ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY (PAPA)

A Lecture Notes Prepared By

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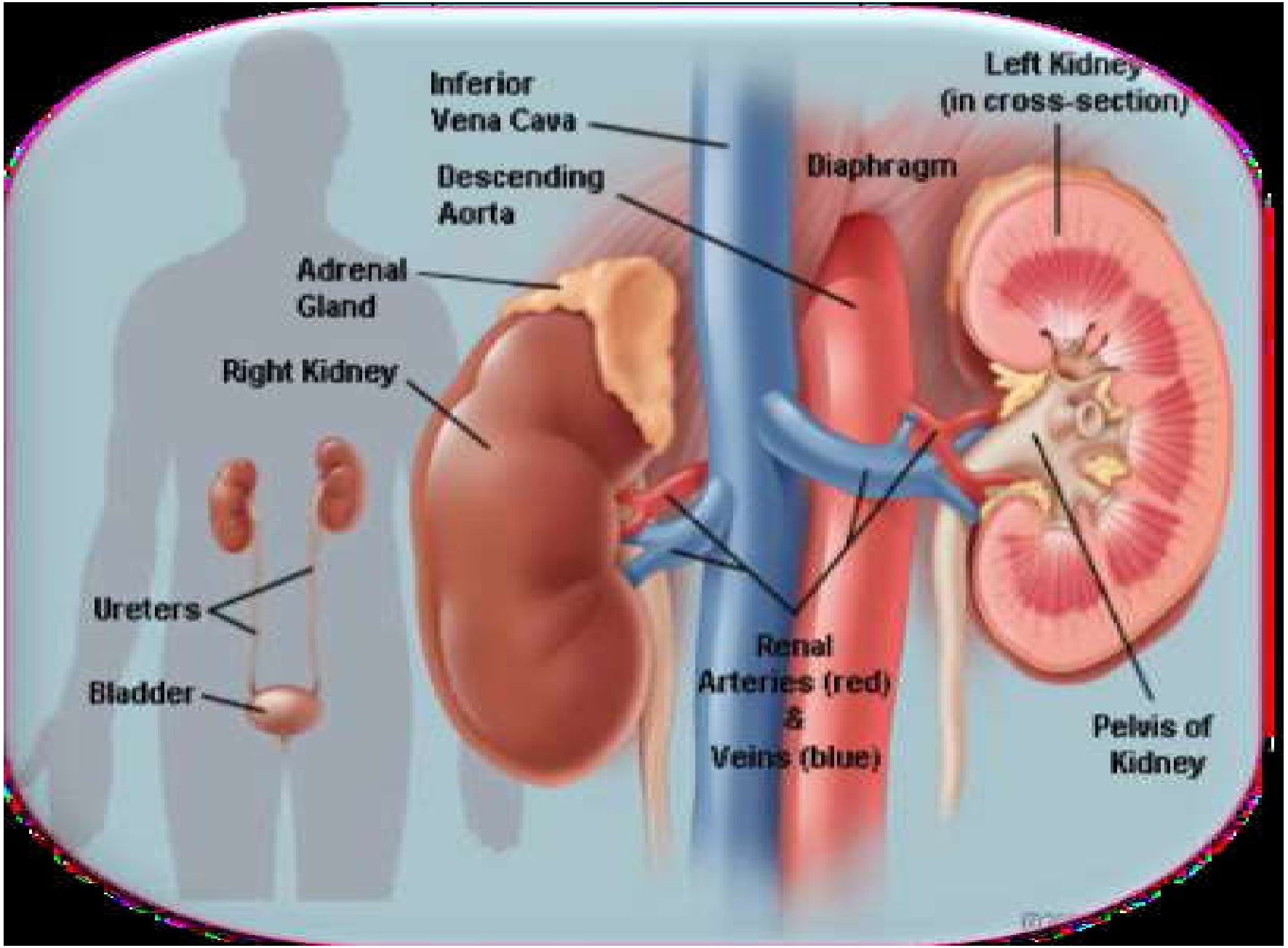
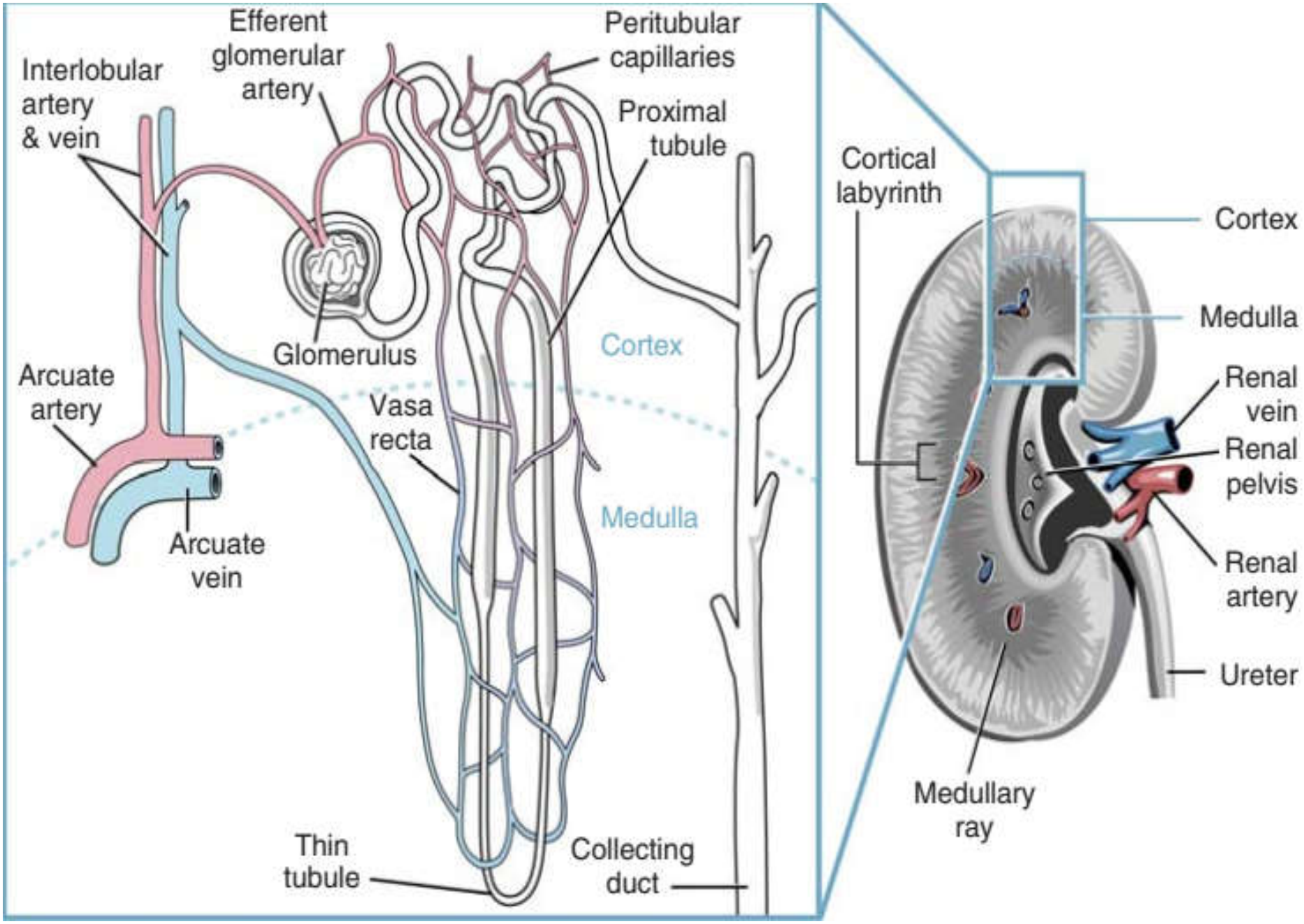
Kidney Function Tests


Inferior
Vena Cava
Descending
Aorta
Adrenal
Gland
Right Kidney
Ureters
Bladder
Left Kidney
(in cross-section)
Diaphragm
Renal
Arteries (red)
&
Veins (blue)
Pelvis of
Kidney


Efferent glomerular artery
Peritubular capillaries
Interlobular artery & vein
Proximal tubule
Cortical labyrinth
Cortex
Medulla
Renal vein
Renal pelvis
Renal artery
Ureter
Glomerulus
Cortex
Arcuate artery
Vasa recta
Medulla
Arcuate vein
Thin tubule
Collecting duct
Medullary ray

Functions of Kidney

Erythropoietin:

- ✓ A peptide hormone, stimulates hemoglobin synthesis and formation of erythrocytes.

1,25-Dihydroxycholecalciferol (calcitriol):

- ✓ The active form of vitamin D is finally produced in the kidney.
- ✓ It regulates calcium absorption from the gut.

Renin:

- ✓ A proteolytic enzyme liberated by kidney, stimulates the formation of angiotensin II which, in turn, leads to aldosterone production.
- ✓ Angiotensin II & aldosterone are the hormones involved in the regulation of electrolyte balance.

INDICATORS OF RENAL FUNCTION

- Urea nitrogen
- Physiology
- Dietary proteins are hydrolysed in the intestines to their constituent amino acids which may, in turn, be degraded to ammonia by the action of gut bacteria.
- The ammonia and amino acids are transported to the liver via the portal circulation where they are utilized in the urea cycle.
- The urea formed in the hepatocytes is excreted via the kidney tubules.
- Urea plays an important role in concentrating the urine the presence of high concentrations of urea and sodium chloride in the renal medullary interstitium creates an osmotic gradient for reabsorption of water.

INDICATORS OF RENAL FUNCTION

- Indications for assay
- The urea nitrogen (urea) concentration is one of the tests used when screening renal function. It is often measured when the clinical signs include vomiting, anorexia, weight loss, polydipsia and dehydration.
- **Analysis**
- Urea can be measured in serum, plasma and urine by spectrophotometry.
- **Reference ranges**
- Dogs 3.0-9.0 mmol/L
- Cats 5.0-10.0 mmol/L
- Interfering phenomena
- lipemia interferes with the analysis and produces variable effects depending on the methodology.

INDICATORS OF RENAL FUNCTION

Causes of reduced blood urea

- Reduced dietary protein intake is associated with a low blood urea.
- In addition, patients with diffuse liver disease have an impaired capacity to synthesize urea and reduced hepatic production.
- Where hepatic disease is suspected, a complete biochemistry profile and a bile acid stimulation test are indicated.
- The marked diuresis associated with some conditions, especially hyperadrenocorticism and diabetes , results in increased urinary loss of urea which, in turn, causes a reduction of the blood urea.

INDICATORS OF RENAL FUNCTION

- **Causes of increased blood urea**

- Increased dietary protein intake produces a high level of urea in the blood. A moderate increase in dietary protein is not commonly associated with a notable rise in urea above the reference range, but high-protein diets can cause significant increases.
- A 12-hour fast is recommended before sampling for measurement of urea.
- Intestinal haemorrhage also results in an increased concentration which is reported to correlate with the severity of blood loss.

Cont…

- Urea is freely filtered at the glomerulus and reabsorbed in the renal tubules.
- The rate of reabsorption is higher at slower urinary flow rates, e.g. in dehydrated patients.
- Blood urea is therefore not a reliable estimate of the glomerular filtration rate (GFR).
- Increased urea concentrations are associated with conditions other than parenchymal renal disease.
- The presence of a concentrated urine sample (urine SG gt 1.030 in dogs, gt 1.035 in cats) supports the diagnosis of a prerenal azotaemia.

Urine Formation

- Nephron is the functional unit of kidney.
- Each kidney is composed of approximately one million nephrons.
- Nephron, consists of a Bowman's capsule (with blood capillaries), PCT, loop of Henle, DCT & collecting tubule.
- The blood supply to kidneys is relatively large.
- About 1200 ml of blood (650 ml plasma) passes through the kidneys, every minute.
- About 120-125 ml is filtered per minute by the kidneys & this is referred to as **glomerular filtration rate** (GFR).
- With a normal GFR (120-125 ml/min), the glomerular filtrate formed in an adult is about 175-180 litres/day, out of which only 1.5 litres is excreted as urine.
- More than 99% of the glomerular filtrate is reabsorbed by the kidneys.
- Urine formation basically involves two steps **glomerular filtration & tubular reabsorption.**

Urine Production

- Urine production is the combination of glomerular filtration, tubular reabsorption, and tubular secretion.

A. Glomerular filtration

- • This process is the filtration of blood from the glomerular capillaries into Bowman's capsule.
- The filtrate consists of a large amount of fluid, free of protein.
- Substrates other than protein are freely filtered.
- The glomerular filter consists of endothelium from the capillaries, a basement membrane, and epithelial cells called podocytes.
- All three of these layers have a negative charge which helps repel protein.

Up..Cont…

B. Tubular reabsorption

- • Transport of material across the tubular epithelial membranes in the renal interstitial fluid occurs through the peritubular capillary membrane back into the blood and can be active or passive.
- Active transport requires energy derived from metabolism of adenosine triphosphate (ATP) or an ion gradient.
- Passive transport occurs by osmosis or electrical potential.
- The passive reabsorption of water is coupled primarily to sodium reabsorption.
- As sodium is transported out of the tubule, their concentration will decrease inside the tubule while increasing outside the cell.
- This creates a concentration gradient so water can then follow sodium.

Up..Cont…

C. Tubular secretion

- Tubular secretion is the active release of substances by the tubular epithelial cells into the lumen of the nephron.

- This is useful to maintain electrolyte balances, primarily in the secretion of potassium, and the maintenance of acid–base balance with secretion of hydrogen ion.

- **Urine** = net effect of ***glomerular ultrafiltration of plasma*** and renal tubular **excretion** and **reabsorption** (into and out of the glomerular filtrate)

Glomerular hydrostatic pressure and GFR

- GFR is the rate at which the glomerulus forms the ultrafiltrate of plasma within Bowman's space.
- Is a passive process that is driven primarily by glomerular hydrostatic pressure.

- The glomerular hydrostatic pressure **directly reflects** the hydraulic pressure generated by:-
  - Cardiac output
  - Local renal vascular tone
- Therefore, extra-renal factors that alter renal perfusion can affect :
  - GFR
  - Elimination of nitrogenous wastes from the body

Histological structure of Glomerulus & it's effect on glomerular functions

Histology ?

- The properly functioning glomerulus → exclude molecules from ultrafiltrate ←size, shape, and charge.
- Smaller molecules freely →filtrate. ← modified by renal tubular **re-absorption** or **secretion**.

Participation of renal tubules in urine and body water balance

❖ The renal tubules function ←multiple hormone (**ADH, Aldosteron**) → alter GFR in a way that maintains homeostasis

How ?

❖ The renal tubules modify the glomerular filtrate either by retention of water, which results in a concentrated urine, or by water excretion, which results in a dilute urine

Renal threshold

- *Renal threshold* = maximal concentration of a given substance that the renal tubules can reabsorb

- **The substance in the urine** → its concentration in the plasma, and in the glomerular filtrate > threshold (reabsorptive capacity).

- Or if **filtration unit damage** → substance in urine

- There are certain substances in the blood whose excretion in urine is dependent on their concentration.

  - Such substances are referred to as renal threshold substances.
  - At the normal concentration in the blood, they are completely reabsorbed by the kidneys.

Renal threshold

- The renal threshold of a substance is defined as its concentration in blood (or plasma) beyond which it is excreted into urine.
- The renal threshold for glucose is 180 mg/dl; ketone bodies 3 mg/dl; calcium 10 mg/dl bicarbonate 30 mEq/l.
- The renal threshold varies considerably with species.
- In dogs, it is 180 mg/dL, cats have a much higher threshold at 280 mg/dL and cattle are much lower, *<100 mg/dL.*
- **Tubular maximum (Tm):** The maximum capacity of the kidneys to absorb a particular substance.
- Tubular maximum for glucose is 350 mg/min.

Kidney Function Tests

Kidney function tests may be divided into 4 groups.

I) Glomerular function tests:

- All the clearance tests (inulin, creatinine, urea) are included in this group.

II) Tubular function tests:

- Urine concentration or dilution test, urine acidification test.

III) Analysis of blood/serum:

- Estimation of blood urea, serum creatinine, protein & electrolyte are useful to assess renal function.

IV) Urine examination:

- Routine examination of urine - volume, pH, specific gravity, osmolality & presence of certain abnormal constituents (proteins, blood, ketone bodies, glucose etc).

Urea and creatinine and their significance to kidney disease assessment

❖ Urea and creatinine → serum biochemical **marker** of nitrogenous waste retention by kidneys

Urea

❖ Urea represents the endpoint of protein catabolism.

❖ Within **liver**, two molecules of ammonium (NH_4^+) are combined to form *urea* (urea cycle).

- BUN concentration → ≈ rate of protein catabolism
- Diseases or physiologic states that alter the rate of protein catabolism can affect BUN.
  - Consumption of high-protein diet
  - Gastrointestinal (GI) hemorrhage
  - Prolonged strenuous exercise
  - Fever/Acidosis/Hyperadrenocorticism/corticosteroid administration/ Infection

Kinetics of urea once it is presented to the glomerulus

❖ The blood urea concentration is equal to that contained within the initial glomerular filtrate

- A. Proximal tubule: urea passively reabsorbed from the filtrate
- B. Descending limb of Henle's loop: urea passively secreted into the filtrate
- C. Thin ascending limb of Henle's loop: urea passively reabsorbed from the filtrate

❖ **During its movement through distal portions** of the nephron [urea] modified → final [urea] within the urine is different than that in the initial glomerular filtrate

Rate of renal tubular fluid flow and BUN

- Passive diffusion **depends on the fluid flow rate through the renal tubules.**
- A more rapid flow rate →less time for passive →more excretion of urea in the urine

Clearance Test

- Clearance is defined as the volume of plasma that would be completely cleared of a substance per minute.
- In other words, clearance of a substance refers to the milliliters of plasma which contains the amount of that substance excreted per minute.

$$C = \frac{U \times V}{P}$$

Where;

- U = Concentration of the substance in urine.
- V = Volume of urine in ml excreted per minute.
- P = Concentration of the substance in plasma.

- The maximum rate at which the plasma can be cleared of any substance is equal to the GFR.
- This can be calculated by measuring the clearance of a plasma compound which is freely filtered by the glomerulus & is neither absorbed nor secreted in the tubule.
- Inulin (a plant carbohydrate, composed of fructose units) and 51Cr-EDTA satisfy this criteria.
- Inulin is intravenously administered to measure GFR.

CREATININE CLEARANCE TEST

- Creatinine is an excretory product derived from creatine phosphate.
- The excretion of creatinine is rather constant & is not influenced by body metabolism or dietary factors.
- Creatinine is filtered by the glomeruli & only marginally secreted by the tubules.

- Creatinine clearance may be defined as the volume (ml) of plasma that would be completely cleared of creatinine per minute.

Procedure:

- In the traditional method, creatinine content of a 24 hr urine collection & the plasma concentration in this period are estimated.
- The creatinine clearance (C) can be calculated as follows:

$$C = \frac{U \times V}{P}$$

- **U = Urine concentration of creatinine.**
- **V = Urine output in ml/min (24 hr urine volume divided by 24 x 60)**
- **P = Concentration of creatinine.**

Activity I (may be quiz)

- 40kg dog has a serum Ct of 1.0mg/dl and in 1 hour produces 30ml of urine that has a urine Ct of 200mg/dl. Calculate Creatinine clearance for this animal in mg/min/kg.

Creatinine

Physiology

- Creatinine is formed from creatine in the muscles in an irreversible reaction.
- The quantity of creatinine produced depends upon diet (small contribution) and the muscle mass.
- Disease affecting the muscle mass may affect the daily creatinine production.
- Both urea and creatinine are freely filtered at the renal glomerulus but urea is subject to tubular reabsorption and thus creatinine is said to be a better indicator of GFR.

Analysis

- Creatinine can be measured in serum, plasma or abdominal fluid by spectrophotometric methods.

Reference ranges

- Dogs 20-110 umol/L
- Cats 40-150umol/L

Modified procedure:

- Instead of a 24 hr urine collection, the procedure is modified to collect urine for 1 hr, after giving water.
- The volume of urine is recorded.
- Creatinine contents in plasma & urine are estimated.
- The creatinine clearance can be calculated by using the formula.
- Reference values:

❖ The normal range of creatinine clearance is around 120-145ml/min.

❖ These values are slightly lower in female.

- Serum creatinine normal range in human:

➢ Adult male: 0.7-1.4 mg/dl

➢ Adult female: 0.6-1.3 mg/dl

➢ newborn: 0.5-1.2 mg/d

CREATININE

- Used as ***a serum biochemical marker of nitrogenous waste retention by the kidneys***.
- *Creatinine* ← phosphocreatine (decompose) which is energy storage molecule found in muscle.
- Creatinine is formed by a nonenzymatic, spontaneous, irreversible cyclization of phosphocreatine, which generates creatinine and free inorganic phosphate.

- ***Creatine*** = Methionine + guanidoacetate (**liver**) → Circulation → Muscle
- ***Phosphocreatine*** **=** Creatine +phosphate group (**creatine kinase - in muscle**)
- ***Creatinine*** **=** *decomposition* of phosphocreatine

- ***Creatinine*** →blood stream →**throughout the total body water → passive movement into the glomerular filtrate (kinetics)**

Factors that influence the generation of serum creatinine

- [creatinine] ← depends on precursor, phosphocreatine.
- Phosphocreatine content depends on:-
  - Dietary intake of creatine and phosphocreatine from meat-based feeds
  - The rate of hepatic creatine synthesis
  - The animal's muscle mass.

Muscle mass & [CK]

❖ Muscle mass is directly proportional to serum creatinine

❖ Cachexia →↓serum [CK]

❖ Cachectic animals with **renal failure →↑ [BUN] but a normal [CK]**.

- *Conversely, increased muscle mass due to physical training can lead to mildly increased serum creatinine*

The effect of renal tubules on the concentration of creatinine in the initial glomerular filtrate

- In veterinary species, [CK] in the glomerular filtrate is considered to undergo **no modification** during its passage
- **Exception**:
  - male dogs & goats/ very minimal tubular **excretion** of creatinine occurs/active process in PCT

The effect of renal tubular fluid flow on [CK]

- Rate of renal tubular fluid flow does *not* affect serum creatinine.
- Rate of renal tubular fluid flow does not affect how much creatinine is removed from the serum and excreted by the kidneys.
- *As an active process, tubular excretion of creatinine is also unaffected by the renal tubular fluid flow rate.*

- ***Therefore the renal clearance of creatinine can be used as an estimate of GFR***

BUN & CK as an estimation of glomerular function

- ❖ BUN is a **less reliable** than CK (estimate of renal function)← **diet, gastrointestinal hemorrhage (↑ BUN), and concurrent liver disease (↓ BUN) & renal tubules effect.**
- ❖ In ruminants and gut fermenters (such as horses)
  - BUN metabolize (enteric bacteria) → amino acid biosynthesis.
- In these animals, creatinine is generally a more reliable indicator of glomerular function.

- Elevations in BUN may not occur until 75% of the nephron mass is nonfunctional.
- BUN will then double each time the remaining functional mass is halved.
- In some cases, creatinine clearance can detect as little as a 20% deficit in renal function.

- *Additionally, measurement of urine albumin and creatinine (urine protein/creatinine ratio) is an easy and convenient way to detect glomerular damage*

- **Dehydrated** patients → ↓renal tubular fluid flow →↑ reabsorption of urea and therefore **less excretion of urea in the urine.**
- Reabsorbed urea enters the renal interstitium, where it may remain as a component of the **medullary solute concentration gradient**.
- A portion of the reabsorbed urea may also reenter the general circulation and contribute to the BUN concentration.

EVALUATION OF RENAL FUNCTION

Urine concentration test

- a. **Rationale**: **Dehydration** increases plasma osmolality, which stimulates the release of antidiuretic hormone (ADH) by the pituitary gland.
- ADH acts on the collecting tubular epithelial cells, causing resorption of water and concentration of the urine (**increased urine specific gravity**).

- b. Clinical indications for using the urine concentration test include the following:
  - (1) Polydipsia and polyuria (in animals lacking azotemia, clinical evidence of dehydration, and biochemical evidence of disease).
  - (2) Repeated random urine samples from a nonazotemic animal in which the urine persistently has a low or isosthenuric specific gravity (1.008 to 1.030 in the dog, 1.008 to 1.035 in the cat, and 1.008 to 1.025 in the horse and cow).

c. The urine concentration test is contraindicated in the following conditions:

- (1) Azotemia or uremia. A diagnosis of renal disease is already established if azotemia accompanies dilute urine (postrenal azotemia is still a diagnostic consideration). Prerenal azotemia is associated with concentrated urine.
- (2) Dehydration. Maximal stimulation of ADH release is already in effect; further water deprivation is unnecessary.
- (3) Sever debilitation.
- (4) Evidence of other metabolic diseases that can cause polyuria and polydipsia

Types of concentration tests

- A. abrupt water deprivation test
- (1) The animal is weighed, abruptly deprived of water, and urine specific gravity is monitored.
  - The test is stopped if adequate concentrating ability (i.e., specific gravity greater than 1.030 in the dog, greater than 1.035 in the cat, greater than 1.020 in bird, and greater than 1.025 in the cow and horse) is demonstrated.
  - The test also is terminated if undesirable clinical signs develop or if 5% of body weight is lost (which indicates that adequate stimulus for ADH release and subsequent [urine] has occurred)

- (2) In cattle, **three to four days** of water restriction (large reservoir of water in the rumen)**.**

B) Gradual water deprivation test

- (1) This test is suggested to be of value when polyuria is associated with medullary washout of solute. In this situation, medullary hypertonicity must be re-established before the renal tubules can respond to ADH and the abrupt water deprivation test.
- (2) While monitoring body weight, the animal is progressively deprived of water until it is completely withheld; the guidelines of the abrupt water deprivation test are then followed

c. ADH concentration test

- (1) This test may be used when water deprivation poses a risk to the patient. This concentration test has been used primarily in the dog and evaluates renal response to exogenous ADH.
- The ADH concentration test also may be used after the water deprivation test to diagnose pituitary-associated diabetes insipidus.
- (2) An exogenous source of ADH is given to stimulate water reabsorption and urine concentration.
- (3) Renal concentrating ability appears to be more effective after water deprivation than after injection of ADH.

3. Interpretation of concentration test result

a. A urine specific gravity greater than 1.030 in the dog, greater than 1.035 in the cat, greater than 1.020 in the bird, and greater than 1.025 in the horse and cow indicates adequate concentration of urine

b. Causes of an abnormal concentration test include the following:

(1) Renal disease

- *(a) Approximately t*wo-thirds of the nephrons are nonfunctional before abnormal concentrating ability of the kidney can be demonstrated

- (2) Pituitary diabetes insipidus
  - *(a)* Pituitary disease causes a lack of ADH secretion. The renal tubules are normal but are not stimulated to reabsorb water.
  - *(b)* The specific gravity is usually in the 1.001 to 1.007 range because the kidney can still reabsorb solute.
  - *(c)* Affected animals respond to exogenous ADH by concentrating the urine

Creatinine clearance test

- Creatinine clearance measures the renal capacity to remove nitrogenous wastes from circulation
- This test is commonly used in animals that have suspected renal disease but that ***are not yet azotemic*** (75% functional deficit for renal azotemia)
- Creatinine clearance may also be used to monitor the progression of renal disease and response to therapy

Exogenous creatinine clearance rate

- Clearance of subcutaneously administered, exogenous creatinine, which requires bladder lavage and **two** 20-minute collection periods separated by 60 minutes

Principles:

- The urine creatinine concentration is compared to the *serum concentration over a specific period,* during which the urine volume, serum creatinine, and urine creatinine are measured.

$$UC = \frac{\text{Urine volume (ml) x Urine creatinine (mg/dl)}}{\text{Time (min) x Serum creatinine (mg/dl) x Body weight (kg)}}$$

A. Indications

- 1. To assess GFR in **nonazotemic**, **non-dehydrated** animal that are suspected of having renal disease, usually because they are polyuric
- 2. To obtain a more objective assessment of the degree of impaired GFR in **azotemic animals**, which may be helpful in predicting **prognosis or monitoring response to therapy**

B. Basics of the procedure.

- ❖ Adequate hydration must be established or confirmed.
- ❖ The urinary bladder must be emptied completely.
- ❖ All urine produced during a specific period (20min to 24Hrs) is collected (catheterization & volume is recorded (it is critical that all urine produced should be collect with no spillage & minimal evaporation)
- ❖ All blood samples for serum [Crt] is collected during the urine collection period
- ❖ The urine is mixed well and urine [Crt] is measured

Endogenous Creatinine clearance rate

- The creatinine clearance test may be performed by measuring the clearance of endogenous creatinine, which requires a **24-hour urine collection period**

- Procedure is the same as the exogenous Crt procedure

- **Advantage of exogenous Crt:** there is increased plasma Crt in exogenous and therefore increase challenge to the kidney, so it is a better assessment of GFR

Causes of low serum creatinine

- Since the daily production of creatinine is dependent upon the muscle mass of the animal, the body condition should be considered when interpreting serum creatinine concentrations.
- A poor body condition may be associated with low concentrations while minor rises in such cases may be more significant than in other individuals.

Causes of increased serum creatinine

- Decreased glomerular filtration is the major cause of raised serum creatinine.
- However, approximately 75% of nephron function must be impaired before serum creatinine (and urea) is increased.
- Creatinine is considered a more reliable indicator of GFR than is urea nitrogen, since there are fewer factors which influence the serum concentration of creatinine.

Urinalysis

Ways of urine collection & influence on results

- Urine can be collected by:-
  - Cystocentesis
  - Transurethral catheterization
  - Free catch of voided sample(manual compression of urinary bladder)

A. Voided specimens

- 1. Contamination of urine from the lower urinary tract and reproductive tract may occur in mammals. Common contaminants include bacteria, leukocytes, spermatozoa, and epithelial cells.
- 2. Midstream ("clean catch") collection of urine may reduce contamination. This is the preferred method of urine collection in large animals.
- 3. Birds lack a urinary bladder. Urine is discharged through the cloaca, a common opening for the urinary, digestive, and reproductive tracts of birds.
  - a. Contamination of avian urine is common and usually obscures evaluation of its physical, chemical, and microscopic characteristics.
  - b. Urine contains a solid component of **urate**s, a pasty white to yellow substance. Urates may impede detailed microscopic examination of avian urine. Several drops of sodium hydroxide may be added to a wet mount of avian urine to dissolve urates; however, this basic solution also may dissolve casts

B. Catheterized specimens

- 1. Care must be taken to clean the urethral orifice and surrounding area to reduce contamination of the urine specimens and prevent the introduction of pathogens into the urinary tract.
- 2. Traumatic catheterization may dislodge transitional epithelial cells from the urethra and cause iatrogenic hemorrhage.

C. Cystocentesis specimens

- Cystocentesis is the preferred method of urine collection in small mammals such as dogs and cats.
- Urine obtained by *cystocentesis* is free of bacteria and may contain low numbers (<5 per high-power field) of epithelial cells.
- iatrogenic **microscopic hematuria** is frequently induced by cystocentesis
- Cannot be distinguished from disease-induced hematuria.

- The collection method also influences the results of urine culture and sensitivity
- Ideally, urine culture and sensitivity are performed on samples collected by cystocentesis. However, this is not always possible.
- When necessary, quantitative urine culture and sensitivity can be performed on samples collected by catheterization or free catch.

Optimal method of urine sample handling

- A. The urine specimen should be collected prior to medical therapy or contrast radiography, if possible.
- B. Collection vessels should be clean, detergent and reagent free, and sterile if microbiological culture is indicated.
- C. Ideally, the urinalysis should be performed **within 30** minutes of specimen collection.
- 1. The urine specimen may be refrigerated, but **not frozen**, up to 12 hours if urinalysis is delayed.
- 2. The urine specimen should be re-warmed to room temperature before analysis.
  - a. Precipitates that form at lower temperatures will redissolve.
  - b. Inhibition of enzymatic activities by low temperatures will be avoided

❖ Urine should be collected in

- an opaque
- airtight container

❖ Avian urine

- The liquid component of “urine” should be collected from an impervious surface as soon as possible after excretion.

- The **container** in which the urine is placed can also affect UA results. Ideally, urine should be collected in:-
  - Sterile container
  - Airtight container (evaporation of volatile substances, such as the ketone, acetone, and to prevent loss of carbon dioxide, which raises the pH)
  - Opaque container (photo degradation of light-sensitive analytes such as **bilirubin**)

Artifacts induced by storing urine for prolonged period at room temperature

- Prolonged storage at room temperature may cause:-
  - Increase in pH (escape of carbon dioxide)
  - *In vitro* bacterial overgrowth
- The bacterial overgrowth affect:
  - Falsely increase the turbidity of the sample
  - Glucose in vitro reduction of analyte (bacteria consume glucose)
  - In vitro change in pH (acidic pH / catabolism of glucose /alkaline-urease)
- Alkalinization of urine may cause:
  - False-positive dipstick protein reaction
  - lysis of cells, and degeneration of casts
  - alter the type and amount of crystals present.

Effect of Storage on urine:

1. **Increase in pH:** Due to production of **ammonia from urea** by urease producing bacteria.
2. **Formation of crystals:** Precipitation of **phosphate and calcium.**
3. **Loss of ketone bodies**: since they are **volatile.**
4. **Decrease in glucose:** Due to glycolysis and **utilization of glucose** by cells and bacteria.
5. **Oxidation of bilirubin to biliverdin: false-negative** test for bilirubin
6. **Oxidation of urobilinogen to urobilin: false-negative** test for urobilinogen.
7. **Bacterial proliferation**
8. **Disintegration of cellular elements** (Especially in alkaline and hypotonic urine.

PHYSICAL AND CHEMICAL ASPECTS OF URINALYSIS

- Urinalysis (UA) is performed in healthy patients to screen for:
  - **occult** (undetected) disease.
  - is an essential component in the **diagnostic evaluation of a diseased patient**.
  - can also be used **to monitor**
    - **disease progression**
    - **response to therapy**
    - **safety of potentially nephrotoxic drugs**.

- A complete UA includes:-
  - Gross inspection of the urine
  - Specific chemical testing
  - Microscopic examination of sediment and cultures for microbe

- ***Performance of a complete UA allows effective interpretation of data obtained from measurement of serum biochemical markers of renal function and of the individual components of the UA.***

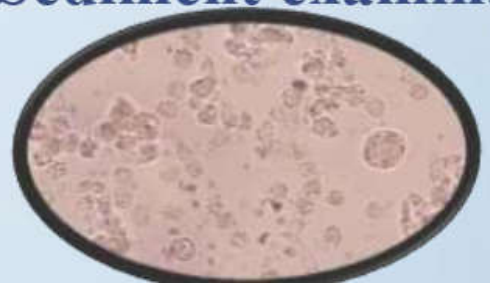
The basic (routine) urinalysis consists of four parts:

1. **Specimen evaluation**

2. **Physical examination**

3. **Chemical examination**

4. **Microscopy of urine sediment/ Sediment examination**



PHYSICAL EXAMINATION OF URINE

Urine color (pigments)

- ***A. Physiologic processes***
  - Normal yellow to amber color (due to urochrome /poorly defined urine pigments. eg. riboflavin)
  - Pale yellow urine is usually less concentrate than dark yellow urine
- ***B. Abnormal colors***
  - **Red urine**: erythrocytes, hemoglobin and myoglobin
  - **Red-brown**: erythrocytes, hemoglobin, myoglobin or methemoglobin
  - **Brown to black**: methemoglobin
  - **Yellow-orange**: bilirubin
  - **Yellow-green**: bilirubin and biliverdin

Urine clarity

- Physiologically clear urine is expected, but may have **mild turbidity** (epithelial cells & crystals)
- Equine urine is turbid or cloudy (mucoproteins/kidney and calcium carbonate crystals)
- **Cloudy urine-** common causes of cloudy urine
- Urine is made cloudy by the presence of particulate matter that **scatters light**, such as cells, crystals, lipid, mucus, and bacteria

Odor

- An odor of ammonia if urea is being converted to ammonia (bacterial)
- Ketone bodies, characteristic sweetish, fruity odor (pregnancy diseases, acetonemia, and diabetes mellitus)

Foam. When shaken after collection, normal urine produce a white foam (limited).

- If there is proteinuria, the amount of foam produced is in excess and slow to disappear.
- If bile or bile pigments are present, the foam may be green, yellow or yellow brown
- If hemoglobin is present, the foam is red to brown

Urine specific gravity

- *USG (also called relative density)* is the **ratio** of the density(weight) of urine to the density(weight) of equal volume of distilled water
- Urine is denser than pure water because it contains excreted solutes (> 1.000).
- Specific gravity is affected by:
  - The number of solute particles in solution
  - Molecular weight of those solutes
- It is therefore a way to estimate a solution's osmolality, which is affected only by the number of solute particles in solution.

- To determine **the clinical significance** of a given USG measurement, the value must be **interpreted in light** of :
    - The patient's hydration status
    - Blood urea nitrogen (BUN)
    - Serum creatinine.
- The measurement of true urine SG is an absolute procedure & SG is a unitless ratio.

Refractive index as an estimation of USG (USG_{ref}) *indirect*

- Measured with a refractometer and used as a routine clinical examination of USG.
- Refractive index = the ratio of the speed of light in a **vacuum** to the speed of light in the solution.
- As solute is added to water, the degree to which the light refracted increases proportionately to the increase in solute concentration
    - **UG also increase proportionately to the solute concentration, so UG correlates with refractive index if the types & proportion of solute remain similar**

Note: Refractometers measure the refractive index of the soluble solids in the fluid

- ***Suspended particles (cells, casts, and most crystals) do not refract light and so do not alter the refractive index of urine***
  - 1.025 to 1.030 in horses and bovines
  - 1.030 to 1.035 in dogs
  - 1.035 to 1.045 in cats **suggests that the kidneys likely have adequate capacity to concentrate the urine.**
- USG much lower than this may be seen in randomly collected samples, depending on the patient's **hydration status**.
  - **The concentrating function of the kidneys must be compromised by at least two thirds before an abnormal result may be detected.**

Reagent strip for estimating USG

- **Principle**: the ionic strength of urine is related to total solute concentration.
- The reagent system has indicators that produce different colors when dipped in urine samples **of different ionic strength.**

Isosthenuria, hyposthenuria, and hyperosthenuria.

- Usg is classified relative to the Sg of **glomerular filtrate**.
- The specific gravity of glomerular filtrate ranges from 1.008 to 1.012.
  - USg within this range is classified as *isosthenuria*
  - USg less than this range is referred to as *hyposthenuria*
  - USg that is greater than that of the glomerular filtrate is classified as *hyperosthenuria*.
- However, hyperosthenuria does not necessarily indicate adequate concentrating function by the kidneys.
- The patient's signalment and **hydration status** and the degree of hyperosthenuria need to be considered when making this determination

Urine specific gravity & localization of azotemia

Azotemia can result from the following:

- ***Prerenal causes***:
  - decreased delivery of nitrogenous waste to the kidneys due to renal hypoperfusion
  - increased generation of nitrogenous wastes
- ***Intrarenal causes***:
  - decreased removal of nitrogenous wastes from circulation due to primary renal dysfunction

- ***Postrenal causes***:
  - decreased elimination of nitrogenous wastes due to urethral obstruction or bladder rupture
- Any *combination* of prerenal, intrarenal, or postrenal causes
- USg can be a useful tool in helping to localize the cause of azotemia.

- With *prerenal azotemia,* adequately functioning kidneys respond to decreased perfusion by producing a **concentrated urine** with Usg:
  - >1.035 in dogs,
  - >1.045 in cats, and 1.030 in cattle and horses.
- With *renal azotemia* usg is usually
  - between 1.008 and 1.029 in dogs and cattle and between 1.008 and 1.035 in cats.

- Therefore, renal azotemia is often found concurrently with inadequately concentrated urine.
- The Usg associated with *postrenal azotemia* is variable.
  - Other clinical findings, such as oliguria or anuria with a firm, possibly distended bladder, can be used to distinguish this cause of azotemia.

Chemical examination of urine

Urine pH

- **Reagent strip**
- **Principle:** based on the double indicator system that is sensitive to change in $[H^+]$ but not measure bounded $[H^+]$ in $NH4^+$, $H2P04^-$ & H_20)
- The reagent strip has pH indicator pad with range of 5.0-8.5.(reported to the nearest 0.5)

- Healthy urine pH in dogs & cats ranges from 6.0-7.5, and horse and cow ranges from 7.5-8.5
- Urine pH is variable and reflects the animal's
  - Diet (vegetable → alkaline, and meat → acidic urine)
  - The timing of urine collection relative to eating
  - The patient's acid-base status, which can be affected by various disease states

Diseases associated with alkaline urine include:

- Urinary tract infection by urease-producing bacteria (primarily *Staphylococcus* and *Proteus* spp.)
- Metabolic or respiratory alkalosis
- Vomiting

- ***In vitro conditions***
  - overgrowth of urease-producing bacteria
  - loss of carbon dioxide (prolonged storage before analysis)
  - exposure to detergent residues (storage container)

Conditions that causes urine to be acidic (aciduria)

- Meat-based diets tend to result in acidic urine.
- Many diseases such as severe diarrhea, diabetic & ketoacidosis
- Renal failure
- Severe vomiting, and protein catabolism

- Administration of certain drugs, such as furosemide and methionine, can also acidify urine.
- **In vitro conditions**
  - overgrowth of bacteria that metabolize glucose and create acidic metabolic byproducts.

Urine glucose (glucosuria)

Physiologic process

- Glucose (small molecule) → enters the ultrafiltrate.
- Glucose is resorbed (PCT via a Na^+-glucose cotransport/ to peritubular fluid)

Analytical concepts

- 1. ***Reagent strip method***
- Principle: conversion of glucose to gluconic acid is catalyzed by glucose oxidase with liberation of H202, which reacts with an indicator to give a color change in the reagent pad

- The degree of color change is proportional to the concentration of glucose

Causes of false results in glucose measurement by the dipstick method

❖ The presence of pigmenturia and certain drugs, may interfere with interpretation of the dipstick test

❖ False-negative results may occur, presence of **ascorbic acid or the ketone concentration being greater than 40 mg/dl**.

- During the glucose dipstick test reaction, the presence of glucose results in the generation of hydrogen peroxide.
- This reaction causes oxidation of an indicator dye, which results in a visible color change.
- False-positive results may occur when:
  - **hydrogen peroxide**
  - **bleach, chlorine**
  - **other oxidizing substances have contaminated** the reaction.
- Proper storage and handling of urine samples and dipsticks decrease the risk of contamination

2. Copper-reducing method

- Principle: Cu^{2+} reacts with a reducing substance (glucose, fructose, lactose, maltose) to produce cuproud (Cu^{+}) oxide and cuprous hydroxide and these a color change

- The method is probably more accurate than strip method but requires a greater concentration for detection

Glucosuria (glycosuria) disorders

- **1.Hyperglycemic glucosuria**
  - transient or persistent hyperglycemia results in more glucose in the ultrafiltrate than can be resorped by proximal tubules
- **Causes of hyperglycemic glucosuria**
  - Diabetes mellitus
  - Hyperadrenocorticism
  - Acute pancreatitis

2. Renal glucosuria (normoglycemic glucosuria)

- ✓ transient or persistent
- ✓ caused by damaged or abnormal proximal tubules

Causes of normoglycemic glucosuria

- Primary renal glucosuria(toxicosis or ischemia)
  - e.g., aminoglycoside administration
  - Fanconi's syndrome (congenital)
  - Transient stress

3. Glucose related polyuria

- ❖in the tubular fluid will cause osmotic dieresis (water is "held" by the glucose in the tubular fluid, especially in the proximal tubules)

- ❖cause decreased renal concentrating ability and increased urine volume (polyuria)

Causes of Increased blood Urea

Pre-renal conditions:

- Dehydration: Severe vomiting, intestinal obstruction, diarrhea, diabetic coma, severe burns, fever & severe infections.

Renal diseases:

- Acute glomerulonephritis
- Nephrosis
- Malignant hypertension
- Chronic pyelonephritis

Post-renal causes:

- Stones in the urinary tract
- Enlarged prostate
- Tumors of bladder

Causes of Decreased Blood Urea:

- Urea concentration in serum may be low in late pregnancy, in starvation, in diet grossly deficient in proteins and in hepatic failure.
- Azotemia:
- Increase in the blood levels of NPN (creatinine, urea, uric acid) is referred to as azotemia & is the hallmark of kidney failure.

Protein in urine

Physiologic processes

- Many small proteins (relative molecular mass <68,000kd) can pass via the glomerular filtration barrier.
- In most healthy animals, the proteins are resorbed in the proximal tubules (little to no protein in urine)

Analytical concepts

1. Reagent strip method

- **Principle:** the reagent pad contains a colorimetric pH indicator (tetrabromphenol blue) at acidic pH.

❖ Amino group → bind the dye and change the pad's color

❖ Abnormal urine color(pigmenturia) may interfere with reagent pad color

❖ Readings may be falsely increased in highly buffered alkaline urine (>8.0), in moderately alkaline urine if highly concentrated

2. SSA (Sulfosalicylic acid) turbidity

- **Principle:** proteins are denatured by acids and form a precipitate that is seen as increased solution turbidity.
  - Urine that is hazy to cloudy should be centrifuged prior to SSA turbidity testing
- Results may be expressed on a visual turbidity scale (1+ to 4+) or visually compared against standard solution to interpolate concentration
- There are also spectrophotometric SSA methods that provide more quantitative results

Sensitivity of dipstick protein reaction and SSA

- The protein reaction on the dipstick is more sensitive to albumin than to globulins, hemoglobin, immunoglobulin light chains (Bence Jones proteins), and mucoproteins.
- The color change ←depends on the binding of free amino groups of the proteins to the indicator dye.
- Albumin →most abundant free amino groups →interaction with indicator dye(2x globulin →a similar color change)

- SSA test is able to precipitate most proteins, including Bence Jones proteins, resulting in cloudiness that is approximately proportional to the quantity of protein present.

- False-positive reactions can be seen with the dipstick method when the urine is alkaline or if cleansers have contaminated the urine sample or dipstick (e.g., chlorhexidine, quaternary ammonium disinfectants).

- A false-negative protein dipstick reaction can be seen with a pure Bence Jones proteinuria.
- Highly alkaline urine can have the opposite effect on the sulfosalicylic acid test, causing a false-negative reaction.
- False-positive results can occur with this method if the test is performed using uncentrifuged urine or if exogenous substances are present.

3. Robert's Test

Principle: precipitation of protein by strong acids (con. nitric acid).

- A positive test is indicated by a **white ring** at the zone of contact of acid & urine, which should be read against a dark ground.

Proteinuria

1. Prerenal (overflow, overload, & preglomerular) proteiuria

- A pathologic state increases the plasma concentration of small proteins that pass via glomerular filtration barrier and results in that the amount of filtrate exceed than resorption
  - Eg. Hemoglobinuria, myoglobinuria, postcolostral proteinuria and light-chain proteins

2. **Glomerular proteinuria-**damaged filtration barrier & decreased selective permeability & **larger or negatively charged** plasma protein pass easily.

- The glomeruli of kidney are not permeable to substances with molecular weight more than 69,000 & plasma proteins are absent in normal urine.
- The smaller molecules of albumin pass through damaged glomeruli more readily.
- When glomeruli are damaged or diseased, they become more permeable & plasma proteins may appear in urine.
- **Albuminuria is always pathological.**
- Large quantities of albumin are lost in urine in nephrosis.
- Small quantities are seen in urine in acute nephritis, strenuous exercise & pregnancy.

3. **Tubular proteinuria**-proximal renal tubules defective , so proteins that normally are resorbed from ultrafiltrate (albumin & smaller globulin) are not & excreted in urine

- This occurs when functional nephrons are reduced, GFR is decreased & remaining nephrons are over-working.
- The tubular reabsorption mechanism is impaired, so low molecular weight proteins appear in urine.
- They are Retinol binding protein (RBP) & α-1 microglobulin.

4. Nephron loss Proteinuria

- In CKD, there is a decrease in the number of functioning nephrons.
- The compensatory rise in glomerular filtration by other nephrons increases the filtered load of proteins.
- Even if there are no glomerular permeability changes, tubular proteinuria is seen.

5. Urogenic Proteinuria

- This is due to inflammation of lower urinary tract, when proteins are secreted into the tract.
- Accumulation of proteins in tubular lumen can trigger inflammatory reaction.

6. Hemorrhagic or inflammatory proteinuria

Hemorrhage into GIT(impaired hemostasis, inflammation & blood vessel damge, neoplasm)

Inflammatory: exudation of plasma protein via vessels walls into the genitourinary tract

- **Urine protein/creatinine ratio and indications for glomerular disease**

❖ A small amount of protein is excreted along with other solutes in the urine daily

❖ Therefore, to **qualify the degree of proteinuria** and **determine its significance**:

  - the urine protein concentration can be compared to that of creatinine
  - Crt passes freely through the glomerulus and is not modified by tubular excretion or reabsorption.

- UPC is generally performed when the urine sediment is inactive (no cells or bacteria) during the initial evaluation of proteinuria.

- ***The UPC can be measured serially to stage the progression of disease and to evaluate the response to therapy.***

- The UPC should be less than one.
- Values greater than one raise concern for glomerular disease (glomerulonephritis, glomerulosclerosis, canine amyloidosis), Bence Jones proteinuria, or less often tubular proteinuria.

Ketones in urine and ketonuria

- Listed in decreasing concentration, β-hydroxybutyrate (78%), acetoacetate (20%), and acetone (2%) are ketones excreted in the urine
  - Diminished use of carbohydrates (diabetes mellitus)
  - Increased use or loss of carbohydrates (lactation, pregnancy, renal glucosuria, fever)
  - Severely decreased dietary intake of carbohydrates (high-protein and high-fat diets)

- Ketone bodies are not expected in the urine of healthy mammals that have an **adequate intake of nutrients**
- Ketone bodies may enter the urine by both
  - glomerular filtration of plasma
  - by tubular secretion. The tubular secretion process probably shares a transport process with other organic anions
- After entering the tubular fluid, ***acetoacetate and β-hydroxybutyrate are nonresorbable***

Analytic concepts

1. Reagent strip method

- **Principle:** acetoacetate (mostly) and acetone (10%) formed colored complexes with nitroprusside.
- The amount of color change reflects the amount of ketones present
- The reagent system does not react with **β-hydroxybutyrate**, the ketone body that does not have ketone chemical structure

The relative detection sensitivities for the different ketones by the dipstick reaction

- Beta-hydroxybutyrate, which is the predominant ketone present in urine, is not detected by the ketone dipstick reaction.
- The dipstick reaction is most sensitive to acetoacetate and is mildly sensitive to acetone.

❖ Approximately 96% of the color change associated with a positive reaction is caused by the presence of acetoacetate.

- Interpretation of the color change can be complicated by the presence of pigmenturia.
- Ketones in the sample can be decreased by the presence of:
  - urinary tract infection
  - in vitro bacterial contamination
  - in vitro evaporation of acetone.

Ketonuria

- Ketonuria ←mobilization of lipids (shift of energy production from carbohydrates to lipids)
  - diabetes mellitus, starvation & hypoglycemic disorders.
- Excessive β-oxidation of fatty acids **in hepatocytes** generates more **acetyl-coenzyme A** than can be used for gluconeogenesis and triglyceride synthesis.
- Excess acetyl-coenzyme A → hepatic ketogenesis→ ↑ ketoacids, →**ketonemia**.

- Ketone bodies are easily cleared from blood & are excreted in urine
- Decreased insulin activity & increased glucagon activity promotes ketogenesis
- *Ketones can be monitored in diabetic animals to assist in monitoring the effectiveness of insulin therapy.*
- *Routine checking of the urine of unregulated, clinically ill diabetic animals for the presence of ketones can assist in the diagnosis of diabetic ketoacidosis*.

MICROSCOPIC ASPECTS OF URINALYSIS AND DISCUSSION OF SELECTED DISEASE PROCESSES

- **Urine sediment formation**
- ***Preparation of specimen***
  - Take 5 ml of well-mixed urine sample
  - Centrifuge for 3-5 minutes at 1,000 – 2,000 rpm
  - Decant the supernatant and break up the sediment by shaking
- ***Put a drop on glass slide cover with cover slip or make smear and stain it***
- Use subdued light

The urine sediment may be divided into:

- **Organized elements***
  - Epithelial cells
  - Leukocytes
  - Erythrocytes
  - Casts
  - Bacteria, yeast, fungi, protozoa, parasite ova and sperm
- **Unorganized elements**
  - crystals,
  - pigments and fat droplets.

Clinical significance of crystalluria

- Crystalluria occurs when urine is saturated with crystallogenic substances.
- Various ***in vivo*** factors:
  - urinary tract infection/diet
- ***In vitro*** factors dictate whether crystal formation will occur:
  - duration of sample storage/ storage temperature
  - evaporation of water from the sample
  - urine pH, and the overgrowth of bacterial contaminants that may alter urine pH (e.g., urease-producing organisms).

- Fresh urine samples should be analyzed within 1 hour of collection
- Increased duration of storage time, especially when samples are refrigerated, significantly increases in vitro crystal formation.
- When crystalluria is detected in a refrigerated urine sample
  - (it is prudent to verify the finding by prompt analysis of a freshly obtained sample).

- A small amount of struvite or amorphous phosphate crystalluria can occur in clinically normal animals (dogs and cats).
- Calcium carbonate crystalluria is a common finding in equine, goat, rabbit, and guinea pig urine.

- Detection of crystalluria may be diagnostically useful when abnormal crystal types are identified
  - e.g., **ammonium urate, calcium oxalate monohydrate**
- when large aggregates of struvite or calcium oxalate crystals are found, or when crystalluria is observed in a patient that has confirmed urolithiasis.

- **Crystals that formed in acidic urine.**
  - a. Ammonium urate (ammonium biurate)
  - b. Amorphous urates
  - c. Bilirubin
  - d. Calcium oxalate monohydrate/dihydrate
  - e. Cystine
  - f. Sulfa metabolites
  - g. Uric acid
- **Crystals that formed in neutral pH.**
  - a. Ammonium urate (ammonium biurate)
  - b. Calcium oxalate monohydrate/dihydrate
  - c. Cystine
  - d. Magnesium ammonium phosphate (struvite)
- **Crystals that formed in alkaline urine.**
  - a. Amorphous phosphates
  - b. Calcium carbonate
  - c. Magnesium ammonium phosphate (struvite)

Magnesium ammonium phosphate crystals

- are referred to as *struvite* crystals or "triple phosphate" crystals (a misnomer)

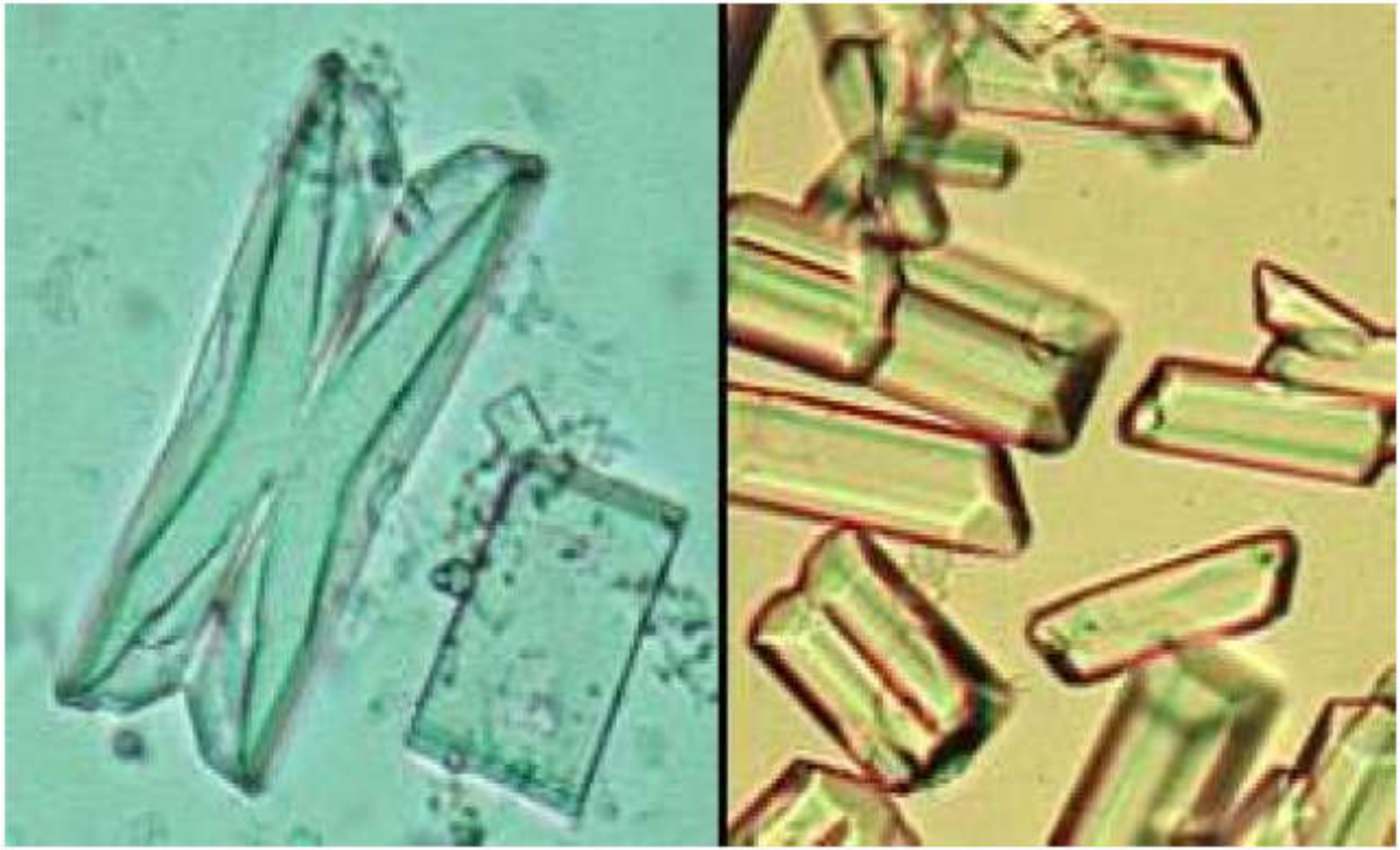
  - These are colorless
  - frequently form variably sized, **coffin lid–shaped crystals**

- However, struvite crystals can have a variable appearance and may occur as **three- to eight-sided prisms, needles, or flat crystals with oblique ends.**

- They form most often in alkaline urine.
- Struvite crystalluria may form in vitro in refrigerated, stored urine samples or in those that become alkaline with storage
- **When struvite crystals are detected in a stored urine sample, the finding should be verified by examination of a freshly obtained urine sample.**

- *Seen frequently in dogs and occasionally in cats.*
- *When found in significant number, struvite crystals are most often associated with bacterial infection (urease-Staphylococcus or Proteus spp).*
- In cats, in the absence of infection, likely due to ammonia excreted by the renal tubules.
- Struvite crystals may be seen in clinically normal animals that have:
  - alkaline urine
  - animals that have sterile or infection-associated uroliths of potentially mixed mineral composition
  - or those with urinary tract disease in the absence of urolithiasis.

Struvite crystals (magnesium ammonium phosphate, triple phosphate)

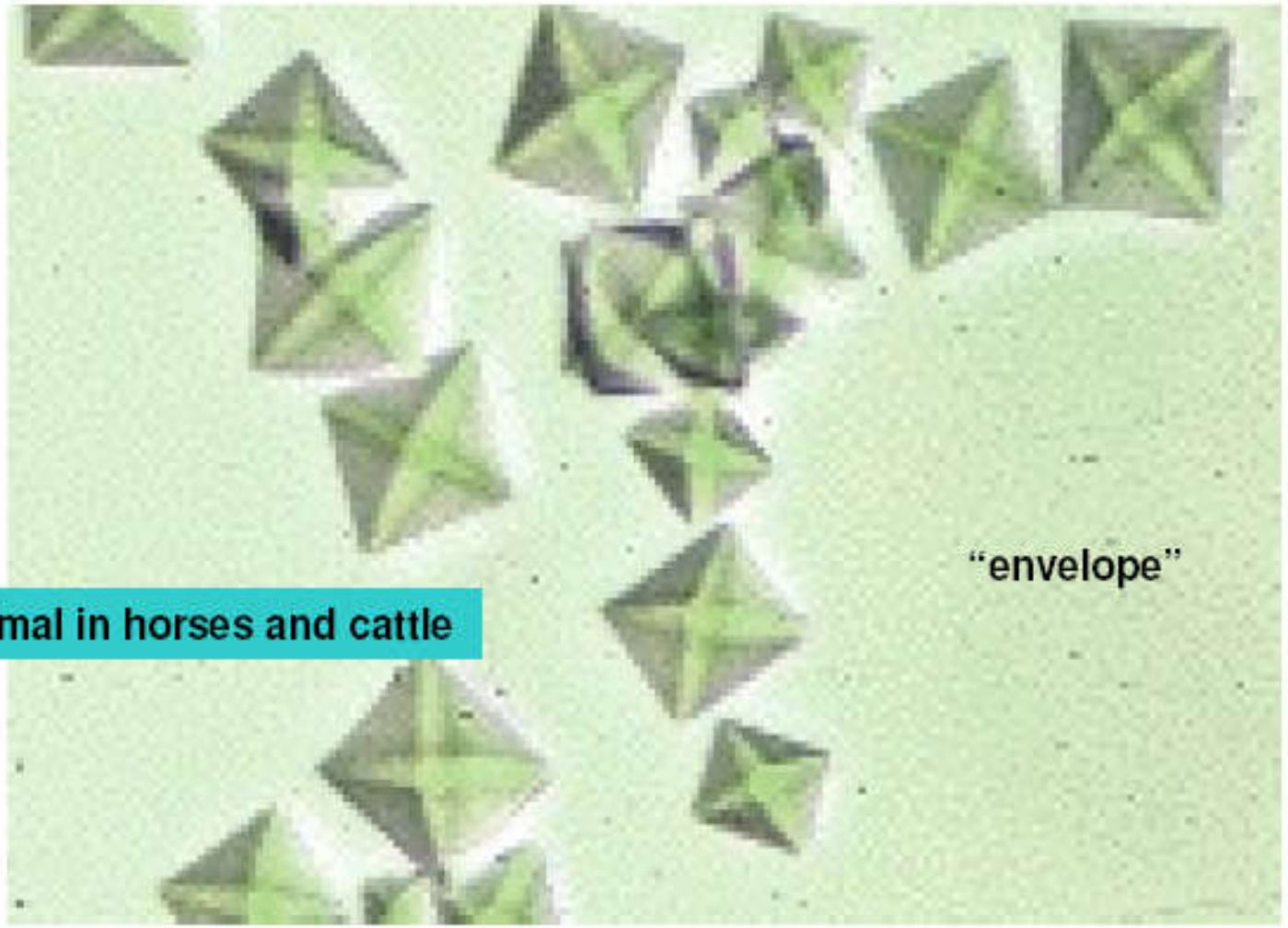


Calcium oxalate dihydrate crystals

- Calcium oxalate dihydrate crystals occur as colorless, variably sized **octahedrons** that resemble an **envelope or a Maltese** cross
- They form most often in acidic urine.
- Prolonged storage, especially with refrigeration, significantly increases the potential of in vitro calcium oxalate formation.

- A sample that becomes acidic during storage also may lead to in vitro calcium oxalate formation
- These crystals may be seen in clinically normal animals, in those:
  - with calcium oxalate urolithiasis
  - with hypercalciuria, (e.g., due to corticosteroids)
  - or with hyperoxaluria, (e.g., ingestion of vegetables high in oxalates, or infrequently with ethylene glycol toxicosis)
- Calcium oxalate dihydrate crystals have been reported with increased frequency in cats as a complication of urine acidification to manage struvite formation.

Calcium oxalate dihydrate crystals

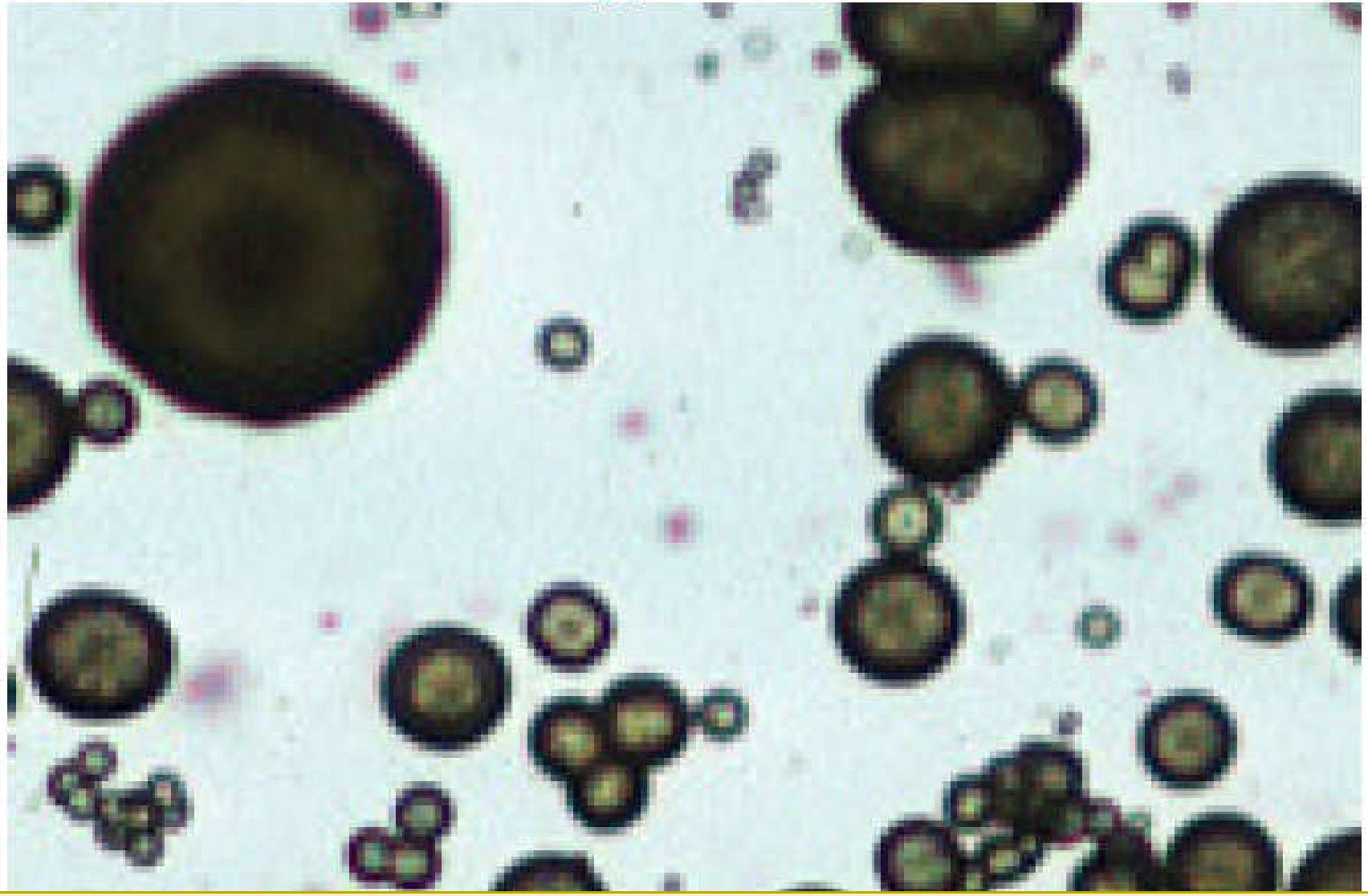


normal in horses and cattle

Calcium carbonate crystals

- ❖ Calcium carbonate crystals occur individually or in clusters and are:
  - variably sized, yellowbrown or colorless
  - radiant spheres or dumbbell shaped
- They usually form in **alkaline urine** and are seen in clinically normal horses, goats, rabbits, and guinea pigs.
- Calcium carbonate crystals have rarely been observed in dogs.

Calcium carbonate crystals: spheroids with radial striations (**cartwheel** appearance)



Casts

- Formed from a combination of **protein and mucopolysaccharide**
- Fairly common in concentrated acid urine, but uncommon in dilute alkaline urine
- indicates a pathological change in the kidney*
- Absent or very few in urine samples from normal animals

Cylinduria and its clinical significance

- *Cylinduria* denotes the presence of renal tubular casts in the urine sediment.
- Tubular casts are **imprints of renal tubules** that form due to the accumulation of a proteinaceous matrix (Tamm- Horsfall mucoprotein) within LH, DCT, CT) of the nephron, → **hyaline casts.**

- The **proteinaceous** matrix can be modified by the addition of **renal tubular cells** that exfoliate or by **leukocytes.**

- Once cells accumulate within a tubular cast, they are thought to undergo degeneration and progress from a **cellular cast→ granular cast→** finally to **waxy cast.**

- <2 per high-power field of hyaline or granular casts can be seen in animals without renal disease.

- when granular casts are present in increased numbers or when cellular casts are found →presence of renal disease (degeneration and necrosis of renal tubular epithelium).

- Renal tubular inflammation is suggested when leukocytes are found within the casts.

- The number of casts present does not reflect the duration, severity, or reversibility of the renal disease.

- Casts are formed on a regular basis and in increased numbers during renal tubular disease.

- When and whether they are dislodged depend on the renal tubular fluid flow rates.

- *Hemoglobin casts (hemolysis) red blood cell casts (renal hemorrhage), bilirubin casts (bilirubinuria).*

Granular versus cellular versus waxy cylinduria and the clinical significance

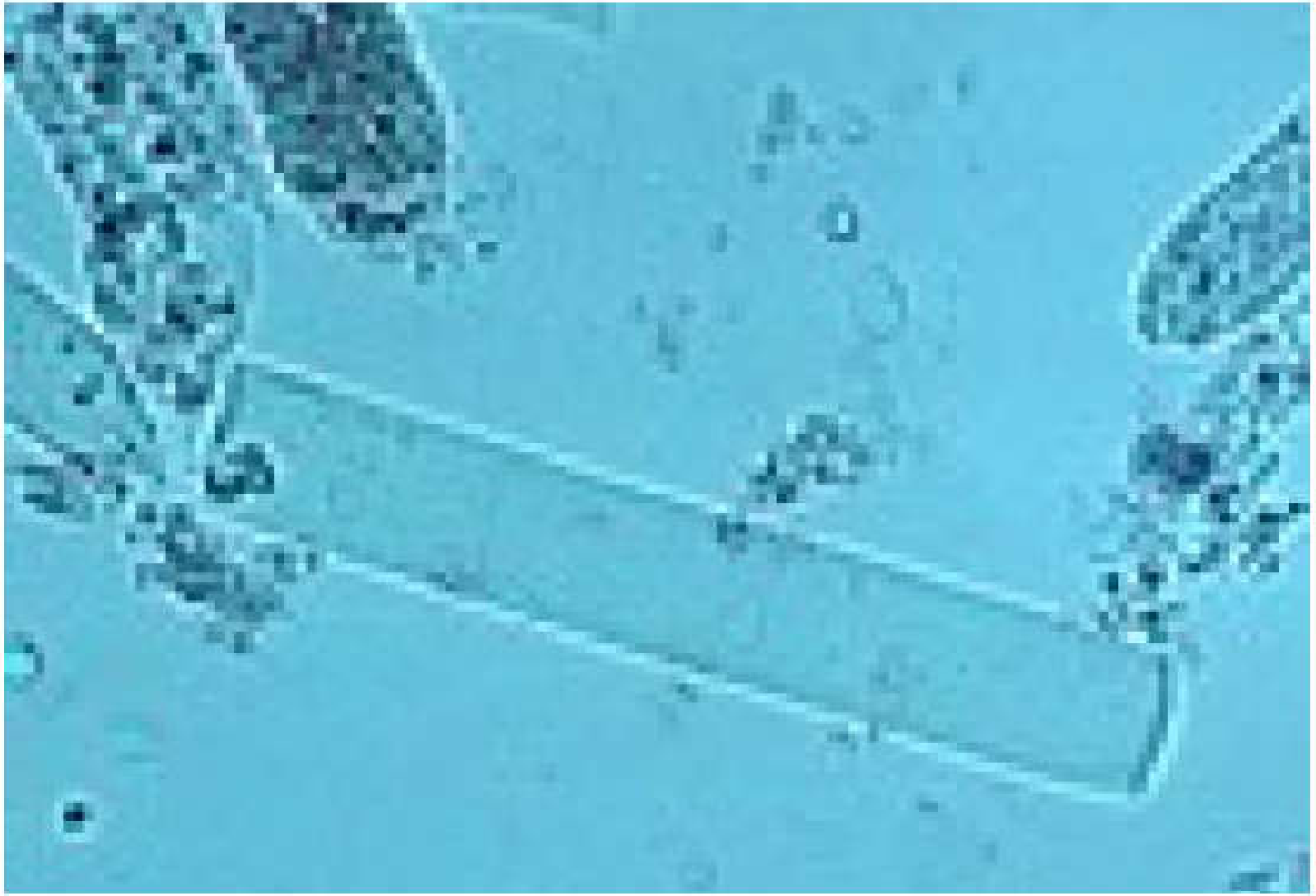
- The type of cast present does not necessarily indicate the severity of the disease and should not typically be used as a prognostic indicator for the response to therapy or the potential for recovery.

- Rather, these types of casts represent **various stages of development.**

- Cells in a cellular cast degenerate, →amorphous granular substance (**granular casts**)→membranes /cellular material → homogeneous, cholesterol containing substance →**waxy cast**.

- Therefore, these stages of cast development more accurately predict the **length of time** the cast has been lodged in the renal tubule, not necessarily the severity of the disease condition.

"Waxy" cast



STAY HOME PROTECT COVID-19

Veterinary Clinical Pathology II (Vetm5212)

DVM 5rd year (18.75%)

BY Debella Taweya (BSC, MSc, Lecturer)

April, 2020

LIVER FUNCTION TESTS

(Tests for evaluation of liver disease)

DVM 5th year

Functions of liver

① **Excretory function:** bile pigments, bile salts and cholesterol are excreted in bile into intestine.

② **Metabolic function:** liver actively participates in carbohydrate, lipid, protein, mineral and vitamin metabolisms.

③ **Hematological function:** liver is also produces clotting factors like factor V, VII. Fibrinogen involved in blood coagulation is also synthesized in liver. It synthesize plasma proteins and destruction of erythrocytes.

Storage functions: glycogen, vitamins A, D and B12,and trace element iron are stored in liver.

⑤ **Protective functions and detoxification:** Ammonia is detoxified to urea. kupffer cells of liver perform phagocytosis to eliminate foreign compounds. Liver is responsible for the metabolism of xenobiotic.

What are the LFTs?

- LFTs include liver enzymes, albumin and other proteins, and bilirubin.

- The liver enzymes are produced by cells within the liver. They include alkaline phosphatase (ALP), ɣ–glutamyl transpedtidase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), but the combination of liver enzyme results you receive depends on your local laboratory.

- The protein components comprise total protein, albumin and globulin [N.B. Total protein = Albumin + Globulins]. The globulins are a mixture of globular proteins such as immunoglobulins, enzymes, carrier proteins and complement.

Indications for liver function tests

1. To assist in the differential diagnosis of ictrus (hemolytic crisis or an intrahepatic or extra hepatic obstruction)

2. In primary liver diseases that are present either with or without ictrus (infectious hepatitis, suppurative hepatitis, hepatic fibrosis, toxic necrosis, neoplasms…etc)

3. In secondary liver diseases (infiltrative and degenerative lipidosis ← diabetes mellitus, pancreatic fibrosis, starvation and hypothyroidism).

4. In the evaluation of prognosis of hepatic diseases and evaluation of therapy

Limitations of liver function tests

1. Extensive damage is required

2. The tests are lacking sensitivity or too sensitive

3. Functions tests do not indicate the functional status of the entire organ

4. Specific hepatic functions are greatly affected by a wide variety of pathologic conditions of extrahepatic origin

Types of LFT

Classified based on the major functions of liver:

① **Excretion:** Measurement of bile pigments, bile salts.

② **Serum enzymes:** Transaminase (ALT, AST), alkaline phosphate(ALP), 5'-nucleotidase, LDH isoenzyme.

③ **Synthetic function:** Prothrombin time, serum albumin.

④ **Metabolic capacity:** Galactose tolerance and antipyrine clearance

⑤ **Detoxification**

Classification of liver function tests

1. Tests dependent primarily on hepatic secretion and excretion
   A. Bile pigments
   B. Clearance of foreign substances

2. Tests dependent upon measurement of serum enzyme activity

3. Tests dependent up on specific biochemical functions
   A. Protein metaboliasm test
   B. Carbohydrate metabolism
   C. Lipid metabolism

1. Excretion : Bile pigments

A. Bilirubin

- Bilirubin is the product of haemoglobin breakdown.
- Lipid-soluble, unconjugated bilirubin is conjugated in the liver, making it water-soluble, and then excreted into bile.
- When a raised bilirubin or clinical jaundice is found we should consider haemolysis (production of unconjugated bilirubin), liver cell function (conjugation and excretion of bilirubin) and biliary tree function (excretion of bile).

- A raised bilirubin level is a strong indicator of underlying pathology and should always be investigated with a careful clinical history and appropriate investigations. A liver ultrasound is usually necessary.

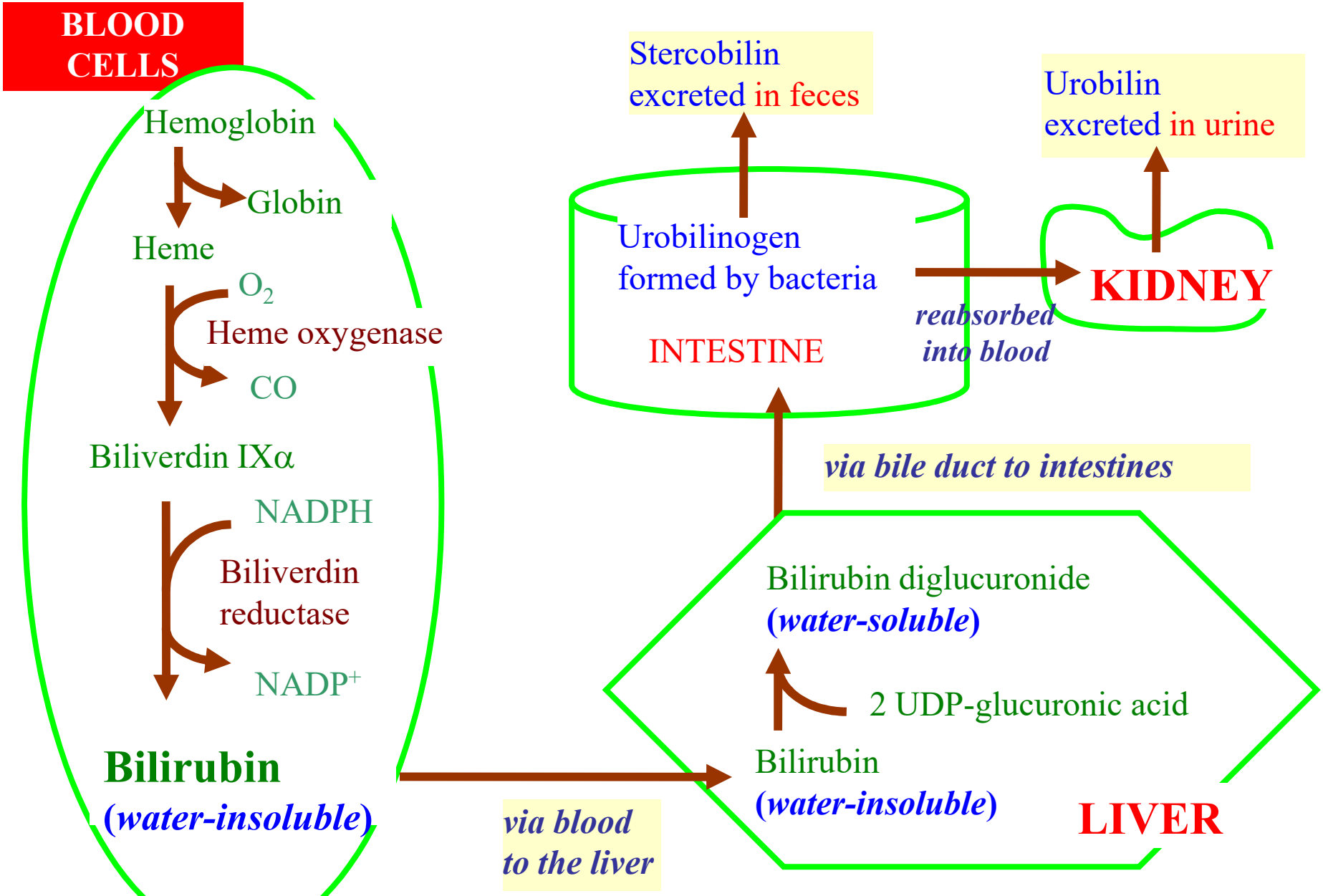


Fig. 2 metabolism of bilirubin

1. serum bilirubin:

- Normally, a small amount of bilirubin circulates in the blood. Serum bilirubin is considered a true test of liver function, as it reflects the liver's ability to take up, process, and secrete bilirubin into the bile.

    1. Indirect **bilirubin**
    2. Direct **bilirubin**
    3. Total **bilirubin**

A. urobilinogen :

Conjugated bilirubin is excreted via bile salts to intestine. Bacteria in the intestine break down bilirubin to urobilinogen for excretion in the feces

B. Urobilin

- Urobilin is the final product of oxidation of urobilinogen by oxygen in air. The amount change with the amount of urobilinogen excretion .

➢C. bilirubinurine:

- Bilirubin is not normally present in urine and faese since bacteria in intestine reduce it to urobilinogen. The kidneys do not filter un conjugated bilirubin because of its avid binding to albumin.
- Conjugated bilirubin can pass through glomerular filter.
- Bilirubin is found in the blood in obstructive jaundice due to various causes and in cholestasis.
- **Note:**
- Bilirubin in the urine may be detected even before clinical jaundice is noted.

- Bilirubin is used to diagnosis of jaundice. Abnormal bilirubin levels can be found in many disorders, including: blocked bile ducts, cirrhosis and hepatitis. Jaundice can be divided into three types

Hemolytic Jaundice

Hepatic Jaundice

Obstructive jaundice (Cholestasis)

Hyperbilirubinemia

- Occurs when the rate of Bu (bilirubin unconjugated) production exceeds the rate of Bu uptake hepatocytes

Bu Production ↑ = Bu uptake by Liver hepatocyte

(Hemolytic jaundice)

- Or the rate of Bc (bilirubin conjugated) formation in hepatocytes exceed the rate of Bc excretion in bile

Bc Production ↑ ⇌ ↓ Bc excretion by Liver

Increased Bu production

1. Hemolytic or prehepatic ictrus-hemolytic disorders

- Immune mediated/ bacteria(Bacillary hemoglobinuria) or virus (FeLV, EIA)/hemangiosarcoma, protozoan parasites…etc

- Pathogenesis → rate of Bu formation exceeds the hepatobiliary systems capacity for Bu uptake or Bc excretion

Other expected laboratory findings with hemolytic ictrus

1. Anemia, regenerative if of sufficient duration
2. Hemoglobinuria/ hemoglobinemia
3. If plasma hepatic enzyme activities are increased, hepatocyte damage or cholestasis could be due to anemic hypoxia

Decreased Bu uptake by hepatocytes

1. Fasting Hyperbilirubinemia

❖ The fasting → lipolysis in adipocytes →↑[fatty acids] in the blood → interfere with Bu uptake by hepatocytes →[Bu]

❖ The interference is competitive because fatty acids and Bu binds to the same cytoplasmic receptor proteins(Z-protein /fatty acid and Y-protein / also known as glutathione S-transferase B)

2. Decreased functional hepatic masses

❖ A marked reduction in functional hepatocytes →↓ Bu uptake →↓ Bu conjugation ↓decreased Bc excretion

❖ **Decreased Bu conjugation**

- A marked reduction in functional mass would reduce Bu conjugation.
- When cytoplasmic recptor proteins are saturated, the uptake of Bu by hepatocytes decreases.

Decreased Bc excretion

- **Obstructive cholestasis** ← obstructs bile flow (bile canaliculi or bile ducts (hepatic or posthepatic))

- Lesions that impair bile flow include
  - hepatocellular swelling that compresses canliculi,
  - periportal lesions that compress bile ducts
  - infections and other process that damage bile ducts
  - blockage of bile ducts by stones, parasites or neoplasms

- A persistently increased plasma [Bc] results in increased urinary excretion of Bc (bilirubinuria)
- 1. Increased [Bc] in systemic and thus sinusoidal blood saturates bilirubin receptrors on hepatocytes, and thus Bu uptake is impaired
- 2. With time, increased [Bc] leads to increased [Bu], but the [Bc] is expected to remain greater than the [Bu]

Other expected laboratory findings

- Increased serum activities of enzymes associated cholestasis (ALP & GGT)
- Bilirubinuria

Icterus index

- Definition: a value that represents an estimation of the yellow discoloration of plasma caused by Hyperbilirubinemia
- Methods ***(analytical measurements)***

1. Potassium dichromate method

- Comparing the color of a patient's plasma to a set of standard solution containing ***potassium dichromate***.
- The plasma of healthy herbivores is expected to be more yellow because carotenoid pigments from plants

2. Icterus index determination by chemistry analyzer

3. Van den Bergh tests & 4. Urobilinogen test

Principle of Van den Bergh tests

- Bilirubin from obstructive icterus (conjugated) reacts with Ehrlich's diazoreagent while serum with hemolytic jaundce (un conjugated) requires alcohol addition

- Comparison of results of the direct and indirect van den Bergh tests is often significant in assisting to classify icterus

- The indirect bilirubin value is calculated by subtracting the direct bilirubin value from the total bilirubin value

Interpretation of the Van Den Bergh reaction

Dog. The percentage of conjugated or uncongugated bilirubin is more important in interpretation of results than is the total concentration of each

- Less than 20% conjugated bilirubin are indicative of hemolytic diseases of dog
- If more than 40% of the total bilirubin is of the conjugate type hepatocellular disease is probably present

- Conjugated bilirubin levels 25-35% of the total may occur when there is hemolysis plus hepatocellular disease
- Obstruction of the bile duct system (cholestasis) results in a greater increase of conjugated bilirubin in the serum (55-90%)
- An additional factors that must be considered in interpretation of bilirubin levels is the functional status of the kidney

Horses- The greater portion of serum bilirubin found in horses with either hemolytic or hepatic icterus is **unconjugated**

- Uncojugated bilirubin levels of up to 25mg/dl of serum may occur in the liver disease in the equine
- If conjugated bilirubin is greater than 30% of a total serum bilirubin cholestasis should be considred

Urinary urobilinogen (Ehrlich's Test)

- Urobilinogen reabsorbed from the intestine & passes unchanged via the liver & the general circulation → kidney →via urine.
- It may be decreased in chronic disease (dilute it).
- It may also increase in association with hemolytic diseases.

B. Bile Acid Production, Concentration and tests

(Group assignment and presentation)

- Cholesterol (degraded) $\rightarrow 1^0$BA (cholic or chenodeoxycholic acid) $\rightarrow 1^0$BA $\rightarrow$ conjugated to (1^0BAc)

- A 1^0BAc is secreted into the biliary system and transported via the bile ducts ***to the intestine*** $\rightarrow$ Absorption by the intestinal mucosa and entrance into the portal blood
- BA molecules that escape the enterohepatic circulation $\rightarrow$via glomerular filtration (excretion)

Note:-

A) in health, the enterohepatic circulation of bile acids is highly efficient, and nearly all bile salts excreted in bile are returned to the liver via intestinal absorption and portal flow

B) Ingestion of a meal →released cholecystokinin →gallbladder contraction after → After intestinal absorption of BAs, the resulting higher [BA] in portal blood may exceeds the liver's ability to extract BAs, and thus there is a **postprandal increase in [BA]** in systemic blood

Hypercholemia - Increased bile acid concentration in serum or plasma & is called *hypercholemia*

Fasting hypercholemia

❖ There are two major pathological process that increase serum [BA] in fasting

1) Decreased BA clearance from portal blood

2) Decreased biliary excretion BA

Accordingly increased **Fasting hypercholemia** supports the conclusion that an animal has ***decreased hepatobiliary function***.

Hypercholemia due to diseases (Diseases or conditions that cause an increase [BA])

❖ Decreased BA clearance from portal blood

- Decreased functional hepatic mass: diffuse hepatocellular disease (decreased uptake BA from the sinusoids blood because of decreased functional mass) or
- Decreased portal blood flow to the liver: congenital and acquired portosystemic shunts

❖ Decreased BA excretion in bile

- Obstructive cholestasis
  - Hepatic cholestasis: lipidosis, diabetes, lymphoma
  - Posthepatic cholestasis: cholangitis, bile duct carcinoma, liver flukes, cholelithiasis, and cholecystitis

Bile acid challenge test for dogs and cats

- *Of the other tests, the serum bile acids test is the easiest to perform, the most sensitive, and liver specific.*

- Therefore the bile acids test has largely replaced BSP (Sulfobromophthalein) and ammonia tolerance tests.

Procedure

- A. fasting sample is collected from the dog or cat after 12hr fast. Then, the animal is observed while it ingests food containing protein and fat and then A 2hr latter postprandial sample is collected
- *An increase in [BA]>5µmol/L than normal range with fasting and an increase in [BA]>10 µmol/L 2hr post prandial indicates liver disease and/or portosysytemic shunt*

Clearance of foreign dyes from the serum

- Prior to availability of spectrophotometric assay for BA
- Sulfobromophthalein (BSF)- is widely used index of hepatic function in domestic animals
- When injected intravenously this dye is taken up rapidly, concentrated by the liver and excreted into the bile

❖ Note: this dye compete for hepatic uptake with bilirubin and this test is not indicted if there is icterus and high level of serum bilirubin

Interpretation

- **Dog-**
  - Prolonged BSP retention dogs has been reported in hepatobiliary diseases
- **Cow**-the BSP clearance technique sufficiently sensitive to detect the presence of hepatic disease

2. Tests based on serum enzyme activity

The major categories of enzymes used to evaluate liver disease

Two major categories

- **Leakage enzymes**
- **Cholestatic enzymes**.

Leakage enzymes → into the plasma ← hepatocyte injury / death

Therefore, high activities in serum are an **indication of hepatocellular injury.**

Commonly measured **leakage enzymes**

- Alanine aminotransferase (ALT; also alanine transaminase)
- Aspartate aminotransferase (AST; also aspartate transaminase)
- Sorbitol dehydrogenase (SDH)
- Lactate dehydrogenase (LDH)

Serum enzymes

- A large number of enzyme estimations are available which are used to ascertain liver function. They are be divided into two groups:

I: most commonly and routinely done in the laboratory.

Serum transaminase(ALT/AST)

Serum alkaline phosphate(ALP)

II: not routinely done in the laboratory.

- Sorbitol dehydrogenase (SDH) A Reading assignment
- Lactate dehydrogenase (LDH)

Alkaline phosphatase (ALP)

- ALP occurs in all tissues, especially liver and bone.
- The alkaline phosphatase test is often used to help diagnose certain liver diseases and bone disorders .

Mechanism of increase in ALP in liver disease:

- Increase in the activity of ALP in liver disease is not due to hepatic cell disruption , nor to a failure of clearance , but rather to increased synthesis of hepatic ALP .
- The stimulus for this increased synthesis in patients with liver disease has been attributed to bile duct obstruction by stone, tumors , intrahepatically by infiltrative disorders or space-occupying lesions.

Cholestatic enzymes synthesis is increased ← **bile retention or administration of drugs.**

- Bile retention usually results from intrahepatic or extrahepatic bile duct obstruction.
- Commonly measured cholestatic enzymes include the following:
  - Alkaline phosphatase (ALP)
  - Gamma-glutamyltransferase (GGT)

- Enzyme tests provide information about hepatocellular injury or cholestasis but do *not* define how much functional liver is present.
- Therefore, specific liver function tests are needed to assess liver function.

3. Tests based on specific biochemical functions

- Plasma proteins & analytical principles
- Total protein

Hepatic insufficiency (hepatic failure) & decreased protein synthesis

❖ **A** marked reduction in functional hepatic mass (<20% remaining) →↓ the synthesis of nearly all plasma proteins except immunoglobulin.

❖ Disorders: Cirrhosis, hepatic necrosis, hepatic atrophy and neoplasms that damage liver extensively

Tests based on specific biochemical functions

Formation of prothrombin by liver

-Plasma proteins & analytical principles

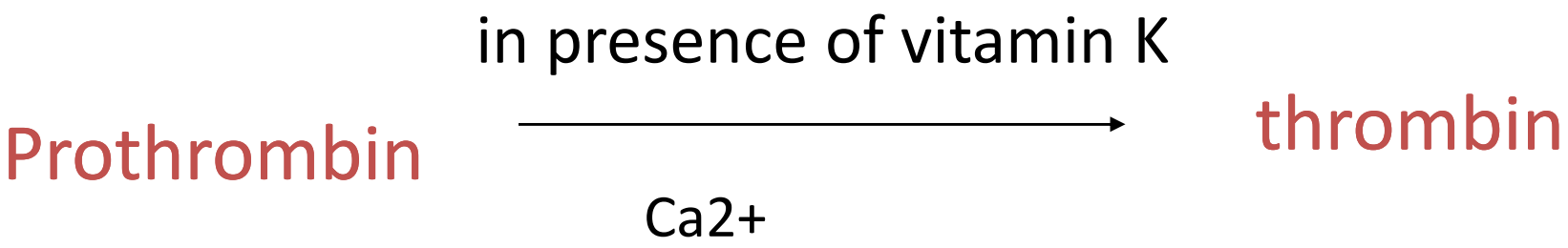
- Total protiens (Group assignments)

At least 12 different proteins are involved in clotting. Blood clotting factors are proteins made by the liver and are associated with the incorporation of vitamin K metabolites into a protein. When the liver is significantly injured, these proteins are not normally produced.

Prothrombin time (protime or PT)

★ Prothrombin is a plasma protein that is converted into thrombin during blood clotting.

★ Prothrombin is formed in the liver from inactive "preprothrombin" in presence of vitamin K.



What is prothrombin time?

- Prothrombin time is measured as prothrombin activity. The term prothrombin time was given to time required for clotting to take place in plasma to III factor and Ca+ have been added.
- ★ PT is used to assess the activity of extrinsic blood clotting pathway .
- ★ PT is also a useful test of liver function, since there is a good correlation between abnormalities in coagulation measured by PT and the degree of liver dysfunction.

Thank you

INTESTINAL PROBLEMS PANCREATIC FUNCTION TEST

Signs associated with GIT (intestines)

- The most common is diarrhea, which is an increase in the frequency, volume, and consistency of bowel movements. Less common signs include
- vomiting (although vomiting may be common in cats) especially in cases of inflammatory bowel disease
- weight loss (with chronic diarrhea), melena, hematemesis, polyphagia, coprophagia, pica, abdominal distention, abdominal pain.

Malabsorption & maldigestion

- ***Malabsorption*** *is a failure to either digest or absorb nutrients.* Even though maldigestion is defined as a failure of food digestion and malabsorption as a failure to absorb what was digested, the differentiation between the two is somewhat subjective.

- For several authors, the global term ***malabsorption*** *refers to a process where food is either not digested or absorbed* properly.

Causes –divided *into* ***luminal***, ***mucosal***, *or* ***postmucosal*** *(* overlap between categories).

- ***Luminal causes*** - exocrine pancreatic insufficiency EPI/ cholestatic /liver disease, and small intestine bacterial overgrowth.

- ***Mucosal causes*** - villous atrophy, and inflammatory bowel disease .

- ***Postmucosal causes*** -lymphangiectasia, and neoplasia.

Clinical signs expected in malabsorption

- The most common clinical sign of malabsorption is probably **chronic diarrhea** associated with **weight loss** & **malformed feces (bulk, soft, abnormal)**
- Vomiting may also be present. However, it is important to note that diarrhea is *not always present in cases of malabsorption.*
- ***Acute enteric diseases that causes diarrhea for few days causes temporary malabsorption & are not tipically considred under malabsorption***

Clinicopathologic tests used to diagnose malabsorption

- Clinicopathologic tests for malabsorption depend on the suspected underlying cause.
- The two most common broad causes are **intestinal** and **pancreatic** problems .

Pancreatic function tests

Pancreas

- The pancreas is a compound gland. It is both an exocrine gland, and an endocrine gland. The **exocrine** pancreas plays a crucial role in *food digestion* by secreting *digesting enzymes* and bicarbonate into the duodenum portion of the small intestine.
- The endocrine part of the pancreas is responsible for secreting like insulin and glucagon, play an important role in plasma glucose homeostasis.

What Is Exocrine Pancreatic Insufficiency?

- Exocrine pancreatic insufficiency (EPI) causes problems in how the animal digest food. Their pancreas doesn't make enough of the enzymes that their body needs to break down and absorb nutrients.

- Enzymes speed up chemical reactions in their body. The enzymes made by their pancreas move into their small intestine, where they help break down the food they eat.

- Exocrine pancreatic insufficiency (EPI) is a condition characterized by deficiency of the exocrine pancreatic enzymes, resulting in the ***inability to digest food properly***, or *maldigestion*. The etiology of this deficiency includes both ***pancreatic*** and ***non-pancreatic(Intestinal-problem)*** causes.

- EPI occurs when the part of the pancreas (acinar tissue) that produces digestive enzymes no longer functions properly. As a result, the animal can't digest its food.

- EPI is sometimes also referred to as PAA (Pancreatic Acinar Atrophy), Pancreatic Hypoplasia, Malabsorption, or Malassimilation.

- The exocrine pancreas produces 3 main types of enzymes: amylase, protease, and lipase. Under normal physiologic conditions, the enzymes (specifically, lipase) break undigested triglycerides into fatty acids and monoglycerides, which are then solubilized by bile salts.

- Because the exocrine pancreas retains a large reserve capacity for enzyme secretion, fat digestion is not clearly impaired until lipase output decreases to below 10% of the normal level.

- The diagnosis of exocrine pancreatic insufficiency (EPI) is largely clinical. It may go undetected because the signs and symptoms are similar to those of other GI diseases or because the signs and symptoms are not always evident, due to dietary restrictions.
- A complete laboratory evaluation (including pancreatic function testing) is required not only to diagnose **EPI** but also to determine the **extent of the malabsorption** and assess the manifestations of the underlying disease.

causes of exocrine pancreatic insufficiency (EPI)

- Most common cause of EPI is chronic *acinar atrophy(idiopathic*).
- Genetic predisposition for an immune-mediated condition called *atrophic lymphocytic pancreatitis, resulting also in* ***chronic acinar atrophy.***
- *Pancreatic hypoplasia is a possible but rare* cause of EPI in dogs.
- **Chronic pancreatitis *seems to be the major cause of EPI.***

Pancrititis- inflammmation

- ***In acute pancreatitis*** (mild edematous to severe necrotizing or hemorrhagic) the release of cytoplasmic enzymes from the damaged acini cells can result in:
  - increased serum activity of **AML**, & **LPS**, **[TLI]** & increased urine & plasma [TAP/trypsinogen activation peptode]
- ***Chronic pancreatitis-*** recurrent episode of acute pancrititis → slow progressive destruction of pancratic acinar cells (common in cats).

Intestinal malabsorption- could be localized or diffuse

- ❖ Involves malabsorption of many nutrients (sugars, proteins & fat)
- ❖ Specific diagnosis of suspected primary intestinal diseases usually requires histologic examination of intestinal tissue & other tests

Eg. Tissue of intestinal disease.

Clinical signs expected in cases of EPI

Dogs - large volumes of semiformed feces or ***diarrhea***, often accompanied by ***weight loss*** and polyphagia (although anorexia and hyporexia may be).

- Feces can have a ***greasy or discolored appearance***(not always present).
- ***Coprophagia and pica*** may also be reported. Animals are rarely depressed or lethargic.

Cats- signs are similar to dogs (vomiting and anorexia seem to be more common in feline).

Clinical signs

- Eating stools / feces (coprophagia)
- Eating non-food items (pica) *Some EPI dogs will eat sticks, stones, paper, dirt, their toys, etc.*
- **Rapid weight loss:** *Normally occurs since the dog is literally starving to death*
- **Diarrhea that won't go away** *Watery or very loose diarrhea also occurs with some frequency.*

Clinical signs of EPI can resemble with intestinal diseases

Tests used for diagnosis of EPI

- Tests include trypsin-like immunoreactivity (TLI),PLI, pancreatic elastase, oral triglyceride challenge (corn oil) test,, tests for fecal fat.
- serum amylase and lipase are unreliable indicators of pancreatic function and should not be used to diagnose EPI

- Serum TLI is the most sensitive and specific test for exocrine pancreatic insufficiency in dogs and cats.
- TLI assays are species specific, it is important to perform the tests in veterinary and not human laboratories.
- Serum TLI measures the amount of trypsinogen that normally leaks from the pancreas into the bloodstream.
- Because trypsinogen is pancreas specific, the TLI test provides a good indication of the pancreatic tissue function.
- Sensitivity and specificity for TLI in cases of EPI are reportedly close to 100%.

TLI values expected at EPI

- *Dogs: less than 2.5 µg/L (reference value 5-35 µg/L)*

- *Cats: less than 8 to 10 µg/L (reference value 17-49 µg/L)*

- Tests for serum TLI concentrations considered diagnostic for exocrine pancreatic insufficiency should be performed on an animal that has been ***fasted for at least 12 hours***

Pancreatic elastase Test in stools

- is used as a gold standard for exocrine pancreatic insufficiency

❖ *Pancreatic elastase is a zymogen* produced **exclusively** by pancreatic acinar cells and eliminated in feces, and its value should decrease in cases of EPI.

- This zymogen is very stable and is resistant to proteolytic degradation in the gut.
- Determination of fecal elastase and chymotrypsin (2 proteases produced by the pancreas) can be used to try to **distinguish between pancreatic causes and intestinal causes of malabsorption**.

❖ Recently, a sandwiched-ELISA test has been developed for use in dogs and shows very good sensitivity and specificity.

Gelatin (or x-ray film) fecal digestion

- Fecal proteolytic activity has been used for many years in veterinary medicine. However, this measurement is plagued by:
  - wide variation in results depending on the method used
  - autodegradation of proteases during the interval between stool sample collection and analysis is important.
- The test is easy to perform but unfortunately is frequently unreliable because this procedure does not seem to be standardized among laboratories.

- Also, gelatin digestion is difficult to interpret, resulting in many false results.

- False-positive -intestinal bacterial proteolytic activity.

- False-negative -a delay between sampling and analysis or from severe diarrhea diluting pancreatic enzymes.

Oral triglyceride challenge (corn oil) test.

- The test is based on the assumption that dogs with EPI or primary small intestinal disease will have fat malabsorption.

- Dogs are given corn oil orally after a **12-hour fast**, and if either a pancreatic or an intestinal problem is present, preprandial and postprandial serum **triglyceride** values should basically be the same.
- The triglyceride challenge (corn oil) test does not seem to be used in cats.

- When the test is repeated with pancreatic enzyme supplementation, postprandial triglycerides should increase at least **twofold** over baseline values if the animal has EPI.

- Unfortunately, false-positive and false negative results are possible, and the corn oil test should not be used to **confirm** EPI.

Fecal fat tests and results interpretation

- Tests for fecal fat can be either ***qualitative*** or ***quantitative.***

- The test should be standardized by feeding the animal a diet containing moderate amount of fat (about 8%) for at least 48 to 72 hours before analysis and by analyzing at least ***two fecal samples.***

Qualitative fecal fat testing can be divided into **direct** and **indirect** tests.

- The direct test detects undigested fecal fats

- The indirect test detects split fats (e.g., fatty acids).

- The *direct test is performed by placing a small piece of fresh feces on a microscope* slide mixed with a drop of **Sudan III or IV**, placing a coverslip, and examining at **10×**.

- Positive results show more than **three large, refractile orange droplets per field** at low magnification.

- The *indirect test is performed by adding 1 or 2 drops of* ***glacial acetic acid*** *to the edge of the* coverslip of the slide used for the direct test.

- The slide is then ***heated to near boiling***, and heating is stopped when a few bubbles start to appear underneath the coverslip.

- The slide is examined under the microscope when still warm. ***Heat and acetic acid convert fecal soaps into insoluble free fatty acids that aggregate into large globules.***

❖ Dogs with intestinal malabsorption should have ***more than three globules per field*** at low magnification.

- Theoretically:
  - animals with EPI should be positive for both the direct and indirect tests
  - whereas intestinal problems should result only in a positive indirect test

❖ Practically, however, the fecal fat test does not seem to differentiate between pancreatic and intestinal causes of steatorrhea in either dogs or cats.

- The quantitative fecal test is rarely used because it is time consuming, expensive, and unpleasant (feces collected for 72 hours).

- Fecal fat tests cannot be used for a final diagnosis or even recommended as a screening test for EPI.

Tests for intestinal malabsorption

- *If EPI has been ruled out based on laboratory testing, and now small intestinal disease is suspected, the following tests can be used*
- If parasites (fecal examination), systemic diseases and intestinal accidents (radiography, ultrasound) have been ruled out, more specific tests are required.
- These tests may include D-xylose absorption, combined xylose/3-*O-methyl-D-glucose, oral trigylceride challenge (discussed earlier with EPI),*

- Fecal fat excretion (described with EPI), and serum folate and cobalamin (vitamin B12) concentrations should be tested

D-xylose absorption test

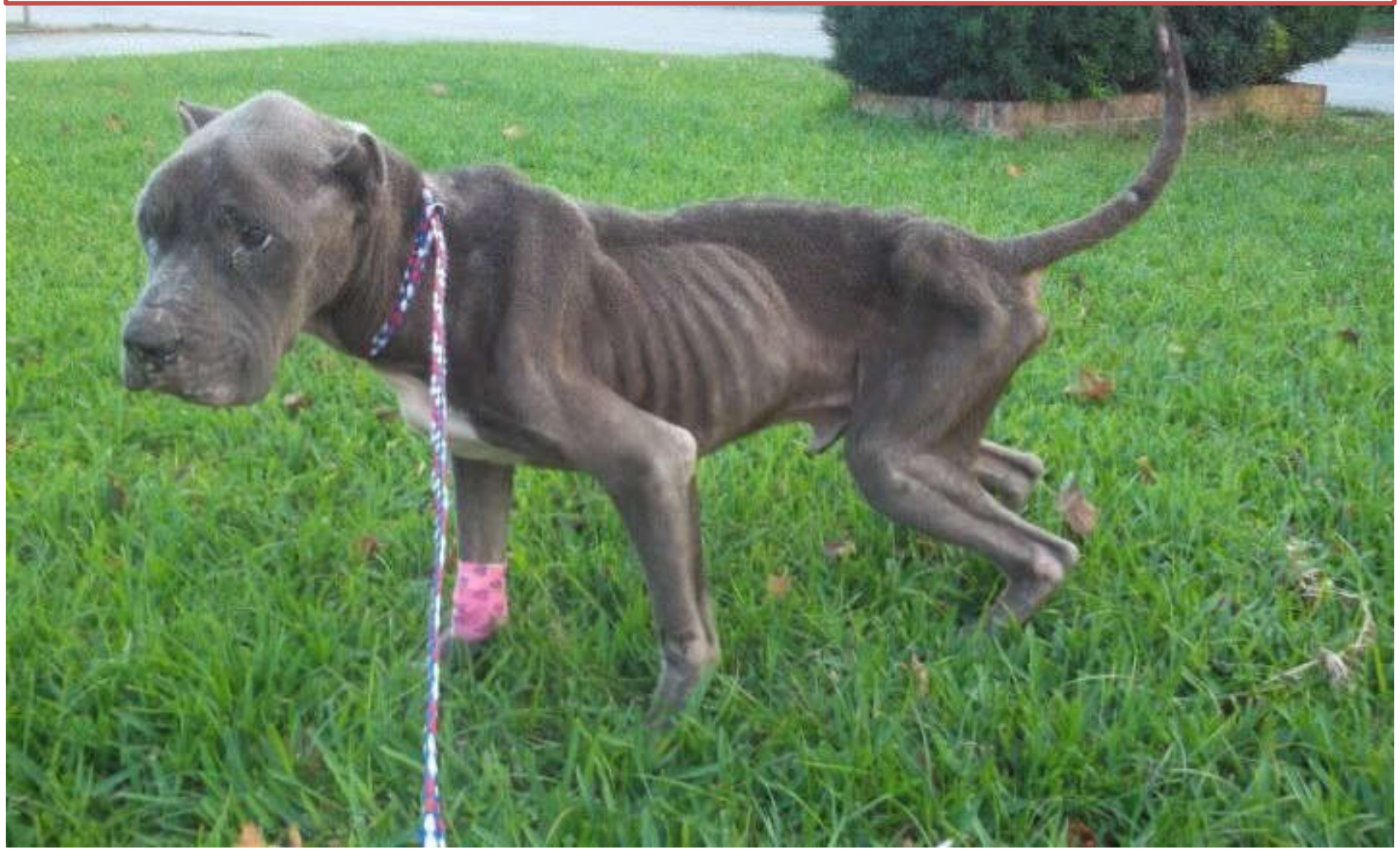
- *D-xylose is an exogenous pentose sugar that is absorbed by the small intestine through* carrier-mediated transport.
- **It is not metabolized after absorption, however, and is excreted intact in the urine**.
- The test is performed by measuring either blood xylose after 3 hours or its urinary excretion after 5 hours

- If the 72-hour fecal fat collection results demonstrate fat malabsorption, the D-xylose test is used to document the integrity of the intestinal mucosa.

- If absorption of D-xylose is impaired by either a luminal factor (eg, bacterial overgrowth) or a reduced or ***damaged mucosal surface*** area (eg, from surgical resection or celiac disease), urinary excretion will be ***lower*** than normal.

- Cases of pancreatic insufficiency usually result in normal urinary excretion because absorption of D-xylose is still intact.

Please discuss the types of sample collection and related tests better used for this animal? And also write the clinical signs and diseases you expect (5%).



EXAMINATION OF MILK

Mastitis is inflammation of mammary gland. It is a common condition of milch cattle. Mastitis can be classified into clinical and sub clinical. Clinical form of disease can easily be recognized by the clots or flakes formed by millions somatic cells that move into the milk. However sub clinical form remains unnoticed because gross sign of inflammation are not observed. Milk and udder appear normal. Sub clinical mastitis (SCM) is far more important than clinical form because:

- It is usually the basis of herd problem when mastitis out breaks occurs.
- It usually precedes the clinical form.
- It reduces milk yield drastically.
- It has long duration (months or even years).
- It is 15-40 times more prevalent than clinical form

DIAGNOSIS - Clinical diagnosis of acute bovine mastitis can be done easily, as there are gross abnormalities in milk. However, detection of sub- clinical mastitis by some reliable diagnostic test is more difficult but is an important part of any herd survey to establish disease incidence. Increasing emphasis on the detection of abnormal milk has resulted in the development of a large number of indirect tests utilized to recognize the presence of inflammatory exudates and cells in market milk. Veterinarians are seldom called upon to participate in these evaluations of market milk, as their problem is to detect, treat and prevent diseases in dairy herds under their supervision.

The various lab tests used in diagnosis of mastitis may be divided into chemical, microscopic and culture. Some tests may be conducted in the field, whereas other must be made in the laboratory. Our discussion will be confined to tests that are used principally for the detection of clinical and subclinical mastitis. Many of the screening tests that have been developed primarily for laboratory detection of mastitis milk in bulk samples are not included.

1. Physical examination of udder - Best conducted on empty udder immediately after milking. The gland is carefully palpated between both hands for atrophy, hardening, thickenings of ducts and fibrosis.

2. <u>Milk - Strip cup test</u>: First streams of milk is milked to the surface of a cup having flat black plate of metal or plastic and examined for the presence of clots, flakes, wateriness or discoloration .The strip cup serves a number of useful purposes such as: i. The detection of abnormal milk ii. Removal of the first milk helps in stimulating for milk let down, iii. Discarding of first streams leads to lowering of bacterial count since the first few streams of milk commonly has the largest number of bacteria per ml. of milk.

2. **Chemical Tests** – The majority of the chemical tests for diagnosing mastitis depends upon the demonstration of abnormalities in milk composition and thus are indirect tests for mastitis. Since the use of indirect test is based on the demonstration of abnormal substances in the milk, samples from animals with clinical mastitis will usually react positively. However, abnormal changes may not appear with regularity in the milk of all cows having an udder infection. In most instances, a positive test indicates an infected quarter, but a negative test does not indicate that the quarter is not infected. The only positive method for detecting udder infection is bacteriological examination of a properly collected sample.

The most commonly used indirect tests for the existence of mastitis include pH determination, and chloride, catalase and Whiteside tests and the California mastitis test or a variation of this technique.

1. pH determination - The pH of normal milk varies from 6.4- 6.8. Milk from mastitis udder is abnormally alkaline, with the degree of alkalinity depending upon the severity of inflammation. Abnormal milk may have pH as high as 7.4. In a mastitis udder, due increased vascular permeability, there are changes in bicarbonate, chloride concentration in milk. This can be detected with the help of bromothymol blue (BTB) and bromocresol purple (BCP) tests.

The reaction of milk may be determined by several different methods, the most common of which is the use of indicators that change color at or near normal pH. The pH should be determined on freshly drawn milk, although milk held at refrigerator temp for 24-48hrs may be used. Contaminated milk sample or samples containing a large no. of bacteria are not suitable for testing after being exposed to a warm temp. for a few hrs, as lactose-fermenting bacteria alter pH. Several commercial tests have been devised for detecting the pH of milk by impregnating absorbent heavy filter paper with an indicator. These test blotters are used in the field by placing a small quantity of milk on the spot of indicator and noting the color change. These tests are not accurate as those in which small quantities of dye are placed in a test tube

and the milk added. With the latter technique, alterations in color are more easily discernible. Both bromothymol blue (BTB) and bromocresol purple (BCP) have been widely used for the detection of pH alterations in mastitic milk.

Bromothymol blue (BTB) **test:** Reagent: Bromothymol blue – 1 gm, N/100 NaOH- 160 ml, Distilled water - 590 ml. One ml of this reagent is pipetted into a test tube having a capacity of 8-15 ml. Five ml of milk from a sample bottle is added with a pipette or more conveniently, tubes may be marked to the 6-ml level and filled to that mark directly from the udder. If tubes are filled directly from udder, care should be taken to avoid foaming. When bromothymol blue is added to normal milk, a yellow color appears, but a sample containing abnormal milk from an infected quarter will be green/dark green/greenishblue, depending upon the amount of alkalinity. Blue/bluishgreen color is indicative of mastitis. This increase in alkalinity is is due to the presence of exudate containing unusually large amounts of alkaline salts derived from blood and lymph. Care must be taken in interpreting pH changes as cows in late stages of lactation may give false-positive reaction. Milk at this stage of lactation being normally more alkaline than during other stages of lactation. This test will indicate alterations associated with most acute or subacute cases of mastitis. But in chronic conditions there may not be sufficient pH change to be detected. In chronic mastitis, there is so little active inflammation that that exudates is not produced in a quantity sufficient to cause a pH change.

For performing the test on plate, one drop of reagent is added to eight drops of milk. For strip test, paper strips are impregnated in the indicator solution and the milk drops are used on this strip to detect mastitis. The pH tests are not sufficiently sensitive for detection of all forms of mastitis and stages of mastitis.

Bromocresol purple (**BCP) tests**- (BCP is used in the same manner as BTB for determining milk pH. BCP has the advantage of becoming yellow in a pH range below 5.2 and thus abnormally acid milk may be detected. In addition, this indicator is used in the Hotis test so that a pH determination using BCP and the Hotis test may be conveniently combined. For its preparation: Take BCP powder 0.9gm and dissolve in 100 ml of distilled water. In both the test for pH and Hotis determination, 0.5ml of BCP solution is added to 9.5 ml of milk. If Hotis test is to be conducted, the BCP should be sterilized in the test tube prior to addition of milk. With normal milk, addition of BCP solution produces a pale grayish-purple color, whereas abnormal

milk become a deep purple with increased alkalinity, the intensity of color varying with the degree of alkalinity.

Determination of pH is of limited value in detecting the existence of udder inflammation, and other screening tests have, for the most part, replaced the pH determination.

2. Chloride test - This test is dependent upon the determination of an abnormal quantity of chloride in the milk. Composition of the solutions used in chloride test is as:

Chloride test solutions:	Solution A:	Silver nitrate	- 1.3415g
		Distilled water	- 1000.0 ml
	Solution B	Potassium chromate	- 10.0g
		Distilled water	- 100.0 ml

Normal milk contains 0.08 -0.14% chloride. Abnormal milk contains a greater quantity of chloride because of the presence of inflammatory exudates. These exudates contain a considerable amount of chloride, and even a small amount of exudate in milk will result in a positive chloride test. Test is conducted by adding 5 ml of silver nitrate solution to 1 ml of milk, followed by the addition of 2 drops of pot chromate solution and mixing by inversion of the tube. The appearance of a yellow color indicates that more than 0.14% chlorides are present in the sample. And a brownish red color indicates that the sample contains less than that amount. Cows in either early or late lactation may give false positive reaction to chloride test because of normal physiological processes of the udder.

3. California mastitis test (CMT): The CMT is a simple, inexpensive, rapid screening test. Like whiteside test, It has a specificity for leucocytes in the milk. The reagent used consists of an anionic surface active agent and an indicator, Bromocresol purple. In this test a white plastic paddle with 4 receptacles/cups into which milk may be drawn is used. CMT reflects the SCC quite accurately and is reliable indicator of the severity of infection. CMT reagent is added in an equal quantity to milk in each cup. Reactions occur immediately and are graded as the milk and reagent are mixed by a gently circular motion of paddle. The total cell count of milk is reflected

by the degree of precipitation or gel formation that occurs. The pH change associated with abnormal milk is indicated by a color reaction with bromocresol purple present in CMT reagent.

CMT may be used with fore milk or strippings from individual glands. It is also applicable to bucket milk for rapid screening of herds for mastitic cows and to bulk milk as delivered to processor for selection of herds that have a high degree of udder infection. The appropriateness or value of CMT evaluation of bulk milk decreases as the herd size increases. Cow in the first few days after calving and the last stage of lactation may give false positive reaction. CMT reactions are scored as follows:

Negative – The mixture remains liquid with no evidence of formation of a precipitate.

Trace – A slight precipitate that tends to disappear with continued movements of the paddle.

1+ A distinct precipitate but no tendency toward gel formation.

2+ The mixture thickens immediately, with a suggestion of gel formation. As the mixture is caused to swirl, it tends to move in towards the centre, leaving the bottom of the outer edge of the cup exposed. When the motion is stopped, the mixture levels out again, covering the bottom of the cup.

3+ A distinct gel formation that tends to adhere to the bottom of paddle and during swirling a distinct central peak forms. Alkaline milk is indicated by a plus sign, which is used when the mixture is distinctly purple as indicated by contrasting purple color. The symbol Y for acid milk is used when bromocresol purple becomes yellow.

Relationship between CMT score and Somatic cell count

California mastitis test scores correlation of CMT score With Somatic cell count	
CMT Score	**Somatic cell range**
Normal (N)	**0 – 2,00,000**
Traces(T)	**2,00,000 – 4,00,000**

1	**4,00,000 – 12,00,000**
2	**12,00,000 – 50,00,000**
3	**Over 50,00,000**

4. Modified white side test: This test also gives an indirect indication of number of inflammatory cells in milk. Test requirements include: A plate or cup with black background, Sodium hydroxide 4% solution, dropper or Pasteur pipette, a slim wooden applicator stick 6" size, and a good light source for observation and interpretation of results. To perform the test,

i. Thoroughly mix each individual milk sample, being careful to avoid violent shaking.

ii. Place 5 drops of milk on a glass plate with black background. Avoid spreading of milk over too great area, as proper mixing with reagent may prove difficult.

iii. Two drops of 4% NaOH solution are added to the milk and mixture is stirred with an applicator stick for 20-25 sec.

iv. Milk from normal animal will have no change after the addition of NaOH. Milk from a cow suffering from acute or subacute mastitis will become thick and viscid, but that from a animal with chronic mastitis, may have only a few white flakes.

Laboratory tests

1. Somatic cell count (SCC): Somatic cell count is performed to detect leukocytes as an indicator of mastitis and also as a measure of the quality and market acceptability of milk. Some other body cells always are included in this test. So the term SCC is used to be accurate. Presence of more than five lacs cells per ml of mixed herd milk is suggestive of significant incidence of mastitis in a given herd. Somatic cell counts can be run on milk from the bulk tank (this indicates herd mastitis status) or from individual cows (detects a specific cow with possible mastitis). Somatic cell counts of less than 100,000 to 200,000 cells/ml of milk are not indicative of mastitis. As cell counts increase so does the chance that a bacterial infection is present.

3. <u>Cultural examination of milk</u> - Bacteriological examination of milk is a specific test for diagnosis of sub-clinical mastitis as it identifies specific causative agent. Proper collection of milk samples is essential for accurate diagnosis of intramammary infections. The milk for cultural testing for mastitis should be collected and dispatched as follows:

Sample collection – Since absolute diagnosis of mastitis and identification of its causative agent are based on the isolation and identification of bacteria, all specimens for lab examination should be collected in such manner so that as little contamination as possible. Sample should consists of fore milk taken at least 6 hrs following a regular milking. Fore milk is preferred, as the first few milliliters of milk will usually have the greatest change if the udder is in any way abnormal. Sample should be collected methodically as follows:

- Wash hands with soap and water.
- Clean the udder well by brushing off any dirt, loose straw or mud that is clinging to the skin.
- Wash the udder thoroughly with a clean cloth soaked in a disinfectant solution.
- Allow the udder to dry, and treat the teat orifice with tincture of iodine solution/spirit or 70% alcohol and allow it to dry.
- While the tincture of iodine is drying, label the tubes as to cow and quarter from which the sample will be taken. One procedure commonly used is to label the tubes by the symbols RH, LH, LF and RF (for right hind, left hind, left fore and right fore quarters). Test tubes used for collecting specimens should be sterilized by autoclaving. Small screw-cap vials with a frosted area that can be used for labeling are preferred.
- The cap of the sterile tube is carefully removed and held between the fingers in such a manner that the inside of the cap is facing downward. The tubes should be held at a slight angle to prevent contamination of the sample by falling particles. In order to avoid contamination of the teat orifice, the specimens should be collected in the following order: LF, LH, RH and RF. Do not allow anything to come in contact with the mouth of the tube.

- Collect 1 to 2 streams of milk from each quarter, starting in the given order. Close the container before removing it from beneath the teats.
- Immediately following collection, refrigerate the samples for transportation to the nearest diagnostic Laboratory. (preferably on ice particularly in summer season. Samples can be refrigerated for 24 hours).
- In herd surveys, it may not be necessary to collect individual quarter samples and composite samples may be used. In collecting a composite sample at least 5 ml of milk should be collected from each quarter, the collector should be careful to avoid contamination.

Limitations of cultural examination: i. Strict aseptic conditions are required for collection of milk samples. Ii. Specific laboratory facilities are required. Iii. Time consuming and expensive.

BASIC CULTURE MEDIA

Nutrient broth, general purpose medium suitable for the cultivation of less fastidious organisms and stock cultures. It consists of : Beef extract - 10 g; Peptone - 10 g; Sod. Chloride- 5 g; D.W.- 1000 ml. Dissolve the ingredients in water, adjust pH 7.6 and finally sterilize in the autoclave at 15 lb for 15 min.

Nutrient agar is nutrient broth jelled with 1.5 -2% agar.

Blood agar, an enrich media suitable for the growth of most fastidious organisms and also acts as an indicator of hemolysis. For preparation of media proceed as for nutrient agar. After sterilization, cool melted agar to 50^0 C & add 5 % defibrinated sheep/calf blood, mix carefully and distribute in Petri dishes.

MacConkey's agar, a differential media which distinguish between lactose fermenter and non-lactose fermenter. It consists of : Sod. Taurocholate- 5 g; Peptone- 20 g; Sod. chloride 5 g; D.W. - 1000 ml. Dissolve the ingredients in water and adjust pH 8.0, cool and filter.

Edward's medium – This medium inhibits Staphylococci and Coliform bacteria are readily distinguished by their characteristic black colonies. Various sps of Streptococci produce characteristic colonies as: Streptococcus agalactiae- Gray-blue colony, Streptococcu dysgalactiae - Gray or grayish-blue colonies, Streptococcus uberis – brown colonies. If by experience, Vet knows that streptococci are a problem in herd, this medium can be used for isolation.

Sodium azide-crystal violet Blood agar – This medium inhibits growth of almost all bacteria except Streptococci and particularly used for culturing badly contaminated samples. Streptococcus colonies are quite small and have varying amount of hemolysis characteristic of their sps. In addition to streptococci, certain sps of micrococci will also grow on selective medium.

Salt agar - Sodium chloride is added to any common media at a conc of 7.5%, makes a selective media for isolation of staphylococci, which are not inhibited by a conc of salt as high as 7.5% but most other bacteria fail to grow. Any colony appearing on salt agar must be identified by staining, hemolysis and coagulase production before diagnosis of staphylococcal infection is made.

Glycine-Tellurite agar – is also a selective media that will allow staphylococci to grow but inhibits many other organisms. On the surface of glycine-tellurite agar, staphylococci appear as black colonies, whereas other organisms produce a clear to colorless colony.

Examination of culture plates after incubation - After incubation, cultures are examined by naked eyes and description of colony morphology is noted. Smears are made from single colony and Gram stained. On the basis of colony characteristics, Gram's reaction and morphology, now it is possible to decide what further tests are necessary for final identification these organisms. Organisms that require biochemical testing or susceptibility testing are sub cultured so that these tests can be performed on a guaranteed pure culture.

HOTIS TEST – it provides considerable information regarding the condition of a milk sample. Milk (9.5ml) for Hotis test should be collected in a sterile test tube containing 0.5 ml of bromocresol purple. Following observations may be made:

1. pH of milk may be determined by noting the color produced after addition of milk.
2. A portion of milk sample may be used for the isolation of pathogenic organisms.
3. The milk may be utilized for examination of an incubated sample.
4. A direct leucocyte count may be made.
5. Hotis test itself is useful determining the presence of streptococci and staphylococci.

Following the collection of 9.5ml of milk in 0.5ml of bromocresol purple, the sample is incubated at 37^0C for 24 hrs. The appearance of canary yellow colonies of organisms along the sides of tubeor on the bottome indicates the presence of streptococcus agalactiae in the sample.

This color change is due to production of acid from milk lactose by the action of this microbe, which grow in colony form on the sides or bottom of tube. If sample is incubated for another 48 hrs, entire sample often turn yellow.

Staphylococcus aureus produces a characteristic change in some , but not all tubes. The organism grow in small agglutinated colonies, producing a rust-brown color. This organism may also digest milk. If the presence of *Staphylococcus aureus* is suspected, sample may be incubated for 72 hrs. Such prolonged incubation yields higher % of positive reactions with this organism.

If more than one type of organism is present or sample is contaminated, a combination of changes may obscure the typical Hotis test reaction. If such a change occurs, other techniques such as direct smear examination or culture examination must be used to make a accurate diagnosis.

Examination of Cerebrospinal Fluid

Removal and laboratory examination of CSF are indicated whenever there is:
i. clinical evidence suggesting CNS disease ii. Removal of CSF for alleviation clinical sings with CNS disorders iii. Estimation of intra cranial pressure using manometer iv. Introduction of material for radiographic exam of CNS v. For treatment of diseases by injecting drugs, serum or anaesthetics.

In Veterinary Practice, few reports exist about use of CSF exam in diagnosing Animal Diseases.
Contra indications : If a localized skin infection exists over area where puncture is to be made for collection of CSF.

Lesions involving the CNS do not consistently or uniformly cause changes in the CSF. The results of CSF analysis may be within normal limits in many instances of neurologic disease, and even when CSF cytologic abnormalities are present, they are often nonspecific. Alterations of CSF probably depend more on the location and extent of the CNS lesion than on cellular abnormalities. Thus, disease of the meninges produces greater alterations in CSF than do most diseases of CNS parenchymal tissue. Septic meningitis may cause suppuration within the CSF;

inflammatory cells may be numerous and levels of exudative protein markedly increased. In contrast, viral disease affecting the CNS, such as Canine distemper usually cause only a mild increase in CSF nucleated cell numbers.

Collection of CSF : i. Lumbar Puncture/Lumbo-sacral Puncture: Widely used and recommended method for bovines. Use sterile 5'', 14-16 gauze needle with stylet. The area should be clipped, shaved and disinfected before puncture. Insert needle in depression located between dorsal processes of last lumbar vertebrae and ant. end of Median sacral crest. Resistance/pressure is released upon entry of needle in subarachnoid space. Collect 10-15 ml of CSF for examination.

The limitationis only difficulty in entering the sub arachnoid space, if animal is in lateral recumbency.

ii. Sub-occipital puncture - we can collect CSF from an animal in standing position or in lateral recumbency. For this, use sterile 3 to 4'', 16 gauze needle complete with stylet. The head of animal should be fully flexed and firmly held with assistants. The area should be clipped, shaved and disinfected before puncture. Puncture the skin at a midline at the level of a line that joins anterior borders of of the wings of atlas. In most animals, CSF flows readily from needle following removal of stylet. If it does not occur spontaneously, 5-10ml of CSF can be collected by a sterilized syringe.

In ovines, Sub-occipital puncture is most satisfactory technique. Lumbar puncture technique is similar to that for Cows. But inadequate amount of CSF is obtained unless animal is held in a sitting position.

For Porcines, sub-occipital puncture is too difficult to use in swine. To obtain adequate restraint, a general anaesthesia is essential. Lumbosacral puncture is the method of choice to obtain CSF. But inadequate amount of CSF is obtained unless animal is held in a sitting position. In Equines, Sub occipital puncture is the the method of choice. However puncture is made between the atlas and axis vertebrae.A general anaesthesia is usually necessary. A needle and stylet that are approx. 3.5 '' long and 14 to 16 gauze are recommended.

In Canines, lumbar puncture is extremely difficult in dog due to the lumbar vertebral arches and small subarachnoid area. CSF is removed from dog at the cisterna magna at the atlanto-occipital articulation for sub occipital puncture. Light anaesthesia is recommended. A 20 gauze, 2 to 5

inch spinal needle with stylet should be used. For Felines, sub-occipital puncture is recommended in cat. Not more than 0.5 to 1.0ml of fluid should be removed from cat. As this animal is quite susceptible to Meningeal hemorrhage, if too much CSF is withdrawn.

Collected CSF should be placed in EDTA anticoagulant at the same concentration as used for blood samples. Refrigeration at 4^{o} C also aids cell preservation. Because cells in CSF sometimes degenerate rapidly, cell counts and cytologic examination should be performed within 30 minutes of collection.

Routine exam of CSF should include:A. Physical Examination – i. Colour ii. Turbidity iii. Coagulation B. Chemical Examination – i. Protein ii. Glucose iii. Sodium iv. Enzymes C. Cytological/Microscopic Examination - i. Total cell count ii. Differential count.

1.Physical Examination

a.**Color**: i. The normal CSF is crystal clear and colorless and resembles distilled water. In viral encephalitis or meningitis, the CSF may remain clear. ii. Hazy pink CSF may be associated with a traumatic tap or intracerebral or subarachnoid hemorrhage. iii. Bright red CSF indicates fresh blood from iatrogenic hemorrhage. Centrifugation of the fluid will produce a clear, colorless supernatant. Blood contamination will invalidate the results of CSF analysis but a crude correction can be made by discarding 1 WBC and **1 mg%** protein for every **1000 RBCs** present. iv. Dull red or brown fluid indicates previous hemorrhage. v. Xanthochromia **(pale orange or yellow)** is due to bilirubin from degenerating **RBCs.** It indicates hemorrhage of at least 2-4 days and can persist for up to 40 days. It may be associated with tumors, trauma, spinal cord compression and abscesses and can have protein concentrations greater than **400 mg%.** Xanthochromia may also be due to altered permeability of the blood brain barrier allowing influx of pigments from blood plasma **(e.g., unconjugated bilirubin).**

b.Turbidity: Turbidity will be apparent when the CSF contains 300-500 cells/ul or more. Bacteria or mycotic agents can contribute to turbidity. Bacterial meningitis may be only slightly turbid to almost pure pus which may clot. Viral encephalitis, trauma, tumor, abscess may show turbidity due to large amounts of protein, fibrin and/or cells.

c.**Coagulation :** Normal CSF does not coagulate. Coagulation may occur if damage to the blood brain barrier permits fibrinogen to enter the CSF or if CSF collection results in hemorrhage.

2. Chemical Constituents

a. **Protein:** 1. CSF protein levels are normally very small and consist mostly of albumin. 2. Globulin protein is of interest in that normal CSF is relatively free of globulins and their concentrations are increased in pathological conditions. The Pandy test is a qualitative test for high molecular weight protein (globulin). Add 1-2 drops of CSF to 1 ml of Pandy reagent (10 g phenol crystals/100 ml of distilled water) and observe for white turbidity. Normal CSF produces only faint turbidity. With abnormally large amounts of globulins, the solution becomes cloudy.

3. Urine protein reagent strips may be used to grossly detect protein. An elevated protein level would usually be represented on reagent strips as >100 mg/dl .

4. Quantitative tests are colorimetric or turbidometric. In contrast to cell evaluation, protein quantitation need not be done immediately. Fluid may be frozen and evaluated at a later date. The Coomassie brilliant-blue technique is the more commonly used spectrophotometric assay. Normal values are <48 mg/dl for dogs and cats, **<70 mg/dl** for horses and **<60 mg/dl** for ruminants.

5. Quantitation of protein fractions in both serum and CSF may be necessary to differentiate leakage of plasma protein across the blood-CSF barrier from increased synthesis of immuno-globulins **(IgG)** within the **CNS**. Correlation between CSF albumin and CSF globulin levels indicates a blood-CSF barrier dysfunction with both albumin and globulin entering the CSF with equal facility, but at a greater than normal rate. In contrast, low serum IgG concentrations, high **CSF IgG** levels and normal CSF albumin levels indicates a local CNS IgG production and not leakage across the blood-CSF barrier.

6. Increased total protein or globulin occurs in: a. Encephalitis or meningitis which increases the permeability of the blood-brain barrier to plasma proteins. Levels are generally markedly increased by bacterial meningitis and somewhat less altered by viral meningitis or encephalitis. Toxoplasma infections may also greatly increase CSF protein levels. b. Brain or spinal cord abscess c. Hemorrhage or blood contamination during a traumatic tap. d. Tissue destruction e. Neoplasm - total protein greater than **100 mg%**f. Convulsive states g. From high intracranial pressure due to a brain tumor or intracerebral hemorrhage. 7. The pathologic mechanisms altering CSF protein levels are complex and it must be remembered that CSF protein content may increase without a concomitant increase in cell numbers.

b. **Glucose:** i. Normally averages 75 mg% and is dependent on blood glucose concentrations (should be measured concurrently). Normal CSF glucose values are 60-80% of the blood glucose level. ii. Decreased levels are seen in hypoglycemia (normal CSF/blood glucose ratio) or in the presence of pyogenic organisms or rapidly growing tumors which utilize glucose. (decreased CSF/blood glucose ratio). iii. Increased levels are seen in hyperglycemic states (normal CS/blood glucose ratio).

c. Sodium: 1. CSF sodium levels are slightly less than that of blood. 2. The levels are increased in CSF (more than 160-200 mEq/L) in water deprivation (Salt poisoning syndrome of swine).

d. Enzymes : 1. Increased CSF Creatine kinase activity has been reported in a wide variety of neurologic disorders. Elevated CSF CK levels in dogs (normal values = 3 IU/L) appears to be a nonspecific but sensitive index of CNS disease.
2. **SGOT (AST)** is found to be elevated in canine distemper, purulent meningitis, or damage to brain issue.
3. SGPT **(ALT)** is found to be elevated in canine distemper.

3. Cytologic examinationa:

a. Total Nucleated Counts - Total cell counts of CSF can be determined by using a hemocytometer with a Neubauer ruling. A capillary pipette is used to place CSF on one chamber of the hemocytometer. The cells in all 9 squares are counted and the total multiplied by 1.1 to determine the total cell count/µl. Differentiation of RBC and nucleated cells is done using the

high-dry objective of the microscope. Nucleated cells are larger than RBC and have a granular appearance.

Normal CSF is free of RBCs and contains <8 nucleated cells/ul in dogs and cats and <5/ul in all large animal species. Total nucleated cells counts in the CSF are mildly elevated (pleocytosis) in a variety of disorders, and, though cytologic findings may suggest that inflammation is chronic (primarily macrophages) or chronic-active (nearly equal numbers of macrophages and neutrophils), no specific etiology may be apparent. Nucleated cell counts and cytologic findings of CSF examination can be difficult to fit into classifications conforming to specific disease. All classifications appear to overlap. Pleocytosis of CSF indicates abnormality, but CNS disease may exist in which there is no pleocytosis and the cells which are present are normal.

b. Differential Cell counts - The nucleated cell population of normal CSF is predominantly mononuclear consisting of small lymphocytes and a few macrophages, endothelial cells and histiocytes. Neutrophils are not normally found in CSF unless the tap is traumatic. They should not constitute >10% of the total cell count.

Neutrophilic pleocytosis usually indicates pyogenic or bacterial infection, an abscess, bacterial encephalitis or meningitis. Under these conditions, counts may vary from 50 to over a 1000 cells/ul. Elevated neutrophil numbers may also occur in noninfectious inflammatory conditions. Attempts should be made to identify bacteria and the fluid should be cultured. In ruminants, pigment granules are common and must be distinguished from bacteria.

The CSF of animals with mycotic and protozoan encephalitis will have varied pleocytosis, consisting of neutrophils, mononuclear cells or eosinophils. An increase in TNCC consisting primarily of lymphocytic cells may indicate viral infections, uremia, fungal infections, postvaccinal inflammation, chronic infection and toxic conditions. Monocytes/macrophages are often associated with lymphocytes and will increase in conjunction with lymphocytes. They may predominate in the CSF in FIP.

Pleocytosis is rarely observed in noninflammatory conditions involving the CNS. In animals with spinal trauma, a slight increase in cell numbers may occur. Animals with congenital malformations of the CNS generally have normal CSF. Animals with cerebral infarcts may have

increased numbers of RBC in the CSF and erythrophagocytosis by macrophages is a diagnostic feature of this condition.

C. Neoplastic cells may be evident if the meninges are involved. Filtration techniques are usually needed to demonstrate these cells. In most CNS tumors, CSF samples will have a normal or only slightly increased nucleated cell count consisting predominantly of mononuclear cells.

Histopathology Techniques: Tissue Processing and Staining

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Sampling for Histopathological Examination:

- Tissue submitted for histopathology must not be more than 3 mm thick and not larger than the diameter of slides used. Most specimens from solid tissues are cut in the form of pieces measuring 10 to 15 mm on the slides and 2 to 3 mm in thickness. Adipose tissue must be cut even thinner.
- Discrete areas of calcification or ossification should be taken out and should be decalcified in nitric acid.
- Small fragments of tissue must be wrapped in thin paper.
- If the fragments are very small, it should be stained by haematoxylin to facilitate their identification by the histopathologist.
- All tissue should be submitted in a diagnostic endometrial curettage. However, if the procedure was done for incomplete abortion and gross examination shows obvious product of conception, one representative section is more than adequate.
- Determination of surgical margin is helpful by painting them with India ink or a similar pigment before sectioning.

Methods of Sample Collection:-

1. Biopsy
2. Operation
3. Necropsy

I. Method of Biopsy Sample Taking:

1. Incisional biopsy
2. Excisional biopsy
3. Punch biopsy
4. Core needle biopsy
5. Curettage biopsy

Incisional biopsy:

In this method only a portion or wedge of tissue from a large lesion is taken and therefore, the procedure is strictly a diagnostic nature. It is performed when removal of entire lesion is impossible and often performed prior to major surgical procedure.

Excisional Biopsy:

In this technique, the entire lesion is removed, usually with a rim of normal tissue and therefore, the procedure serves the diagnostic and therapeutic function. Excising the entire lesion ensures sufficient tissue for histopathological examination, lessen the risk of tumour dissemination and eliminate sampling errors. It is performed when the lesion is smaller in size.

Punch Biopsy:

It is done by biopsy forceps. It is performed in the lesion of uterine cervix, oral cavity, esophagus, stomach, intestine and bronchus.

Core Needle Biopsy:

It is done with special type of wide bore biopsy needle. It permits a percutaneous approach to internal structures. Sampling errors are a significant problem in needle biopsy.

Curettage Biopsy:

Curetting are usually done for diagnosis of endometrial disease.

Choice of Biopsy Procedure:

The choice of appropriate procedure is dictated by anatomic consideration, biology of tumour type and by the request of pathologists.

Some General Rules for the biopsy Procedure:

1. The larger the lesion, the numerous the biopsies that should be taken from it because of the variability in the pattern that may exists and the fact that the diagnostic areas may be present only focally.
2. In ulcerated tumor, biopsies of the central ulcerated areas may show only necrosis and inflammation. Biopsies should be taken from the periphery that includes normal and diseased tissue.
3. The biopsies should be deep enough that the relationship between tumor and stroma can be properly assessed.
4. Deeply seated lesions are sometimes accompanied by a prominent peripheral tissue reaction which may be characterized by chronic inflammation, hyperemia, fibrosis, calcification and metastatic bone formation. If the biopsy is too peripheral, this may be the only tissue obtained.
5. When several fragments of tissue are obtained they should be sent to the pathology laboratory and all of them submitted for microscopic examination.
6. Crushing or squeezing of the tissue with forceps should be carefully avoided.
7. Once the biopsy is obtained, it should be placed immediately into container with adequate volume of fixative

II. Operational Method of Sample Taking

The collection of the sample is done at the moment of releasing surgery by local or general anesthesia. The part of the organ collected for sample is trimmed and sent to pathology

department for gross study by the pathologist and then to the histopathological laboratory for further investigation at the level of the cells.

III. Necropsy

Examination of a cadaver of dead animal, in particular the internal structures to obtain valuable information related to pathological disorders to make a definitive diagnosis.

3.1. Types of sample that could be collected

3.1.1. **Hair**: Collection includes the root.

3.1.2. **Skin**: Samples are collected at the periphery of the lesion, because it is the site where the multiplication take place

3.1.3. **CNS**: Routine sampling at hippocampus for diagnostic purpose or serial sampling (closely) for research work.

3.1.4. **Bone**: Use fine saw to cut a bone and place it in appropriate fixative.

- To collect the bone marrow cut the bone horizontally and /or longitudinally.
- Decalcification is done using Perenyl's solution.

Calcification is a term applied to organic tissues which have been infiltrated with calcium salts. These salts provide hardness and rigidity to the bone and must be removed to assure that the specimen is soft enough to allow cutting with the equipment available. In some cases, only when special microtomes are available for sectioning undecalcified specimens could the complete histologic picture be made possible.

Bone and other calcified material should be cut into small pieces (approximately 5mm) with a fin saw before fixation. After adequate fixation, place in a large quantity of decalcifying solution, at least a quarter, for blocks of average size.

Stirring, agitation, and the use of vacuum hastens decalcification and should be employed when possible. Tissues suspended in the upper third of the solution during decalcification will allow the calcium salts to sink to the bottom of the container as they are dissolved.

Suspension of the tissue can be accomplished in a number of ways:

1] Place tissue in a gauze bag suspended with dental floss or string which has been dipped in hot paraffin; or

2] Place in a perforated porcelain dish on the bottom of the container.

Since decalcification acids continue to act on tissue specimens during any subsequent tissue handling (including paraffin storage) it is important that every trace of decalcifying solution be removed by washing the specimens in running water for several hours before processing can take place. For determining the decalcification end point methods see the following:-

PERENYL'S METHOD

1] Place calcified specimen in a large quantity of PERENYL'S solution until decalcification is completed.

Preparation of PERENYL'S solution

A] 10% Nitric acid……..……. 40 ml

B] Absolute Alcohol (Ethanol) ……..……..30 ml

C] 0.5 chromic acid (aqueous)……..30 ml

The various ingredients may be kept in stock, and should be mixed immediately before use. This solution may aquire a blue violet tinge after a short while, but this will have no effect on its decalcifying properties.

2] Wash in running water for 2 hours. Store in 95% alcohol if processing cannot continue following the step.

3] Dehydration, clearing and impregnate with paraffin or processing as desired.

3.1.5. **Adrenal gland:** Do not remove the fats, because it will be autolysed within 1-2 hours.

3.1.6. **Gastro-intestinal tract** (GIT):

A] Inject Formalin into the GIT by tube.
B] Inject the fixatives intraperitoneally
} To avoid autolysis

Any tubular organ must be place on a carton to stretchened the tissue

3.1.7. **Amputated limbs**:

- ✓ Preserved and fixed in formalin tank with 10% Formalin.
- ✓ Decrease the temperature up to 4^0C.
- ✓ One can put the sample in chill room of -20^0 C to -40^0C

3.1.8. **Eyes:** It should be sampled within an one hour, otherwise, retina, cornea, crystalline and etc will be autolyzed.

Procedure of eye sampling

- ❖ Remove the fat around the eye
- ❖ Never infiltrate fixative into the eye ball, because it may destroy the retina
- ❖ Place in fixative for 48 hours
- ❖ Dissect horizontally up to the optic nerve by razor blades
- ❖ After sectioning the sample place again in appropriate fixative for not less than 7 days
- ❖ Prepare each structure of the eye the same as other organs for the following step

3.1.9. **Lungs:** Inject fixative to the lung through the tracheal tube at 100 Hgmm, otherwise, above this pressure destruction of lung tissue will occur.

3.1.10. **Lymphatic tissue**: This includes lymph node and Spleen

Procedure

- ✓ Take knife for sampling
- ✓ Take a thin sliced part which is about 5 cm or 3 cm, because lymph node is covered by capsule, hence it will not allow easy penetration of the fixatives.

3.1.11. **Pituitary:** Found in hypothalamus

3.1.12. **Muscles**:

a) Cardiac: Take the sample at the vertex of the heart for parasite

b) Skeletal: Take 5cm x 5cm including the lesion or the parasite, like cysts.

c) Smooth: Take 5cm x 5cm of the desired lesion and place it on a "carton".

3.1.13. **Liver:** Take 5cm x 5cm of the desired lesion and put it in appropriate fixative.

3.1.14. **Reproductive organs:**

a) Male:

- Taken to test for artificial insemination (AI) evaluation.
- In case of the whole testes, sample the central part.

b) Female: Take ovaries, uterus, cervix and the mammary glands from the affected areas

3.1.15. **Kidney**: Take 5cm x 5cm of the desired lesion

Handling of Specimen

Specimen should be transported in glass, plastic or metal container or in a plastic bag in 10% formalin. If formalin is not available at hand, place the specimen in refrigerator at 4^0C to slow down autolysis. The container should have an opening larger enough so that the tissue can be removed easily after it has hardened by fixation. However, fresh material is needed for the following purpose:

1. Frozen section
2. Immunocytochemistry
3. Cytological examination
4. Microbiological sampling before histopathology
5. Chromosome analysis
6. Research purpose
7. Museum display

Requirement in Histopathology Dissection Room

1. A cutting board designed in such a fashion that all the fluids will flow directly into the sink.
2. Selves for specimen container.
3. Ready access to sink with hot and cold water.
4. Ready access to formalin.
5. Box of instruments including heavy and small scissors, different sized smooth and

toothed forceps, a malleable probe, a scalpel handle, disposable blades, a long knife and pins for attaching specimen to a cork surface.

6. Labels.
7. A large formalin container.
8. Container with other fixative with instruction on how to mix them at the time of use.
9. Hard saw
10. Balance.

General Principle of Gross Examination:

1. Proper identification and orientation of the specimen.
2. Unlabelled specimen should never be processed.
3. A properly completed histopathology requisition form containing patient's name, age, sex, relevant clinical data, surgical findings, nature of operation and name of tissue submitted.
4. Careful search and examination of all the tissue submitted in order.
5. Surgeon should be instructed to submit all the material that they have removed, not the selected portion from it.
6. Place the specimen on cutting board in an anatomic position and record the following information:
    a. Types of specimen
    b. Structure included.
    c. Dimensions
    d. Weight
    e. Shape
    f. Colour
    g. Consistency
    h. Surgical margin, whether included or not involved by tumour.

7. Measurements are usually given in centimeter unless the specimen is very small in which mm can be used.
8. Endometrial and prostatic tissue should be measured by aggregate pieces in volume.
9. Endoscopic biopsies should be numbered.

Trimming of samples

- ✓ Fixed samples for 48 hours must be trimmed naturally by the Pathologist or if not by Clinician.
- ✓ Samples must be trimmed as small as 3 mm x 3mm or 1mm x 1mm in size.

Histopathological Technique:

Histological technique deals with the preparation of tissue for microscopic examination. The aim of good histological technique to preserve microscopic anatomy of tissue and make them hard, so that very thin section (4 to 5 micron) can be made. After staining, the section should represent the anatomy of the tissue as close to as possible to their structure in life. This is achieved by passing the total as selected part of the tissue through a series of process.

These processes are:

1. Fixation
2. Tissue processing
    2. 1. Dehydration----- removal of water from the sample by ethanol
    2. 2. Cleaning --------- removal of ethanol from the sample by Xylene
    2.3. Impregnation --- removal of Xylene from the sample by paraffin
4. Embedding
5. Sectioning or Cutting
6. Staining

Fixation:

This is the process by which the constituents of cells and tissue are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with various reagents

with minimum loss of architecture or to preserve cells or tissues making its constituents in a condition which is identical to the cell when at live one. This is achieved by exposing the tissue to chemical compounds, call fixatives.

Mechanism of action of fixatives:

Most fixatives act by denaturing or precipitating proteins which then form a sponge or meshwork, tending to hold the other constituents.

Good fixative is most important factors in the production of satisfactory results in histopathology. Following factors are important:

- Fresh tissue
- Proper penetration of tissue by fixatives
- Correct choice of fixatives

No fixative will penetrate a piece of tissue thicker than 1 cm. For dealing with specimen thicker than this, following methods are recommended:

1. **Solid organ**: Cut slices as necessary as but not thicker than 5 mm.
2. **Hollow organ**: Either open or fill with fixative or pack lightly with wool soaked in fixative.
3. **Large specimen**, which require dissection: Inject fixative along the vessels or bronchi as in case of lung so that it reaches all parts of the organs.

Properties of an Ideal Fixative:

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissue in their natural state and fix all components.
3. Make the cellular components insoluble to reagent used in tissue processing.
4. Preserves tissue volume by coagulation.
5. Avoid excessive hardness of tissue.

6. Allows enhanced staining of tissue.
7. Should be non-toxic and non-allergic for user.
8. Should not be very expensive.
9. To change semi-fluid to semi-solid

Temperature:

The fixation can be carried out at room temperature. Tissue should not be frozen once it has been placed in the fixative solution, for a peculiar ice crystals distortion will result.

Speed of fixation:

The speed of fixation of most fixative is almost 1 mm/hour. Therefore, a fixation time of several hours is needed for most specimens.

Amount of fixative fluid:

This should be approximately 10-20 times the volume of the specimen. Fixative should surround the specimen on all sides.

Factor affecting fixation:

1. Size and thickness of piece of tissue.
2. Tissue covered by large amount of mucous fix slowly.
3. The same applies to tissue covered by blood or organ containing very large amount of blood.
4. Fatty and lipomatous tissue fix slowly.
5. Fixation is accelerated by agitation.
6. Fixation is accelerated by maintaining temperature around 60oc.

No single type of fixative is used; however, they are used in combination.

Classification of Fixatives:

A. Tissue fixatives

a. Buffered formalin

b. Buffered gluteraldehyde

c. Zenker's formal saline

d. Bouin's fluid

B. Cytological fixatives

a. Ethanol

b. Methanol

c. Ether

C. Histochemical fixatives

a. Formal saline

b. Cold acetone

c. Absolute alcohol

D. Cold usage

Refrigeration at -4^0C and at time -2^0C is used to preserve samples.

Routine Formalin:

Formalin is sold as 40% w/w which is approximately equivalent to 100% solution of formaldehyde gas in water. It is used as 10% solution in water or normal saline. It does not precipitate protein but combine with NH_2 group to form an insoluble gel, preserve particularly all elements including fats. It keeps phospholipids insoluble in fat solvents. Tissue can remain in it for prolonged periods without distortion. It is compatible with most special stain. It has pungent smell, cheapest and most popular fixative.

It can be prepared with:

- ✓ Magnesium carbonate
- ✓ Carbon dioxide
- ✓ Monobasic salts

Preparations of solutions

Formalin 10%

Formalin 37 – 40% ----------10 ml

Distilled or Clean Tap water ----------90 ml

Formalin Saline 10%

Formalin 37 – 40 % ----------100 ml

Tap water ---------- 900 ml

Sodium Chloride ---------- 9gm

Buffered neutral formalin 10%:

37 – 40 % Formalin ---------- 100.0 ml

Distilled water ---------- 900.0 ml

Sodium phosphate monobasic ---------- 4.0 gm

Sodium phosphate dibasic (anhydrous) ------ 6.5 gm

The best overall fixative, therefore strongly recommended for routine use. The neutrality of formalin is needed, because there is a base and an acid in the tissue.

Ethyl Alcohol:

It is colorless, inflammable, boils at 78^0C, used in 90-100% strength, mix with Xylene, powerful dehydrating agent, it precipitates protein in particular albumin but not nucleoprotein. It causes shrinkages and hardening of tissues, hence causes hepatic cirrhosis. It destroys mitochondria, preserves glycogen and is useful for histochemistry of glycogen, uric acid, iron etc.

Tissue Processing:

In order to cut thin sections of the tissues, it should have suitable hardness and consistency when presented to the knife edge. These properties can be imparted by infiltrating and surrounding the tissue with paraffin wax, colloidin or low viscosity nitrocellulose, various types of resins or by freezing. This process is called tissue processing. It is done in stages. It can be subdivided into dehydration, clearing, impregnating and embedding. It is important that all specimens are properly labeled before processing is started.

For labeling, pen containing ordinary ink should not be used. Printed, graphite pencil written, type-written or India ink written labels are satisfactory.

A system of transportation is required to carry the tissue through various steps in processing. The cut specimens are put in muslin cloth together with their labels and are then transported from reagent to reagent in metal containers that have perforated walls, so that the reagent enters into the tissues.

Tissue processing is a long procedure and required 24 hours. Tissue processing can be done by manually or mechanically.

Manual Tissue Processing:
In this process the tissue is changed from one container of reagent to another by hand.

Mechanical Tissue Processing:
Automatic tissue processors are available. In this processor, there are different jars containing reagents. These are arranged in a sequence. The tissue is moved from one jar to another by a mechanical device. Timings are controlled by a timer which can be adjusted in respect to hours and minutes. Temperature is maintained around $60^{o}C$.

The processing, whether manually or mechanically, involves the same steps.

Sequence of manual tissue processing:

A. Dehydration:
Dehydration is the removal of all extractable water by dehydrants like ethanol which is most commonly used, isopropyl alcohol, dioxane, acetone, and tetrahydrofuran. Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%. The duration for which tissues are kept in each strength of alcohol depends upon the size of tissue, fixative used and type of tissue; e.g. after fixation in aqueous fixative delicate tissue need to be

dehydrated slowly starting in 50% ethyl alcohol directly whereas most tissue specimens may be put into 70% alcohol. Delicate tissue will get high degree of shrinkage and distortion by too great concentration of alcohol. Acetone provides a rapid method, when required as “stat” methed.

The volume of alcohol should be 50-100 times that of tissue.

B. Clearing:

During dehydration water in tissue has been replaced by alcohol. The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble, ie, the clearing reagent must be miscible with the dehydrants and the paraffin wax.This step is call clearing.

Clearing of tissue is achieved by any of the following reagents:

- *Xylene* is most commonly used. Small piece of tissue are cleaned in 0.5 – 1 hour; whereas larger (5cm or more thick) are cleaned in 2-4 hours.
- *Chloroform* is the clearing agent of choice in our laboratory
- *Benzene* and *Toluene* are difficult to remove during the paraffin impregnation.
- *Carbon tetrachloride*

C. Impregnation with Wax:

This step is the complete removal of the clearing reagents, by substitution, as the paraffin penetrates the tissue with use of no less than two and preferably, paraffin baths. three This is allowed to occur at melting point temperature of paraffin wax, which is 56 – 58^0C or 54-60^oC. Frequent check of the paraffin temperature of the paraffin baths is a *must* , since more than 50C above melting point of the paraffin will cause excessive tissue shrinkage and hardening. Volume of wax should be about 25-30 times the volume of tissues. The duration of impregnation depends on size and types of tissues and the clearing agents employed. Longer periods are required for larger pieces and also for harder tissue like bones and skin as compared to liver kidney, spleen, lung etc. Xylene is easiest way to remove. Total duration of 4 hours is sufficient for routine impregnation.

Types and recommended paraffin Wax to be employed for Impregnation:

1. Paraffin wax
2. Water soluble wax
3. Other material, like colloidin, gelatin, paraplast, Bioloid, etc.

Paraffin wax is used routinely. It has hard consistency, so section of 3-4 micron thickness can be cut. However, *Paraplast* is perhaps the best embedding medium for use in conventional histopathology slide preparation. But, it should not be used, when thin – walled circular specimens like veins, eyes, trachea, and cysts. It prevents complete expansion of the lumen.

Straight paraffin is not recommended, because the cutting consistency of the product is not firm enough. The sections compress and wrinkles are difficult, if not impossible, to remove. It lacks the elasticity that aids in obtaining wrinkle – free ribbons. But it can be used if *10 -20 % beewax* is added to overcome these deficiencies.

D. Blocking:

Impregnated tissues are placed in a mould with their labels and then fresh melted wax is poured in it and allowed to settle and solidify. Once the block has cooled sufficiently to form a surface skin it should be immersed in cold water to cool it rapidly.

After the block has completely cooled it is cut into individual blocks and each is trimmed. Labels are made to adhere on the surface of the block by melting the wax with a metal strips sufficiently wormed.

Summary of Paraffin Wax Embedding: There are many, but this is the commonest.

Dehydration

70% alcohol 1 hour

90% alcohol I 1 hour

90% alcohol II 2 hours

100% alcohol I 1 hour

100% alcohol II 2 hours

100% alcohol III 2 hours

Clearing

Xylene I 2 hours

Xylene II 2 hours

Wax Impregnation

Paraffin wax 1 1 hour

Paraffin wax II 1 hour

Paraffin wax III 1 hour

EMBEDDING IN PARAFFIN

Embedding is the orientation of tissue in melted paraffin, which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut by microtome sharpest knife. Each laboratory has a preference as to the method used for embedding with the two most often used being lead L's and the Tissue Tech embedding system.

PREPARATION OF SECTIONS

CARE AND USE OF MICROTOME ANT ITS KNIVES

The cutting of good sections greatly depend upon practical experience and a complete thorough knowledge of the equipment used. *Manual dexterity a must*; without it one may face a difficult task in handling the fine manipulative detail required in section cutting. Since the results produced by histologic technique depend greatly upon the sharpness of the knives used to cut the sections, it is imperative that each well trained technician know how to care for his knife as well as how to use. A perfect edge on a microtome knife is difficult to describe, however, with a good

knife edge, sections of 3 microns in thickness should easily be cut from well-processed, average-sized, tissue blocks. The ribbon from the sample block should be flat and unwrinkled fashion, as much as paper comes off a printing press. Microscopically, the section must show no vertical lengthwise scratches or horizontal thick and thin areas.

Hurried and inadequate introductory and/or initial training will reflect badly for years afterward; conversely a high standard of training will prevail admirably throughout one's career. *Speed* in performing any phase of histologic technique should never be a primary objective since, it only leads to unsatisfactory processing, cutting, and staining of the tissue sections. A well trained tissue technician will produce first rate sections in a far shorter time than one who always is aiming primarily at speed.

Commonly encountered problems in cutting and their causes

N$^{0.}$	**Problems in cutting**	**Causes**
1	***Crooked or uneven ribbons***	Knife and block not parallel Blocks not square or rectangular Irregular knife edge Nonhomogenous or impure paraffin
2	***Compressed, wrinkled or jammed sections***	Dull knife; warm knife and / or block Loose microtome set screws, too vertical knife….. and too thin sections
3	***Crumbling or tearing of sections***	Incomplete dehydration, clearing, and/or infiltration Paraffin too hot during infiltration and/or embedding
4	***Split ribbons or lengthwise scratches***	Nicks in knife, dirt on block or knife, knife tilt too great, artifacts, such as dirt, foreign bodies, grit
5	***Lifting of sections or ribbon on upstroke***	Too vertical a knife tilt, diry knife edge, dull knife
6	***Thick and thin sections***	Too large a block, loose set screws for

		blocks and/or loose knife holder, block too hard to section without soaking, Tilt of knife, insufficient to clear bevel with resultant compression of tissue.

Staining:

Staining is a process by which we give colour to a section. There are hundreds of stains available.

Classification of Stains:

Generally the stains are classified as:

A. Acid stains

B. Basic stains

C. Neutral stains

All dyes are composed of acid and basic components. Dye is a compound which can colour fibres and tissue constituents.

Acid Dyes:

In an acid dye the basic component is coloured and the acid component is colourless. Acid dyes stain basic components e.g. eosin stains cytoplasm. The colour imparted is shade of red.

Basic Dyes:

In a basic dye the acid component is coloured and the basic component is colourless. Basic dyes stain acidic components e.g. basic fuchsin stains nucleus. The colour imparted is shade of blue.

Neutral Dyes:

When an acid dye is combined with a basic dye a neutral dye is formed. As it contains both coloured radicals, it gives different colours to cytoplasm and nucleus simultaneously. This is the basis of Leishman stain.

Special stains:

When a specific components of tissue e.g. fibrous tissue, elastic tissue, nuclear material is to be stained, certain special stains are used which specifically stain that component tissue.

Procedure of staining:

Every stain is to be used according to a specified method. Staining can be done either manually or in an automatic stainer.

Manual Staining:

In a small laboratory when a few slides are stained daily, this is the method of choice. Although it is time consuming it is economical. Different reagent containers are placed in a special sequence and the slides are removed from one container to another manually.

Automatic staining:

In a busy histopathology laboratory when hundreds of slides are stained daily, an automatic stainer is required. This method has different containers of staining reagents. They are arranged according to the desired sequence. It has a timer, which controls the time for stay of slides in a given container. It has a mechanical device which shifts the slides from one container to next after the specified time. Advantages of automated stainer are:

a. It reduces the man power
b. It controls the timing of staining accurately
c. Large number of slides can be stained simultaneously
d. Less reagents are used

Slides stained either manually or by automatic stainer, pass through same sequences.

Haematoxylin and Eosin staining:

It is the most common used routine stain in histopathology laboratory.

Reagents:

1. Mayer's Haematoxylin
2. Acid alcohol
3. Ammonia water
4. Alcoholic eosin solution

There are two methods of staining when haematoxylin is employed. They are *Progressive* and *Regressive*.

The progressive method is uses Haematoxylin sol with excess Aluminum salts, thus increase selectivity for nuclei. Progressive can be washed in water before second staining material. This method eliminates the necessity for differentiation and bluing the sections. In this method the background is completely colourless The stained slides often fade after one to three years . This problem can be eliminated, if the slides are washed in running water for a minimum of twenty minutes. This simple technique is good for teaching others for giving a consistent results even if two different persons stains sections from the same block. The sectioned sample can be left in Haematoxylin for hours without over stains. Any well fixed tissue samples take short time to stain. Therefore, there is definite reduction in time performance of the stain itself. Furthermore, the sample can be embedded in paraffin, celloidin or frozen sectioned.

In case of regressive, neutral Haematoxylin is used. There is over staining. It is washed by acid alcohol, which requires Ammonia water or Lithium carbonate to neutralize. In addition, during staining it requires experienced and trained eyes to stain properly or it is obligator to use microscope to control this circumstances

.

Preparation of Mayer's Haematoxylin

- ✓ Haematoxylin crystals ---------- 1.0 gm
- ✓ Distilled water ---------- 1000.0 ml
- ✓ Sodium iodate ---------- 0.2gm
- ✓ Ammonium or Potassium alum----------50.0 gm (phosphate monobasic or dibasic (anhydrous)
- ✓ Citric acid ---------- 1.0 gm
- ✓ Chloral hydrate---------- 50.0gm

Procedure for the mixture of ***Mayer's Haematoxylin***

- ❖ Prepare all the general equipment like bench, rack, Bunsen burner, scale and the like.
- ❖ Dissolve Alum in distilled water from measured one (1000 ml) without hearting.
- ❖ Add Haematoxylin in this solution.
- ❖ Add Sodium iodate, Citric acid and Chloral hydrate.
- ❖ This staining solution should be kept in brown bottle well topped and protected from direct sunlight. Keep / store in dark place while not in use.

Haematoxylin staining is an *indirect staining*, which requires mordant like Aluminum Ammonium Sulfate or Aluminum Potassium Sulfate to form tissue – mordant – dye complex before satisfactory staining. Mordant dye act as basic stains in indirect stain methods. Certain accentuaters are required to increase the staining power. In case of the staining of central nerve system accelerators like chloral hydrate is essential. To hold the dyes trapping agents like Tannic acid can be implemented. Haematoxylin solution can get ripened if put in air, light, warmth, an use of oxidants like Sodium iodate (NaI_2), Potassium permanganate (K_2MnO_2), and Mercuric oxide (HgO_2). On the hand, Simple aqueous or alcoholic solution dyes such as Eosin, Methylene blue are directly staining tissue perfectly, hence, they are *direct stains*

In the case of direct staining

PREPARATION OF COUNTER STAIN FOR HAEMATOXYLIN

Many counter stains could be listed here which would give good and almost identical results. It is for this reason that we will only list those counter stains which give different staining results.

1% STOCK ALCOHOLIC EOSIN

- ✓ Eosin Y, water soluble ----------1.0 gm
- ✓ Distilled water ----------20.0 ml : dissolve and add:
- ✓ Alcohol, (Ethanol or Ethyl) 95% ----------80.0 ml

WORKING EOSIN SOLUTION

- ✓ Eosin stock solution…….... 1 part
- ✓ Alcohol (Ethanol or ethyl) 80 %..……3 parts

Just before use, add 0.5 ml of Glacial Acetic acid to each 100 ml of stain and stir.

Counter stains is referring to stains applied to render the effects of another stain more discernible or separate or make clear, to show inclusion bodies, pigments, minerals or cytoplasmic changes to arrive at the definitive diagnosis. With this in mind, the most common errors that occurs in eosin staining are improper use of alcohol after eosin, alcohol one in dehydrative use, during the removal of excess eosin, to avoid the overshadowing of inclusion bodies, pigments, minerals or cytoplasmic changes and passing through alcohol rapidly. Therefore, proper differentiation of the eosin by alcohol is *MUST*.

Staining Procedure:

1. Put the sections fixed on slides in xylene for 3 minutes.
2. Then transfer to absolute alcohol for 3 minutes.
3. Transfer to 80% alcohol for 2 minutes.
4. Place in 50% alcohol for 2 minutes.
5. Wash the slide in running tape water for 1 minute and put in Harris's Haematoxylin for 5- 7 minutes.

6. Wash in running tape water for 30 seconds
7. Wash excess dye in 1% acid alcohol by continuous agitation for 15 second.
8. Wash in running tape water for 30 seconds
9. Give 2-3 dips in ammonia water solution until tissues attain a blue colour.
10. Wash in running tape water for 30 seconds
11. Counter stain with eosin for 3-5 minutes.
12. Wash in running tape water for 30 seconds
13. Dehydrate by keeping in increasing concentration of alcohol (2-3 minutes in 50%, 70%, 95% and absolute alcohol).
14. Clear it in xylene and mount with DPX or Canada balsam.

Result:

Nuclei…….... Bright blue
Muscle, keratin…….. Bright pink
Collagen and cytoplasm……. Pale pink
Erythrocytes……... Orange red

In our condition the following H-E staining procedure is helpful.

- ✓ Xylene 1, 2.
- ✓ Absolute alcohol 1,2,3,4.
- ✓ Alcohol 95% 1.
- ✓ Alcohol 70% 1
- ✓ Water
- ✓ Haematoxylin
- ✓ Water
- ✓ Eosin
- ✓ Water
- ✓ Alcohol 70% 1
- ✓ Alcohol 95% 1.

- ✓ Absolute alcohol 1,2,3.
- ✓ Xylene 1, 2.3.
- ✓ Mount by DPX, Canada balsam…
- ✓ Labeling
- ✓ Interpretation
- ✓ Filing in dark place or in slide box.

CHEMICAL DYE STAINING REACTIONS

A dye must ionize in solution to produce coloured cations or anions which are capable of uniting with protein and other tissue constituents to form coloured compounds. A typical basic dye is positively charged (cationic) at a pH rages that are used in histology, and an acidic dye is negatively charged (anionic); an amphoteric dye has an uncharged point (isoelectric point) within the pH range and is basic below and acidic above this pH.

Table ------ Ionization of basic, acidic, and amphoteric dyes

Dyes	**pH**								pH affinity
	3	4	5	6	7	8	9	10	
Crystal violet	+	+	+	+	+	+	+	+	Basic dye (Cationic
Orange G	_	_	_	_	_	_	_	_	Acidic dye (anionic)
Haematein	+	+	+	+	_	_	_	_	Amphoteric dye

In the same way, electrically charged groups are present in the proteins and other components of tissues. At the ordinary pH range tissue constituents, like dyes, are basic, acidic, or amphoteric (Table -----). DNA, RNA, and phospholipids are acidic due to their phosphoryl groups, while mast cells, cartilage and some mucous secretions of glands contain acidic sulphuryl and carboxy groups. Collagen, red blood corpuscles, and the granules of eosinophil leucocytes are basic due to the predominance of basic amino groups, while the amphoteric proteins of cell cytoplasm and muscle contain a balance between acidic carboxyl and hydroxyl groups and basic amino groups.

Small changes in pH will make amphoteric substances in tissues basic or acidic, whilst larger changes alter the electrical charges of basic or acidic tissue components.

Table : ---- Ionization of tissue components

Basic (+)	Acidic (-)	Amphoteric (+/-)
Collagen	DNA , chromatin	Cytoplasm
Red blood corpuscules	RNA	Muscle
Granules of eosinophil	Myelin	
Leucocytes	Cartilages	
	Mucous secretions	
	Mast cell granules	

As dyes and tissues are both ionized, they will react together, and do so direct staining techniques; electropositive stains unit with electronegative tissues and the vice versa. Mordanted dyes act as basic stains in indirect staining methods. The differentiation of acidic and basic staining by acids and alkines is due to ionic charges brought about by the charges in pH; both the stains and the tissue will be affected. For instance, the addition of alkaline diminishes the ionic association of basic amino groups and increases the dissociation of carboxyl groups, thus making the proteins of cell cytoplasm, and muscle negatively charged; acidic will have the reverse effect and proteins will become positively charged. The intensity and 'fastness' or grip of a stain depends upon the avidity of its ionized radicals for tissue components and from the number and the strength of these chemical bonds. Carboxyl groups are only weakly acidic whilst phosphoryl groups are stronger and the sulphuryl groups are stronger still; thus sulphonated dyes (acid fuchsin, Orange G) have strong affinity for the basic groups of proteins, which are in plentiful supply, and will unite with them to form powerful bonds that require strong differentiation. These sulphonated dyes will successively compete for ion in tissues, and will even displace less avid dyes that have already combined with tissues, thereby staining or differentiating the first dye. Mordanted dyes combine with tissue in the same way as direct basic stains, but the metallic mordant forms a firm tissue-metal-dye linkage.

Successful dye staining by chemical interaction can be briefly described as a presentation of fixed tissues with ionized chemical groups to staining solutions with different and opposite charged radicals that have an affinity for each other.

PHYSICAL DYE STAINING

Dyes can combine with tissues by *adsorption*, which is the propertyof solid objects, especially when in a state of fine division, to attract and hold other substances on their free surfaces (Bayliss, 1906) ; such stains can usually be differentiated in water or alcohol. Physical factors that are important include the *density* and *permeability* of the tissues. Dense tissue substances that have a large amount of protein chain per unit volume hold a large amount dye and will remain coloured after these dense tissues have been discolourized by differentiation. Similarly, highly permeable tissues will be quickly stained and quickly differentiated, whilst less permeable substances require longer to take up a stain and more resistant to differentiation. Closely woven or ; 'close knit' tissues (Gurr, 1962) have a tight meshwork of molecular protein chains that first resist penetration, then resist differentiation of dye. Moreover, tissue substances may change their permeability with age; Lendrum et al., (1962) described staining methods for fibrin of different ages which are based upon the fine structure of fibrin becoming more open as the fibrin ages and thus more capable of holding dyes with large molecules. This principle has also been applied to the staining of amyloid (Lendrum, Slidders, and Fraser, 1972). It has already been seenthat dye staining is sometimes due to solubility, the Sudan fat stains are the best examples of these and they will not be discussed further here. Finally, it must be re-emphasized that dye staining is usually brought about by a mixture of physical and chemical affinities between the dye and the tissue constituents.

THE SCIENTIFIC CONTROL OF STAINING METHODS

It has been shown that histological staining can be accomplished by precise chemical reactions, but is still carried out by less exact chemical and physical processes. This means that staining methods will vary considerably according to a large number of different factors and explains the

numerous modifications of staining techniques that have been developed. The techniques described in the following chapters have all been tested, but every histologist has had the discouraging experience of a method that gives poor or even negative results. This may be due to unreactive tissues; unsuitable fixation; to differences in composition and solubility of the dyes; to the inadequate ripen or the deterioration of a staining solution; to variations in the temperature, pH of the water or stains; and to many other factors.

This lack of scientific precision in dye staining means that histology must be prepared to control his own methods in order to obtain the good results.

A method which works well in one country may not be satisfactory in another country, or even in a different part of the same country, if climatical condition varies or the composition or pH of tap water alters. The direction given in a staining technique should be carefully followed, but usually represents a compromise and should not be regarded as inviolable. The laboratory should hold a stock of mounted unstained paraffin sections of tissues known to contain special tissue elements, pathological changes, bacteria, metals and the like. These are used as *control solutions* and are stained at the same time and also exactly the same way as the sections under investigation. This is best achieved by placing the slide with the test section, back to back with slide of *control section*; the two slides are treated identically, being stained, differentiated, etc., for the same lengths of time. These controls avoid frustration of staining for special structures, such as glycogen or acidic bacilli, and then being unable to tell whether the tissue contained no glycogen or acid-fast bacilli, or whether the technique was a failure; a glance at the control section will decide this. Every laboratory worker should be able to make controlled variations of a technique. Multiple haphazard alterations staining method may be chance give excellent results, but it is then usually impossible to reproduce the technique. Changes in a staining method must be made one at a time, in an orderly and carefully recorded fashion. A large number of sections should be cut from the same tissue block, and a series of variations of each step of the method should be made, one by one, with one --- change in each section. By keeping the rest of the technique constant the sections will show which part of the method was defective and how it can be corrected. Occasionally it is required to stain a foreign substance or a pathological change which is not present to any appreciable amount in not tissue or in the laboratory control sections. In these cases an artificial ‘tissue block’ can be made of gelatin containing the substance under

examination (Cameron, 1930), or the material may be injected into a laboratory animal ---- the tissues used for control sections.

In the laboratory it is essential to appreciate the strengths and the limitation of the methods in use. By an understanding of the modes of action of stains and their applications to histology, it is possible to obtain the best results to recognize the cause and the cure of failures when they occur.

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Please refer the following in books or google.

Semen examination

Indication and sample collection

Volume and gross appearance of ejaculate

-Motility of spermatozoa and sperm density

-Microscopic evaluation of semen/**-Biochemical tests**

Evaluation of reproductive performance in male animals

GOOD LUCK!!!