presence of organic matter.

Hydrogen peroxide: Active against bacteria, spore forming bacteria and viruses.

Virkon: Active against many viruses, bacteria including some spore forming bacteria such as Clostridium, and fungi.

Postmortem examination in large animals

- ✓ Place animal on left side (Ruminants)
- ✓ Place horse on right side and dog on vertebral column. Make mid ventral incision with knife from chin to anus. Surround the prepuce, scrotum/mammary gland.
- ✓ Remove skin dorso ventrally. 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 subcutaneous tissue, muscles, superficial lymph nodes prescapular, prefemoral supramammary, etc.
- ✓ Open abdominal cavity by cutting muscles and peritoneum. 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 and other animals like Monogastric animals: Stomach Liver, Pancreas, Intestines, Mesenteric lymphnodes, Spleen, Kidneys, Ureter Pelvic cavity: Urinary bladder, uterus

Postmortem examination of poultry

- ✓ 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, and 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 skull 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.

The most common postmortem observed from poultry are listed as:

Stripes in leg- or breast-muscle: Sarcosporidiosis diagnosed by cytology of such a stripe reveals the bradyzoites.

A large dark spot distal to the keel: Swollen liver

Changes of the skin: Cnemidocoptes, yeast-infection

Opaque air sacs or (fibrinous) inflammation: Chlamydiosis

Opaque air sacs or obvious inflammation: Bacterial infection: rods or

Cocci in cytology smear; culture and sensitivity test.

Airsacs covered with white/yellow plaques: Fungal infection; diagnosis: wet mount (heated with chlorallactophenol), showing hyphae, culture.

Air sacs solid with white/yellow material: chronic fungal infection, mostly aspergillosis; diagnosis: wet mount showing hypha, culture.

Air sacs, esp. cervical and prescapular, with small black dots in passerines and small psittacines: Sternostoma tracheocolum infestation; diagnosis: magnifying-glass and wet mount.

Air sacs filled with food: forced feeding; diagnosis: wet mount and histology.

Pericardial sac filled with fluid: inanition, cachexia; diagnosis: muscle wasting, oedema and gelatinous fat-tissue.

Pericardium covered with white chalky deposits: visceral gout; diagnosis: wet mount with crystals; often in combination with nephritis

Crop:

- ✓ Thickened wall with white material: yeast infection; diagnosis: smear of the material; culture.
- ✓ Thickened wall with mucous material: capillaria infection; diagnosis: smear of scraping of the epithelium; histology.
- ✓ Thickened wall with grey/yellow material, sometimes with trapped air bubbles; trichomoniasis; diagnosis: wet mount; cytology; histology.

- ✓ Local yellow necrotic ulceration: pox-lesions; diagnosis: Macroscopic (gross) examination; histology; virus culture.
- ✓ Local red mucosal thickening: papilloma: diagnosis: histology.

Stomach (proventriculus and ventriculus):

- ✓ Dilated proventriculus and gizzard, often stuffed with seeds (sunflower): gastric dilatation syndrome; diagnosis: histology; (ganglio) neuritis, lymphoid infiltrates in the adrenals.
- ✓ An empty proventriculus with excess of mucous: *Macrorhabdus ornithogaster* (formally “megabacteria”); diagnosis: wet mount and cytology.
- ✓ Swollen red glands in proventriculus: *Tetrameres* spp; diagnosis: parasitological examination. Intestines

Haemorrhagic contents duodenum: Coccidiosis; diagnosis: wet mount, cytology.

Haemorrhagic, black contents in the entire small intestine: Haemorrhagic diathesis; diagnosis: history (fasting during high energy need for over 24 hours), macroscopic (gross) examination.

Pseudomembranous covering of the duodenal wall: Hexamitiasis; in cranes; diagnosis: wet mounts, cytology and histology.

Thickened wall with or without blood in the lumen: Enteritis; diagnosis: wet mount and cytology; parasitology; microbiology. Beware: in psittacines very rarely coccidia, often ascaridia; in small passerines rarely worms, often coccidian spp.

Haemorrhagic contents: lead intoxication, clostridium infection, pseudomonas infection, *Giardia* spp.; diagnosis: lead in gizzard; lead analysis liver and kidneys; cytology, culture.

Clear watery contents in small intestine with flabby wall: Hexamitiasis; diagnosis: fresh wet mount, cytology, histology.

Yellow non-digested starch and broken seeds in small passerines: *Cochlosoma* or *Campylobacter* spp.: diagnosis: fresh wet mount, cytology, and selective culture.

Enlarged caeca with pseudomembranous to necropurulent content: Typhlitis; diagnosis: galliformes: histomoniasis (“blackhead”); diagnosis: cytology, Histology (often with liver lesions).

Tongue with yellow “abscesses” at the location of the salivary glands in psittacines: Metaplasia, due to vitamin A deficiency; diagnosis: wet mount, diet history, histology.

Chronic, necrotic lesions especially in commisures: Tuberculosis; diagnosis: cytology (acid fast stain), histology, culture.

The presence of turbid mucus: Sinusitis; diagnosis: wet mount, cytology, culture.

Steps of postmortem examination

Before starting postmortem examination collection of information from owner is the first option. The anamnesis should be taken careful with date and time of death of animals. The post-mortem record includes the aspects of animal identification, illness, therapeutic and preventive measures adopted and date and time of death.

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 specially examined for the presence of blood and any other lesion. The unclotted 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 example: wound, fracture, cuts, etc.
- ✓ Fungal infection example: Ringworm.
- ✓ Parasitic infestations example: Mange, lice, ticks Side of animal is lying down on earth.
- ✓ Discharges from openings.
- ✓ Burn, ulcers, erosions etc.

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 muscle hardening, calcification. In ruminant animals tongue shall be palpated to detect: abscesses, actinobacillosis, an incision shall be made through the center of the each internal pterygoid and external masseter muscles (*Cysticercus bovis*).

The medial retropharyngeal, lateral retropharyngeal, parotid and mandibular lymph nodes are to be exposed, examined visually and carefully incised. Two to three incisions/slices right through the nodes is considered sufficient. In equine the inspection of the head must also include the guttural pouch. Careful examination must be made of the abdominal walls for encysted parasites, of the neck region for fistulous conditions near the first two cervical vertebrae, and of the axillary and subscapular spaces of white and gray horses for melanosis.

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, fibrinous or fibrous adhesions, Parasites, Abscess and tumour.

Respiratory system

Organs tissue to be examined: External nares, nasal passage, larynx, trachea, bronch, lungs, air sacs (poultry) mediastinal lymphnodes, pleura. Lungs- Congestion, consolidation, nodules, presence of exudate on cut surfaces, oedema, atelectasis, emphysema, haemorrhage, and necrosis. Mediastinal lymph nodes- Oedema, hardening, calcification, congestion, haemorrhage
Lesions to be observed: Discharge from external nares. Growth (granuloma/polyp) innasal passage if there is blood-mixed nasal discharge. Trachea and BronchCongestion, haemorrhage, presence of caseous exudate, frothyexudate (Figure 1,2).

Cardiovascular system

Organ tissue 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. The techniques how to incise and examine of the heart is detailed in figure 3,4.

Digestive system

Organ tissue to be examined: Mouth cavity, esophagus, crop, proventriculus, gizzard (poultry), rumen reticulum, omasum, abomasum (ruminants), stomach, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), cloaca, vent (poultry), anus, liver, pancreas, gall bladder, mesenteric lymph nodes etc.

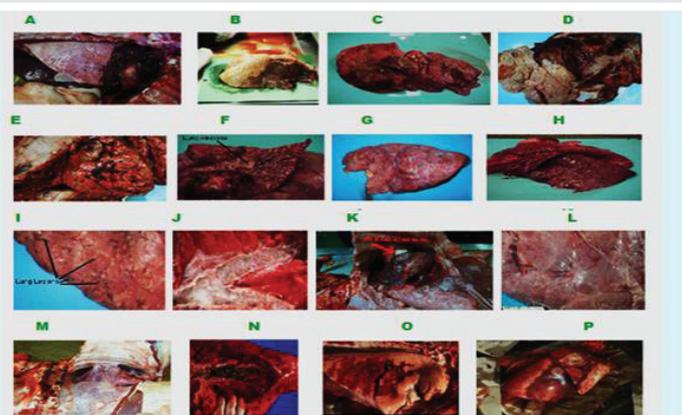


Figure 14.1: Abnormal of lung. **A:** bronchopneumonia. Notice how the bottom-front of the lung is darker than the rest of the lung. Usually this part of the lung will be heavier than the more normal, pink lung to the left. **B:** Bronchopneumonia. **C:** chronic pneumonia. Notice how the lung looks darker (or redder) than normal pink lung tissue. This lung may be heavier than normal lung and will not have the "spongy" feel to it. **D and E:** the diseased, darker, redder lung to the right compared to the more normal lung tissue to the left. The diseased lung is heavy compared to the light, spongy normal lung tissue. **F:** An abscess will have a liquid to "cottage cheese" like appearance in the middle. A tumor or other growth will usually be harder in the middle **G:** pulmonary (lung) emphysema. When pinched, this lung will "pop" like bubble-wrap. Air has accumulated in areas where there is normally only tissue. **H:** Pulmonary consolidation. This lung will be heavy and "wet". It is also much darker than normal lung tissue. The lung may contain blood or other types of cells due to inflammation, or other disease process. **I:** Presence of lung worm. **L:** The lung is adhered to the rib cage. These types of adhesions are commonly found in cattle. **N and P:** Pneumonia. The red areas are the most affected and there may be some collapse of the lung in these areas.

Figure 1:



Figure 2: Tuberculosis lesion on lungs and other organs under postmortem inspection.



Figure 3: Procedure heart incision and diagnosis.

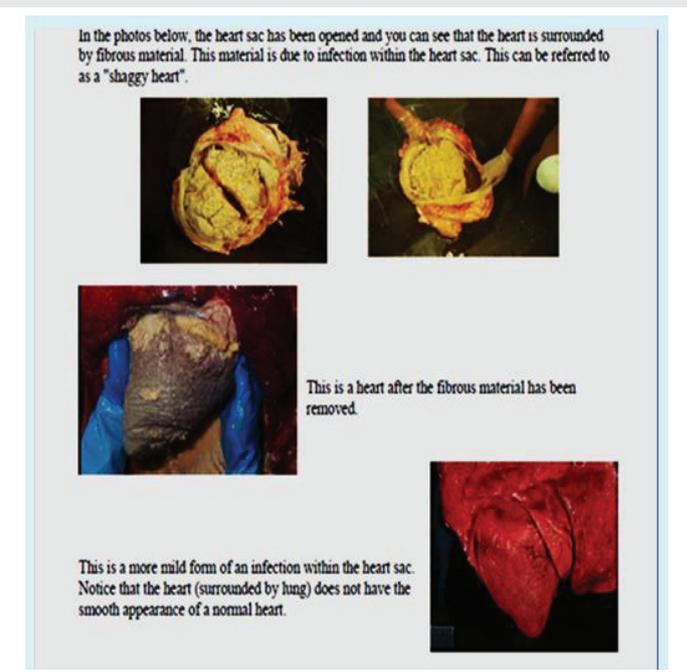


Figure 4: Abnormality of heart.

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 (Figure 5,6).

Urinary system

Organ tissue to be examined: Kidneys, ureter, urinary bladder, urethra.

Lesions to be observed: Congestion, haemorrhage, infarction, oedema. Necrosis, hardening, nodules and deposition of salts, calculi, Obstruction (Figure 7).

Genital system

Organ 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. The step how to open udder and examine (Figure 8,9).

Immune system

Organ tissue to be examined: Spleen, lymph nodes, bursa and thymus (poultry), bone marrow, Peyer's patches, GALT and RALT. The retropharyngeal lymph nodes shall be incised except for partially dressed carcasses, where it may be easier to incise the parotid lymph nodes. The mesenteric lymph nodes,

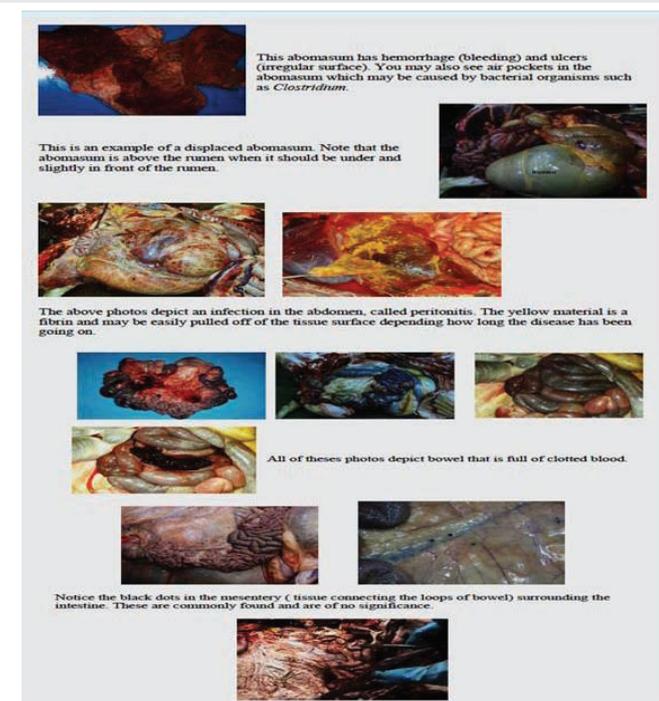


Figure 5: Abnormality of stomach and intestine.

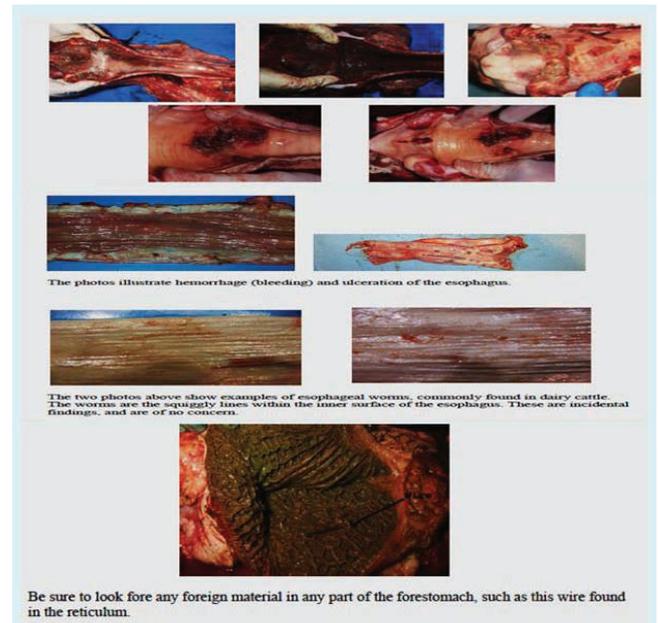


Figure 6: Abnormality of esophagus and reticulum of animals.

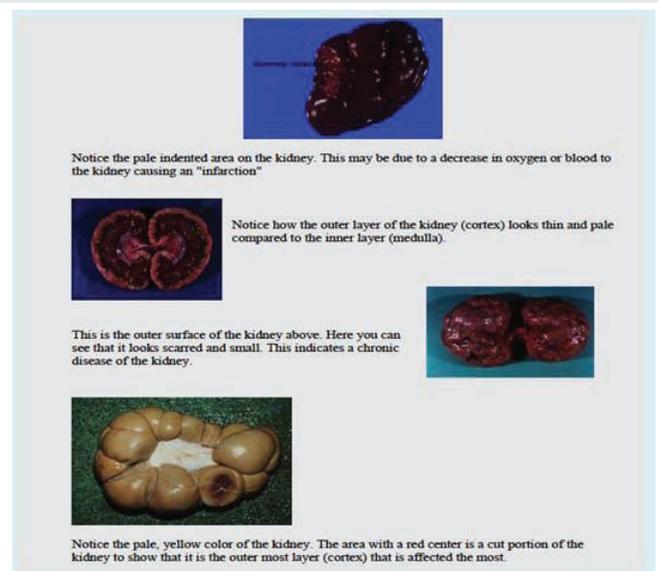


Figure 7: Abnormality of kidney of domestic animals.

the bronchial, mediastinal, hepatic, and superficial body lymph nodes (subiliac, superficial inguinal or mammary, superficial cervical) are to be routinely visualized and palpated. Lymph nodes shall be incised whenever palpation is inadequate to determine the absence of abscesses indicating caseous lymphadenitis or if granulomas are suspected and granulomas consistent with a Tuberculosis lesion shall be observed. Lesions to be observed: Size, shape, atrophy, hardening, Oedema, congestion and haemorrhage.

Nervous system

Organ tissue to be examined: Brain, Spinal cord, nerves, meninges.

Lesion to be observed: Congestion, haemorrhage, hema-



Figure 8: The step how to open udder of cattle.

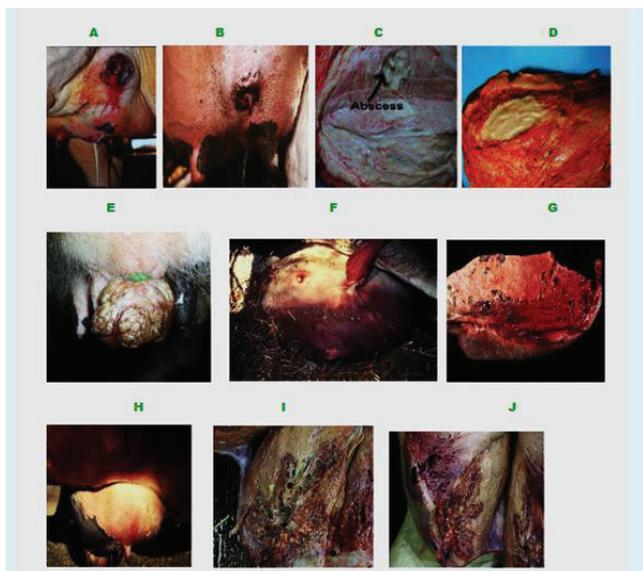


Figure 9: Abnormality of udder A and B: Draining of abscess from udder. C and D: Mammary gland abscesses. These will usually be confined to one quarter. E: Tumor in udder. F,G,H,I and F, J: Progressive Mastitis.

toma. Oedema, swelling, Abscess. Hypoplasia.

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ANNEX

General Characteristic and Miscellaneous Examination of Microorganism

Some important characteristics of the organisms are given in each genera which may serve as key for their identification.

Gram Positive Cocci

- * *Staphylococci*: Gram positive cocci arranged in bunches, catalase positive, acid from glucose. Pathogenic staphylococci are coagulase positive.
- * *Streptococci*: Gram positive cocci, arranged in chains, catalase negative, beta hemolytic.
- * *Micrococcus*: Gram positive cocci, single or small clusters, catalase positive, maltose (-), mannitol (-), coagulase (-), nonhemolytic.

Gram Positive Rods

- * *Bacillus anthracis*: Gram positive rods, cylindrical rods, spores are centrally located, short chains, square ends of rods, capsulated, reduce methylene blue, non-hemolytic, produces dull, opaque, greyish colour colonies.
- * *Listeria*: Beta hemolytic, catalase (+), nitrate not reduced, motile at 25°C, acid from glucose. Single or short chains, noncapsulated, on blood agar circular, transparent colonies, pathogenic for guinea pigs leading to death in 3-4 days.
- * *Eysipelothrix*: Alpha hemolytic, small round, translucent, discrete colonies with entire edge, 1 mm dia, organisms are short rods, thickened filament, non-motile, guinea pigs are resistant, glucose fermented, catalase negative, nitrate not reduced. *Actinomyces*: Gram positive coccoid, branching filaments in media; in tissues sulfure granules, club like process.
- * *Corynebacteria*: Gram positive, coccoid or pleomorphic, non motile, clumps or single, on blood agar minute pin

point colony, beta hemolytic, cream to orange coloured colonies.

- * *Lactobacillus*: Long, slender, Gram positive rods, motile, catalase negative.
- * *Nocardia*: Gram positive rod, acid fast, branching filaments, rod or coccoid, catalase positive.
- * *Clostridium*: This group consists of anaerobic bacilli with rounded ends, spore formers, Gram positive rods.
 - *Clostridium chauvoei*: Gram positive rods with rounded ends, single or short chains, long filament, spores are elongated or oval and sub terminally or terminally located and are wider than width of organism which gives it a tennis racket like appearance.
 - *Cl. septicum*: Cylindrical Gram positive rod, single filaments.
 - *Cl. Novyi*: Largest size, parallel edge, rounded ends, long jointed filaments, Gram positive.
 - *Cl. Perfringens*: Gram positive rods with square ends, nonmotile.
 - *Cl. botulinum*: Gram positive large rods with rounded ends, short chains, oval spores and terminally located.
 - *Cl. tetani*: Gram positive large slender rod, short chains, rounded ends; spore 2-3 times larger than diameter of rod, located terminally giving the organism drum stick like appearance.
 - *Cl. hemolyticum*: Gram positive rods, oval or elongated, spore terminally situated.

Gram Negative Rods

- * *Yersinia*: Small, Gram negative rods or coccobacilli, catalase (+), oxidase (-), motile at 25°C, urease (+), reduces nitrate.
- * *Pasteurella*: Small Gram negative rods or coccobacilli,

oxidase (+), opaque whitish colonies on blood agar, bipolar staining from blood or tissues.

- * *Brucella*: Gram negative coccobacilli, non-motile, noncapsulated, oxidase positive (Br. ovis oxidase (-). Urease positive (Br. ovis urease (-), produces small delicate semitransparent colonies on tryptose soya agar.
- * *Pseudomonas*: Gram negative rods, catalase positive, oxidase positive, motile (*pseudomonasallei* non-motile), produces translucent large, irregular and spread colonies, blue-green water soluble pigment in media.
- * *Actinobacillus*: Gram negative rods, oxidase positive, catalase positive, non-motile, produces flat colonies, transparent, slightly bluish in colour.
- * *Moraxella*: Gramnegative rods, non-motile, catalase positive, oxidase (+), does not grow on McConkey agar, short, diplococoid rods, produces small, round, greyish white colonies, translucent, beta hemolytic.
- * *Escherich coli*: Produces circular, convex, smooth translucent colonies having wet appearance, lactose fermenter, non-motile, Gram negative coccobacilli, Indole (+), MR (+), VP (-), citrate not utilized, produces metallic sheen in EMB agar.
- * *Salmonella*: Gram negative rods, non-lactose fermenter, produces white colonies on McConkey agar and pink on brilliant green agar (BGA), produces homogenous, smooth, glistening, greyish colonies.
- * *Protocus*: Gram negative rod, motile, thin transparent colonies, swarming on agar plates, MR (+), VP (-), citrate not utilized, 3s (+), urease positive.
- * *Klebsiella*: Gram negative, coccobacilli, mucoid colonies, indole (+), MR (i), VP (+), citrate utilization (+), H₂S (-), ureases (+).
- * *Shigela*: Gram negative rod, non-motile, pleomorphic, long filaments, occurs in chain, rough, dry, circular, mucoid colonies, opaque, raised colony on agar surface.
- * *Hemophilus*: Gram negative rod or coccobacilli, non-motile, semitranslucent, flattened, circular, greyish colony with sharp edges.
- * *Vibrio or Campylobacter*: Gram negative, non-capsulated, 's' shaped, bluish pin point size colonies, slightly raised on thiol medium or blood agar.
- * *Mycobacteria*: An acid fast bacillus, capsulated, slow growth, takes at least a week's incubation period for growth.
- * *Leptospira*: Spirochete Long spiral rods, having many curves, ends look like hook, demonstrated in dark field microscope, difficult to grow.
- * *Rickettsia*: Rod shaped, coccoid or pleomorphic, Gram

negative, intracellular organism, does not grow on artificial media. Grows well in yolk sac of 5-7 days old chicken embryo; after 4-5 days' incubation inclusions in yolk sac smears stained with Geimsa or Macchiavello stain. Intra-cytoplasmic inclusions can be demonstrated in liver impression smears of guinea pig inoculated I/P with rickettsia.

- * *Chlamydia*: Intra-cellular organisms, produce intracellular inclusions, do not grow in artificial media, and grow in seven day old chicken embryo through yolk sac route, inclusion in yolk sac membrane.
- * *Mycoplasma*: Pleomorphic, small coccobacilli, ring form, filamentous, produces fried egg colony, grows on PPLO broth or agar containing horse serum, produces nipple shaped colonies, small colonies seen by stereoscope, for cultivation incubated at 2-3°C for 24-48 hours in PPLO broth, then transferred 0.1 ml to solid media. Incubated in humid chambers at 37.5°C. Growth occurs in 2-4 days, flood the plate with 1-2 ml PPLO broth, incubate further for 2-3 days, if no growth for 14 days then declare as negative.

Some importance veterinary laboratory test

- * **Catalase test**: On a clean and dry glass slide, place a drop of 30% hydrogen peroxide. Take bacterial culture with loop from a slant or colony of plates and mix it with hydrogen peroxide on glass slide. The production of gas characterized by appearance of bubbles is indication of a positive reaction.
- * **Coagulase test**: Take 0.5 ml of rabbit plasma diluted (1:5) with sterile normal saline solution. Add 2-3 drops of overnight incubated broth culture and mix well. Incubate at 37°C a positive reaction is indicated by coagulation of plasma within 2-3 hrs.
- * **Oxidase test**: Prepare a 0.5% solution of N, N, N: N Tetramethyl-p-phenylenediamine dihydrochloride and put a drop of this on suspected colony on the plate. The colour of colony will become dark purple in case the oxidase test is positive.
- * **Nitrate reduction test**: In a test tube, take 5 ml trypticase broth culture and add 1 ml of 0.8% sulfanilic acid in 5N acetic acid. Add drop by drop 0.5% alpha naphthylamine in 5N acetic acid. Development of red/pink/maroon colour is an indication for the positive nitrate utilization reaction. However, in negative test reaction add zinc dust in the tube of test culture as well as in broth for control. The colour of positive reaction is characterized as red or pink while the control will remain the same.
- * **Indole test**: Take a 48 hour growth of organism in brain heart Infusion (BHI) broth. Add 1 ml ether in 5 ml culture and mix well. Keep the tube on stand for a few minutes. Add 0.5 ml of Kovac's reagent slowly on the top of the test tube contents. If indole is present, a red colour ring is formed just below the ether layer in the culture.

- * **MR Test:** Take 5 ml growth of organism in MR-VP broth and incubate for 4-5 days. To this add a few drops of MR reagent. In positive test, red colour will develop in the culture. Yellow colour is indication of a negative reaction.
- * **VP test:** Take 2 ml growth of organism in MR-VP broth incubated for 2 days and to this add 0.6 ml of 5% alpha naphthol in ethanol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine and shake well. Place the test tube on stand for 5-10 min. In positive reaction, orange red colour will develop.
- * **Urease test:** Inoculate the organism on urea agar slants under sterile conditions. Incubate for 12 hours at 37 °C. In case of positive test, the media turns into pink colour.
- * **Citrate Utilization test:** Inoculate the organisms on Simmon's citrate agar slants and incubate for 24 hours at 37 °C. The development of a blue colour is indication of citrate utilization.
- * **Hydrogen sulfide gas production test:** The organism is inoculated on TSI agar slants with a straight wire of the bacteriological loop. Inoculate the slant as well as butt and incubate the culture for 18-24 hours at 37 °C
- * **Hemolysis:** Inoculate the organism in pure culture on blood agar plate in straight line and incubate for 24 hours at 37 °C. If there is greenish narrow zone around the colony, it is alpha hemolytic. Clear zone indicates beta hemolysis and no zone indicates gamma hemolysis.
- * **Sugar fermentation tests:** The organisms are inoculated in the sugar fermentation media (sugar + peptone water) (Apyendix). The fermentation of sugar is indicated by development of pink colour in the sugar tube. The development of gas is detected by placing Durham's tube in the sugar tube. The sugars which are generally used for fermentation tests are glucos, lactose, fructose, mannose, mannitol, dulcitol, sucrose, rhamnose, etc.

Fungi

Skin scrapings or suspected clinical samples are inoculated on Sabouraud's dextrose agar containing anti-bacterial drugs and cycloheximide in order to prevent the growth of bacteria and saprophytic fungi. Media may be placed in plates or tubes.

Incubate the culture media at room temperature for several days and examine this daily for fungal growth.

- * **Trichophyton:** It takes 7-10 days to grow on Sabouraud's dextrose agar. Colonies are white which become tanned after sometime and have a granular pigment. Smear made from the colonies may show the presence of numerous microconidia with some macroconidia; these can be stained with lactophenol cottonblue which enhances the visibility of fungus.
- * **Microsporidium:** It grows well in 3-4 days and produces flat colony, white or yellow. Microscopically, few microconidia and numerous macroconidia seen;

macroconidia are large, spindle shaped, multiseptate, thick walled bodies having a knob at the tip.

- * **Candida:** It produces soft, cream coloured colonies, with yeasty odour.
- * **Blastomyces:** White colonies which turn brown after some time. Branching septate hyphae containing lateral oval conidia.
- * **Cryptococcosis:** Thick walled, oval to spherical budding cells surrounded by gelatinous capsule, growth in 10-15 days, colonies are white, glistening, mucoid, which turn brown with age.
- * **Histoplasma:** On Sabouraud's dextrose agar, it produces white, cottony colonies which become brownish after some time. Branching septate hyphae having microconidia; in older cultures macroconidia are seen.
- * **Coccidioids:** It produces fluffy white colonies on Sabouraud's dextrose agar. The hyphae contain rectangular arthrospores, which float in the air. It should be grown only in sophisticated laboratories because there is danger of air transmission.

Virus

The virus can be cultured in diagnostic laboratories, but it requires trained and specialized personnel. The culture of virus can be done in embryonated chicken eggs or in cell culture systems.

Chicken Eggs

The clinical samples (feces/ tissues/secretion/excretion) are collected in sterile containers. These are processed in order to prepare bacteria free filtrate for inoculation in chicken eggs or cell culture. For this, prepare 1:5 or 1:10 suspension of clinical samples in HBSS, which is centrifuged at 10000X for 30 min at 4°C. The supernatant is collected and filtered through EK grade Seitz filter. If the material is less in quantity (5-10 ml), it can be filtered through nitrocellulose membrane filter having 0.22 micrometer pore size. The filtrate is stored at -20°C till use. It can be inoculated (0.1ml) in embryonated chicken eggs through following routes:

- ✓ Chorioallantoic membrane (CAM)
- ✓ yolk
- ✓ Allantoic cavity

The eggs are inoculated under aseptic conditions preferably in laminar flow or sterile hoods. The inoculated eggs are incubated for 2-5 days at 37°C in incubator. During incubation, the eggs are seen daily through candling and dead embryos (within 24 hrs) are discarded. The growth of virus on CAM is observed by appearance of tuckering, congestion, hemorrhage, pock lesions, and necrosis. The allantoic or yolk sac fluid is examined for the presence of virus by using indirect tests like HA or HI. The growth of embryos and lesions on various organs of embryo are also observed.

Cell culture

The cell cultures are primary or continuous cell lines. The primary cell culture can be prepared from chicken fibroblasts, calf kidneys, calf testis, lamb kidneys and lamb testis. For this the tissues or organs are collected under sterile conditions in HBSS. These are cut into small pieces with sterile scissors and forceps. The cell suspension is kept on magnetic stirrer with a bar after addition of trypsin which dissociates the cells. After stirring, the cell suspension filtered through gauze or muslin cloth and the cells are washed with HBSS three times and one time with culture medium (Eagles MEM or medium 199) having 10% fetal calf serum.

A 10% cell suspension is prepared and is poured in cell culture bottles. The bottles are incubated at 37°C to form a sheet on flat surface of glass bottle; it is ready within 2-3 days. Check the cell culture for contamination. After completion of sheet, clinical sample can be inoculated on the cell culture. The

presence of virus in cell culture can be detected by appearance of cytopathic effects, including necrosis, desquamation, aggregation, syncytia formation, plaques and presence of inclusions in the cell. The growth of virus is also observed by immunofluorescent-staining, immunoperoxidase staining and cell culture fluid can be examined for the presence of virus by ELISA, DIA or CIEP. Electromicroscopically, one can demonstrate the virus in cells or in cell culture fluid.

Continuous Cell Lines

These are immortal cancerous cells, which grow fast if proper nutrition is provided. Now a days, these cells are available commercially or in some developed institutes which supply them on request free of cost or with some nominal charge. The cells are maintained and cultured in same way as in primary cell culture. The relevant details of media and/or other growth requirements are supplied with the cells which should be followed for their culture.