The book is a systematic clinical / Laboratory diagnostic manual for most of the bacterial and parasitic fish diseases. A brief introductory section on applied anatomy of fishes is offered in this manual . The last section of the manual is a colored atlas of selected fishes' anatomy. The manual is recommended for both undergraduate and graduate students who study Fish Health, Fish Diseases , Fish Bacteriology , Fish Pathology and Fish Anatomy. The book is also very useful for aquatic veterinarians , fish pathologists, fisheries scientists and marine biologists.

Dr. Alaa Eldin Eissa has earned Ph.D. in Aquatic Animal Health from Michigan State University, USA in 2005 and a Masters in Fish Diseases from Cairo University in 1997 and a Bachelor of Veterinary Sciences from Cairo University in 1993. He is currently working as an associate professor of Fish Diseases at Faculty of Vet. Medicine Cairo University.

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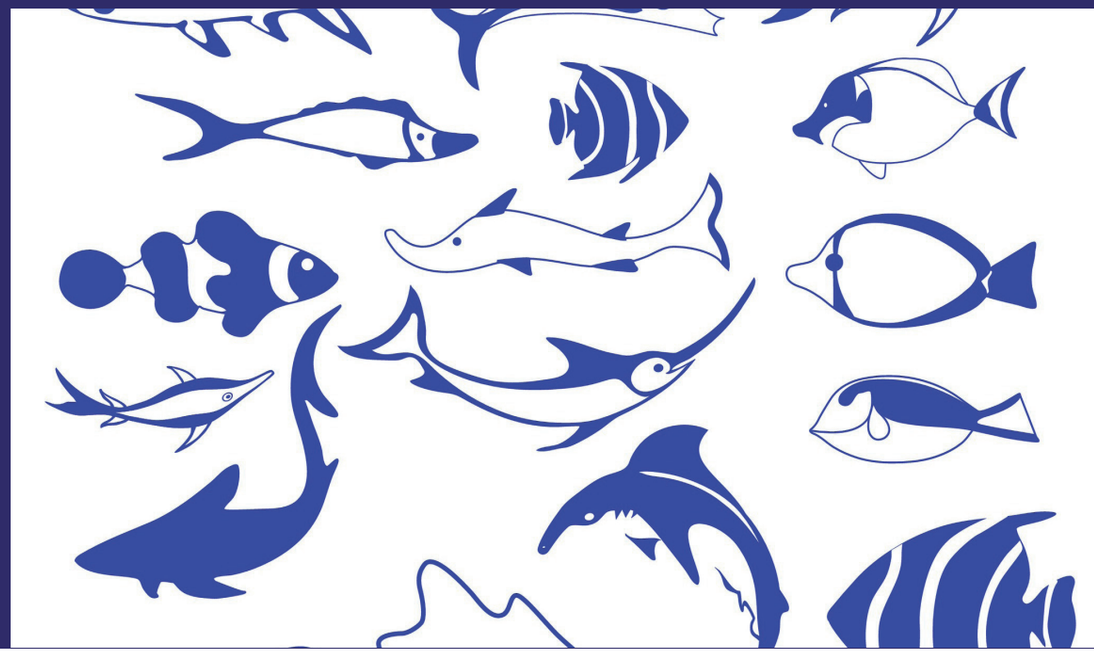
Practical Manual of Fish Diseases

Eissa



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Clinical and Laboratory Manual of Fish Diseases

Dr. Alaa Eldin Eissa, D.V.M., Ph.D.

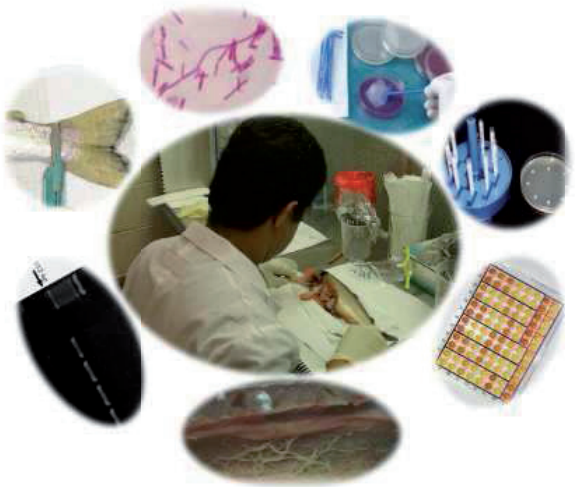
Associate Professor

Department of Fish Diseases and Management

Faculty of Veterinary Medicine

Cairo University

Giza, Egypt



2016

PREFACE

The aquatic veterinary practice have witnessed the publication of many diagnostic manuals that mainly focused on specific species , specific group of fishes , specific group of pathogens or specific diagnostic procedures. However, comprehensive forms of diagnostic manuals are relatively scarce. Thereby, a wide scope of clinical and laboratory procedures have been addressed in the current manual. The manual contain detailed information about the finfish anatomy, farm visits, fish reflexes/ behavior , possible etiologies / description of the lesions of the most common finfish diseases, transportation, anesthesia , sampling , laboratory bacteriological and parasitological examination. The last chapter of the manual is a briefed colored atlas of fish anatomy. Ultimately, the manual will be very useful guide to aquatic veterinarians , fish biologists , veterinary students , wildlife / fisheries students , biology students and aquatic veterinary technicians .

NOTE: The mention of products brand-names in the text of this manual is not a recommendation to any of these products, but rather used as a descriptive model for the reader.

Alaa Eldin Eissa

April, 5, 2016

Index

CLASSIFICATION OF FISHES

Fishes can be classified according to the following

1. Skeleton nature

a. **Bony (Osteichthyes):** Cichilids, Cyprinids, Mugillids, Clarids and Salmonids

b. **Cartilaginous (Chondrichthyes):** as Sharks, Skates, Sea lamprey.

2. Presence or absence of Jaw

a) Jawed Fishes: Cichilids, Cyprinids, Mugillids, Clarids and Salmonids, Sharks, Skates

b) Jawless Fishes (Cyclostomata): Sea Lamprey and Hag Fish

3. Temperature

Able to live, feed, grow and reproduce in the following water temperature average:

a) **Cold water fish:** 0℃ -15℃ such as Salmonids.

b) **Temperate water fish:** temperature range from 15℃ -35℃ while optimum temperature of (22℃ -25℃) such as tilapia , carp and *Mugil*.

c) **Warm water fish:** more than 35℃, such as *Oreochromis mozambicus*.

4. Salt tolerance

a) **Freshwater fishes:** lives at salinity less than 0.5 ppt (0‰ - 0.5‰), e.g. Carp, Clarias (catfish), Nile tilapia and Nile perch.

b) **Brackish (estuarine) water fishes:** lives at salinity range (2‰ to 12‰) such as grey mullet (*Mugil*).

c) **Marine fishes:** lives at salinity range 17ppt to 40ppt (17‰ - 40‰) e.g. Sea bass, Sea bream, grouper...etc.

5. Feeding habits

a) Carnivorous as Nile perch, sea bass.

b) Herbivorous as grass carp.

c) Omnivorous as tilapia, clarias and common carp fish.

d) Detritus (Plankton feeders) such as mullet (*Mugil*).

6. Position in water while feeding

a) Surface Feeders: eats from the surface of the water as tilapia

b) Eats along the zone of water column: such as carp

c) Bottom Feeders: eats from the bottom such as catfish

7. Migration

a. **Non Migratory Fishes:** spend their life in the same water habitat

b. Migratory Fishes: The physiological adaptation of different fish species and searching for food and light let some fish to migrate between water of various salinities.

c. Acc. to migration patterns:

i. Anadromus: migrates from marine to freshwater such as salmonids.

ii. Catadromous: migrates from fresh water to marine such as eels (anguillids).

iii. Amphidromus: migrates from both marine to freshwater then from freshwater to marine such as *Mugil* (mullet).

8. Type of breeding

a. Although most species have male and female sexes, hermaphrodites might occur.

b. External Fertilization (Oviparous): Most fishes are oviparous, where eggs and sperms may be discharged into the water for external fertilization.

i. **Mouth breeders:** takes the eggs and larvae and brood them in mouth (*Sarotherodon gallilaeus*).

ii. Substrate breeders: breed in nests that made on the pond bottom (*Tilapia zilli*). Zilli is a mouth breeder.

iii. Substrate and mouth breeders: Breed on the pond bottom but brood the eggs and larvae in the mother ´S mouth while she is fasting during that time (*O. niloticus, O. aureus, O. spilurus and O. mozambicus*).

c. Internal Fertilization (Viviparous or live bearers): Swordtail and guppies possess internal fertilization behavior where male fish deposit milt (semen) into the female tract where sperm fertilizes the ova over there and larvae get out after a while.

ANATOMY AND PHYSIOLOGY OF FISHES

EXTERNAL ANATOMY (TOPOGRAPHICAL ANATOMY) OF FISHES

1. Morphology of fishes (body shapes)

Biological importance of fish body shapes: The shape of fish body plays an important role in swimming and survival of fish in their habitat.

- **Fusi-form** as salmonids
- **Compressi-form** as tilapias, common carp and seabream.
- **Depressi-form** as solea spp.
- **Angulli-form** as eels
- **Globi-form** as gold fish.

2. Regional anatomy of fishes

Fish body is divided into three main body regions which are the head region (anterior part), trunk region (middle part) and tail region (posterior part).

- **Head region**

  It includes:

  1. Mouth opening, mouth cavity and pharynx ----- -Digestive system .
  2. Gills, gill cover (operculum) and gill slits----------Respiratory system.
  3. Brain and part of spinal cord---------- -----Nervous system.
  4. Eyes, nostrils and barbels in some fishes----------sensory organs.
  5. The head topographic regions are:

     a. Snout : The most anterior part from nostrils to mouth opening

     b. Occiput: The area started from after the nostrils and dorsally covers the brain

     c. Isthmus: the area that lies ventrally between the two opercula starting from the mouth opening till the end of opercula.

- **Trunk region**

1. It extends from the posterior margin of operculum (gill cover) to the constricted part of the body (caudal peduncle).

2. The trunk of fish includes some important topographical areas as belly in ventral aspect and nuchal region in dorsal aspect.

3. The fish trunk carries a clear sensory organ, the lateral line, which is:

   a. well developed: in (Nile perch) Lates niloticus

   b. ill-developed: in (Nile tilapia) Oreochromis niloticus

   c. completely absent: in Sardine

4. Finally, different types of fins, the main organs of locomotion, and different openings as vent, urinary and genital openings are recognized.

- **Tail region**

1. It is the most posterior part of the fish body.

2. It is usually termed the caudal fin.

3. Different shapes of tail fins, namely:

   a. **Forked:** Mugil (Mugilids)

   b. **Truncated:** Tilapia (cichlids)

   c. **Rounded:** Catfish (clarids)

   d. **Lunar:** Carp (cyprinids)

4. The shape of the caudal fin is correlated to the swimming speed of the fish (Forked > lunar > rounded > truncated)

Figure (1) From left to right, caudal fin of Carp (semilunar), Mugil (forked) and Tilapia (truncated).

3. Body appendages

They constitute the organs of locomotion (fins) and sensation (barbels)

Fins

- **Paired fins**

They are located symmetrically on both sides of fish body, they include:

1. Pectoral fin

- Location: just behind the operculum.
- Function: carry chemo-and touch receptors

2. Pelvic fins

- Location: found on the ventral aspect of the belly anterior to the vent.
- Function: stabilizing and braking of fish during swimming.

- **Unpaired fins**

1. Dorsal fin

- Function: stabilization and changing the direction during swimming.

2. Anal fin

- Function:
  - ✓ Braking of fish.
  - ✓ Modified in swordtail fish to act as male copulatory organs.

3. Tail fin

- Function: act as a propeller of fish during swimming.

4. Skin (Integumentary system)

- Unlike the mammalian skin, the fish skin is living and non-keratinized.
- Moreover, it is considered the primary barrier against the environmental pollutants.
- Histologically, the skin layers are cuticle, epidermis, dermis and hypodermis.

Cuticle layer

- It is mainly composed of mucopolysaccharide that formed largely from epithelial surface.
- It contains specific immunoglobulins, lysozymes and free fatty acids that usually react destroy and digest the invasive micro-organisms.

Epidermis layer

- It is the cellular layer of skin and composed mainly of stratified squamous epithelium with mucus secreting cells, club cells and some macrophages and lymphocyte cells.

The dermis layer

It is consists of two layers:

1. *Stratum spongiosum* which is formed of loose collagen fiber with pigment cells (chromatophores), mast cells and scales with scales pockets.

2. *Stratum compactum* which is formed from dense collagen fiber and blood vessels, nerves and sensory nerve ending.

The hypodermis layer

- It is vascular and a looser adipose tissue.
- It is the best site of development of infectious processes.

The significant functions of fish skin

1. Primary barrier against noxious, harmful parasite and micro organisms through:

   a. The physical barrier achieved by its skin intactness and scales.

   b. The mucus barrier helps in getting off the noxious accumulated chemicals and micro organisms away from fish body.

   c. The immunological barrier through the presence of immunoglobulin, lysozymes and free fatty acids in cuticle layer.

2. The mucus of skin minimizes the friction between the fish body and water during swimming, these consequently saving the energy loss.

3. The skin has a respiratory, excretory and osmoregulatory functions

4. The skin contains sensory and chemo receptors.

INTERNAL ANATOMY OF FISHES

1. Respiratory system

The main components of the respiratory system in fish are gills.

I. Gills

- **Location:**
  - Located on both sides of fish head in the branchial cavity.
- **Function:**
  - Respiration (Gas Exchange)
  - Osmoregulation
  - Excretion of the main nitrogenous waste products (Ammonia).
- **Structure:**
  - ✓ **Holobranch:**
    - The gills of fish comprise two sets of four holobranch, forming the sides of pharynx.
  - ✓ **Hemibrach:**
    - Each holobranch consists of two hemibranch projecting from the posterior edge of the gill arch.
  - ✓ **Primary Lamellae (Primary Gill Filament):**
    - Each hemibranch consists of a row of long, thin filaments called **primary lamellae, which stand up like the teeth on a comb.**
  - ✓ **Secondary Lamellae:**
    - The surface area of each primary lamella is increased further by the formation of regular semilunar folds called secondary lamellae.
    - For maintaining the gas exchange and the flow of blood through the secondary lamellae, they are formed from a single and thin layer of epithelium supported and separated by pillar cells.

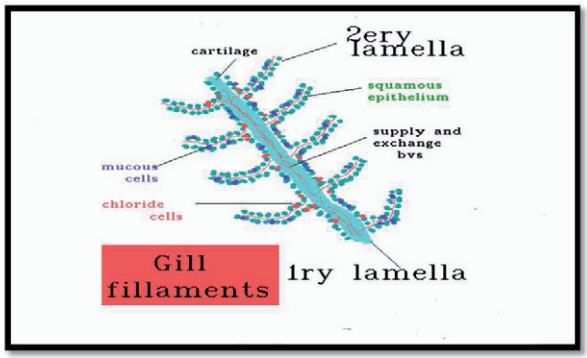


Figure (2) Gill filaments cellular components

- ✓ **Gill Rackers:**
  - The anterior faces of the gill arches are modified into numerous thorny projections called
  - Function: prevent food and debris from reaching the respiratory components of the gills by means of cough reflex.

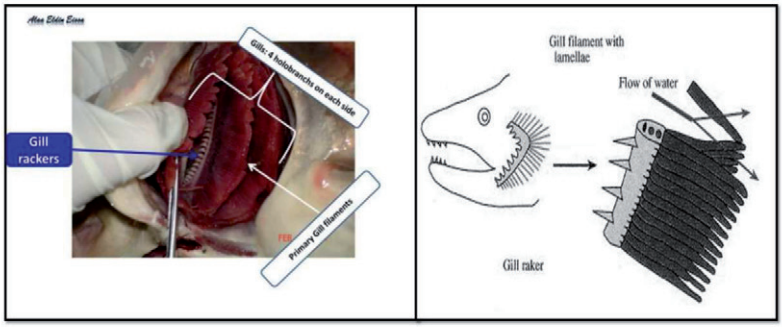


Figure (3) Gill rackers

II. Modified Respiratory Organs

1. Accessory Lung

- Only present in catfish (clarids)
- Location:
  - Located under the gills
- Structure:
  - A very thin sac that is formed of an arborization (tree) of highly vascularized tissue.
- Function and significance:
  - The tissue has the capability to get the atomospheric oxygen from outside the water.
  - The accessory lung is responsible of keeping the fish alive for relatively long period during the drought season and for a shorter period if they jumped out of an aquarium.

2. Lung

- Present in African lungfishes and whales.
- Lungfish must return to the surface to breathe air.
- A lungfish swallows air to fill up an air sac or "lung" This lung is surrounded by veins that bring blood to be oxygenated.
- Its gills alone can't bring enough oxygen to keep them alive.

2. Cardiovascular system

- The circulatory system of fishes is relatively simple, and composed mainly of two main components, the pumping heart and the peripheral blood vessels.
- Fish circulation: The typical fish circulation is a single circuit; heart-gills-body-heart.
- Mammalian circulation: In contrast, mammals have two circuits; heart-lungs-heart and heart-body-heart.

Pumping heart

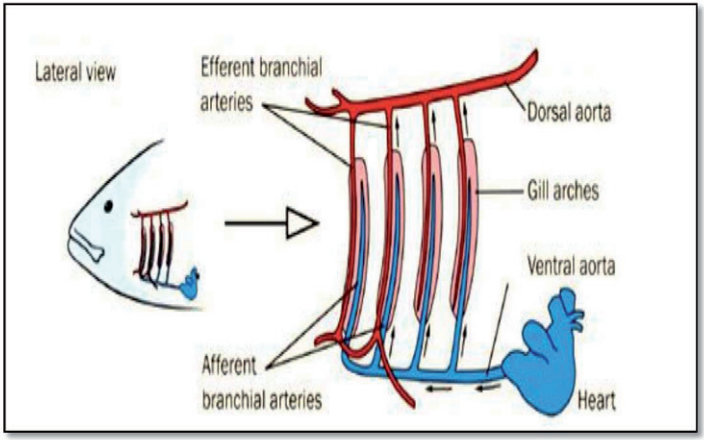


Figure (4) Dorsal aorta , ventral aorta and branchial circulation.

- **Location:**
  - ✓ The heart is situated inside the pericardium anterior to the main body cavity and usually ventral to the pharynx.
- **Structure:**
  - ✓ It has four chambers namely, Sinus venosus, Atrium, Ventricle and Conus- or bulbs arteriosis through which the deoxygenated blood flows in simple succession (one direction).
- **Blood Succession (Circulation):**
  - ✓ The non oxygenated blood is collected from the anterior part of fish via two ducts of Cuvier, and from posterior part of fish through hepatic veins to enter the sinus venosus. A valve at the end of the sinus venosus opens into the atrium.
  - ✓ The atrium has thick, muscular walls. The atrium receives deoxygenated blood and pumps it into the ventricle.

- ✓ The ventricle is the largest and most muscular chamber of the heart. When the ventricle fills with blood it constricts and forces the blood through the *bulbus arteriosus*.
- ✓ The bulbus (conus) control blood flow out of the ventricle and into the ventral aorta.
- ✓ Blood passes through the *bulbus arteriosus* to the ventral aorta.
- ✓ From the ventral aorta blood flows to the gill filaments.
- ✓ The ventral aorta distributes blood to efferent branchial arteries.
- ✓ The efferent branchial arteries, that carry oxygenated blood, join to form the dorsal aorta.
- ✓ Along the length of the dorsal aorta there are lateral branches to the body musculature, while the viscera being supplied mainly by the anterior mesenteric artery.
- ✓ The continuation of the dorsal aorta to the posterior part of the body is called caudal artery while that of the ventral aorta is called caudal vein and together can be called caudal vessels.

Methods of blood sampling (blood collection)

1. Two Ducts of Cuvier

- The blood can be collected from a Duct Cuvier which is blue vessel found under the dorsal end of each 5th rudimentary gill arch.
- This method is preferred in bilaterally compressed such as tilapia.

2. Heart puncture

- It is a simple method for blood collection from large fusiform fishes such as catfishes (*Clarias* and *Bagarus* species).
- The position of heart is determined by making two intersecting imaginary lines. The first line is a sagital line that is passing through mid plane of the abdomen dividing the fish body into two similar halves, while the second one is a perpendicular line that passes between the roots of the pectoral fins.

- Small gage needle is inserted at the point of intersection in upward and slightly forward direction to obtain the blood slowly.

3. Caudal vessels puncture

- It is one of the least traumatic methods for blood collection.
- The site of collection can be approached laterally or ventrally.
- The needle is gently inserted through the skin near the base of the caudal peduncle.
- After feeling the contact with the vertebral column, the needle directed slightly ventrally and lateral to the vertebral column, while the syringe gently aspirates the blood.
- It may be necessary to slowly rotate the needle before blood can be withdrawn.

Figure (5) Caudal vessels puncture in carp (left) and Tilapia (right)

4. Tail amputation

- This method is used to bleed small and young fishes.
- The base of the tail is severed with a scalpel blade.
- An anticoagulated capillary tube is quickly applied to the caudal vessels, and the blood is collected in the tube by the capillary action.

Important facts to be considered during blood sampling

- Fish blood is rapidly coagulated (clotted) due to high fibrinogen, high calcium, and high thrombin contents.
- For making blood films for further blood picture, blood parasites and other blood pathogens, blood should be anticoagulated.
- The anticoagulant of choice for fishes is Sodium citrate 3.8 % (does not affect cell morphology and does not chelate heavy metals.
- To obtain serum sample for serology, blood sample should be left in an oblique position in a cool place (fridge) to ensure good coagulation and good serum separation.

3. Hemopoietic system

❖ Fish hemopoietic system major characteristics

- Fish have no lymph nodes
- Fish have no bone marrow
- Haemopoietic tissues are usually located in the stroma of the spleen and the interstitum of the anterior kidney and to lesser extent, in the periportal areas of the liver and the specialized lymphoid organs like thymus.

❖ Anterior (head kidney)

- The anterior kidney is the primary site for haemopoiesis.
- It is dark red to brown, soft amorphous tissue that has similar structure to the mammalian bone marrow.

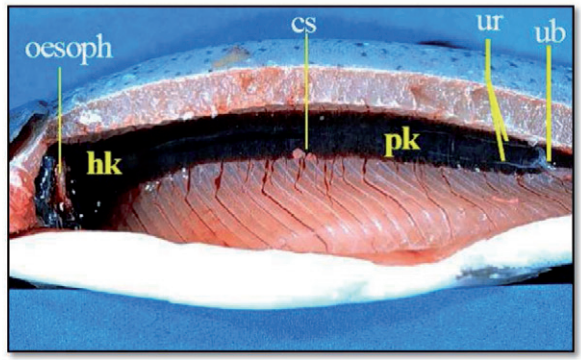


Figure (6) Head Kidney (hk), Posterior Kidney (pk), Corpuscle of stannous (CS),Ureter (ur) and Urinary bladder (ub)

❖ Spleen

- It is the only lymph node like organ to be found in fish.
- It is dark red in color and usually has sharply defined edges.
- It is situated near to the greater curvature of the stomach or the flexure of the intestine.

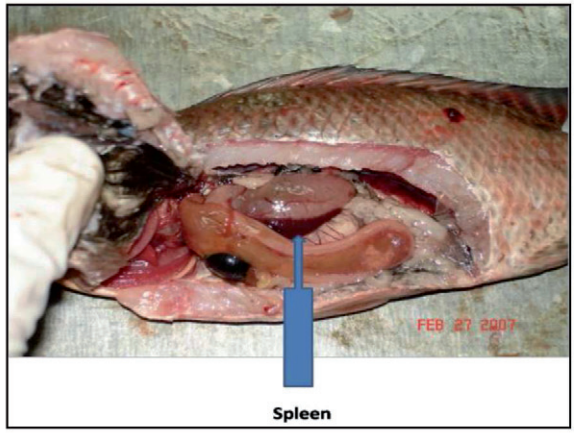


Figure (7) Spleen of Nile tilapia.

❖ Thymus

- Fish have a distinct thymus that is highly vascularized, paired, bilateral organs situated subcutaneously at the dorsal commissure of the operculum.
- In contrast with the mammalian thymus, the fish thymus is difficult to locate in young fishes, but it can be seen grossly in fish older than 5 months.
- In air breathing catfishes, the thymus is caudal to the accessory respiratory organ.

4. The excretory systems

❖ What the excretory and osmoregulatory organs of fish?

1. Skin in young fish
2. Gills
3. Kidney.

❖ Kidney

- The kidney of fish is a mixed organ comprising haemopoietic, reticulo-endothelial, endocrine and excretory elements.
- **Location:** It is usually located in a retroperitoneal position up against the ventral aspect of the vertebral column.
- **Morphology:** It is a light to dark brown organ normally extending along the length of body cavity.
- **Structure:** It is usually divided into anterior or head kidney, which is largely composed of haemopoietic elements, and posterior or excretory kidney.

- ❖ The ureters, which conduct the urine from the collecting ducts to the urinary papilla
- ❖ They may fuse at any level and may be dilated, after fusion, to form a bladder.
- ❖ The urinary duct opens to the outside, posterior to the anus.

5. Osmoregulation

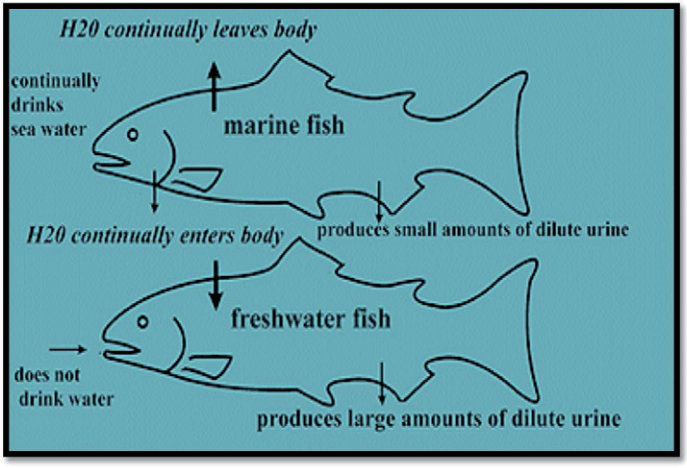


Figure (8) Schematic presentation of osmoregulation in freshwater and marine fishes.

1. Both marine and freshwater fishes must regulate the movement of water across their body surfaces.

2. Marine Fishes: The tissues of marine fishes are less salty than the surrounding water.

a. Water is continually leaving the body of a marine fish through its skin and gills.

b. To keep from becoming dehydrated, a marine fish drinks large amounts of water and produces a small amount of concentrated urine.

c. In addition, its gills are adapted to secrete salt.

3. Freshwater fishes: The tissues of a freshwater fish are saltier than its surrounding environment.

a. Water is continually entering the body of a freshwater fish through its skin and gills.

b. Freshwater fishes do not drink water, and they produce large amounts of dilute urine.

6. The reproductive system

- The fish show greater diversity in the reproductive patterns than does any other group in the animal kingdom.
- An understanding of fish reproductive anatomy and physiology and its patho-physiology is particularly important in cultured species, where the egg production and larval stages are normally the most critical in terms of economic as well as biological efficiency of the system.

- **Testes:**
  - The testes are paired organs, suspended by mesentries from the dorsal abdominal wall, alongside or below the swim bladders.
  - They vary in size from small strands tissue in juvenile to large organs approaching 12% of the total body weight.
  - There is a main collecting duct (the vas deferens) which conducts the mature spermatozoa to an excretory meatus at the urinary papilla.
- **Ovaries:**

- The female genital tract varies in structure from simple cluster of ovarian follicles found in oviparous species to the very complex organ found in viviparous species.
- This not only produces eggs but also act as a spermatozoa store, a vagina and uterus where the embryo can be nourished (live bearer fish).
- The mature ovaries can represent as much as 70% of the total body weight.
- They are also suspended at the abdominal wall by mesenteries.

Figure (9) Arrows to testes exposed in a dissected whitefish and ovaries packed in a plastic zip lock bag.

6. The digestive system

Consists of three main parts:

- Digestive tract
- Digestive glands
- Digestive appendages

❖ Digestive tract

- **Mouth cavity**

According to the feeding habits, type of available food and nature of the aquatic habitat the following adaptations were developed:

1. Position

(a) Terminal---à cichlids (tilapia)

(b) Sub-terminal---> cyprinids (Carp)

(c) Inferior -----> Sharks and dogfish

(d) Superior----> Gourami

2. Teeth

(a) Large and well developed (predator)

(b) Small in carnivorous and Omnivorous fishes

(c) Completely absent in small herbivorous fishes

3. Barbells (will be discussed in sensory organs section)

4. There is no salivary gland although considerable mucus produced by the oral mucus gland.

5. Gill rackers act as pharynx (very large teeth in predators to very long filamentous in plankton feeders)

6. Mouth function

- **Esophagus**
  - **Carnivorous Fishes:** in the form of short, straight muscular tube passing from the mouth to the stomach.
  - **Herbivorous Fishes:** in the form of tune connecting directly to the intestine.
  - The presence of longitudinal folds in the inner surface of esophagus enables fishes to swallowing the larger food particles.

- **Stomach**

✓ The stomach varies in size and shape according to the feeding habits of fishes.

  - **Clarias (Catfish):** It is usually U- or V- shaped, sac like
  - **Mugil (Mullet):** Heavy muscular walled, gizzard like stomach
  - **Tilapia:** Small rudimentary, sac like stomach.
  - **Goldfish and Common carp:** completely absent

- **Pyloric ceca**
  - ✓ The demarcation between the stomach and small intestine may be completely absent, except if numerous blind sacs (pyloric caeca) are attached at the pyloric end of the stomach.
  - ✓ **Function:**
    - Digestive function
    - absorptive functions
    - Their histological features resemble those of the intestine.

- **Intestine**

Its relative length and shape may vary according to diet. They vary from very long and coiled to short tube or straight, sigmoid or coiled depending on the shape of the abdominal cavity. Intestine is very long and coiled in herbivorous fishes while remarkably short in carnivorous fishes.

Digestive glands

Liver

- ✓ **Size:** The liver is a relatively large organ.
- ✓ **Location:** It is localized, and predominantly located in the left side of the abdomen.
- ✓ **Lobation:** Liver is bi-lobed in most fishes, but in some fishes it is only one lobe or sometimes three lobes.
- ✓ **Color:** The liver of various fishes can be quite pigmented. It is usually reddish brown in carnivorous and lighter brown in herbivorous species, but at certain times of year it may be yellow or even off white (wintering season or dry season).
- ✓ **Function:** The main hepatic functions are secretion of bile, detoxification of the toxins and glycogen storage.

Pancreas

- ✓ **Hepatopancreas or splenopancreas:** In most fishes, no discrete pancreas will be found on gross examination, it is diffusely distributed in hepatic tissue (hepatopancreas) or in spleen (splenopancreas).
- ✓ **Discrete pancreas:** It present as a discrete organ only in the sharks and lungfishes.
- ✓ **Function:** Pancreas secret several digestive enzymes directly to the digestive tract (exocrine gland), and insulin hormone for regulation of blood glucose level (endocrine gland).

Digestive appendages

Swim bladder (gas bladder, air bladder or pneumatic tract)

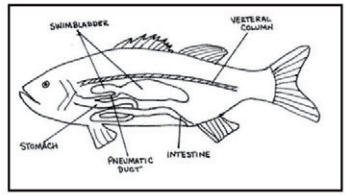


Figure (10) showing the retroperitoneal location of the swim bladder and its connection to fore-stomach using a pneumatic duct

- ✓ The gas filled swim bladder constitutes up to 7% of body volume.
- ✓ **Development:** It develops as diverticulum from the foregut during the embryonic life.
- ✓ **Location:** The swim bladder is a thin walled retroperitoneal organ.
- ✓ **Shape:**
  - Short torpedo shape duct with only one chamber----> tilapia
  - Divided into cranial and caudal chambers connected with isthmus------ > carp

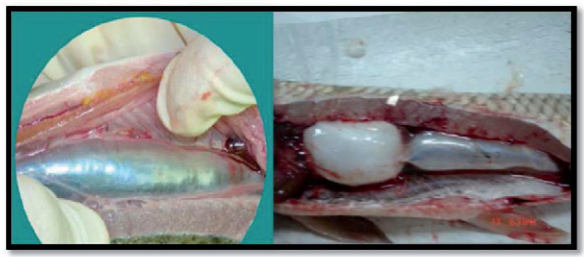


Figure (11) Swim bladder of salmonids (left) and cyprinids (right).

- ✓ The swim bladder is absent in many pelagic or bottom feeder fishes such as African catfish (Clarius species).

- ✓ **Function:**
  - Storage of fat in some fishes.
  - Maintaining the buoyancy of fish.

- Sound and pressure reception
- In some species is equipped with drumming muscles for sound production.

7. Special sense organs in fishes

I. Eyes

- ✓ The eyes of fish are bilaterally placed in bony field of mono-ocular vision and free movement.
- ✓ Fish eyes are slightly bulged.
- ✓ Bilaterally placed except in *Solea* fish (flounder), both eyes are dorsally located.
- ✓ Fish have no eyelids except grey mullet has fatty membrane around the eye.

II. Olfactory and gustatory senses

- ✓ **Olfactory organ:**
  - Olfaction in fish is centered in the nasal cavity, where water is circulated between the pairs of nasal openings as the fish swims.
  - The two nasal sacs are lined with olfactory epithelia that are connected to the olfactory nerve.
- ✓ **Gustatory (Taste Buds):**
  - The taste buds are found on lips, head, barbells, gill rackers, gill arches and mouth cavity.
- ✓ **Temperature and touch receptors:**
  - They are many free neurons scattered within the fish epidermis to give it the sense of temperature and touch effects.

III. Labyrinth (inner ear)

- ✓ **Function:**
  - The labyrinth is an amazing development of the anterior lateral line, forming a complex sensory organ associated with maintenance of equilibrium and hearing.

- ✓ **Structure:**
  - o It consists of two connected parts; the semicircular canals and the otolith organs.

IV. Lateral line system:

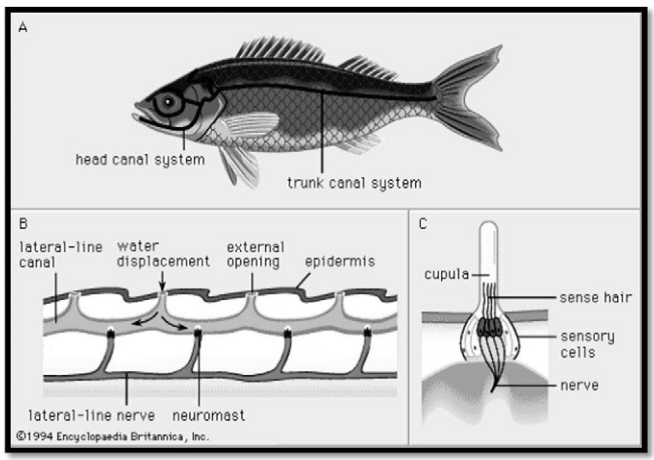


Figure (12) Ultra-structure of lateral line.

- ✓ **The lateral line is a distinct organs found only in lower vertebrates.**
- ✓ **Topographic anatomy:**
  - o Its main components are the paired lateral line canals along the trunk of fish.
- ✓ **Function and significance:**
  - o This canals are microscopical pores or openings containing mechanoreceptors, so that, the lateral line is very sensitive to any low frequent motions in water (water vibrations).
- ✓ **Development:**
  - o Well developed ------> Nile Perch
  - o Ill developed ----------> Nile tilapia
  - o Absent ----------> Sardine

8. The endocrine system

- The endocrine system of fishes has the same basic components as that of mammals, although the presence of some endocrine structure as the corpuscles of stannous has no apparent analogues in the mammals

Gland	Location	Function
The pituitary	Ventral to the brain	Releasing of stimulating hormones that stimulate other endocrine organs. E.g. thyroid stimulating hormone.
Thyroid	Distributed along ventral aorta and branchial arteries	Release thyroxine which promotes the growth in juvenile fish
Ultimobranchial bodies	Near to the gill slits	Secrete calcitonin which regulates serum calcium in fish.
Inter-renal	Anterior kidney	Equivalent to adrenal cortex. It secretes steroid hormones which are involved in mineral metabolism, response to stress and fat utilization.
suprarenal	Anterior kidney	Equivalent to adrenal medulla secrete adrenaline as stress response, regulates the pigment deposition in the melanophores
The corpuscles of stannous	Kidney of bony fish only	Teleocalcin hormone whish blocking calcium absorption by gills, osmoregulation and electrolyte balance.
The urophysis	At the posterior end of spinal cord In shark and bony fish	Secretes urophyseal hormones have a role in osmoregulation.

Figure (13) Fish endocrine organs.

9. The nervous system

- The nervous system of fishes is composed primarily of brain and spinal cord.
- The brain and spinal cord are protected by a single primitive meningeal layer, enclosing cerebrospinal fluid.
- **Brain:**

  The brain is the lightest organ in the fish body as it does not exceed more than 1% of the fish body.

  The main brain regions are:

  - **Fore brain:** it is responsible for olfaction, aspects of color vision, memory, reproductive and feeding behavior.
  - **Mid brain:** it is also called optic lobes, which are involved in the function of visions.
  - **Hind brain (the cerebellum):** it is concerned with coordination of movements, muscle tone and body balance.

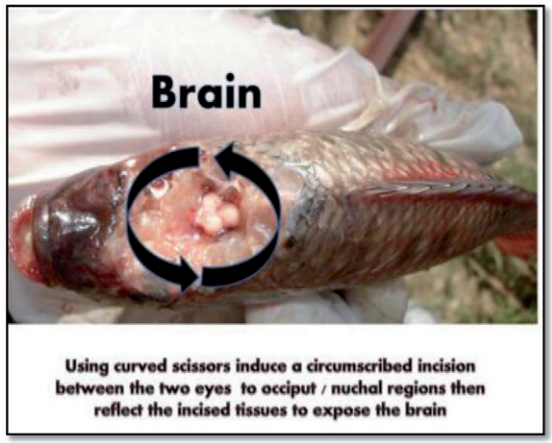


Figure (14) A circumscribed incision made in the head of Nile tilapia to expose the brain .

CASE HISTORY

Questions flow in the following three pathways

- Basic facts questions (e.g. genus, sex, species, age….etc)
- Open ended questions (e.g. give the owner chance to answer)
- Specific questions (e.g. feed husbandry, water quality, previous disease…etc.)

I. Fish

1. Species: Some diseases are restricted to certain spp than others.

i. SVC: affect cyprinids only, so when you ask a question restrict it to cyprinids and not other spp like cichlids.

ii. Carp Pox: affect carp only, so don't ask questions related to other spp.

iii. Furunculosis: affect salmonids, so be restricted in your questions to diseases of salmonids and not other spp.

2. Sex: Some diseases are only affecting females and others affect males. Also, incidences of some diseases are much higher in females than males (visa versa).

3. Age: Some diseases are only affecting the young fishes and others affecting adults, so when you ask a question focus on those restricted to each age. For example, whirling disease only affects young salmonids (fingerlings) and early mortality syndrome only affect yolk sac and fry stages.

4. Weight (Size): large fishes do need more dietary requirements than small ones, so when diet is deficient in one of these ingredients or in amount, then a disease problem will develop. Thus, knowing the standard requirement for each stage of fish (weight) will be clue for solving such disease problem.

5. Source of Fish: Knowing the source of fish (marine, brackish, freshwater, sewage, underground water, earthen pond, agricultural drainage water…etc.) will help in directing the question to follow up the disease problem. Streptoccocosis is a disease arising from sewage problem so when considered you have to ask about the source of fish.

6. Date of introduction of new fish species: will help in determining if the disease is endemic or brought up with fishes coming from another fish farm.

II. Husbandry and Management

1. Type of aquaculture facility

i. Glass aquarium

ii. Earthen pond

iii. Green house

iv. Fiberglass tank

2. Feed

i. Type : Some disease are related to feeding the faulty type of pellets to the fish e.g. tilapia fish is a surface feeder fish that fed floating pellets so when sinking pellets rendered to them , they will not feed and pellets will accumulate in the pond, purified leading to high organic matter and gill problems.

ii. Amount: If fish feed 1 kg three times daily, so when if 3 kg of fish were thrown in pond will be accumulated, purified, high organic matter, gill problem develops. Also, if small amounts of food than required were thrown to fish in pond, then after certain period fish will suffer from dietary deficiency problem.

iii. Method: Manual or automatic feeders.

iv. Storage: Mycotoxin problems due to high temperature and high humidity.

3. Water

i. Source

ii. Depth

iii. Rate of water change

iv. Analysis: (Temp., O2, pH, Salinity, Ammonia)

III. Disease itself

1. Course of the disease (acute, sub-acute, chronic course).

2. Morbidity, Mortality, Case fatality.

3. Control measures (vaccination, disinfection, etc.).

4. Previous occurrence of the disease.

Remote Examination (Visual Examination)

Farm Visit

1. Inspect normal (Fish reflexes)

Types of Fish Reflexes

A. Inside water

i. Escape reflex: Fish are responsive to different physical stimuli; fish escape and hide away in a hiding place while sick fish cannot hide or response to stimuli.

ii. Feeding reflex: Fish normally adapted to come through the water (jumps, make bubbling in water) once they smell

food or hear the one who feed them at certain times. Sick fish are not responsive to that type of reflex and they are off food (anorexic).

B. Outside Water

i. Eye reflex: The eye moves freely toward the side you turn to. When fish are sick they lose the ability to move their eyes to each of these directions.

Figure (15) Eye reflex in rainbow trout. The picture is a courtesy of Dr. Ehab Elsayed (FDML, Cairo University).

ii. Tail reflex: The tail moves freely toward right and left in healthy fish when hold in vertical position. Sick fish are not able to move their tail.

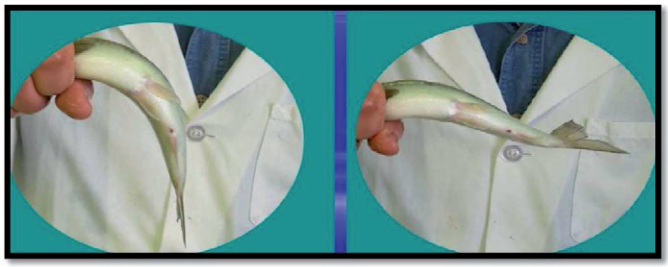


Figure (16) Tail reflex. The picture is a courtesy of Dr. Ehab Elsayed (FDML, Cairo University).

iii. Defense reflex: Fish erect their fins (dorsal fin and pectoral fin) as a mean of defense against aggressors and flap up on board. Unhealthy fish appears lethargic and unable to defend.

2. Compare the case history with the actual finding on the farm visit.

3. Observe the abnormal behavioral changes in the fish population within water.

A. **Surfacing, gasping, piping, rapid opercular movement** and aggregation at water inlet (Gill problem, Low dissolved oxygen).

B. Darkening (generalized as in severe stress due to cannibalism or at posterior part only as incase of whirling disease (black tail)).

C. Loss of buoyancy or balance: Swim bladder diseases.

D. Flashing movement: external and internal parasites.

E. Whirling (circling): e.g. whirling disease, streptococcosis, enteric septicemia of catfish and viral nervous necrosis.

F. **Resting at the bottom of pond with erected pectoral fin:** water pollution.

G. **Rubbing body against fixed object and walls of pond:** External parasitic infestation.

4. Mortality Patterns.

Different fish mortality patterns and their possible causes

Mortality pattern	Possible causes
Extended course of mortality	Internal and external parasites
Mortality in the form of bell-shaped curve	Bacterial, viral and fungal infections
Mortality rises in ascending manner reaching a peak then remains high.	Nutritional deficiency, including deficiency in quantity and quality or complete food deprivation
Mortality occurs in early morning with physical changes in water	Low dissolved oxygen
Mortality occurs abruptly in the fish pond involving all fish	Poisoning

TRANSPORTATION

Objectives

1. Transporting fish sample to the lab for diagnosis

2. Stocking of new fish ponds with fingerlings or frys at the beginning of the rearing cycle.

3. Importation and exportation of fish

Methods (tools) of transportation

1) Plastic bags:

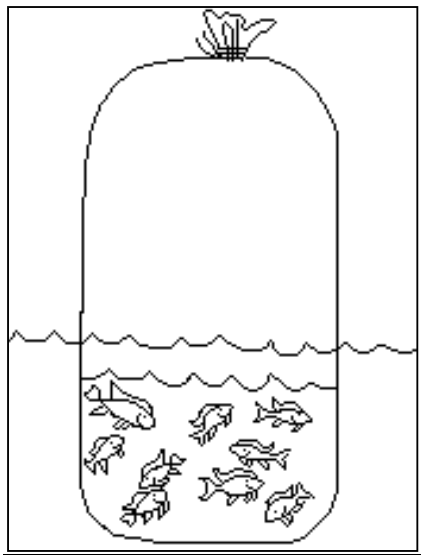


Figure (17) a sketch showing the oxygen : water + fish ratio (2/3 oxygen :1/3 water +fish)

2) Plastic containers:

- A large container that is provided with oxygen source
- Mainly used for large sized fishes and broodstocks

3. Styrofoam containers:

- Used mainly for airborne and maritime fish shipping
- Dark, light in wt, insulated, anti-concussion

4. Plastic buckets:

- Should be supplemented with oxygen source

Precautions adopted in transportation

- **Before:** Fasting 24 – 48 hrs
- **During:**
  - Avoid agitation (low speed vehicle)
  - Sedation (low dose anesthetic) or by adding ice (hypothermia)
  - Keep fish in dark environment
- **After:** Acclimatization

Acclimatization

1. Plastic Bags

- Put closed bag on water surface of water for 20 – 30 minutes.
- Make small opening for gradual water exchange for 15 minutes
- Make large opening till fish start to move to outside the bag then remove the bag

2. Plastic containers or buckets

- Gradual transfer of water from pond to container and vice versa for about 30 minutes.

ANESTHESIA

Objectives of Anesthesia

1. Fish transportation.

2. Handling of broodstocks during egg/ semen collection during artificial insemination.

3. Before blood sampling.

4. Before minor surgery.

5. Before I / M and I / P injection.

Stages of Anesthesia

1. Sedation-------- Motion and breathing reduced

2. Anesthesia

    i. Partial loss of balance

    ii. Slight reactivity to stimuli

    iii. Rapid opercular movement

3. Surgical Anesthesia

    i. Total loss of balance

    ii. No reactivity to stimuli

4. Death

    i. Complete stop of respiration

    ii. Eventual death

Types of Anesthetics

1. MS 222 (Tricaine Methane Sulfonate)

Effective dose: 100 – 200 mg/L

Induction time: 5- 10 minutes

Advantages:

- Easily soluble in water
- Suitable for wide variety of spp

Disadvantages:

- Expensive
- Change blood parameters
- Hypoxic agent
- Produce acid pH: Tricaine Methane Sulfonate-------- Sulfonilic acid------- acid pH. Thus should be neutralized by adding 100-200mg Sodium Bicarbonate to the soln.

2. Benzocaine

Effective dose: 100 – 200 mg/L

Advantages:

- Cheaper than MS222
- Less stressful than MS222

Disadvantages:

- Insoluble in water but soluble in fat solvents (ethanol) so stock solution should be prepared in stock soln.

3. Clove Oil

Effective Dose: 20 – 40 mg/L

Advantages:

- Cheap
- Rapid induction
- Wide safety margin

Disadvantages:

- Insoluble in water so should be prepared in stock solution before use.

SAMPLING FOR DIAGNOSTIC PURPOSES

I. Fish Sample

Sample characteristics

- Sample should be representative
- Moribund fish: at different stages of the disease (early, peak, late stage).

- ✓ **Includes healthy fish:** for comparison with those showing clinical signs of the disease.
- ✓ **Freshly dead fish:** (2-4 hrs after death as maximum)
- ✓ **Size of sample varies according to :**
  - Age
  - Size
  - Suspect disease

    1. Acute toxicity (1-5 fish)

    2. Infectious diseases **(**small fish: 10-15 fish & large fish: 5-10 fish)

- ✓ **Sample should not include dead fish due to the following**
  - Fish tissues rapidly decomposed (PM autolysis)
  - Once fish started decomposition protozoa and viruses leaves fish body, so you will not be able to diagnose those diseases.
- ✓ **Signs of freshness**

Item	Fresh	Decomposed	Purified
Eye	Slightly bulged Transparent	Slightly sunken Slightly opaque	Deeply sunken Complete opacity
Scales	Firmly attached	Loosely attached	Detached
Vent	Closed	Open	Open and prolapsed
Color of gills	Rosy red	Pale red	Whitish or grayish
Dorsal Muscles	Firm (elastic)	Soft (non elastic)	Very soft (doughy)

II. Water Sample

Sample characteristics

- Sample should be representative collected from three points (inlet, outlet and the middle of the pond).
- Sample should be taken in a separate container
- Sample should be taken under complete aseptic condition using sterile glass bottle (500 ml).
- Bottle should be only opened under water surface at depth not less than 0.5 meter.

Parameters to be examined in the water sample

1. pH: measured on spot using pH meter

2. Temperature: measured on spot using the water thermometer

3. Dissolved O_2: measured on spot using oxygen meter

4. Turbidity: measured using Sicchi disc

5. Ammonia: measured on spot to avoid the rapid conversion to nitrite and nitrates using the nitrosomonas and nitrosobacter bacteria.

6. Salinity: measured using refractometer

CLINICAL EXAMINATION OF FISHES

SKIN

ULCERS

1) Non Specific ulcers

Non specific shape or location on fish and can be caused by many different etiologies.

- Septicemic Diseases (MAS, Vibriosis, *Pseudomonas* septicemia, ERM, Streptococcosis……etc).
- Mycobacteriosis (*M. marinum , M. ulcerans* , *M. fortuitum*)
- External Parasites (Monogeneans, Trichodiniasis, Parasitic crustaceans).
- Chemical (Strong alkalis and acids)
- Mechanical injury

2) Specific ulcers

Specific shape, specific location on fish and specific etiology

- **3 Zone Ulcer**
  - Occurs on the caudal peduncle of carp in case of the chronic stage of MAS (due to the effect of dermonecrotoxins produced by *Aeromonas hydrophila*).
  - The 3 zones are: External red (hyperemic zone), Middle white (necrotic zone) and inner bluish (gangrenous zone).

- **Saddle Back Like Ulcer**
  - Produced on the dorsal and back area of fishes infected with *Flavobacterium columnare* (Columnaris disease).

- The ulcer is produced due to the effect of proteolytic (hyaluronidase, protease…..etc.) enzymes produced by *Flavobacterium columnare*.

- **Nodular granulomatous raised surface ulcer**
  - Due to female *Lernea cyprinicea*e head penetration into the carp fish skin with consequent inflammatory and fibrous tissue formation around the head of the crustacean.

FURUNCLES

Furuncles are abscess like lesion that produced on the skin of Salmonid fishes affected with *Aeromonas salmonicida* (Freshwater furunculosis) or marine/ brackish fishes affected with *Vibrio* species (Salt water furunculosis or vibriosis).

PHYSIOLOGICAL RED RASHES

This is a physiological condition in which skin at isthmus and belly regions of ♂ and ♀ tilapia appear intense red due to some hormonal changes associated with fish maturity (spawning).

SKIN HEMORRHAGES

- Hemorrhages are different sizes of blood leaks over the surfaces of the skin of fish.
- According to size, hemorrhages can be classified into: Peticheal (pin point), ecchymotic (few mm to cm), patchy (few cm at certain region) and Extensive or generalized (involve the entire fish body).

- **Possible causes of hemorrhages in fish are :**
  - **Septicemic Diseases** (e.g. MAS , Vibriosis…etc, mainly due to hemolysin and other toxins)

- **Viremic viruses** (e.g. SVC, ISA…etc, mainly due to direct effect of viral replication inside the endothelial lining of the blood vessels).
- **External Parasites** (e.g. monogeneans and parasitic crustaceans).
- **Linear Hemorrhage**: is a linear type of hemorrhage that appear on isthmus and belly of scaly fishes or at different fish skin locations in scales fishes due to fish lice (Argulus) biting.

SPOTS

1) White spots/ nodules

1. **Parasitic** (white spot disease: white spots are CT produced as a host tissue reaction against the mature trophozoite of Ichthyopthirius multifilis) infestation to the skin of fishes.
2. **Chronic granulomatous diseases** (due to granulomatous reaction of the pathogen in the skin of fish)
    a. Bacterial (Mycobacteriosis, Nocardiosis and BKD)
    b. Fungi (Ichthyophonosis)

2) Black spots

- Black spots scattered allover the fish's skin surfaces
- Caused by the presence of EMC of digenetic trematodes (Diplostomum species).
- The black spots are mainly derived by the recruitment of the melanophores that encircles the EMC of the digenetic trematodes with an ultimate development of black spot.

- IMH: wild ranging cichlids (Lake Nasser tilapias) and earthen pond raised tilapias.
- FH: Aquatic birds

SKIN DARKENING

- **Generalized:**
  - Late stages of septicemic diseases.
  - Late stages of toxemic diseases (clostridia, mycotoxins and heavy metals).
  - Severe physiological, biological and natural stresses:
    - Cannibalism
    - Spawning in migratory fishes
    - Migration for a very long distance
- **Posterior (tail):**
  - Mainly known as (Black tail Diseases or Whirling disease) a disease caused by *Myxosoma cerebralis*.
  - Due to the damage of vertebral cartilage number 26 and the consequent pressure on spinal nerve number 26 which result in complete loss of control of posterior part (tail region) pigmentation.
  - IMH : *Tubifex tubifex*
  - FH: young salmonids (3-6 months)

TUMOR LIKE LESIONS

- Proliferative, Nodular and wart like: hard pearl like growths over the fish skin (Lymphocystis Disease Virus – LDV which belongs to the family Herpesviridae).
- Hyperplastic , extensive and soft waxy : its color ranges from white to pink (Carp Pox which belongs to the family Iridoviridae).

MACROSCOPICAL PARASITES

- Parasitic crustaceans (Lernea spp and Argulus spp)
- Water leeches (act as IMH for trypansoma and trypanoplasma).

MOUTH

- **Hemorrhages:** (Enteric Red Mouth Disease (ERM) due to *Yersinia ruckeri* infection).
- **Necrotic stomatitis :**
    - Freshwater fish (primarily due to *Flavobacterium columnare* which get complicated with secondarily invasion with *Saprolgenia parasitica* causing what is called Mouth Fungus Disease).
    - Marine fishes (primarily due to *Flavobacterium maritimus* which get complicated with secondarily invasion with *Aphanomyces spp* causing what is called Mouth Fungus Disease).
- **Macroscopical parasites:**
    - Isopoda
    - Water leeches

EYE

- **Hemorrhages:**
    - **Bilateral:** systemic infection with septicemic (e.g. Streptococcosis, MAS) or viremic diseases (e.g. SVC, ISA)
    - **Unilateral:** (Traumatic injury)
- **Sunken Eye:**
    - Chronic PCBs toxicity
- **Corneal Opacity:**
    - **Central:** Cercaria of digenetic trematodes embedded in the lens of eye without encystations due to the glassy nature of lens (Diplostomum species)

- **Peripheral:** Nutritional deficiency (tryptophan and pantothenic acid deficiency)

- **Exophthalmia (Pop eye)**
  - **Bilateral :**
    - **Systemic infectious diseases**
      - Septicemic diseases (due to the effect of bacterial toxins endothelial lining of BVS)
        - MAS
        - Vibriosis
        - *Pseudomonas* septicemia
        - ERM
      - Viremic diseases (viral replication inside endothelial lining)
        - SVC
        - ISA
        - VHS
      - Chronic Infectious diseases (due to the associated hypoproteinemia)
        - Mycobacteriosis
        - Nocardiosis
        - Bacterial Kidney Disease
        - Ichthyophonosis
    - **Hypoproteinemia (Cashexia)**
    - **Chronic toxemic diseases** (Mycotoxicoses, heavy metal toxicity (organic mercury).
    - **Gas bubble Diseases** (Gas supersaturation).

HOLE –IN – THE - HEAD

- Catfish (*Edwardsiella* Septicemia of catfish – *Edwardsiella ictaluri*).

- Cichlids / Cyprinids (Systemic form of hexamitiosis – *Hexamita intestinalis*)

GILLS

Same lesions and possible causes mentioned in the skin section can be applied to that of Gills.

- **Change of Color**
  - Pale red (Asphyxia)
  - Intense rosy red (Cyanide toxicity)
  - Brown chocolate color (Nitrite toxicity)
  - Congested dark red color (Septicemia)
- **Spots**
  - Black
  - White
- **Change in size**
  - Increase in size (lamellar hyperplasia and clubbing of gill filaments together) due to Ammonia toxicity (EGD).
  - Sloughing of gill filaments (Apical filaments necrosis) due to Columnaris (*F. columnare*) or Bacterial gill disease (*F. branchiophilum*).
- **Macroscopic parasites**
  - Crustaceans (*Ergasillus*) associated with marbling and damage of gills and Egg sacs of the parasites appears in between gill filaments.
  - Yellow grub disease (EMC of a digenetic trematodes known as *Clinostomum* spp)

SKELETAL SYSTEM

- Lordosis, scoliosis , kyphosis, bowed mandible, short lower or upper mandibles, parrot shape skull, stump body, dwarfism, fused fins.
- **Possible causes:**
    - **Infectious:**
        - Chronic granulomatous bacterial diseases (Mycobacteriosis, Nocardiosis and BKD).
        - Chronic granulomatous mycotic diseases (Icthyophonosis).
        - Granulomatous parasitic diseases (Whirling disease).
    - **Non infectious:**
        - Nutritional deficiency (tryptophan and Vitamin C deficiency).
        - Toxic (heavy metal toxicity (cadmium and lead).
        - Traumatic injury.
        - Strong water current at the early life sages.
        - Genetic (hereditary)

ABDOMINAL ENLARGEMENT

- **Anterior Abdominal Enlargement :** (Mainly due to enlargement of swim bladder induced by an inflammatory condition known as Swim bladed Inflammation (SBI) or air saculitis or aerocystitis :
    - Viral air saculitis: Spring Viremia of Carp (SVC)
    - Mycotic air saculitis: *Phoma herbarum*
    - Parasitic air saculitis: Swim bladder nematodes
- **Middle Abdominal Enlargement:** (Mainly due to accumulation of ascitic fluid in the abdominal cavity (Ascitis)):
        - **Systemic infectious diseases**
            - Septicemic diseases (due to the effect of bacterial toxins endothelial lining of BVS)
                - MAS

        - Vibriosis
        - *Pseudomonas* septicemia
        - ERM
    - Viremic diseases (viral replication inside endothelial lining)
        - SVC
        - ISA
        - VHS
    - Chronic Infectious diseases (due to the associated hypoproteinemia)
        - Mycobacteriosis
        - Nocardiosis
        - Bacterial Kidney Disease
        - Ichthyophonosis
- **Hypoproteinemia (Cashexia)**
- **Chronic toxemic diseases** (Mycotoxicosis, heavy metal toxicity (organic mercury).

- **Posterior Abdominal Enlargement** :
    - Mainly due to enlargement of gonads during spawning stages.
        - Ripened mature ovaries.
        - Ripened mature testis.

LIVER

- **Hepatogmegaly**
    - Acute septicemic diseases
    - Hepatic tumors (Chronic heavy metal toxicity –Aflatoxicosis)
- **Change in color**
    - Congested liver (Acute septicemic diseases – Acute viremic diseases)

- Yellow liver (Fasting – Cashexia- BKD – chronic eimeriosis – chronic hexamitiosis)

- **Nodules**
  - **Whitish nodules**
    - Chronic granulomatous diseases
    - Chronic hexamitiosis
    - Chronic eimeriosis
    - EMC of digenetic trematodes
  - **Blackish or reddish nodules**
    - Nematodes cysts

SPLEEN

Same lesions and causes described in liver.

KIDNEY

- **Change in color**
  - Grayish discoloration
  - Congested kidney
- **Nodules**
  - **Whitish nodules**
    - Chronic granulomatous diseases
      - ✓ BKD
      - ✓ Mycobacteriosis
      - ✓ Nocardiosis
      - ✓ Ichthyophonosis
    - EMC of digenetic trematodes
    - Myxosporidial cysts (*Myxosoma tilapiae*)
    - Nephrocalcinosis in salmonids
  - **Blackish or reddish nodules**
    - Nematodes cysts

- **Friable texture**
  - Acute BKD
  - Ochratoxicosis
  - Acute septicemic diseases

SWIM BLADDER INFLAMMATION

AIRSACULITIS – AEROCYSTITIS

- **Viral**
  - **Spring Viremia of Carp (SVC):** Virus targets endothelial lining of swim bladder causing its rupture with consequent hemorrhagic patches and severe inflammation together with increase in size (very pathgnomonic lesion to this virus).
  - **Large Mouth Bass Virus:** starts from mild inflammation extending with acute hemorrhagic inflammation and ending with chronic inflammation manifested by yellow exudates and crusts lining the inner wall of swim bladder.
- **Mycotic**
  - ***Phoma herbarum*** infection in salmonids (starts from the early yolk sac stage and extends till later stages if the fish still alive)
- **Parasitic**
  - **Swim bladder nematodes**
    - *Cystidecola* (salmonids)
    - *Anguillicola* (eels)
- **Bacterial**
  - ***Flavobacterium psychrophilum*** infection in young salmonids and whitefishes (yellow crusts lining inner wall of SB).

LABORATORY PARASITOLOGICAL EXAMINATION

Selection of Samples for Parasitological Examination

- Sample should be moribund and representative (number , stage and distribution).
- It is preferred that fish got examined on spot or submitted live to ensure retrieval of larger portion of the parasites.
- Macroscopic parasites will still be identifiable, and often alive, if whole fish or affected parts are swiftly submitted refrigerated (not frozen).
- Even with fish submitted live, protozoa may occasionally be lost during transport, especially where the temperature is lowered by the addition of ice to the container. Such cases illustrate the need to send fixed tissue and smears, especially of gills.
- Freshly dead fish should be examined as early as possible (On spot).
- Frozen samples should not be used for parasitological examination except for certain protozoa that resist freezing e.g. tissue sporulated protozoa (myxosporidia and microsporidia).
- Anesthetics should not be used while dealing with external parasites to avoid their detachment from the fish host while being in the water (sample will give false negative results).
- Avoid dryness of fish or the wet mount sample especially if suspecting external protozoa.
- A suitable anticoagulant (e.g. Na citrate) should be used to get whole blood sample to search for blood parasites in blood smears after being stained with Giemsa and Leishman stains.
- Fixed material should include any affected tissue, including parasites *in situ* if sufficient examples (preferably in situ unless prepared as below), smears of gills, blood and suspected parasitic lesions.

A. Examination of skin and gills

1. Microscopic parasites

- Wet mount unstained smears are made from skin and gill mucus of living moribund fishes just after their removal from water facility.
- Dried mucus smears are stained with Giemsa stain.
- The skin and gill wet mount mucus and stained smears are examined microscopically under low and high power for the presence of any microscopical parasite such as protozoan ciliates (*Itcthyopthirius multifillis , cryptocarion irritans , Chilodenella spp, Epistyles spp*); flagellates (*Cryptobia branchialis, Icthyopodo spp*); myxozoan (*Henuguya* spp); monogeneans (*Dactylogurus, Gyrodactyllus , cichlidogyrus spp*) .

2. Macroscopic parasites

- The skin is examined for the macroscopic external parasites such as leeches (IMH for Trypanosomnes) , parasitic crustaceans (Lernea and Argulus spp)
- The gill covers were removed with pair of scissors to expose gills which will then examined for the presence of water leeches and parasitic crustaceans such as Ergasillus species.
- The gills were removed from the body and the post-cephalic portion of the abdominal cavity was examined for the presence of the digenean encysted metacercaria (EMC) of *Clinistomum tilapiae* cysts (yellow grub).
- **Examination of EMC:** The encysted metacercariae were picked up from gills and post-cephalic / post-branchial muscles and incubated at 37^{o}C for 2 hours in pepsin digestive solution for excystation of the encysted metacercarciae which then called excysted metacercariae.

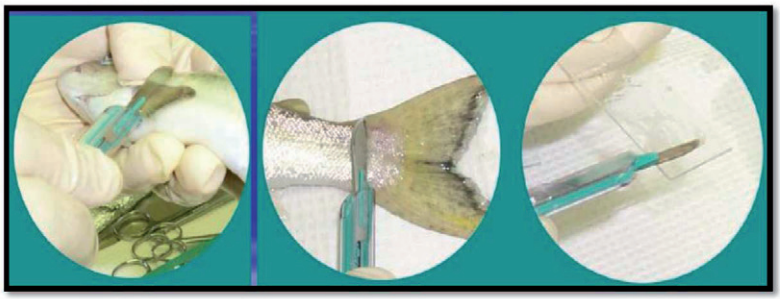


Figure (18) Skin and fin scraping and wet mount preparation from mucus (picture is taken by Ehab Elsayed (FDML-Cairo University) and courtesy of Michigan State Aquatic Animal Health Lab (Dr. Mohamed Faisal).

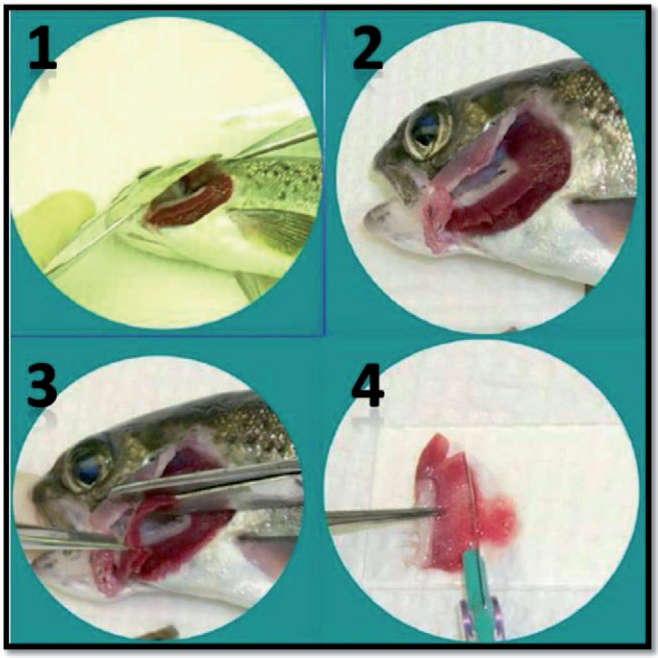


Figure (19) Gill scraping and wet mount preparation from mucus (picture is taken by dr. Ehab Elsayed (FDML-Cairo University) and courtesy of Michigan State Aquatic Animal Health Lab (Dr. Mohamed Faisal).

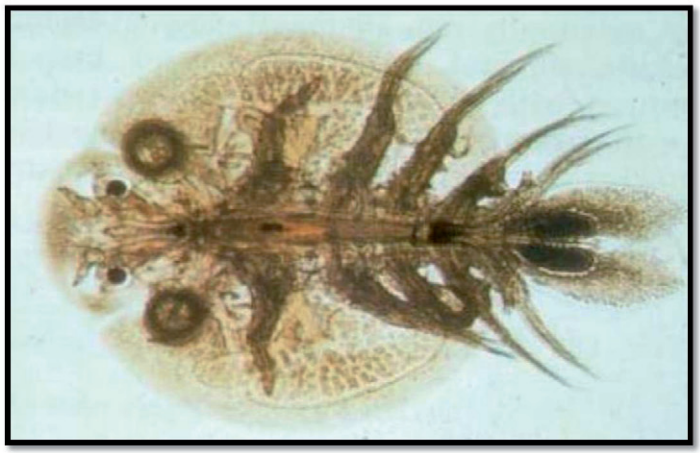


Figure (20) Fish Lice (Argulus spp) ventral view

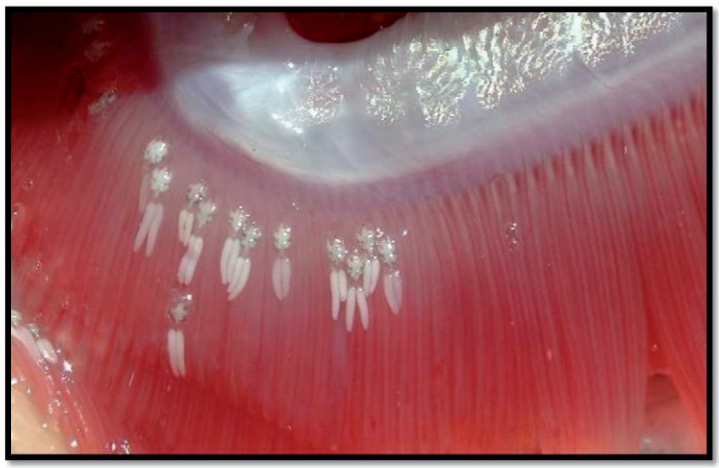


Figure (21) Ergasilus species attached to the gill filaments exposing its long whitish egg sacs .

Figure (22) Lernea species on a Murray cod

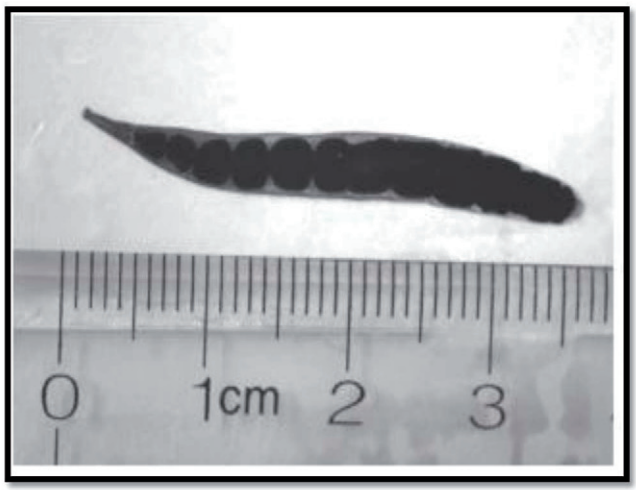


Figure (23) The water leech (Myzobdella lugubris) retrieved from skin of Largemouth Bass Fish (Faisal et al. 2011)

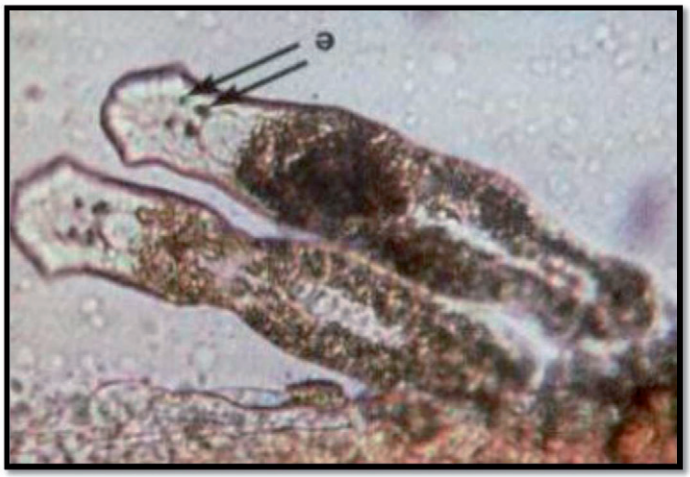


Figure (24) Non stained wet mount showing Dactylogyrus spp with typical 4 eye spots (e)

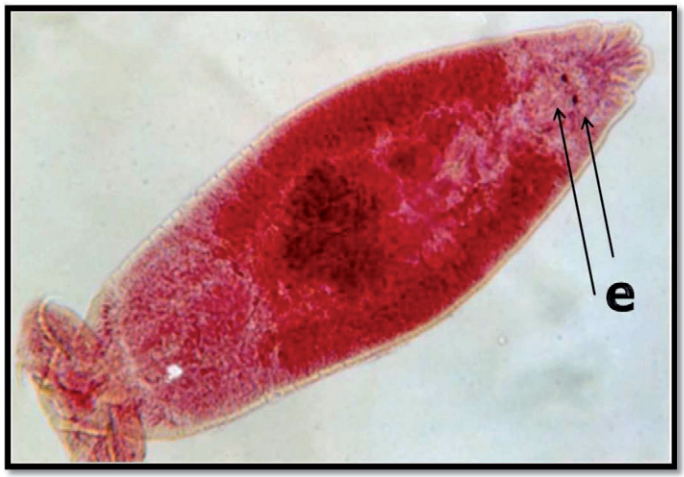


Figure (25) A fixed stained Cichlidogyrus species with typical 2 eye spots (e)

(The picture is a courtesy of Dr. M.S. Marzouk , FDML, Cairo University)

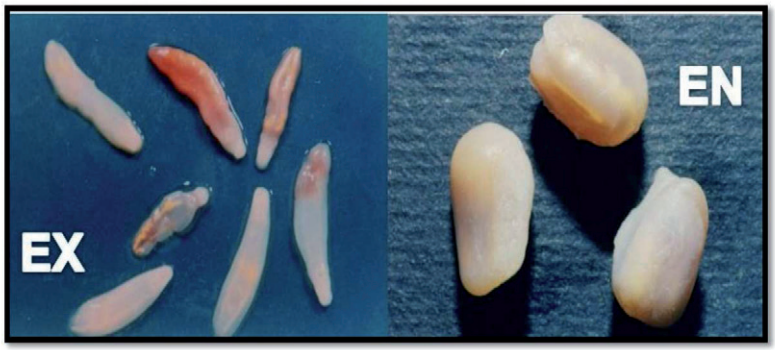


Figure (26) Encysted cercariae (EN) of Clinostomum tilapiae (yellow grub) to the right and excysted cercariae (EX) to the left (The picture is a courtesy of Dr. M.S. Marzouk , FDML, Cairo University)

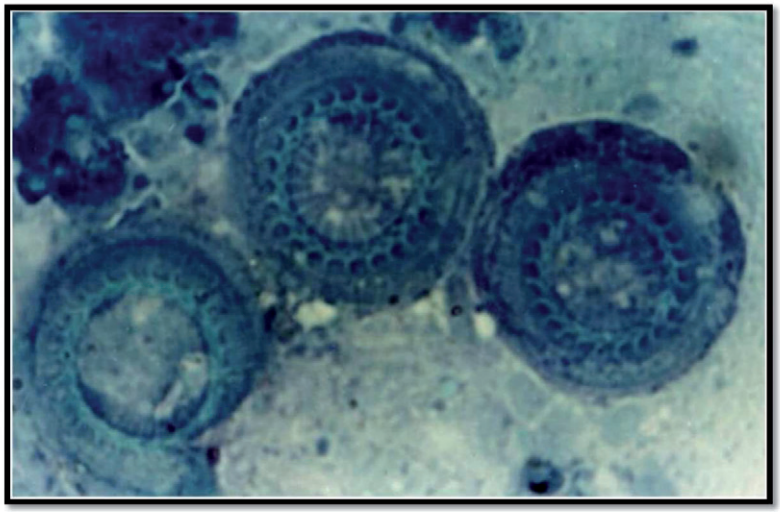


Figure (27) Giemsa stained wet mount from fish skin showing several Trichodina trophozoites.

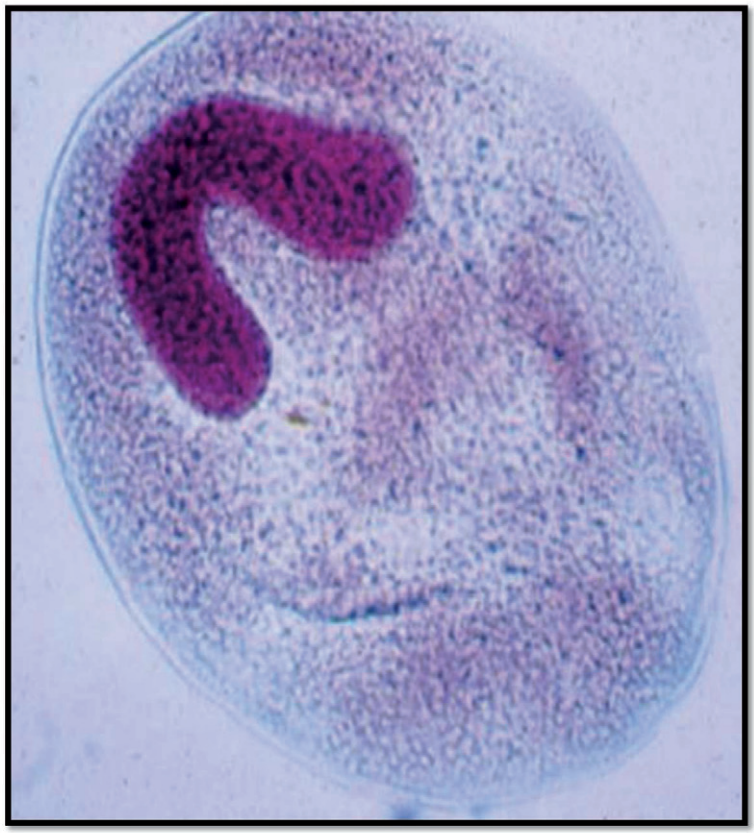


Figure (28) Giemsa stained wet mount from fish skin showing Typical Icthyopthirius multifilis trophozoites.

B. Examination of mouth

Mouth should be visually inspected for any possible leeches infestation (e.g. Large Mouth Bass leech infestation as described by Faisal et al. 2011) or Isopoda infestation where they eat fish's tongues and stay instead as long as the fish live.

C. Examination of eyes

The eye balls are removed using a curved scissors and cut opened using scalpel then the vitreous humor can be examined under low and high power for any possible parasitic cysts (myxoporidia: e.g. *Myxobolus tilapia*, *M. heterosporus*). Further, the eye lens should be macerated and mounted on a clean glass slide to be examined microscopically for eye flukes (*Diplostomum spathecum* cercariae).

D. Examination of brain

Brain can exposed using a circular circumcision between the two eyes and occiput area. Brain should first visually examined for any possible color / consistency change , free worms or parasitic cysts .

E. Examination of muscles, liver, spleen, kidney , gonads and heart

1) Muscles should be grossly examined for any possible parasitic nodules , nematode larvae…etc then squash preparation from muscle (small muscle cube) should be compressed between two slides and examined under microscope at low 10 X / 40 X powers to look for any possible microsporidian spores (e.g. *Haplospora*) , EMC cysts (e.g. *Diplostomum*) or nematode larvae cysts (e.g. *Anisakis*). Change of color / consistency of focal areas in muscle could indicative of muscle break down by microsporidia (e.g. yellow color and soft consistency).
2) Liver, spleen , kidney and gonads should be grossly examined for any change in color / consistency (*Hexamita, Eimeria, Microsporidia, Myxobolus, Ceratomyxa*), free worms (*Anisakis*), or parasitic nodules (nematodes, digenean or myxosporean cysts). Squash preparation from any of these tissues can be made by compressing small piece from the organ containing the nodules / cyst between two slides and examined under microscope at low 10 X / 40 X powers to look for any possible

Hexamita / Eimeria cysts in liver , myxosporidian spores, microsporidian spores, EMC cysts (e.g. Diplostomum) and nematode larvae cysts (e.g. Anisakis).

3) Heart in wild ranging tilapia (Nile or Nasser Lake tilapias) should be visually inspected after triangular incision of the fish has been made to expose internal organs including heart. Long amblicaecum / short contracaecum larvae appear as whitish red nematodes extending from sinus venous and pericardial sac posterior toward abdominal cavity.

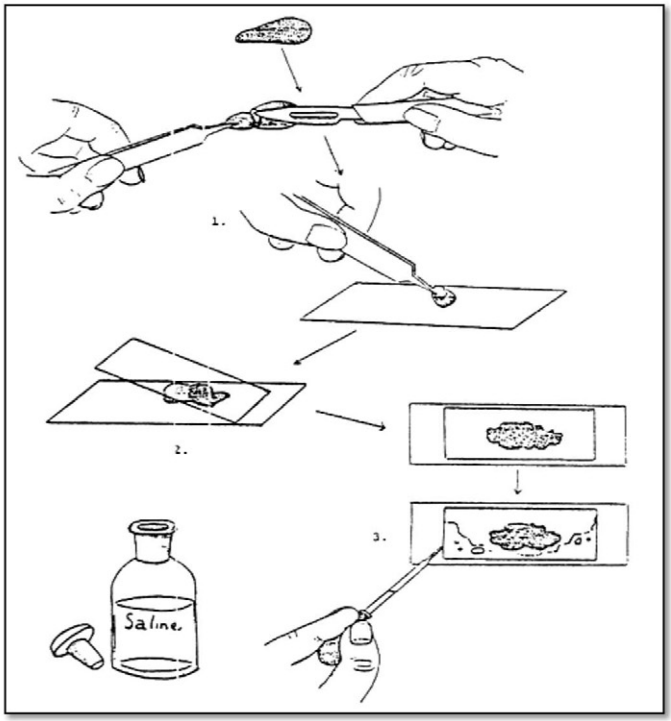


Figure (29) Preparation of a compression smear (squash preparation) from internal organs.

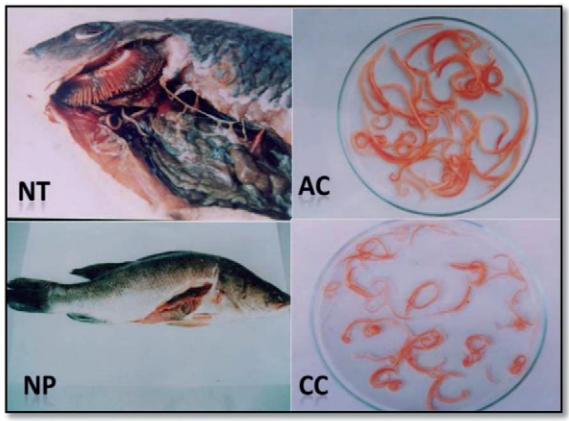


Figure (30) Amplicecum larvae (AC) extending from heart of Nile tilapia (NT) and Contracecum larvae (CC) extending from heart of Nile perch (NP). The picture is a courtesy of Dr. M.S. Marzouk , FDML, Cairo University.

F. Examination of the gastrointestinal tract

The digestive tract of each fresh dead or moribund fish should be separated and placed with its contents into a separate glass jar containing physiological saline and left for sedimentation for 1-2 hours. The supernatant fluid should be discarded and the sediment is washed several times with normal physiological saline to get rid the excess mucus. The clear sediment should then be examined under dissecting binocular microscope. The detected parasites are collected by pipettes and preserved in 10% formol saline (trematodes, cestodes and acanthocephala) and in glycerine – alcohol (1 : 4 for Nematodes).

G. Examination of blood

- Blood smears are prepared from fishes by taking blood samples by different routes especially from the caudal vessel.

- The blood smears are air dried, fixed in methyl alcohol for 3-5 minutes and then stained with Giemsa stain (1ml of stock Giemsa stain added to 9 ml of freshly prepared distilled water) for 20 –30 minutes.
- The stained blood smears are examined for the presence of blood protozoa (trypanoplasma, trypanosoma , hemogregarina, babesiosoma).
- For further identification blood culture can be made to propagate the trypanosomes before further identifications.

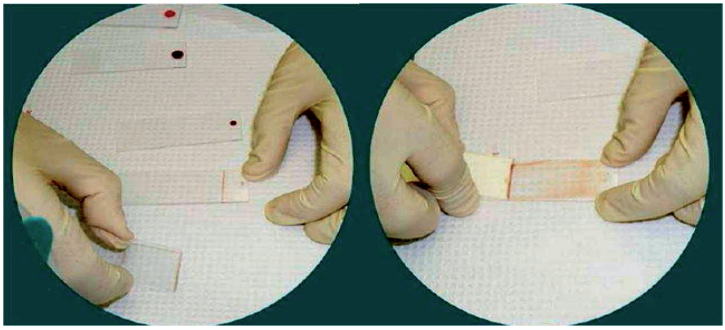


Figure (31) preparation of a blood film as initial step for blood film examination

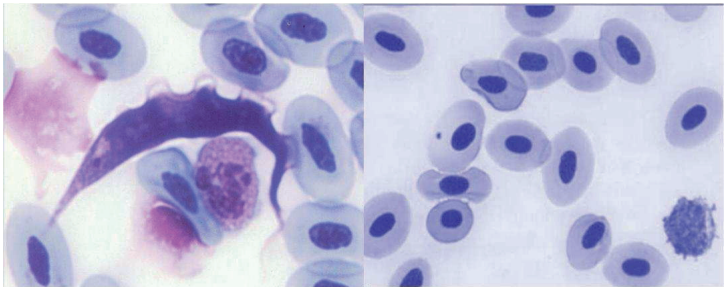


Figure (32) Trypanosoma sp. in a Giemsa stained blood smears (Left) and normal Giemsa stained blood smear (Right)

Preservation of Parasites

Protozoa

1. Permanent preparations of protozoa can usually be made by smearing affected material, for example gills or skin scrapings, thinly over a microscope slide.
2. If specimens fail to adhere, fresh egg-white emulsified in a little distilled water and applied to the microscope slide will help.
3. Let the specimen sit 5-10 min before fixing.
4. Schaudinn's fixative (see below) is most commonly used but Bouin's fluid may also be used.
5. Submerge slides in fixative for 10 min before transferring to 70% ethanol where they may be stored indefinitely.
6. Blood protozoa can be preserved for identification by preparing a thin blood film on a clean microscope slide. This film should be rapidly air dried and fixed in absolute methanol for 5 min.

Trematodes

1. Monogenean flukes from skin or gills should be killed in hot (but not boiling) sea water (for marine fish) or hot tap water (for freshwater fish).
2. They should then be fixed in buffered neutral formalin.
3. Adults of the larger digenean flukes from the gut should be killed in near-boiling saline and then fixed in buffered neutral formalin.
4. Adult and intermediate stages of digenean flukes that are encysted in tissues should be fixed in buffered neutral formalin.
5. AFA fixative has also been used for trematodes, after relaxation in tap water, but may dissolve some hard structures that are useful in identification of these parasites.

Nematodes

1. One method for fixing nematodes is to place them in concentrated glacial acetic acid for 5-10 min, drop into 70% ethanol heated in a water bath to 60^{o}C and allow to cool.
2. This method will cause most nematodes to straighten.
3. Davidson's fixative is less hazardous than glacial acetic acid and if used at room temperature will cause most nematodes to straighten as they fix, which is important to facilitate identification.
4. Alternatively use cold ethanol or 10% neutral buffered formalin for large nematodes since hot fixatives can cause blistering of the cuticle. Remove larval nematodes from cysts if present, before fixing.
5. Store nematodes in 70% ethanol plus 5% glycerol.

Acanthocephalans

1. The proboscis, an important taxonomic character, can usually be made to protrude by placing the specimen in distilled water.
2. Use care so that worm does not become damaged because of excessive swelling.
3. Occasionally some cover slip pressure is necessary to protrude the proboscis.
4. Fix with AFA or neutral buffered formalin and store in 70% ethanol plus 5% glycerol.

Dead Worms

1. Fix dead worms directly in 10% formalin fixatives.

Copepods, Isopods and Other Crustaceans

1. Place directly into 70% ethanol, or 70% ethanol plus 5% glycerol.
2. Parasites may be left attached to gill filaments, skin or tongue in case of isolpodes.

LABORATORY BACTERIOLOGICAL EXAMINATION

Sampling criteria

- Fish must be received either alive or freshly dead (within 1-2 hours) on blue ice in an insulated cooler.
- Fish must not be frozen. However in case of chronic granulomatous bacteria frozen samples can be accepted. Bagged fish should not be in direct contact with blue ice or they will freeze.
- Live fish are preferred for diagnostic samples.
- At least 10 moribund fish should be placed in one or more large leak-proof plastic bags containing hatchery or fish tank water.
- Accurate labeling of the sampling bags is an asset to accurate diagnosis.
- In case of cultured fishes: larger fishes should be individually sampled while smaller fish stages (frys, eyed eggs , eggs) can be pooled together as one sample (5-10 samples / 1 pool) depending on the total number of samples to be examined.
- In case of wild fishes: Each fish should be managed individually even those of smaller sizes within the same examined population. However, in case of mass mortalities occurring within the same fish population , then smaller fishes (frys) can be pooled together on the basis of 5-10 / 1 pool.

Sampling procedures

- The following should be prepared before the onset of sampling process: biosafety cabinet (laminar flow) , sterile dissecting tools, sterile bacteriological loops , ethanol 80 %, Bunsen flame or Bacto-incinerator , biohazard bags for tissue / loops disposal), disposal gloves, clean lab coats and sometimes masks.
- Under the biosafety cabinet , fish should be placed on its left side in a dissecting plate .

- The surface of the fish is flooded with 80% ethanol and left until the surface clouds.

- A triangular incision " 3 line technique" will be applied for opening fish.
- Use one set of scissors and forceps for opening and change the set every 5 fishes in case of pooled samples.
- The scissors and forceps used in opening the fish should not be used for taking bacteriological samples.
- Use a new set of scissors and forceps to open the swim bladder and puncture the peritoneum to get the kidney.

A. Nostrils

1. Gently insert a bacteriological swap into the fish nostrils then swap them in a circular manner .
2. Swaps should be spread onto plates of both enriched media (Brain heart infusion agar (BHI) , Tryptic soy agar (TSA) , Blood agar (BA)) and selective media (Hsu-Shotts agar , Modified Cytophaga Agar, Todd Hewitt agar).

B. Gill, Skin and Fins

1. Skin scraps and smears should be made first then stained with Gram stain.
2. If microscopical examination revealed the presence of any kind of short or long bacilli or cocci then you need to culture from the lesions.
3. Make sure to wash the lesion very well with 80% ethyl alcohol then either use a sterile forceps, scissors or disposable loops for taking the sample.
4. Sample should be spread onto plates of both enriched media (Brain heart infusion agar (BHI) , Tryptic soy agar (TSA) , Blood agar (BA)) and selective media (Hsu-Shotts agar , Modified Cytophaga Agar, Todd Hewitt agar).

5. Other media can be used depending on your suspicion e.g. Rimlers - Shotts medium in case of Aeromonads, *Pseudomonas* isolation agar in case of Pseduomonads.
6. Sodium chloride should be added to media in case of marine fishes (1.5 – 2 %) .

C. Swim bladder

- Puncture the swim bladder using a sterile pointed scissors.
- Take a loopful / swab from the content (crust, blood, exudates) using a sterile bacteriological loop or swab and spread onto three culture plates (BHIA , BA & Hsu-Shotts).

D. Kidney

Kidney can be reached using one of the following approaches:

- **Ventro-lateral approach (Triangular incision):** Upon removing the swim bladder and puncture of the peritoneum, the kidney tissue will appear as long fleshy dark red or brownish tissue resting on the vertebral column of the fish and if you go forward you will find the anterior kidney just lying under the gill arches. Using a sterile disposable loop take a loopful from the kidney and spread it onto the enriched media (BHIA, TSA, BA) , selective media (R-S, CBBA, *Pseudomonas* isolation media, Hsu-Shotts, TCBS, Todd-Hewit) and / or specific media (MKDM and Lowenstein Jensen).
- **Dorsal approach:** using a sterile sharp pair of scissors cut two oblique sections that met each other at one point at the kidney tissue. The lines extend from the site of the dorsal fin or dorsal aspect of the fish till met each other at the head of a triangle that rests in the kidney tissue. The kidney will appear as a red spot when you look from the dorsal view. Introduce the loop into the red spot at the head of the triangle then spread onto the above mentioned plates.

Bacteriological Examination

- **Pack** the inoculated culture plates in well labeled Ziploc plastic bag to minimize the possibility of contamination.
- **Label** the Ziploc bags with the following data : the source, species of fish, type of sample, date cultured .
- **Incubate** the plates according the optimal temperature of the suspect organism (15°C for coldwater organisms, 20-25 °C for temperate water organisms , 25-30 °C for subtropical water organisms and 37 °C for mesophillic organisms) and the season of the year . The duration of incubation extends from 24 hrs to 72 hrs and 5 days in some fastidious organisms. If *Mycobacterium marinum* is a suspect then incubation temperature might extend to 14-21 day.
- **Follow up and purification of the retrieved bacterial isolates:**

1. Record the cultural characteristics (size, shape, color , texture and distribution) of the growing colonies on BHIA, R-S, CBBA, and *Pseudomonas* F after 24-48 hours; on TCBS, Hsu-Shotts after 72 hrs -5 days ; MKDM agar after 7-10 days and Lowenstein - Jensen medium after 2- 3 weeks.
2. Observe the BHIA, R-S, CBBA plates for apparent colonies growth for up to 72 hours and Hsu-Shotts for up to 5 days while MKDM should be observed for up to 10 days and Lowenstein - Jensen for up to 6 weeks. In case of no growth, discard the plates by the end of the previously mentioned incubation period.
3. By the end of the incubation period, pick up a single colony and use it for the following:
    - Purification (1st subculture) by spreading over a new plate using the standard streaking technique
    - Preparing a bacterial smear for Gram staining or (acid fast stain) ZN stain.
4. Incubate the 1st sub-cultured plates at the same incubation temperature and time used for primary isolation then pick up a single colony using

disposable loop and streak onto agar slants and make a bacterial smear using the same colony to be Gram stained later.

5. Examination of the Gram and /or (acid fast stain) ZN stained slide will give us a clue about the degree of purity of your picked colony (if you found more than one type of bacteria e.g. Gram positive, gram negative or bacilli and cocci in the same time that indicates that your colony was not pure and it was a mixed culture and you need to re-culture them and pick definitive single colony).
6. Once you get an individual type of bacteria in your Gram stained slide then you have a pure isolate.
7. Using the same colony used in Gram staining streak two agar slants and store in either the fridge.

- **Identification of the bacterial isolates**

Identification of the pure bacterial colonies includes numbers of sequential tests which start by identifying the phenotypic , cultural , biochemical characteristics, serological profile , and molecular profile.

1. **Cultural, phenotypic and biochemical characteristics:** shape, diameter, size, textures, color, pattern of distribution are all determinative characteristics for primary identification of bacterial colonies. Gram staining and acid fast (Zeil Nelseen) staining are the basic phenotypic or microscopical techniques that specify which direction of identification scheme you should adopt. For example, you will know if your bacterial isolates are mixed infection and need more purification or you have a pure isolate of Gram negative short or long bacilli, Gram positive cocci or bacilli and / or acid fast bacilli or branched bacteria. This will introduce you to other determinative tests which are the biochemical assays. Biochemical tests can be done as individual tests such as (oxidase, catalase, O/F, indole, H_2S….etc) or using kit systems such API 20 E, API 20 NE, API Coryne, API Strept …..etc. This kit system is in the form of micronized test tubes that contains certain reagent that represent each of the above mentioned

biochemical tests in one kit. The results will be matched against a standard biochemical profile index supplied with the kits and based upon the change and intensity of color.

2. **Phenotypic and biochemical identification charts (Schemes):** the above mentioned characteristics are used together in the form of identification charts which are capable of guiding the veterinarian working with such bacterial isolates in reaching a presumptive bacterial identification
3. **Serological tests (Antigen – antibody based tests):** A number of serological tests have been successfully used in detection of fish pathogens. However, in many disease serological tests failed to achieve a determinative final identification criteria due to the presence of heterogenicity or heterogeneity among the bacterial species as in case of *A. hydrophila*. Serological tests such as agglutination test, immune-diffusion, counter-immuno-electrophoresis, FAT, Monoclonal ELISA, and Q-ELISA have been fully used during the past three centuries in detection of fish pathogen with relative degrees of success or failure. Recently, Q-ELISA has been used the main screening tests for certain disease like BKD due to its quantitative nature that is successful in assessing the prevalence and intensity of the infection in certain fish population.
4. **Molecular techniques: (Polymerase Chain Reaction (PCR) based assays): For example the Nested PCR (n PCR):** The nPCR reaction employs oligonucleotide primers to amplify base pair segments of the gene encoding for certain antigen produced by the bacteria of interest. DNA is extracted from the target tissues or bacterial colonies and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a primer for a smaller segment of DNA within the larger segment amplified initially. The DNA products from 2^{nd} amplifications are then visualized using Agarose Gel Electrophoresis. The anticipated nPCR product band size (in bp) is standard for this antigen. The nPCR assay is the most accurate and sensitive method for detection of bacteria even with very low levels of infection (3-4 bacterial cells / gm tissues). Also, the “nPCR-

Secondary amplification" step results in extreme sensitivity in detecting the target DNA. There are a number of few disadvantages: 1. Expensive. 2. Cannot differentiate between dead and live bacteria. 3. Cannot differentiate between different levels of infection (Non quantitative).

The Use of Molecular tools in Diagnosis of Bacterial Fish Diseases

Central Dogma of Molecular Biology

- Central dogma is a complex process in which DNA helix replicates into DNA duplicate passing with the transcription process and RNA synthesis ending with the translation process in which proteins are finally synthesized.

Historical hierarchy of diagnostic techniques from low to high advancement or from old to recent discoveries

1. **Whole Animal based :** (symptomatic and comparative imaging)
2. **Organ based:** Explorative Surgery (endoscopy), Autopsy and Post Mortem Examination (necropsy).
3. **Tissue Based:** Histopathology, Clinical Pathology and Histochemistry.
4. **Cell based :**
    a. **Cellular immunity:** Assessment of cytokines, respiratory burst, antibcaterial peptides, lectins, phagocytic assays ..etc).
    b. **Antigen antibody reactions:** Serology (FAT, AGPT, ELISA), Protein fractionation, 2D Electrophoresis…etc).
5. **Nucleus based (chromosomes, DNA and RNA) based:** Molecular pathology, DNA fingerprinting, RAPD, PCR, RT-PCR, Multiplex PCR, gene cloning, DNA microarrays, Sequencing.

Molecular dissection of a cell

1. Nucleus
2. Chromosomes
3. Nucleoproteins
4. Nucleic acid (DNA and RNA)

Helix of Life

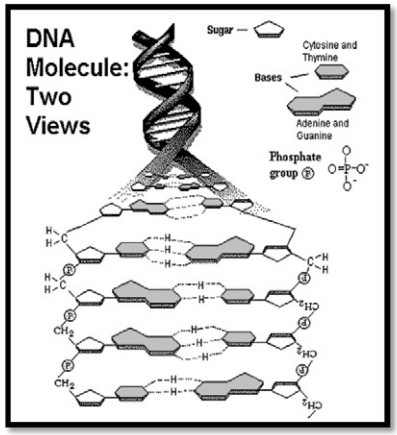


Figure (33) DNA, the helix of life .

Polymerase Chain Reaction

- It is an amazingly simple technique that results in the exponential amplification of almost any region of a selected DNA molecule.
- It works in a way that is similar to DNA replication in nature.

DNA Isolation Protocols

a. Conventional Method

- Phenol / Chloroform / Iso-amyl alcohol
  - Dangerous fumes
  - Less DNA purity
  - High risk of protein and other contaminants

b. Quick Isolation Kits

- Qiagen (DNAeasy Animal and Plant Tissue Kit), Promokine, Biovision, Promega and bioassaysys.
    - Highly safe
    - More DNA yield
    - High DNA purity

Measurement of DNA concentration and purity

- DNA and most of the common contaminants found in DNA solutions, have absorbencies in the region 260nm to 320nm:
    - measurement of the absorbencies in this region allows measurement of the DNA concentration
    - Provides information about the contaminant levels.
- The measurements can be performed on a conventional spectrophotometer
    - **DNA concentration: (260nm)** DNA absorbs light most strongly at 260nm so the absorbance value at this wavelength (called A_{260}) can be used to estimate the DNA concentration using the equation : *Concentration (µg/ml) = A_{260} reading - A_{320} reading) × 50*
    - **Protein contamination :(280nm)** since tyrosine residues absorb strongly at this wavelength, the absorbance at 280nm is used as an indicator of protein contamination.
    - **Turbidity (non specific contamination): (320nm)** A_{320} provides a general measurement of the turbidity of the sample and is normally subtracted from the A_{260} value as a background reading for the calculation of DNA concentration, but excessive values may indicate non-specific contamination.
    - **DNA Purity:** A good quality DNA sample should have A_{260}/A_{280} ratio of 1.7–2.0. Since the sensitivity of different techniques to these contaminants varies, these values should only be taken as a guide to the purity of your sample.

Detection of *Renibacterium salmoninarum (R. salmoninarum)* by the Nested Polymerase Chain Reaction (nPCR)

1. The nPCR reaction employs oligonucleotide primers to amplify base pair segments of the gene encoding for the p57 antigen the major soluble antigen of *R. salmoninarum*.

2. DNA is extracted from fish tissues (Kidney, spleen, ovarian fluid, blood) and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a primer for a smaller segment of DNA within the larger segment amplified initially.

3. The DNA products from both 1st and 2nd amplifications are then visualized using Agarose Gel Electrophoresis

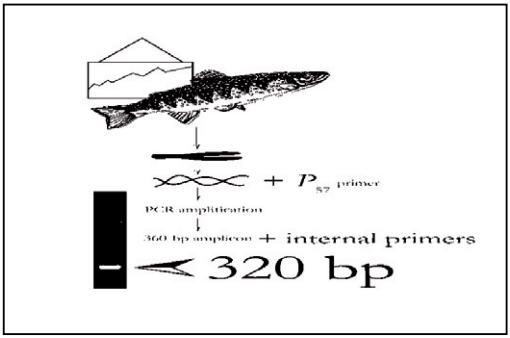


Figure (34) Diagrammatic scheme for nested PCR (Courtesy of Dr. M. Faisal, Michigan AAHL)

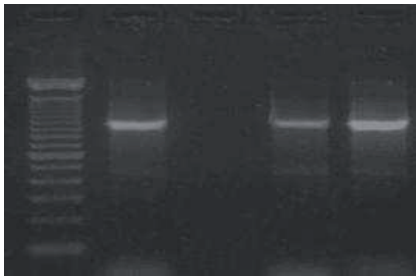


Figure (35) Gel electrophoresis showing the specific 974 bp band of F. psychrophilum isolated from Sea lamprey fish (Elsayed et al. 2007)

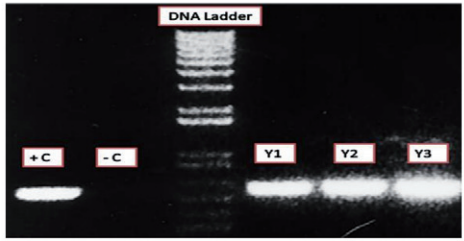


Figure (36) Gel electrophoresis showing 575 bp specific band: Lane 1: +C (DNA from ATCC Y. ruckeri 29473); Lane 2: -C (no template); Lane 3: 100 increment DNA ladder; Lane 4: Y1 (isolate group1); Lane 5 (isolate group 2) and Lane 6 (isolate group 3) showing characteristic 575 bp band (Eissa et al. 2008).

Advantages of Nested PCR

- The nPCR assay is highly accurate, sensitive and specific method for detection of BKD even with very low levels of infection (3-4 bacterial cells / gm tissues).

The use of nested PCR as an epidemiological diagnostic tool

- PCR can be used an epidemiological tool to detect and follow up the spread of certain bacterial pathogens in carrier aquatic animals such (crabs, shrimps, mollusks, water insects, leeches and even in water samples).
- PCR can be used effectively to determine the stage and prevalence of infection of a certain disease among specific fish population.
- PCR can be used as a screening method before egg and semen collection from broodstocks during spawning season.

Rationale for adopting molecular assays instead of other diagnostic methods

- The conventional diagnostic methods are time consuming and less sensitive in detection of latent pathogens.
- Rapid identification of pathogens is crucial for effective disease control in aquaculture.
- Detection of pathogens is important not only in infected fish (clinically and sub-clinically), but also in the environment e.g. between harvesting and re-stocking, and as an 'early warning system.

Multiplex PCR

- Multiplex PCR involves amplifying multiple gene products in a single reaction
- The method has been used successfully to detect number of fish pathogens.
- Agarose gel electrophoresis is typically used to assess results from multiplex PCR

DNA Microarrays

- DNA microarrays offer a more discriminating means to examine reaction products for specific sequences.
- DNA microarrays are important molecular tools that have been applied to studies of gene expression, phylogenetic classification, ecological studies, and the detection and genotyping of bacterial and viral pathogens.
- DNA microarrays consist of ordered sets of DNA fixed to solid surfaces; generally on glass but sometimes on nylon substrates.
- Each spot in a microarray is composed of many identical probes that are complementary to a gene of interest.

DNA Sequencing

- Chemical or enzymatic methods.
- The original technique for sequencing, Maxam and Gilbert sequencing, relies on the nucleotide-specific chemical cleavage of DNA and is not routinely used any more.
- The enzymatic technique, Sanger sequencing: the use of dideoxynucleotides (2',3'-dideoxy) that terminate DNA synthesis

The ideal organization of molecular events in the laboratory in consideration to Quality Assurance (QA) and Quality Control (QC)

1. Separate dissection and tissue sampling room.
2. Separate microbiological and DNA isolation room.
3. Separate PCR master-mixing chamber.
4. Separate amplification and gel electrophoresis room.

CULTURE MEDIA

1) Selective media used for isolation of fish Pathogens

Those are certain media that contains one or a group of chemical reagents that select for or inhibit certain pathogen and inhibit all other competitors.

- **Rimler- Shotts medium (R-S medium):** contains an antibiotic (novobiocin) which inhibits certain bacteria and also contains an indicator (bromothymol blue) which usually change in color into yellow when *A. hydrophila* produce acid from maltose (sugar) so colonies appear yellow on this medium.

Ingredients	Gms / Litre
Yeast extract	3.000
Maltose	3.500
L-Cysteine hydrochloride	0.300
L-Lysine hydrochloride	5.000
L-Ornithine hydrochloride	6.500
Sodium thiosulphate	6.800
Ferric ammonium citrate	0.800
Sodium deoxycholate	1.000
Sodium chloride	5.000
Bromothymol blue	0.030
Agar	13.500
Final pH (at 25℃)	7.0±0.2

- ***Aeromonas* Isolation Agar Base:** It is typically *Aeromonas* Agar Base containing rehydrated Ampicillin and used for selective isolation of Aeromonads.

Ingredients	Gms / Litre
Tryptose	5.000
Yeast extract	2.000
Dextrin	11.400

Sodium chloride	3.000
Potassium chloride	2.000
Magnesium sulphate	0.100
Ferric chloride	0.060
Sodium deoxycholate	0.100
Bromothymol blue	0.080
Agar	13.000
Final pH (at 25℃)	8.0±0.2

- ***Pseudomonas* Isolation Agar Media:** contain rehydrated Ampicillin and used for selective isolation of pseudomonads.

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Triclosan (Irgasan)	0.025
Agar	13.600
Final pH (at 25℃)	7.0±0.2

- **Shotts and Waltman Agar Media:** used for differentiation of *Edwardsiella tarda* from *E. ictaluria* , *A. hydrophila* and *Yersinia ruckeri*

Ingredients	**Gms / Litre**
Tryptone	10.000
Yeast extract	10.000
Phenylalanine	1.25
Ferric Ammonium Chloride	1.20
Bromothymol blue	0.003
Bile salts	1.000
Agar	15.000
Distilled water	980 ml
Final pH (at 25℃)	7.0±0.2

Autoclave for 15 minutes at 121 °C then cool to 50 °C again and add mannitol (filter sterile) to 0.35 (v/v) and colistin sulphate to 10 µg/ml

- **TCBS Medium:** used for isolation of *Vibrio* spp.

Ingredients	Gms / Litre
Yeast Extract	5.00
Enzymatic Digest of Casein	5.00
Enzymatic Digest of Animal Tissue	5.00
Sodium Citrate	10.0
Sodium Thiosulfate	10.0
Ox bile	5.00
Sodium Cholate	3.00
Sucrose	20.0
Sodium Chloride	10.0
Ferric Citrate	1.00
Bromthymol Blue	0.04
Thymol Blue	0.04
Agar	14.0
Final pH: 8.6 ± 0.2 at 25°C	
Heat with frequent agitation and boil for one minute to completely dissolve the medium.	
DO NOT AUTOCLAVE.	

- **Marine Agar (Sea Water Yeast Peptone gar) (SWYP agar):** used for isolation of *Photobacterium damselae.*

Ingredients	Wt / Litre
Peptone	5.0 g
Yeast Extract	1.0 g

Ferric Citrate	0.1 g
Sodium Chloride	19.45 g
Magnesium Chloride	8.8 g
Sodium Sulfate	3.24 g
Calcium Chloride	1.8 g
Potassium Chloride	0.55 g
Sodium Bicarbonate	0.16 g
Potassium Bromide	0.08 g
Strontium Chloride	34.0 mg
Boric Acid	22.0 mg
Sodium Silicate	4.0 mg
Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg
Disodium Phosphate	8.0 mg
Agar	15.0 g
Adjust pH to 7.6	

- **Cytophaga Agar:** contains tryptone, beef extract, yeast extract and sodium acetate which allow the growth of flavobacteria and inhibit others.

Ingredients	**Gms / Litre**
Casein Peptone	0.5gm
Yeast Extract	0.5gm
Beef Extract	0.2gm
Sodium Acetate	0.2gm
Agar	15.0gm
Distilled water	1000 ml
Final pH (at 25℃)	7.2
Autoclave for 15 minutes at 121 °C	

- **TYES:** used for selective isolation of *Flavobacterium* spp.

Ingredients	Gms / Litre
Tryptone	4.000
Yeast extract	0.40
$MgSO_4.7H_2O$	0.50
$CaCl_2.2H_2O$	0.50
Agar	10.000
Distilled water	1000 ml
Final pH (at 25℃)	7.2
Autoclave for 15 minutes at 121 °C	

- **Modified Sheih Medium:** used for selective isolation of *Flavobacterium* spp.

Ingredients	Gms / Litre
Peptone	5g
Yeast extract	5g
Sodium acetate	0.01g
$BaCl_2 (H_2O)_2$	0.01g
K_2HPO_4	0.1g
KH_2PO_4	0.05g
$MgSO_4.7H_2O$	0.30g
$CaCl_2.2H_2O$	0.0067g
$FeSO_4.7H_2O$	0.001g
$NaHCO_3$	0.05g
Agar	10 g
Distilled water	1000 ml
Final pH at 25°C	7.2
Autoclave for 15 minutes at 121 °C then cool to 50 °C again then add 10 units / ml polymyxin (filter sterile) and 5 µg / ml neomycin (filter sterile) . Instead of polymyxin and neomycin , you can only add 1 µg / ml tobramycin.	

- **Hsu- Shotts Medium:** contains tryptone, gelatin, yeast extract, and neomycin sulfate which inhibits all bacterial competitors and allow the growth of flavobacteria. The following is the method used to prepare 500 ml media:
    1. Weigh 1 gram of Tryptone powder, 0.25-gram yeast extract, 1.5-gram gelatin and 7.5-gram agar (The yeast extract is very hygroscopic: tightly close seal the container as soon as possible after use).
    2. Suspend dehydrated media into 500 ml sterile distilled water in 500-ml glass bottle.
    3. Mix thoroughly, heat with frequent agitation using hot plate magnetic stirrer.
    4. Allow mix to boil for 1 minute on the hot plate or for 10 minutes in boiling water bath (100 ℃) to completely dissolve the powder.
    5. Label the media bottles and tape them with the autoclave tape and put them in Nalgen tray then autoclave at 121 ℃ for 15 minutes.
    6. Turn on the water bath and adjust it to 45 ℃ before you start and once the media bottles are autoclaved, put them in the water bath at 45 ℃ for sometime until you feel that bottle temperature is skin touchable.
    7. Add 50 µl filter sterile neomycin sulfate solution to the 500 ml media bottle and mix well then start aliquoting 23 ml of media into each plate.
    8. Leave the plate to cool down until agar solidifies.
    9. Put the solidified Hsu-Shotts agar plates in well-labeled zip lock plastic bags with the name of the media, date, and signature.
    10. Store the Hsu-Shotts agar plates in the cold room.
    11. Pick up one of the plates randomly and keep in the incubator for 2-5 days. If Bacteria grow on this plate, discard the whole batch and prepare a fresh one.

- **Commassie Brilliant Blue Agar (CBBA):** formed of 1 litre TSA and 0.1 g Commassie Brilliant Blue stain which only stains the poly A layer of bacterial pathogen that posses poly A layer, so the bacterial colonies appear blue e.g. *A. salmonicida*. The following is the method used to prepare the CBBA media:
    1. Weigh 0.1 gram of Coomassie Brilliant Blue powder and 20 gram of dehydrated TSA powder media (The media is very hygroscopic: tightly close seal the dehydrated media container as soon as possible after use).
    2. Suspend dehydrated TSA and Coomassie Brilliant Blue into 500 ml sterile distilled water in 500-ml glass bottle or flask.
    3. Mix thoroughly, heat with frequent agitation using hot plate magnetic stirrer.
    4. Allow mix to boil for 1 minute on the hot plate or for 10 minutes in boiling water bath (100 ℃) to completely dissolve the powder.
    5. Adjust pH to 7.2 ± 0.2 using 1 N NaOH and 1 N HCl drop by drop until you achieve the required pH.
    6. Label the media bottles and tape them with the autoclave tape and put them in nalgen tray then autoclave at 121 ℃ for 15 minutes.
    7. Turn on the water bath and adjust it to 45 ℃ before you start and once the media bottles are autoclaved, put them in the water bath at 45 ℃ for sometime until you feel that bottle temperature is skin touchable.
    8. Disinfect hood (Chlorine disinfected and UV was turned on for at least 2 hours) and aliquot 23 ml of media into a sterile 100 ml plastic Petri dish.
    9. Leave the plate to cool down until agar is solidified.
    10. Put the solidified CBBA plates in well-labeled zip lock plastic bags with the name of the media, date, and signature.
    11. Store the CBBA plates in the cold room.

12. Pick up one of the plates randomly and keep in the incubator for up to 5 days. If Bacteria grow on this plate, discard this batch.

- **Selective Kidney Disease Medium (SKDM):** It's a kind of KDM that contains 4 antibiotics (cyclohexamide, cycloserine, oxolonic acid, polymexin sulfate). The media inhibit the growth of all other bacteria and coryne form bacteria and only allow the growth of *R. salmoninarum*.
- ***Pseudomonas* F agar:** contains argazan an antibacterial that inhibits all other bacteria except *Pseudomonas florescence.*
- **Pseudocel medium:** a medium that contain cetrimide which inhibits all other Gram negative bacteria and only allow the growth of pseudomonas spp.

2) Specific media used for isolation of fish Pathogens

These types of media contain specific nutrients, additives, metabolites which are required for the growth of certain fastidious organisms such as *R. salmoninarum*, *Mycobacterium* and *Nocardia* spp.

- **Modified Kidney Disease Medium (MKDM) (Eissa, 2005):** contain L cysteine, 10 % serum, *R. salmoninarum* old spent culture (metabolites) and 4 antibiotics.

Ingredients	Gms / Litre
Peptone	10g
Yeast extract	0.5g
L-Cysteine HCl	1.0g
Agar	15 g
Distilled water	900 ml
Final pH at 20°C	6.8

Autoclave for 15 minutes at 121 °C then cool to 50 °C then add the following ingredients: 100 ml Fetal Bovine Serum (Heat inactivated at 56 ℃), 10 ml previously filter sterilized spent media (Rs metabolite), 50mg previously filter sterilized Cycloheximide, 12.5 mg previously filter sterilized D-Cycloserine, 2.50 mg previously filter sterilized Oxolinic acid and 25 mg previously filter sterilized Polymyxin B sulfate.

- **Lowenstein- Jensen medium and Dorset Egg medium:** are two specific media for *Mycobacterium* and *Nocardia* spp.

Ingredients	Gms / Litre
L-Asparagine	3.600
Monopotassium phosphate	2.400
Magnesium sulphate	0.240
Magnesium citrate	0.600
Potato starch, soluble	30.000
Malachite green	0.400

- Suspend 37.24 grams in 600 ml distilled water containing 12 ml glycerol.
- Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121℃) for 15 minutes.
- Meanwhile prepare 1000 ml of whole egg emulsion collected aseptically. Aseptically add and mix egg emulsion base gently to obtain uniform mixture.
- Distribute in sterile screw capped tubes.
- Arrange tubes in a slanted position.
- Coagulate and inspissate the medium in an inspissator water bath or autoclave at 85℃ for 45 minutes.

BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF GRAM NEGATIVE FISH PATHOGENS

1) CYTOCHROME OXIDASE

Materials

- Filter Paper
- 1 % N, N, N'N' Tetramethyl-P-Phenylenediamine (Oxidase Reagent)

Methods

I. From colonies:

- Pre-wet filter paper with Oxidase reagent.
- Pick a bacterial colony with a sterile plastic loop or wood stick.
- Gently scratch the colony onto the filter paper.

II. From Liquid Culture:

- Spot the filter paper with 10-20μl of culture.
- Add 10μl of freshly made oxidase reagent.

Interpretation:

I. Positive: Purple or blue color within 5-10 seconds (reactions that occur after 10 seconds are negative).
II. Negative: No blue or purple color.

Quality control:

I. Positive: *Pseudomonas* species
II. Negative: *Yersinia* species.

Notes:

- The use of an iron containing metal inoculation loop can lead to a false positive reaction.
- Use freshly prepared reagent. However reagent can be stored refrigerated up to 7 days in dark brown bottles.
- Use young culture (24 hours).

2) MOTILITY TEST

Materials

- A semi solid medium (soft agar) can be used for detection of the bacterial motility.
- Semi solid medium can be formulated by adding 2.5-3 gm/L agar to the medium.

Method

1.Stab the medium with a small amount of inoculum.

2.Incubate overnight at room temperature.

Interpretation:

I. Motile: The medium will become turbid with growth that radiates from the line of inoculum.

II. Non-motile: Only the stab line will have visible bacterial growth.

3) OF BASAL (Oxidation / Fermentation test)

Metabolism of Carbohydrates (Glucose) aerobically (oxidatively) or anaerobically (fermentatively) will result in acid production. The resulting acidic medium will change the color of the Bromothymol Blue PH indicator in the medium from green to yellow. The presence of bubbles in the tube indicates gas production.

Materials

Ingredient	Gms / Litre
Tryptone (trypticase)	2 g
NaCl	5 g
Dipotassium phosphate	0.3 g
Bromthymol blue dye	0.03 g
Agar	2 g
Glucose	10 g
Distilled water	1 liter

1.Fermentative tube is overlaid with sterile paraffin oil.

2. Oxidative tube is not overlaid.

Method

1. Deep culture tube (16X 125 mm tube) is used for the test.
2. With a sterile needle take a small inoculum from an isolates colony and stab to the bottom of the tube.
3. Incubate at 22 °C for 24-48 hours. Check the tubes at 24 hours for acid and / or gas production.

Interpretation:

- **Oxidative Tube:** 1.yellow color on the top of the tube (Acid).
- **Fermentative Tube:** Yellow color with or without bubbles (Acid with or without gas).

Quality control:

- **Fermentative:** *Aeromonas* species
- **Oxidative:** *Pseudomonas* species.

4) TRIPLE SUGAR IRON (TSI): For H_2S production and OF Test

TSI agar contains the three sugars in varying concentrations: Glucose (1X), Lactose (10X) and Sucrose (10X). It also contains the PH indicator Phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Blackened medium is caused by H2S production, which changes Ferrous sulfate to Ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

Method

1) With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube.
2) Incubate at 22 °C for 24 hours.

Interpretation:

Slant / Butt	Color	Interpretation	Quality control
Alkaline slant / No change in butt	Red Slant/ Orange butt (Oxidative).	Only Peptone utilized	*Pseudomonas*
Alkaline slant/ Acid butt	Red slant/Yellow butt (Fermentative)	Only Glucose fermented	*Edwarsiella tarda* *Yersinia ruckeria* *Edwardsiella ictaluri*
Acid slant / Acid butt	Yellow slant / Yellow slant	Glucose + lactose and / or Sucrose fermented	*Aeromonas* species
Gas	Splitting or bubbles	Gas production	*Edwardsiella tarda*
H_2S	Black Butt	H_2S produced	*Edwardsiella tarda*

5) CITRATE UTILIZATION TEST (Simmon's Citrate Agar)

Simmon's citrate agar is a defined medium containing sodium citrate as the sole carbon source and the ammonium ion as the sole nitrogen source. The pH indicator, Bromthymol Blue, will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (basic or alkaline). If the citrate is utilized, the resulting growth will produce alkaline products (pH >7.6), changing the color of the medium from green to blue. In this medium, sodium citrate is the sole source of carbon and energy. This test determines whether or not an organism is able to metabolize citrate for energy. The un-inoculated medium is green. An indicator, Bromthymol Blue, is added to the medium, which changes color based on pH. The citrate will be yellow if the metabolic products are acidic. A color change to royal blue indicates alkaline by-products. Any color change (from green to either yellow or blue) represents a positive test for citrate utilization.

Materials

I. Formula:

Ammonium Dihydrogen Phosphate----------1.0 gm

Sodium Chloride----------5.0 gm

Magnessium sulfate----------0.2 gm

Bromothymol Blue ----------0.08 gm

Dipotassium Phosphate ----------1.0 gm

Sodium Citrate----------2.0 gm

Agar----------15.0 gm

Or

I. Dehydrated media:

1. Suspend 24.2 gm of the medium in 1 Liter of deionized or distilled water
2. Mix well and heat with frequent agitation until completely dissolved.
3. Dispense 7 ml in 16X 125 mm tubes.
4. Sterilize at 121°C for 15 minutes.
5. Cool the tubes in slanted position so that the base is short (1-1.5 cm. deep) and the slant is long.

Method

1. The surface of the slant is inoculated and the butt is stabbed.
2. Tubes are incubated at 22 °C for 48 –96 hours.

Interpretation:

Only those bacteria capable of utilizing citrate as a source of carbon grow on the slant and produce a color change from green to blue (alkaline).

Quality Control:

Positive (Blue)----------*Yersinia ruckeri*
Negative (Green)---------- *Edwardsiella ictaluri*

GRAM STAINING PROTOCOL

Purpose

Gram stain stains the bacterial cells by reacting with cell wall composition .The stain detects the basic in the cell wall components (peptidoglycans) where some bacteria can retain the primary stain (crystal violet) and resist decolorization so they appear blue or purple. Other types of bacteria do not retain the primary stain and they are easily decolorized so they take the counter stain color (safranin) so they appear red.

Method

1. Put 1drop of water on the center of a clean microslide.
2. Pick up a single bacterial colony and evenly spread it using the loop (until slightly turbid).
3. Leave the slide to air-dry and pass it 3-4 times through the flame to heat fix (overheating will damage your bacteria, the slide should not be too hot to touch).
4. Place the slide on the staining rack by the sink.
5. Overlay with the Gram Crystal Violet and wait for 1 minute (use the stop watch during the whole process to judge your time).
6. Wash thoroughly with tape water.
7. Overlay the slide with Gram's Iodine and wait for 1 minute.
8. Wash thoroughly with tape water.
9. Add the Gram decolorizer drop by drop until the slide become clear (15 seconds) (do that until no more violet fluid is noticed). Avoid excessive decolorization, which may change your Gram positive into false gram negative).

10. Immediately rinse the slide with tape water.
11. Overlay with Gram Safranin for 40 seconds.
12. Rinse the slide with tape water and allow to air dry.
13. Put one drop of cedar wood oil on the slide and examine under the oil immersion lens for the presence of GM positive or GM negative.

Interpretation of Results

1. **Gram Negative:** Bacteria do not retain the primary stain (crystal violet). They are decolorized by the Gram decolorizer and develop pink to red color when counterstained with Gram's safranin solution.
2. **Gram Positive:** Bacteria retain the primary stain (crystal violet). They appear purple to blue in color.

Quality Control

A well-prepared gram stained slides can be made from pure culture of bacterial isolates or purchased from ATCC.

GRAM STAIN REAGENTS RECIPES

I. Crystal Violet

1. Weigh 20.0 gram of crystal violet (90 % dye content), and then put in sterile flask.
2. Add 200 ml ethanol 95 % into the flask.
3. Put a magnetic bar into the flask and allow mixing using magnetic stirrer overnight (ripening).
4. In another flask weigh 8.0 grams of Ammonium oxalate and dissolve in 800 ml Distilled water (Ammon.oxalate aqueous solution).
5. In another 1000 ml Flask mix both mixtures (Crystal violet alcoholic solution and Ammonium oxalate aqueous solution).

6. Using Wattman filter paper, filter the whole mixture.
7. Receive the whole mixture filtrate in a dark bottle and keep until use.

II. Gram's Iodine

1. In a sterile flask dissolve 2.0 grams of Potassium iodide in 300 ml distilled water.
2. Add 1.0 grams of Iodine crystals to the previous mixture and then allow to stir using magnetic stirrer until all the iodine crystals are dissolved and the solution appears brown clear.
3. Transfer the Gram's Iodine mixture to a darker bottle and keep away from light until use.

III. Gram decolorizer

1. In a clean bottle mix 40 ml acetone with 60 ml Ethanol (95 %).
2. Tightly close the bottle to avoid evaporation and keep away from heat and fire (highly flammable).

VI. Gram's Safranin

1. Weigh 2.5 grams of Safranin powder.
2. In a clean flask dissolve the safranin in 100 ml of Ethanol (95 %).
3. Add 900 ml of Distilled water to the mixture and stir for 10 minutes.
4. Using Wattman filter paper filters the mixture.
5. Receive the filtrate in another clean bottle and keep until use.

ANTIBIOTIC SENSITIVITY TESTING PROTOCOL IN AQUATIC ANIMALS

Purpose

Antibiotic sensitivity testing is used to determine the susceptibility of bacterial fish pathogens to different antibiotics. This fish adapted standardized test is used to measure the effectiveness of a variety of antibiotics on a specific fish pathogen in order to avoid the use of a non-effective antibiotic, which always results in the development of antibiotic resistant strains. Also, some antibiotic sensitivity tests include some antibiotic discs that have diagnostic value (Novobiocin, 0/129) which help in the diagnosis of certain bacterial fish pathogens like *Vibrio* spp, *Photobacterium* spp and *Aeromonas* spp.

Principle

A series of antibiotic –impregnated paper disks are placed on a plate inoculated to form a bacterial lawn (even, confluent growth). The plates are incubated at 22 ℃ to allow growth of the bacteria and time fo r the antibiotic to diffuse into the agar (time is variable according to the bacterial spp). If an antibiotic is susceptible to an antibiotic or antibacterial, a clear inhibition zone will appear around the disk where the bacterial growth has been inhibited. The size of the zone (Diameter) of inhibition depends on the sensitivity of the bacteria to the specific antibiotic and the antibiotics ability to diffuse through the Agar.

The antibiotic sensitivity discs method is known as Kirby-Bauer test.

Materials

1. Antibiotic dispenser.

2. Antibiotic sensitivity disks: Antibiotic sensitivity disks should be kept in closed containers or bags in the refrigerator. Check expiration day.
3. Muller's Hinton (MH) Media is the suggested culture medium because it results in reproducible zones of inhibition, and it does not inhibit sulfonamides. (If available).
4. Antibiotic Dispenser (If available) or Forceps.
5. McFarland Standard # 0.5 (If available)
6. 100 ml culture plates.
7. 10 ml sterile culture tubes.
8. 25 ml disposable pipettes and Electric pipetor.
9. 1 ml disposable pipettes.
10. L shaped sterile plastic or glass spreaders
11. Sterile disposable transfer pipettes
12. 0.85 % sterile Sodium Chloride (850mg / 100 ml -dd -water).

Methodology

1. Suspend cells from a pure bacterial culture in log phase (no older than 24 hrs) in sterile saline to obtain turbidity equivalent to a 0.5 McFarland standard.
2. Using a sterile 1 ml pipette aliquot 0.5 ml of the bacterial suspension into the MH agar plates.
3. Using L shaped sterile plastic spreader spread the bacterial suspension over the MH agar plate surface (to ensure even distribution of the bacteria on the gar surface).
4. Leave for 5 minutes to give a chance for bacteria to adsorb to the MH agar surface and be sure that the plate surface is dry .If not withdraw the excess suspension with sterile transfer pipette and allow more time for drying.

5. Using antibiotic dispenser or sterile forceps for each antibiotic disc, aseptically place the antibiotic discs to be tested onto the plate. Press onto the agar surface gently if you are using forceps.

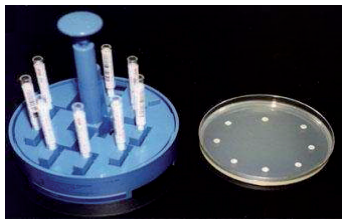


Figure (37) Antibiotic disk dispenser

6. Invert the plates and incubate at 22 ℃ for 18hrs (Rapid growing bacteria) or 48 hrs (slow growing bacteria).
7. Observe and record results by measuring the diameter of the inhibition zone in millimeters around each disc-using ruler.

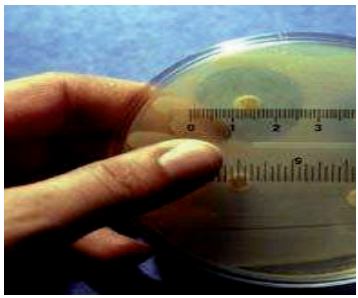


Figure (38) Measuring antibiotic sensitivity zones

Interpretation of Results

There are three possible results for an antibiotic sensitivity test, depending on the diameter of the inhibition zone.

1. **Sensitive**
2. **Intermediate**
3. **Resistant**

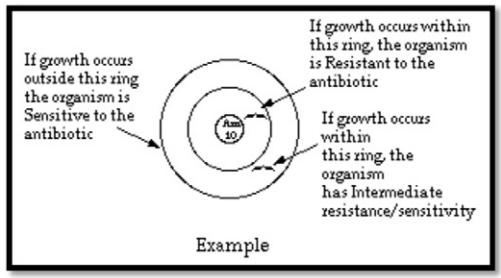


Figure (39) Types of sensitivity zones

- **Interpretation of the antibiotic sensitivity results in pathogens retrieved from aquatic animals can be achieved using the following inhibition zone diameter standard.**

Antibiotic		Inhibition zone diameter standards in mm			
Name	**Code**	**Disk potency**	**Resistant**	**Interm ediate**	**Sensitive**
Terramycin (Oxytetracycline)	TE	30 µg	No zone	<15	≥15
Florofenicol (Aquaflor)	FL	30 µg	No zone	13-17	≥18
Romet 30 (Trimethoprim-Sulfamethoxazole)	SXT	25 µg	No zone	<15	≥15
Erythromycin	E	15 µg	No zone	<15	≥15
Azithromycin	AZM	15 µg	No zone	<15	≥15
Novobiocin	NV	30 µg	No zone	<10	≥10
0/129 (Pteridine)	0/129	0.1 % (W/V)	No zone	< 7	≥7
Ampicillin	AMP	10 µg	≤ 13	14-16	≥17

SELECTED FISH MICROBIOLOGY PICTURES

Figure (40) Chromoshift of Flavobacteria columnare colonies from yellow to pink / brown color after addition of 20 % KOH

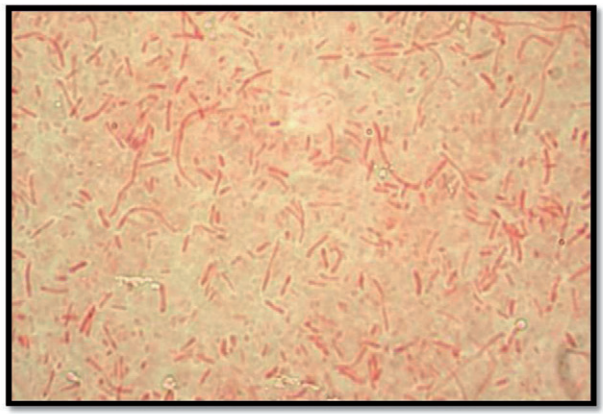


Figure (41)Gram stained smear showing long gram negative bacilli (Flavobacteria spp)

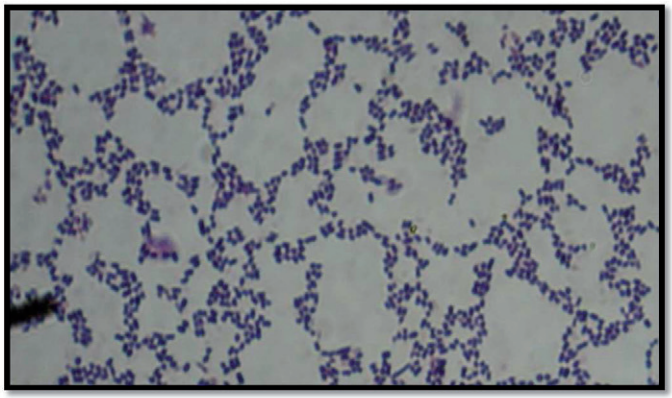


Figure (42) Renibacterium salmoninarum Gram +ve diplobacilli (Courtesy of Alaa Eissa)

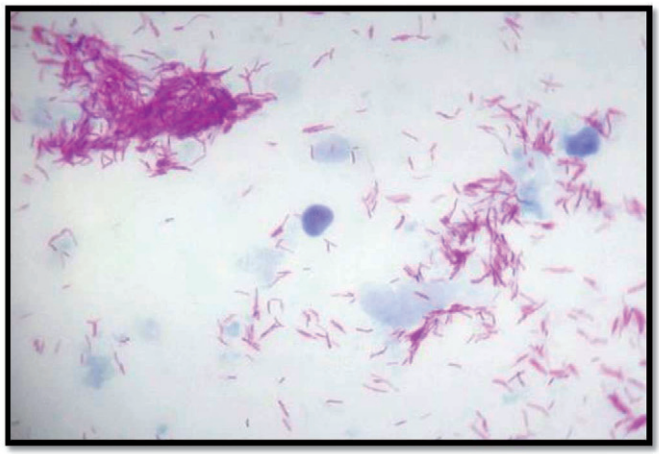


Figure (43) Pink-staining acid-fast rods visible in a bench-top stain of fresh tissue from a fish infected with mycobacteriosis (1000x). Photo courtesy of D. Pouder (http://www.thefishsite.com/articles/1606/mycobacterial-infections-of-fish/)

Figure (44) Lowenstein- Jensen medium

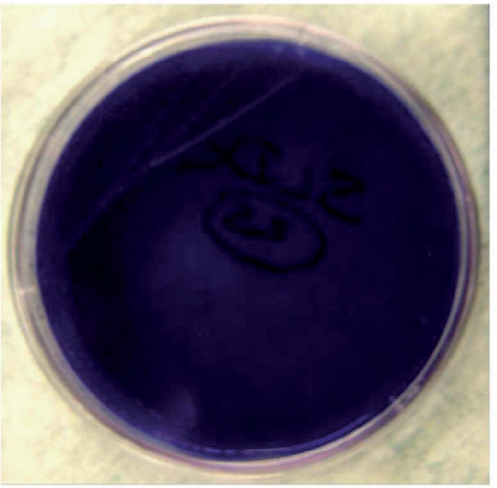


Figure (45) Commassie Brilliant Blue Agar (CBBA)

Figure (46) TCBS Agar

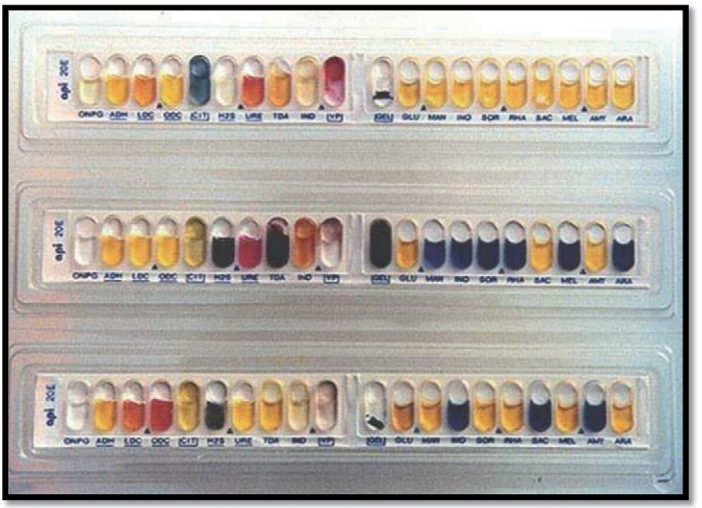


Figure (47) API 20 E Profile (BioMereux)

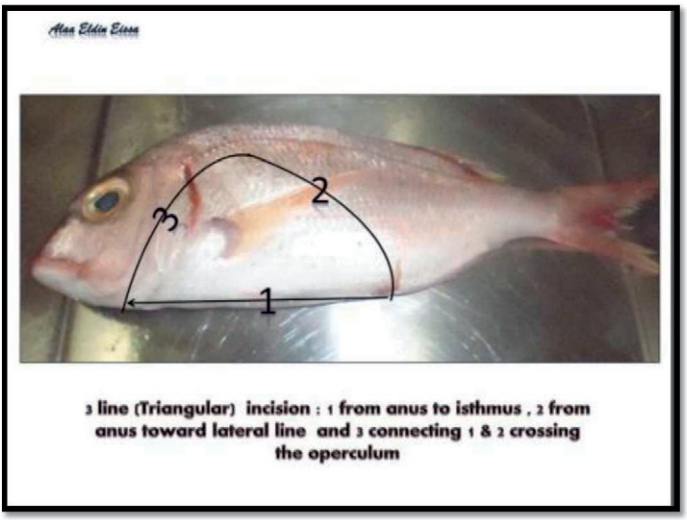
CHEMICALS USED IN TREATMENT OF SOME FISH DISEASES

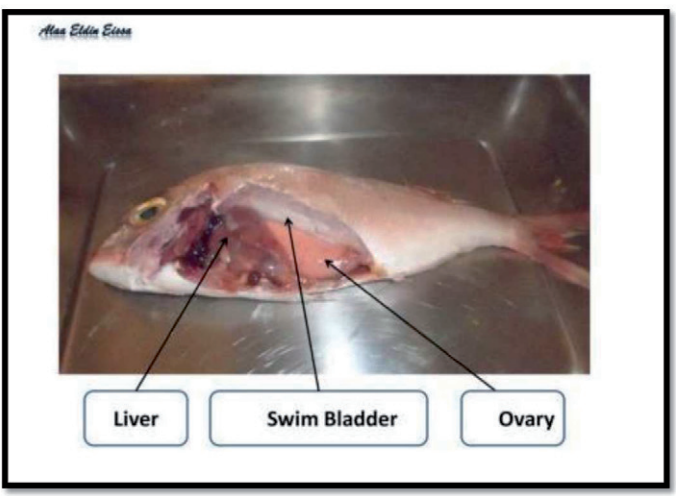
Wide range of chemicals and drugs can be used for treating diseased fishes. If a particular chemical or drug has never been used to treat fish, it is always a good idea to test it first on a small number of fish before an entire pond or holding unit is treated.

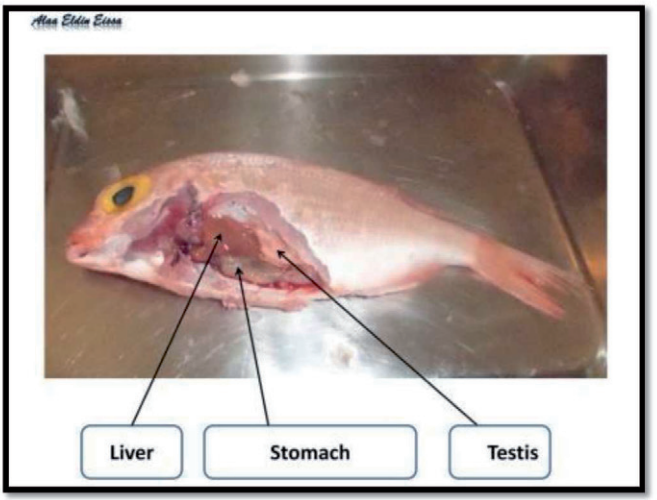
Chemical	Concentration and time of exposure
Calomel	0.2% in food daily for 3 days
Malachite green(oxalate)	2ppm for 30 minutes
Malachite green(zinc-free)	5ppm for 30 minutes
Formalin (37-40%)	25 - 250ppm as bath method for 30 -60 minutes
Malachite green(14 gm) Formalin (one gallon) mixture	25ppm for up to 6 hours daily.
Carbersone	0.2% in food daily for 3 days
Potassium permanganate	3-5ppm as bath method for 15- 30 minutes 5-10 ppm for 1-2 hours
Acriflavin	10ppm for 60 minutes
Sodium chloride	1-2% as dip for 10-20 minutes 3-5% as bath for 1-2 minutes
Copper sulfate	1-2 ppm as bath for 15 minutes
Organophosphorous compounds (masoten, diptrex, diazenon…etc)	16 ppm as bath for 10-20 minutes
Florfenicol (FL)	55-70 mg/kg of fish in food for 10 days
Oxytetracycline (OTC)	55 mg/kg of fish in food for 10 days
Erythromycin phosphate or Erythromycin thiocyanate	55-70 mg/kg of fish in food for 10 days
Sulphonamide e.g. Sulfamethaxzole-Trimethoprim (SXT)	256 mg/kg of fish in food for 3 days followed by 156 mg/kg of fish in food for 11 days

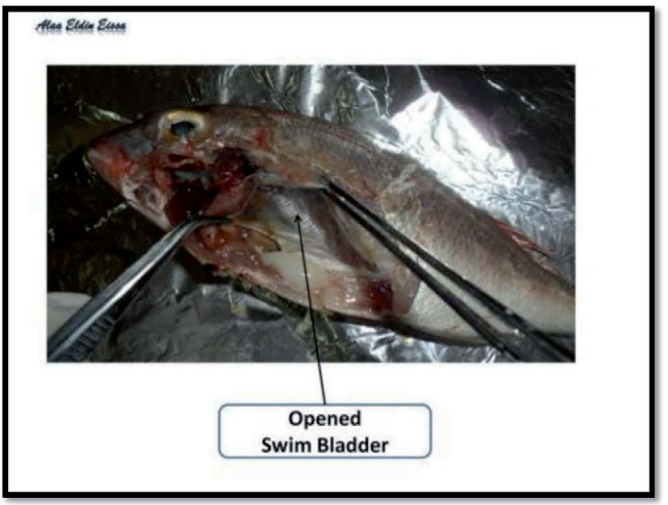
A colored Atlas of Fish Anatomy by Dr. Alaa Eldin Eissa

Sea bream


Alaa Eldin Eissa
3
2
1
3 line (Triangular) incision : 1 from anus to isthmus , 2 from anus toward lateral line and 3 connecting 1 & 2 crossing the operculum

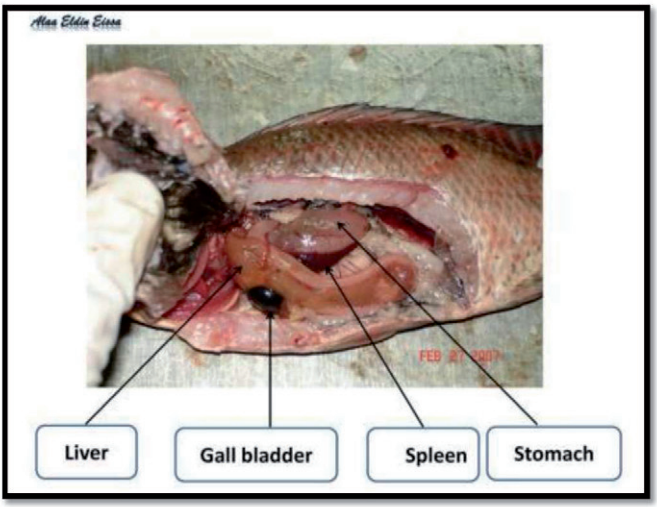

Alaa Eldin Eissa
Liver
Swim Bladder
Ovary

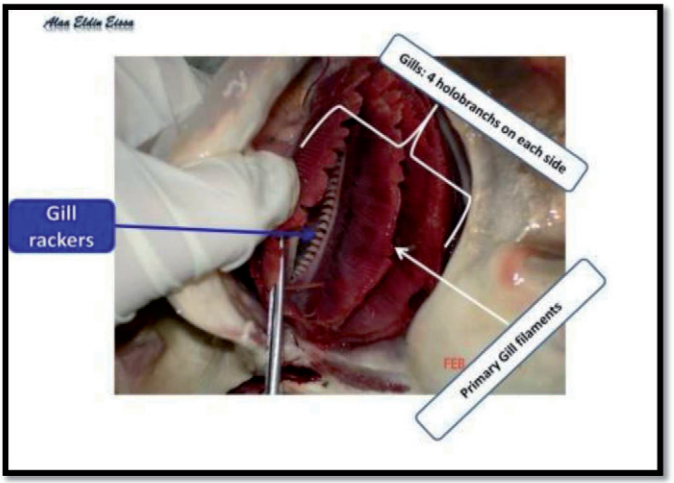

Liver
Stomach
Testis

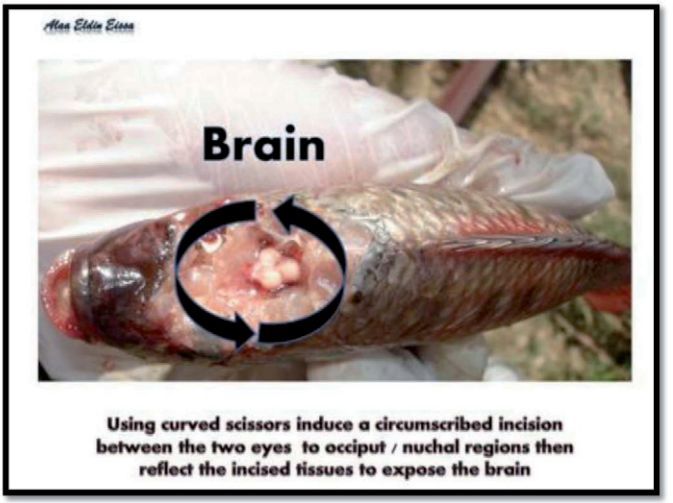

Opened
Swim Bladder

Nile tilapia

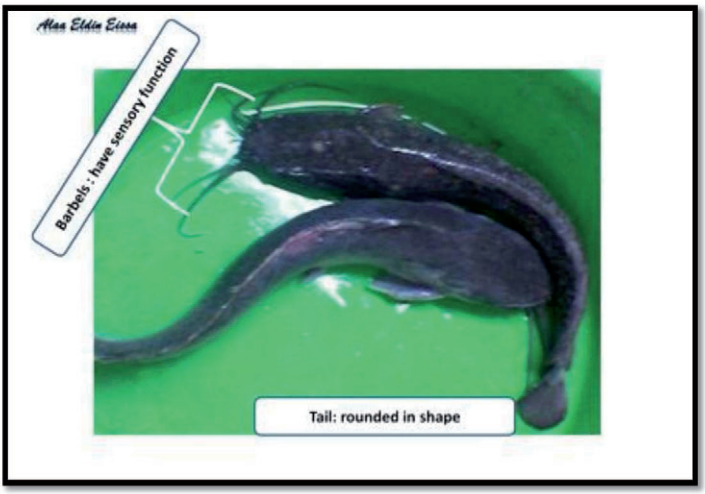

NOV 18 2008

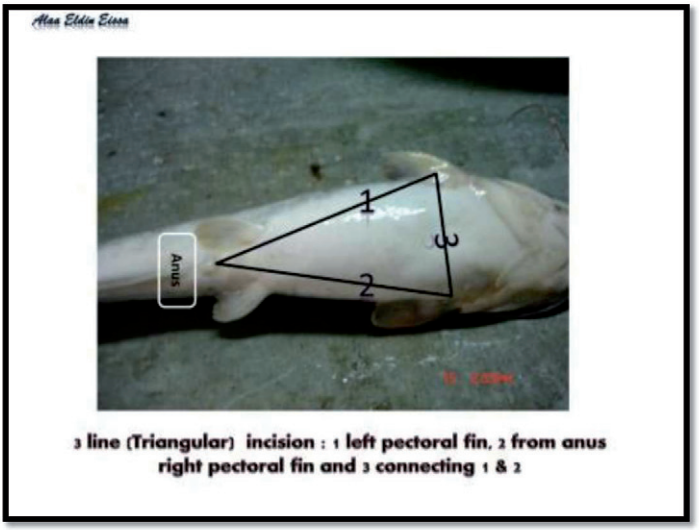

Liver
Gall bladder
Spleen
Stomach


Alaa Eldin Eissa
Gills: 4 holobranchs on each side
Gill rackers
Primary Gill filaments
FEB


Alaa Eldin Eissa
Brain
Using curved scissors induce a circumscribed incision between the two eyes to occiput / nuchal regions then reflect the incised tissues to expose the brain

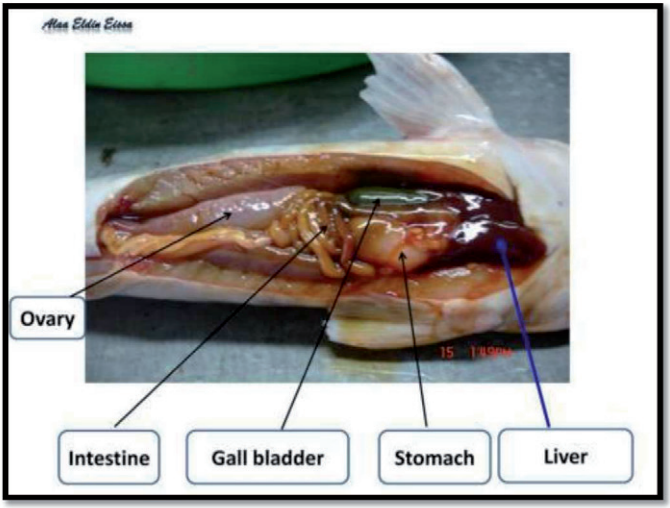
African catfish





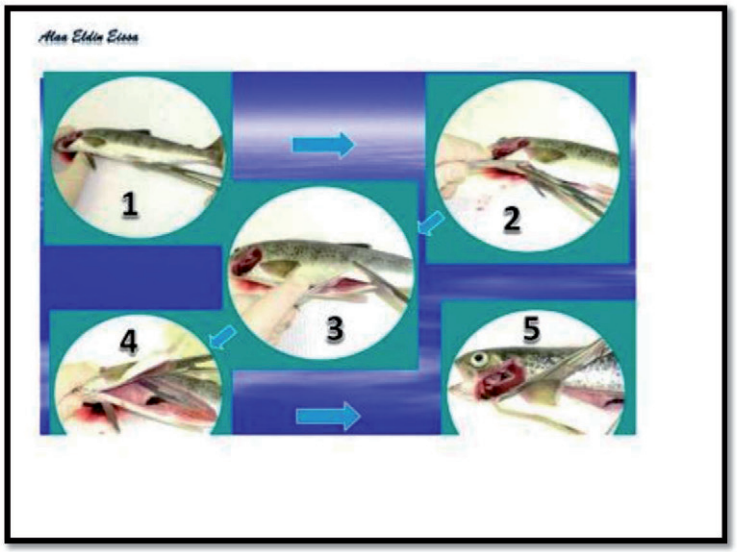
3 line (Triangular) incision : 1 left pectoral fin, 2 from anus right pectoral fin and 3 connecting 1 & 2


Alaa Eldin Eissa

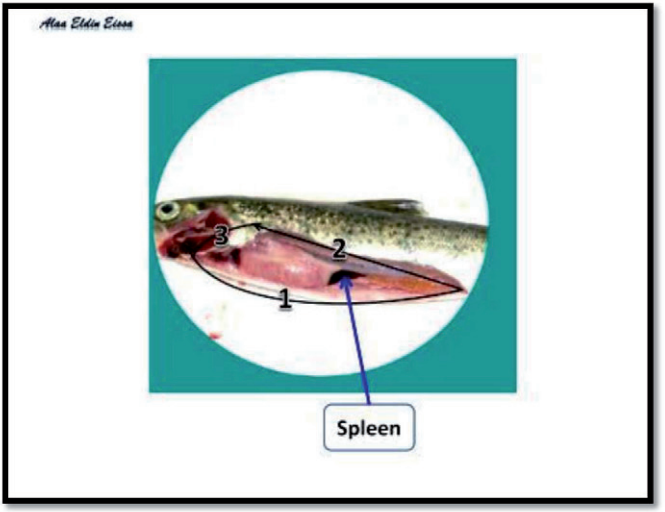

Alaa Eldin Eissa
Ovary
Intestine
Gall bladder
Stomach
Liver

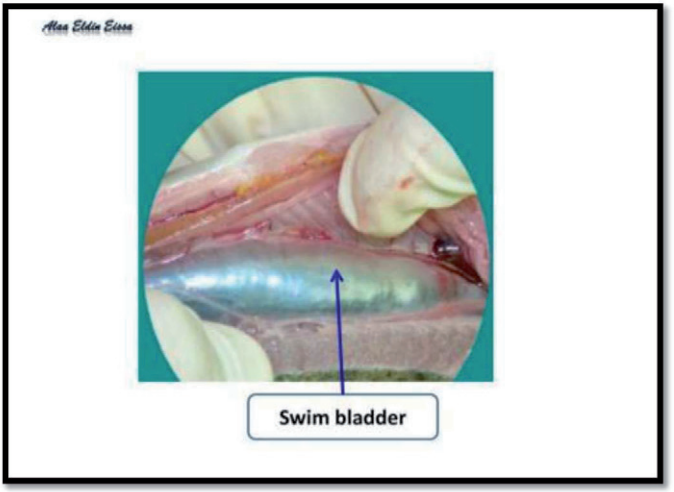
Rainbow trout

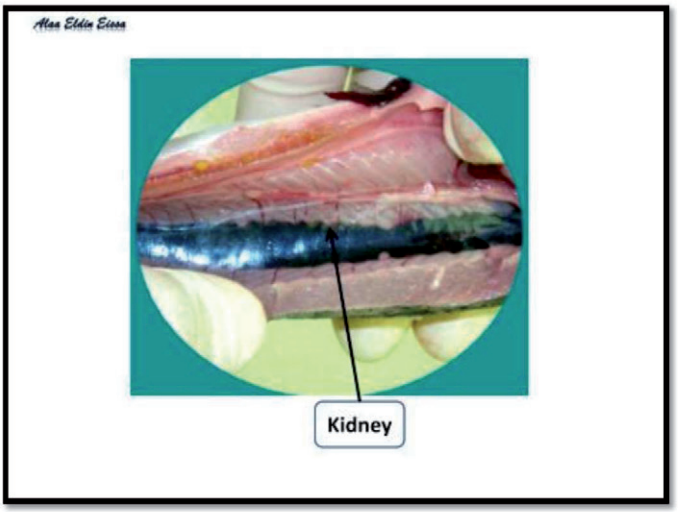

Alaa Eldin Eissa

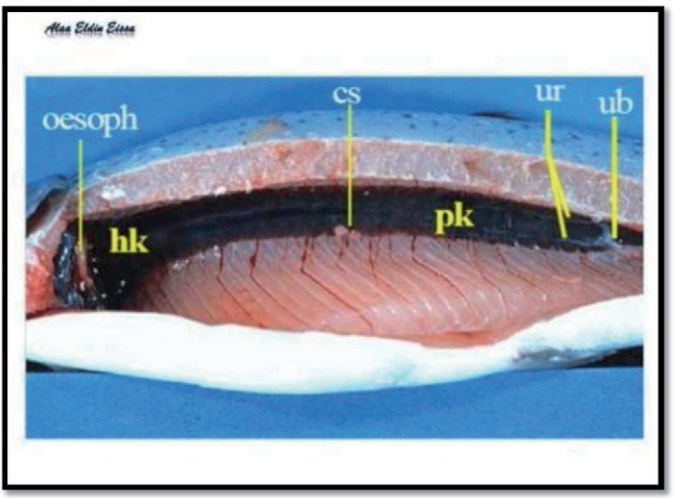

Alaa Eldin Eissa
1
2
3
4
5


Alaa Eldin Eissa


Alaa Eldin Eissa
3
2
1
Spleen


Alaa Eldin Eissa
Swim bladder


Alaa Eldin Eissa
Kidney


Alaa Eldin Eissa
oesoph
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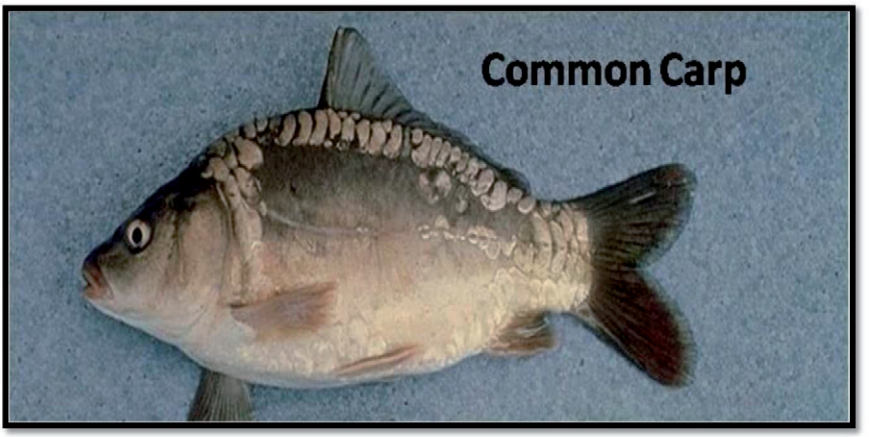
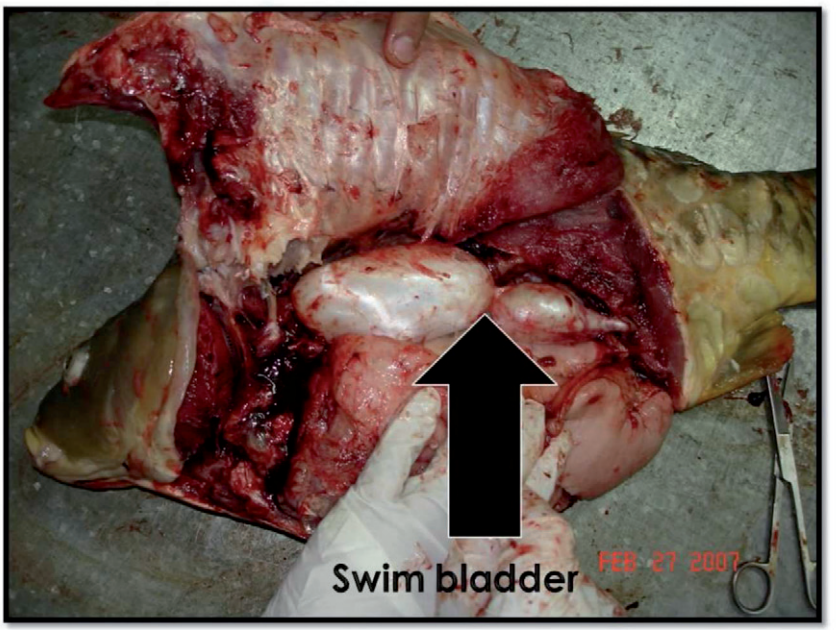
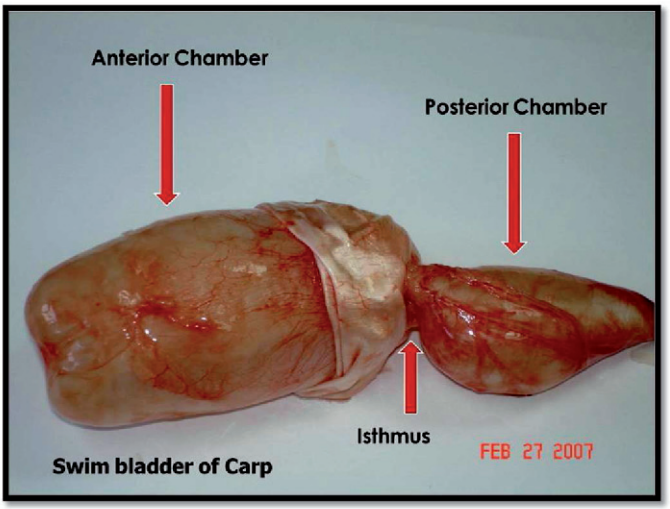
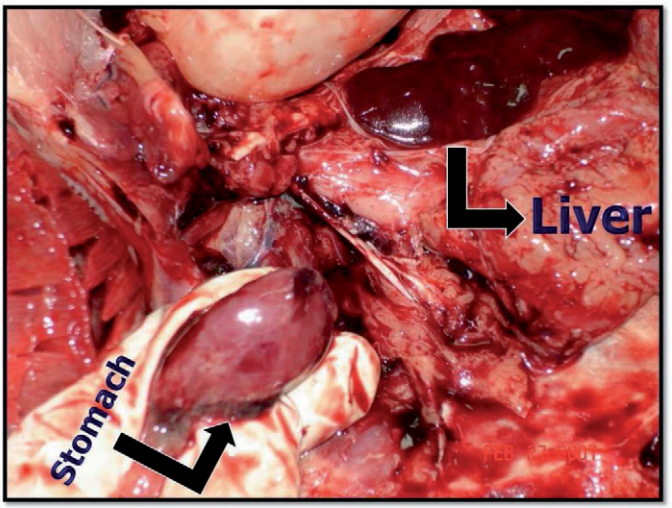
Carp


Common Carp

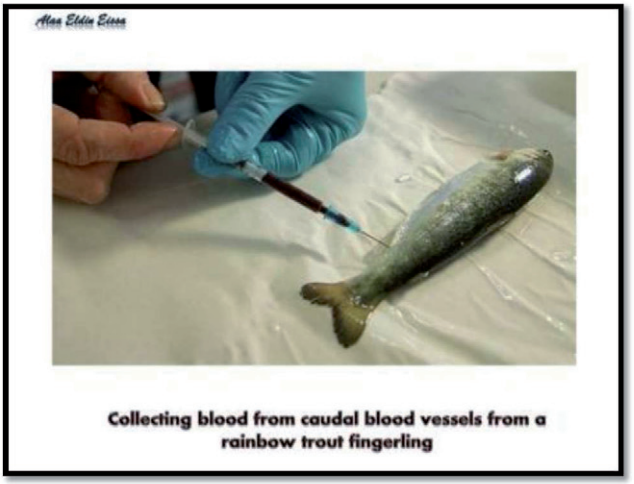

Swim bladder
FEB 27 2007


Anterior Chamber
Posterior Chamber
Isthmus
FEB 27 2007
Swim bladder of Carp


Liver
Stomach

Methods of blood sampling


Alaa Eldin Eissa
Collecting blood from caudal blood vessels from a rainbow trout fingerling


Alaa Eldin Eissa
Collecting blood from caudal blood vessels from a rainbow trout fingerling

Collecting blood from caudal blood vessels from a grass carp fish

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- Dr. Ehab Elsayed (FDML Faculty of Veterinary Medicine, Cairo University).
- Dr. Mohamed Abdelaziz (FDML Faculty of Veterinary Medicine, Cairo University).

REFERENCES

1) AFS-FHS (American Fisheries Society-Fish Health Section) .(2007). FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.
2) Austin B, Austin AD. Bacterial fish pathogens. (2012). Diseases of farmed and wild fish. 5th ed. Chichester, UK: Springer / Prazis Publishing.
3) Bauer, A.W., W.M. Kirby, J.C. Sherris, M. Turck, 10(2): 107-14. (1966). Antibiotic susceptibility testing by a 57. Thune, R.L., L.A. Stanley and R.K. Cooper, 1993. Standardized single disk method. Am. J. Clin Pathol., Pathogenesis of gram-negative bacterial infections 45(4): 49-496.
4) Buchmann K., Bresciani J. (1997). Parasitic infection in pond-reared rainbow trout *Oncorhynchus mykiss* in Denmark. Diseases of Aquatic Organisms, 28, 125-138.
5) Eissa A.E. (2005). Bacterial kidney disease (BKD) in Michigan salmonids. PhD Dissertation. Michigan State University, East Lansing, Michigan, 210 pp.
6) Eissa A.E., Abu Mourad I.M.K., Borhan T. (2006). Contribution on myxosoma Infection in Cultured *Oreochromis niloticus*. Nature and Science, 4(4), 40-46.
7) Eissa, A.E., M. Moustafa, I.N. El-Husseiny, S. Saeid, O. Saleh and T. Borhan. (2009). Identification of some skeletal deformities in some freshwater teleost raised Egyptian aquaculture. Chemosphere, 77: 419-425.
8) Eissa A.E., Moustafa M., Abdelaziz M., Ezzeldeen N.A. (2008). *Yersinia ruckeri* infection in cultured Nile tilapia (*Oreochromis niloticus*) from semi-intensive fish farm in Lower Egypt. African Journal of Aquatic Sciences, 3(3), 283–286.
9) Eissa A.E., Moustafa M., Abumhara A., Hosni M. (2016). Future Prospects of Biosecurity Strategies in Egyptian Fish Farms. Journal of Fisheries and Aquatic Science, 11: 100-107.
10) Eissa, A. E., Zaki, M. M., Aziz, A. A. (2010). *Flavobacterium columnare / Myxobolus tilapiae* concurrent infection in the earthen pond reared Nile

Tilapia (*Oreochromis niloticus*) during the early summer. Interdisciplinary Bio Central, 2(2), 5-1.

11) Eissa, A. E., Zaki, M. M., & Saeid, S. (2011, October). Epidemic mortalities in the dusky grouper, *Epinephelus marginatus* (Lowe, 1834) in Egyptian coastal waters. In Proceedings of the 4th Global Fisheries and Aquaculture Research Conference, the Egyptian International Center for Agriculture, Giza, Egypt (pp. 3-5).

12) Elsayed, E. E., Eissa, A. E., Faisal, M. (2006). Isolation of *Flavobacterium psychrophilum* from sea lamprey, *Petromyzon marinus* L., with skin lesions in Lake Ontario. Journal of fish diseases, 29(10), 629-632.

13) Faisal, M., Eissa, A. E. (2009). Diagnostic testing patterns of *Renibacterium salmoninarum* in spawning salmonid stocks in Michigan. Journal of wildlife diseases, 45(2), 447-456.

14) Faisal, M., Eissa, A. E., Starliper, C. E. (2010). Recovery of *Renibacterium salmoninarum* from naturally infected salmonine stocks in Michigan using a modified culture protocol. Journal of Advanced Research,1(1), 95-102.

15) Faisal M., Schulz C., Eissa A., Whelan G. (2011). High prevalence of buccal ulcerations in largemouth bass, *Micropterus salmoides* (Centrarchidae) from Michigan inland lakes associated with *Myzobdella lugubris* Leidy 1851 (Annelida: Hirudinea). Parasite 18, 79–84.

16) Noga, EJ .(2010). Fish Diseases: *Diagnosis and Treatment*. Iowa State University Press, Ames, Iowa.

17) OIE (Office international des épizooties). (2006). Aquatic Manual: Manual of Diagnostic Tests for Aquatic animals, 5th Edition. Animal World Health Organization, Paris.

18) Post, G. (1987). Revised and Expanded Textbook of Fish Health. NJ: TFH Publications.

19) Stoskopf M .(1993).Fish Medicine. WB Saunders Company, Philadelphia, Pennsylvania.

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