جامعة وهران 1 أحمد بن بلة
Université Oran 1 أحمد بن بلة
كلية علوم الطبيعة والحياة
Faculté des Sciences de laNature et de la Vie

University of Oran 1 Ahmed Benbella

Department : Biology

Teaching handout

Title

Methods for studying protozoa

Course intended for students of

Bachelor's degree (specialty and level) : 'L3 LMD' Parasitology

Dr. HADJ SLIMANE Touria

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Foreword

There are numerous parasitic species and forms that can parasitize various sites of their host, either intracellularly or extracellularly. These parasites cause parasitic infections, some of which are pathogenic or even deadly.

Given the ease with which these parasites spread, it is essential to make a rapid and accurate diagnosis of a parasitic infection as soon as the first symptoms appear to ensure effective treatment.

The purpose of this handbook is to help third-year undergraduate students specializing in Parasitology to master the direct diagnostic techniques used in detecting protozoan parasites responsible for internal human parasitic infections.

The first chapter of this handbook is dedicated to parasitological coprology ; the second to the techniques used for diagnosing blood parasites ; the third to the detection of a sexually transmitted parasite (*Trichomonas vaginalis*), followed by culture methods and ending with molecular biology.

In the first three chapters, the sample collection method specific to each suspected parasite species is detailed.

Summary

Introduction

Parasites are omnipresent, and every living being could be parasitized at some point in its life, regardless of lifestyle or geographic range. Humans host multiple parasitic species and have yet to escape them.

Parasites colonize all ecosystems (aquatic, marine or freshwater, terrestrial, and aerial) and significantly contribute to biodiversity.

Parasites cause human, animal, and plant diseases, the socio-economic impact of which is far from negligible.

More than a billion people worldwide are affected by parasites, with millions of deaths each year, especially on the African continent. In animals, parasites cause decreased productivity or even mortality in livestock, and reduced crop yields in plants.

Parasites belong to very diverse groups (protozoa, helminths, arthropods) that have no common feature other than living at the expense of the human body.

Protozoa are animal-like protists that include rhizoflagellates (amoebas and flagellates), sporozoans, and ciliates. They cause protozoan infections, some of which are endemic and concerning, such as malaria, the world's leading parasitic disease, causing the death of approximately 750 000 to 1 million people annually.

The global population is growing day by day and living longer. In this context, fighting parasites is crucial.

Understanding their morphology and biology would help in their identification, and consequently in combating the diseases they cause. This requires biological diagnosis of parasitic infections, which is most often and as much as possible ensured by detecting the pathogen (direct diagnosis). In some cases, the diagnosis can only be based on indirect data resulting from the host's reactions to the infection (indirect diagnosis).

In this handbook, only the direct detection of the parasite through examination and its DNA are discussed.

The objective of this course is to understand the different methods for detecting parasitic protozoa according to their location

I. Coprological Methods

Digestive parasitic infections are common in developing countries, where they pose a real public health problem. The risk is greater in children and immunocompromised patients. These infections are caused by numerous and diverse parasitic agents, which are mainly transmitted through the ingestion of water or food contaminated with parasitic propagules, and through poor hygiene practices (contaminated hands brought to the mouth). For their diagnosis, the microscopist's goal is to definitively establish the presence of parasites in the stool, whether in cystic, vegetative, or oocyst forms of protozoa, and to correctly identify them.

Coprological methods, as diagnostic techniques, are used to detect intestinal and extra-intestinal parasites that are excreted through feces into the external environment.

In addition to identifying parasites, these methods provide information on:

- The macroscopic appearance of the stools, particularly their consistency, color, and odor.
- The appearance of digestion in the three food categories: proteins, lipids, and carbohydrates.

Every coprological examination must include :

- A direct fresh examination
- A direct examination after parasitic concentration
- A staining procedure that is essential for highlighting the details of parasitic forms.

I.1. Stool sample collection and preparation

Before collecting samples, and to avoid false positives or negatives, the patient must refrain from taking certain medications and consuming specific foods for three days prior to any parasitological examination, including :

- Medications containing activated charcoal, ...
- Opaque products used in radiological exams (barium).
- Fatty substances, particularly laxative oils (paraffin) and suppositories.
- Certain medications that may have a secondary anti-amoebic effect.
- Foods that leave significant residue, mainly :
  - i. Fruits with thick skins that are not digested (peaches, apricots, tomatoes, ...)
  - ii. Rosaceous fruits (apples and pears)
  - iii. Fruits with numerous seeds (figs, pomegranates)

iv. Seeds with husks (beans, lentils)

Sample collection is a crucial step for obtaining quality results. Ideally, the stool should be :

- Collected directly at the laboratory in a clean, dry, sealed container with a tightly fitting lid to avoid any spillage (Figure 1). It is essential to avoid contact with water, soil, and urine (risk of destruction of vegetative forms). The container must be labeled with the prescriber's name, the patient's name, and the date of collection.

  If the stool is collected outside the laboratory, it should be delivered to the lab as quickly as possible to avoid the degeneration of the vegetative forms of protozoa, which are sensitive to temperature variations and dehydration.
- Collected in sufficient quantity (preferably the entire bowel movement).

A negative test result does not rule out parasitic infection due to the parasite's lack of maturation or the intermittent release of different forms. Therefore, three stool samples are collected at 4-5 day intervals between each sample.

Each sample must be accompanied by a properly completed information sheet : clinical signs, age, sex, address, presence of pets, profession (veterinarian, farmer), dietary habits, and immune status.

Figure 1. Stool Collection Containers

A : Without spatula
B : With spatula

I.2. Direct Examination

Direct examination involves both a macroscopic and microscopic analysis of stool samples.

I.2.1. Macroscopic Examination

Macroscopic observation allows for the assessment of :

- Stool consistency (liquid, formed, soft, mushy, hard...) using the Bristol Stool Scale (Annex 1)
- The presence of mucus and/or blood
- Stool color

Upon receipt, stool samples must be fixed immediately if the examination cannot be performed within the following timeframes :

- Liquid stools : within 30 minutes
- Soft or mushy stools : within one hour
- Formed stools : within a few hours

The distribution of protozoa based on stool consistency is shown in Figure 2, indicating that trophozoites (vegetative forms) predominate in liquid stools, while cysts (dissemination stage) are more common in formed stools.

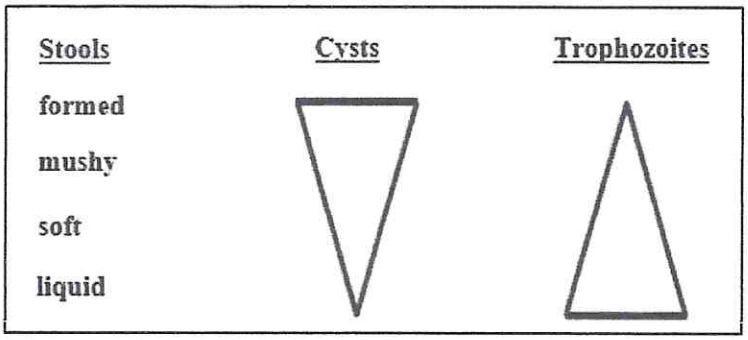


Figure 2. Distribution of the two forms of protozoa according to stool consistency.

Fixatives used include :

- **Sodium acetate - acetic acid - formaldehyde (SAF):**

  Composition:

  Sodium acetate .....1.5 g
  Glacial acetic acid ..... 2.0 ml
  Formaldehyde solution (commercial, 37%) .....4.0 ml
  Water .....92.5 ml

Dissolve and mix all ingredients in the specified order using magnetic stirring. Samples fixed in SAF can be used for permanent staining.

- **10% Formalin :**

  Composition :

  Formaldehyde solution (commercial, 37%) .....100 ml

  Water .....900 ml

  Mix thoroughly.

Both fixatives provide good preservation of protozoan cysts and ensure the rapid death of trophozoites. It is important to store these fixatives in brown bottles, away from heat sources.

I.2.2. Microscopic examination

I.2.2.1. Fresh Examination in Physiological Saline and Lugol's Solution

Fresh preparation is the simplest and easiest method for examining stool samples and should be used in all laboratories. The preparation in physiological saline is mainly used for the initial microscopic examination of stools, especially to highlight the vegetative forms of protozoa. Lugol's solution is primarily used to stain the glycogen vacuole and nuclei of cysts.

- **Method** (Figure 3) **:**
  - **Step 1**
  1. Label the slide with the patient's number and the date on the left side.
  2. Place a drop of physiological saline in the center of the left half of the slide and a drop of Lugol's solution in the center of the right half (Figure 3A).
  3. Using a wooden applicator, take a small portion of the stool sample (about the size of a match head) and mix it with the drop of physiological saline (Figure 3B).
  4. Similarly, take a small amount of stool and mix it with the drop of Lugol's solution.
  5. Cover each drop with a coverslip (Figure 3C). To do this, hold the coverslip at an angle, touch the edge of the drop, and gently lower the coverslip to avoid air bubbles.

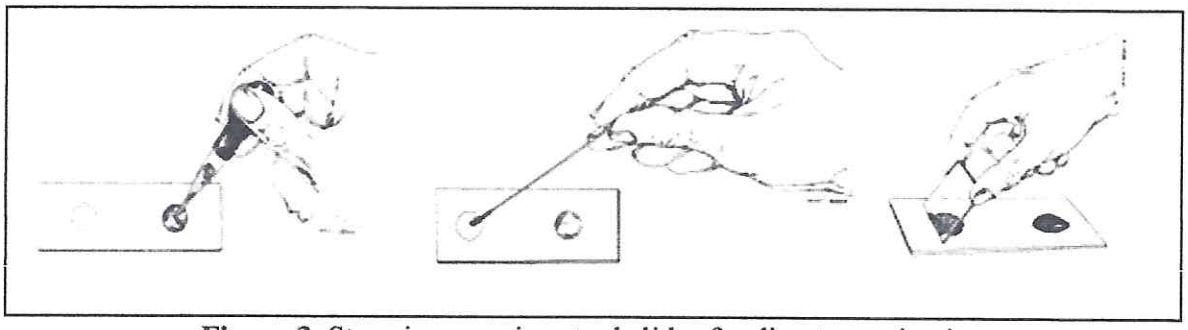


Figure 3. Steps in preparing stool slides for direct examination.

- **Step 2 : Microscopic observation**

1. Place the prepared slide on the microscope stage and focus with the 10x objective.
2. Adjust the light in the field using the diaphragm.
3. Examine the entire area under the coverslip with the 10x objective; focus on the upper-left corner and move the slide in a zigzag pattern (Figure 4).
4. If parasites or suspicious elements are observed, switch to a higher magnification and increase the light by opening the diaphragm to observe their morphology in detail.

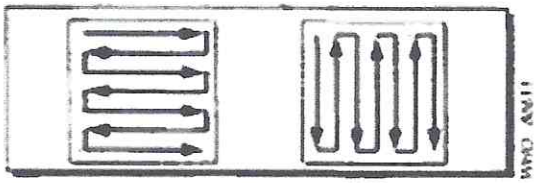


Figure 4. Zigzag pattern of scanning both preparations.

Microscopic examination allows the detection of both parasitic and non-parasitic elements :

- **Non-parasitic elements**

The most common ones are (Annex 2) :

- Leukocytes
- Macrophages
- Erythrocytes
- Epithelial cells : varying in size and shape, which should not be confused with amoebas
- Bacteria
- Fungi
- Food and medication residues

- **Parasite identification**

In physiological saline, one can observe both the vegetative forms and cysts of amoebas and flagellates. Cysts appear as round or oval, refractile structures ; amoebic vegetative forms can be round or irregular, while flagellate forms are typically pear-shaped (elongated and tapering). In fresh stool (within a maximum of one hour), one can observe motile vegetative forms. This motility can be used to identify the species, particularly in the case of flagellates.

Some useful characteristics for identifying the motile forms of intestinal protozoa are shown in Figure 5.

Tables 1, 2, and 3 present the morphological characteristics of the vegetative forms of certain amoeba species.

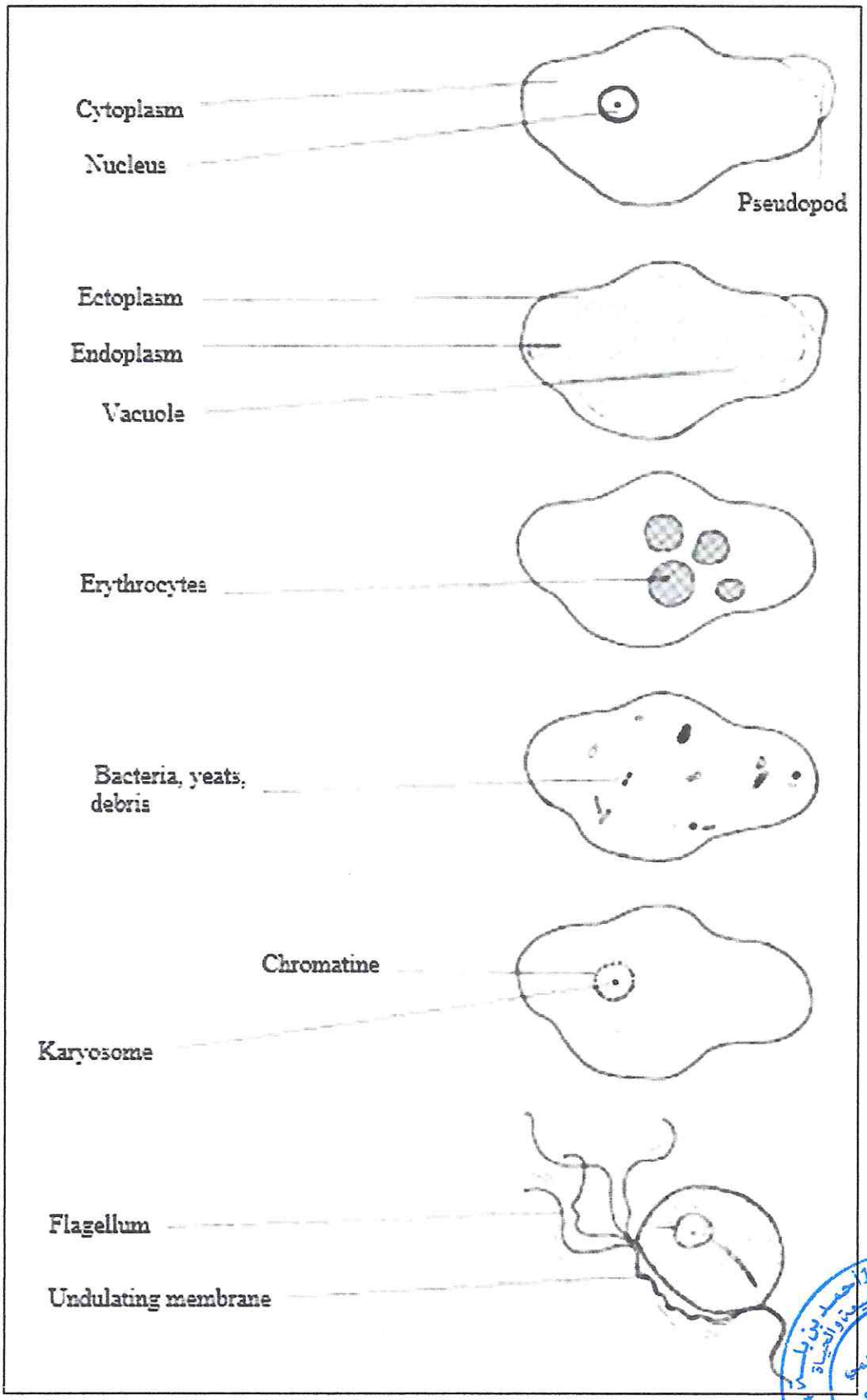


Figure 5. Criteria for Identifying Vegetative Forms of Protozoa

Table 1. Morphological Characteristics of *Entamoeba histolytica*

Characteristic	Description
Size	Variable, from 12 to 35 µm
Shape	Elongated and irregular when moving ; rounded when stationary
Movement	Moves in a single direction ; extends a pseudopod and flows like a slug, relatively quickly
Cytoplasm	Transparent ectoplasm, well-differentiated from the finely granular endoplasm, which may contain vacuoles
Nucleus	Invisible in the motile amoeba, but in amoebae stained with Lugol's solution, a distinct regular membrane and a small, dense central karyosome (like a black dot) can be observed

WHO, 1982

Two mobile forms of *E. histolytica* appear in liquid or diarrheic stools :

(a) a large species, measuring 20 to 35 µm, with vacuoles containing more or less digested red blood cells (Figure 6a), which indicates hemophagic activity (blood consumption) and, consequently, pathogenic properties ;

(b) a small, non-pathogenic species, thriving in the intestinal cavity, where it phagocytizes bacteria or other materials visible inside the vacuoles; it measures 12 to 20 µm (Figure6b).

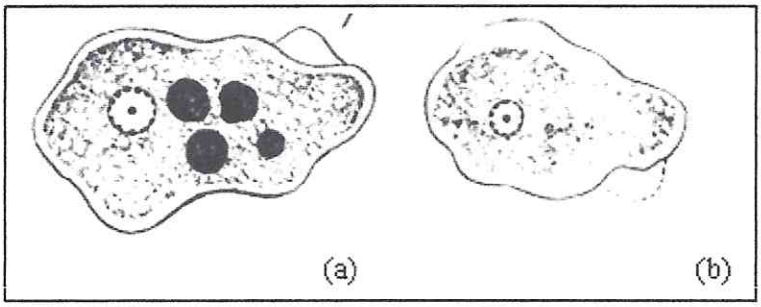
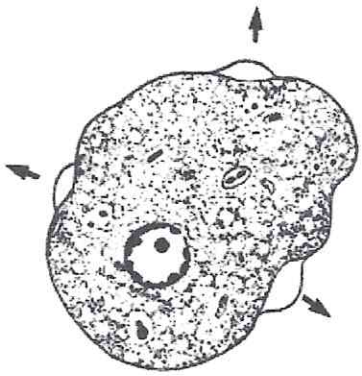


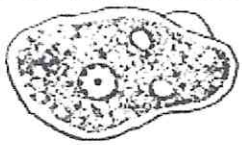
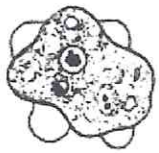
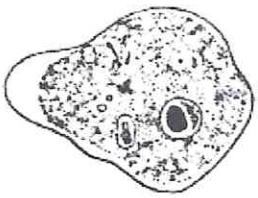
Figure 6. Morphology of *E. histolytica histolytica* (a) and *E. histolytica minuta/dispar* (b)

Table 2. Morphological Characteristics of *Entamoeba coli*

Characteristic	Description	
Size	20 to 40 µm (generally larger than *E. histolytica*)	
Shape	Oval or elongated, fairly irregular	
Movement	Often immobile or moving very slowly with short pseudopodia in all directions, blindly	
Cytoplasm	Granular ectoplasm and endoplasm, difficult to differentiate	
Inclusions	Numerous and varied (bacteria, yeasts, various debris), but never red blood cells	
Nucleus	Visible in fresh samples without staining. The membrane is irregular and granular ("string of pearls" appearance), with a large and eccentric karyosome	

WHO, 1982

Table 3. Morphological characteristics of other amoeba species

Species	Characteristics
Entamoeba hartmani	**Size :** small, always less than 10 µm Same features as *E. histolytica minuta* 
Endolimax nana	**Size :** small, 6 to 10 µm **Movement** : Numerous small, round pseudopodia, moving slowly in all directions **Cytoplasm :** Granular with small vacuoles **Inclusions :** Varied (mainly bacteria) **Nucleus** : Ink-like karyosome (Lugol staining) 
Pseudolimax butschli	**Size :** medium, 10 to 15 µm **Shape :** Compact, resembling a tree leaf **Movement :** Very slow; pseudopodia clear, finger-like or rounded **Inclusions :** Bacteria, large vacuoles **Nucleus :** Large oval karyosome next to a cluster of granules (Lugol staining) 

WHO, 1982

The main characteristics that allow for the identification of intestinal protozoan cysts are presented in Figure 7.

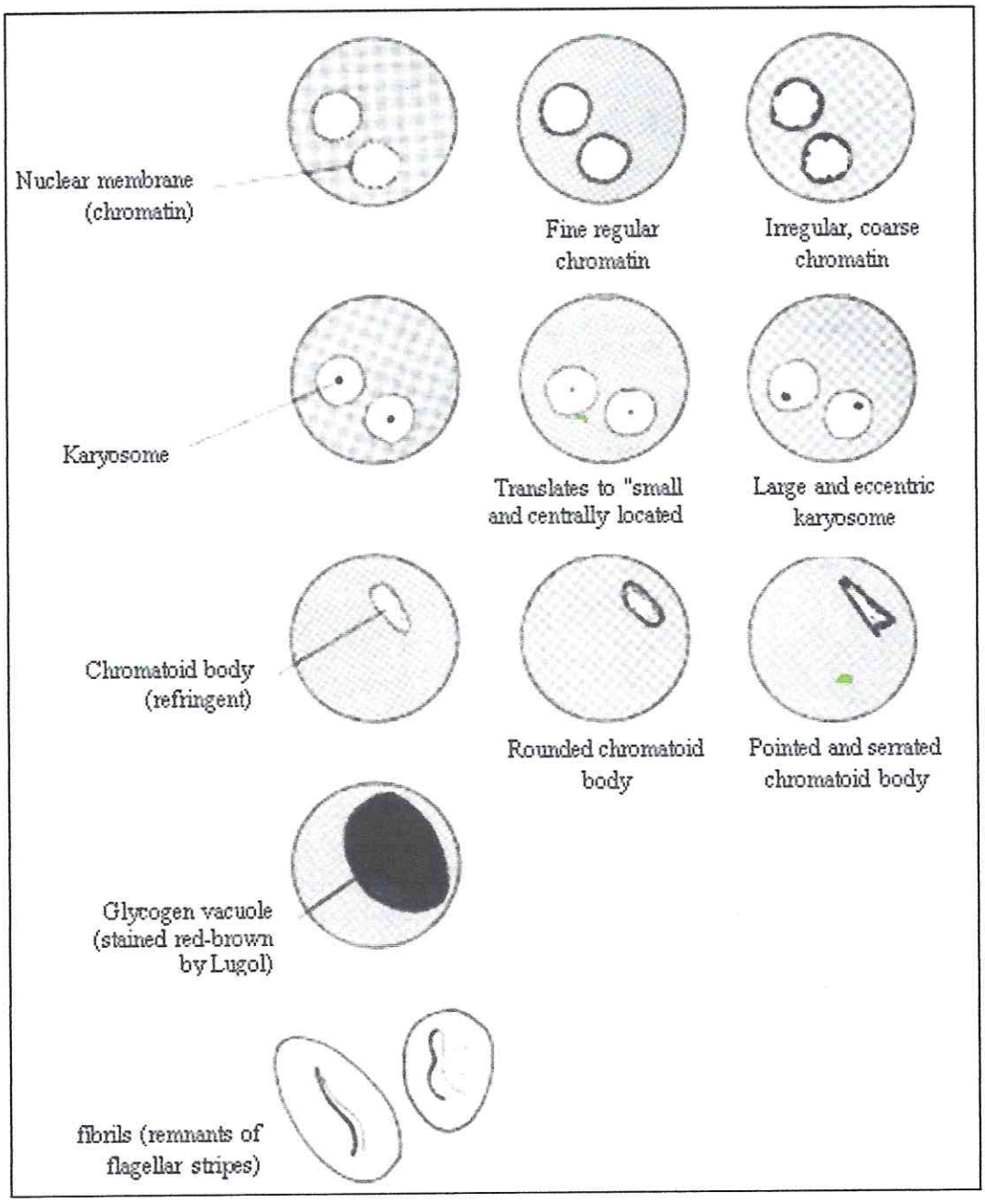


Figure 7. Main criteria for identifying protozoan cysts

The cysts of certain amoeba species are presented in Figure 8.

Entamoeba histolytica

Size : 12 to 15 µm

Shape : round

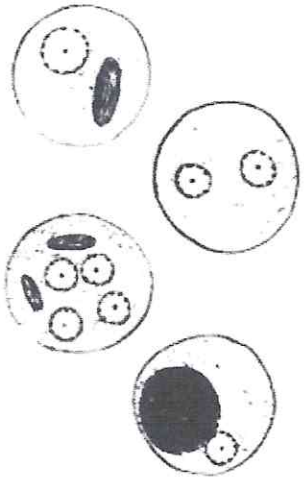
Nuclei : 1 to 4 nuclei :

- Fine and regular chromatin
- Small, dense, and central karyosome

Cytoplasm : (Lugol) yellow-gray, granular

Chromatoid bodies : Rounded ends

Vacuole : Sometimes a large glycogen vacuole (stained red-brown by Lugol)



Entamoeba coli

Size : 12 to 20 µm

Shape : Round or slightly oval, sometimes deformed

Nuclei : 1 to 8 noyaux :

- Irregular and thick chromatin in places
- Large, less dense, often eccentric karyosome

Cytoplasm : (Lugol) light yellow, bright (compared to *E. histolytica* cysts)

Chromatoid bodies : Serrated ends

Vacuole : Sometimes a very large glycogen vacuole (stained red-brown by Lugol)

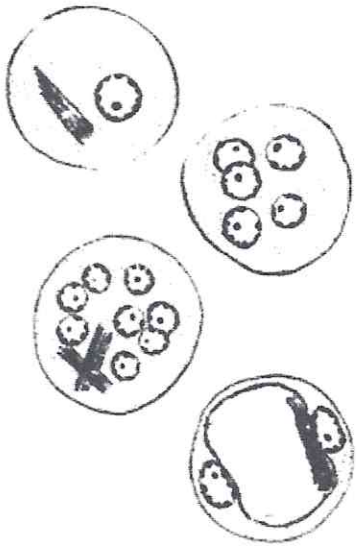


Figure 8. Cysts of some amoeba species

I.2.2.2. Direct examination after parasitic concentration

In cases where the direct examination of fresh samples is negative and depending on the clinical information provided, concentration techniques should be used.

These techniques aim to isolate protozoan cysts from a large quantity of fecal matter by concentrating them into a smaller volume, while eliminating digestion residues. This is achieved by exploiting the different densities of these residues and the target parasites.

A well-conducted parasitological examination should include one or more concentration techniques. Two biphasic techniques are mentioned in this handout.

These methods involve placing fecal samples in contact with two immiscible liquid phases : one aqueous and one organic (such as ether). This creates a partition coefficient for each fecal particle (parasites, food debris) based on its hydrophilic-lipophilic balance, allowing for separation.

The principle of these techniques is to separate the parasites from the fecal debris and concentrate them into a small volume of fixative after centrifugation.

1.2.2.2.1. Modified Teleman Rivas method by Bailenger (1962)

1. Triturate 2 to 5 grams of stool in a conical flask with 10 times its volume of aceto-acetic buffer at pH 5.5.
2. Allow to sediment for 40 to 50 seconds.
3. Pour the supernatant into a 15 ml centrifuge tube.
4. Add sulfuric ether (1/3 of the total liquid volume), ensuring at least 1 cm of empty space at the top of the tube.
5. Shake vigorously until complete emulsion, after sealing the tube tightly.
6. Centrifuge immediately after emulsification for 1 minute at 1500 to 2000 rpm.
7. Discard the supernatant and examine the sediment in a drop of Lugol's iodine solution between a slide and cover slip.

1.2.2.2.2. Modified Ritchie method (1948)

1. In a conical flask, triturate 2 grams of stool in 100 ml of 10% formalin.
2. Strain and pour 2/3 of the fecal dilution and 1/3 of ether into a centrifuge tube.
3. Shake until a homogeneous solution is obtained.
4. Centrifuge at 1500 rpm for 3 to 5 minutes (Figure 9).
5. Discard the supernatant and examine the sediment in a drop of Lugol's iodine solution between a slide and cover slip.

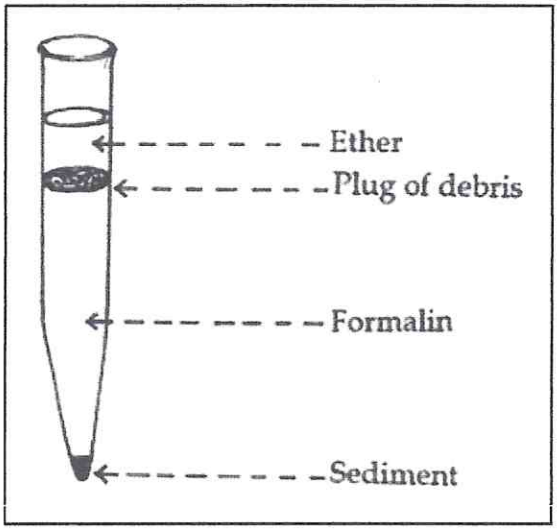


Figure 9. Profile of the Falcon tube after centrifugation

I.2.2.3. Staining techniques

To accurately observe nuclear structures, thick and wet stool smears are prepared, fixed, and stained.

These techniques are :

- Useful when there is doubt in identifying cysts.
- Helpful for diagnosing species of vegetative forms (colored nuclear structures).
- Recommended for preserving reference material or sending samples to another laboratory for consultation.
- Essential for diagnosing certain parasites (e.g., *Cryptosporidium*; microsporidia).
- Useful for detecting parasites that are less well-concentrated.

1.2.2.3.1. Wheatley Trichrome staining

This is a quick and easy staining method that yields satisfactory results. It colors the cytoplasm green and the chromatin red, and is particularly indicated for flagellates.

- **Technique**

1. Prepare a dilution of stool in saline.
2. Spread 10 µl of the dilution on a microscope slide to create a fairly thin smear.
3. Place the slide in a Petri dish, cover the dry smear with Schaudinn fixative, and leave it for one hour with the lid on the dish.
4. Stain with Wheatley Trichrome (see dye preparation in Annex 3).

The staining is performed in closed Borel boxes, following these steps :

1. Immerse the smears in 70% alcohol for 1 minute, then drain.

2. Place in another bath of 70% alcohol for 1 minute, then drain.
3. Stain in Trichrome for 30 minutes, then drain the slide.
4. Dip the slide twice in alcohol-acetic acid and drain.
5. Rinse in 95% alcohol by dipping the slide once, then drain.
6. Rinse a second time in 95% alcohol for about 30 seconds.
7. Immerse the slide for one minute in absolute alcohol, then drain.
8. Dip the slide for 2 to 3 minutes in xylene, then drain.
9. Mount in synthetic balsam.
10. Examine under immersion objective (1000x).

I.2.2.3.2. Ferric Hematoxylin staining

This is a standard staining technique for intestinal protozoa in fixed stool samples. It yields excellent results when executed perfectly ; however, it is lengthy, delicate, and the reagents used do not have a long shelf life.

This method takes advantage of the affinity of nuclear components for iron. Hematoxylin subsequently combines with the iron fixed on the nucleus to form a black ferric lake that contrasts against the colorless background of the preparation.

- **Reagents**
  - Mordant :
    - Ferrous alum.....3 g
    - Distilled water.....100 ml

    Freshly prepared ferrous alum crystals are purple. Over time, they develop a yellowish dusty layer. Only the purple crystals are usable. The solution should be prepared cold. It is yellow and does not store well ; however, refrigeration provides excellent preservation.

  - Stain :
    - Crystallized hematoxylin (Mother solution).....10 g
    - 90% alcohol.....100 ml

This solution must be prepared extemporaneously and exposed to diffuse light for a minimum of 15 days to 3 weeks. The solution darkens progressively and stores well.

- Mother Solution.....10 ml

  Extemporaneous solution
- Distilled water.....90 ml

- Differentiator
  - Ferrous alum.....1 g
  - Distilled water.....100 ml

- **Technique**

The mordant and stain are each poured into a container, such as a Borrel tube, and brought to 37°C.

1. **Mordanting** : The fixed smear (either fixed in Bouin's solution (Annex 4) for 15 minutes, followed by two washes in 90% alcohol and one wash in 70% alcohol for a few days; or fixed with Duboscq-Brasil fixative (Annex 4), recommended for cysts, for 15 minutes; or using Schaudinn acetic solution, which seems to be the best option, for 1 hour) is rinsed with distilled water and then immersed in a 3% ferrous alum solution at 37°C for 30 minutes. Quickly rinse with distilled water.
2. **Staining** : Immerse the slide in the hydroalcoholic hematoxylin solution at 37°C and maintain it for 30 minutes. Rinse with distilled water. The parasites will appear well-stained in black.
3. **Differentiation** : Use a 1% aqueous solution of ferrous alum for differentiation, monitoring under a microscope until the nuclei become clearly visible.
4. **Stop differentiation** : Wash abundantly with water.
5. **Dehydrate**.
6. A drop of Canada balsam is added, and a cover slip is placed on top for permanent mounting.

I.2.2.3.3. Modified Ziehl-Neelsen staining

To effectively observe coccidian oocysts, particularly those of *Cryptosporidium* species, and to preserve reference slides, thick and wet stool smears are prepared, fixed, and stained using the modified Ziehl-Neelsen technique by Henriksen and Pohlenz.

- **Method**

1. Prepare a stool smear, preferably from a sediment from the Ritchie method.
2. Allow the smear to air dry.
3. Fix for 5 minutes in methanol.
4. Stain the smear with phenolic fuchsin for 1 hour at cold temperature.
5. Rinse with tap water.

6. Differentiate by passing the smear through 2% sulfuric acid (H_2SO_4) for 20 seconds while agitating.
7. Rinse with tap water.
8. Perform a counterstaining for 5 minutes in 5% malachite green or 3% methylene blue solution.
9. Rinse with tap water and air dry.
10. Observe under an immersion objective (Figure 10).

Cryptosporidia will appear red against a green or blue background, making them easy to identify.

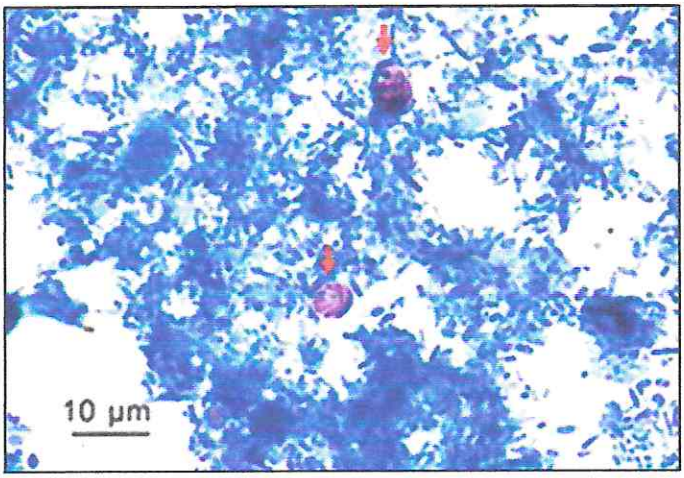


Figure 10. Oocysts of *Cryptosporidium* sp. (arrows) observed under a light microscope (1000x)

II. Hematological methods

II.1. Blood sample collection

Blood parasites are once again becoming a major public health concern due to the increase in travel to endemic areas. In Algeria, two species of *Plasmodium* are the most commonly sought: *Plasmodium falciparum* and *P. vivax*.

In some healthcare facilities, venous blood samples are collected to perform microscopic diagnoses of malaria. Larger volumes of venous blood are collected in EDTA (Ethylene diamine tetra acetic acid) tubes compared to the blood drawn from a fingertip.

II.1.1. Blood sample collection by Venipuncture

1. Label an EDTA tube with the patient's name, the date, and the time of collection.
2. Place a tourniquet around the upper arm of the patient to identify or feel the veins. Ask the patient to clench their fist to make the veins more prominent (Figure 11).
3. With your index finger, locate a vein that is large and stable enough.
4. Disinfect the site with a 70% alcohol swab. Do not touch the disinfected area again.
5. Allow the area to air dry for 30 seconds.
6. Insert a sterile, single-use needle (attached to a syringe or Vacutainer tube) into the vein. Slowly collect 2 to 4 ml of blood.
7. Once enough blood has been collected, release the tourniquet and ask the patient to unclench their fist. Remove the needle and apply firm pressure to the venipuncture site with dry cotton wool. Ask the patient to continue pressing on the site, keeping the arm raised, until the bleeding stops.
8. Transfer the blood into the EDTA tube and gently mix by inverting the tube six times.

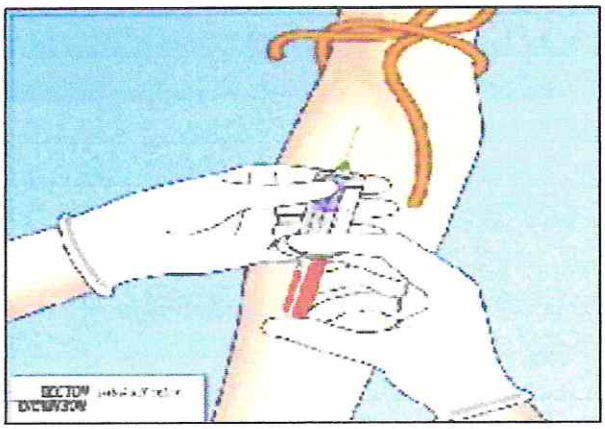


Figure 11. Venipuncture

II.1.2. Capillary Sampling

1. Disinfect the ring finger. (For very young children, the big toe or heel may be used).
2. Let the alcohol dry.
3. Prick the finger with a sterile lancet (Figure 12).
4. Place one large drop of blood in the center of the slide (a) (for the thick smear) (Figure 13) and one small drop 1 cm from the edge of the slide (b) (for the thin smear) (Figure 14).

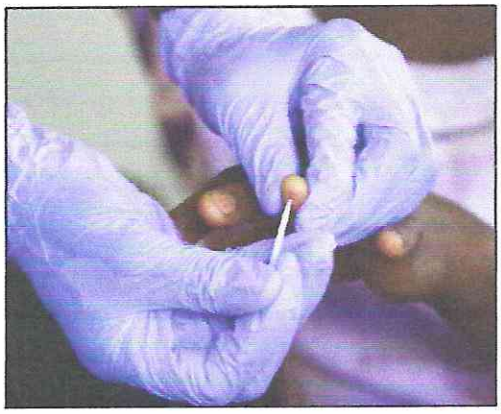


Figure 12. Capillary Sampling

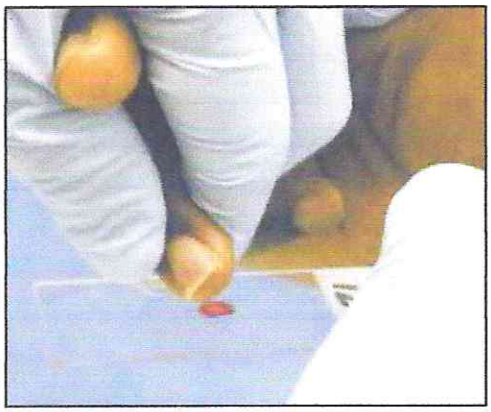


Figure 13. Depositing a Drop of Blood for the Preparation of a Thick Blood Smear

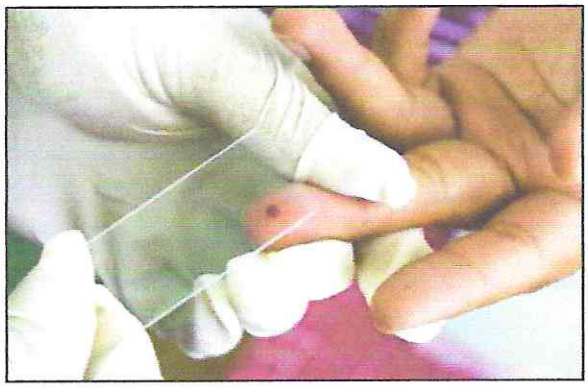


Figure 14. Depositing a Drop of Blood for the Preparation of a Thin Blood Smear

II.2. Preparation and staining of the thin blood smear

II.2.1. Preparation of the thin blood smear (Figure 15)

- **Method**

1. Place the slide with the drop of blood on the workbench.
2. Hold another slide at a 45° angle in front of the drop (1), allowing the blood to spread underneath the edge of the slide by capillary action (2,3).
3. Slide the inclined slide at a 45° angle to spread the drop evenly (4).
4. Shake the slide vigorously to dry the smear.

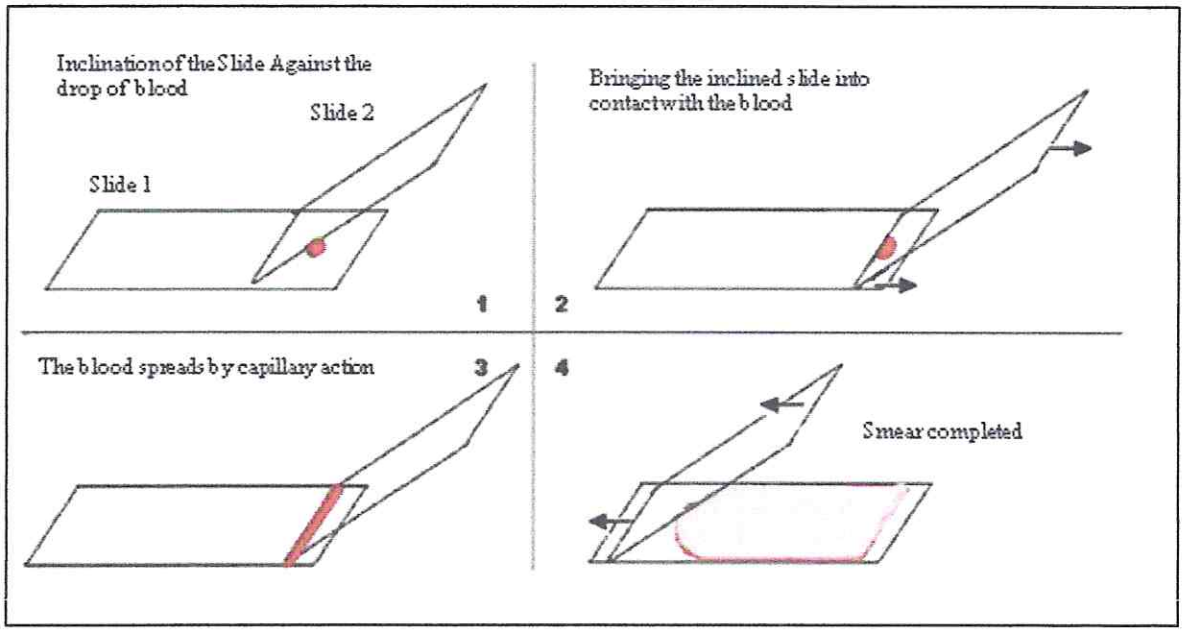


Figure 15. Steps for preparing a thin blood smear

II.2.2. Fixation and Staining with May-Grünwald

- **Method**

1. Place the slide of the smear on a horizontal support above a staining tray.
2. Pour 15 drops of pure May-Grünwald stain onto the slide to completely cover the smear. Allow it to act for 3 minutes.
3. Add an equal number of drops of neutral water as there are drops of stain ; the mixing should be quick.
4. Let it act for 2 minutes. (During this time, prepare a 10% dilution of Giemsa : 1 ml of the stock Giemsa solution + 9 ml of sterile distilled water.)
5. Discard the stain.

II.2.3. Staining with 10% Giemsa

- Method

1. Cover the smear with 10% Giemsa stain.
2. Allow it to act for 20 to 25 minutes.
3. Rinse under a stream of water.
4. Drain and let it dry.
5. Observe under the immersion objective.

II.3. Preparation and staining of the thick blood smear

II.3.1. Preparation of the thick blood smear (Figure 16)

- Method

1. Place the slide (a) on the workbench.
2. Defibrinate for 1 to 2 minutes using the edge of another slide, turning regularly, and spreading evenly with sufficient pressure to promote adherence.
3. Allow to dry flat, either for 24 hours at room temperature or for 1 hour in an incubator set at 37°C.

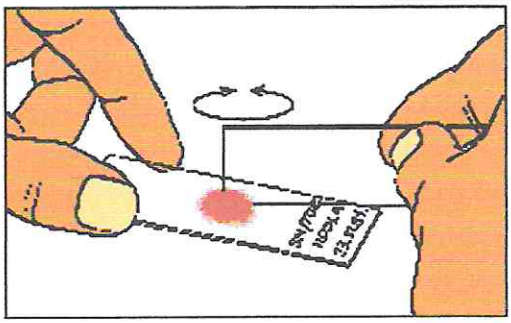


Figure 16. Preparation of the thick blood smear

II.3.2. Dehemoglobinization

This step involves lysing the red blood cells using distilled water or, more simply, tap water. To do this, place the slide vertically in a Borrel tube and let it act for 2 to 4 minutes, depending on the age of the thick smear.

II.3.3. Staining with 10% Giemsa

- Method

1. After removing the slide from the dehemoglobinizing solution and draining it, pour 10% Giemsa solution over the slide and let it act for 20 to 30 minutes.
2. Rinse with tap water and air-dry.
3. Observe under the immersion objective.

Remark

- Blood collected in tubes containing EDTA should not be stored for more than four hours before preparing thin smears and thick blood smears, as the anticoagulant may alter the morphology of the parasite.
- The thick smear may spontaneously fix if exposed to heat.

II.4. Microscopic Examination

Microscopic examination allows for the identification of the *Plasmodium* species and the determination of parasitemia.

In the thick blood smear, the following can be observed :

- White blood cells (leukocytes) ;
- Platelets ;
- Artifacts : debris, crystals.

In the thin blood smear, the following can be observed :

- Red blood cells (erythrocytes) ;
- White blood cells (leukocytes) ;
- Platelets ;
- Artifacts : debris, crystals.

Five species parasitize humans :

- *Plasmodium falciparum* ;
- *Plasmodium vivax* ;
- *Plasmodium ovale*.
- *Plasmodium malariae*
- *Plasmodium Knowlesi*

} Morphologically identical species

All these species, when stained with MGG (May-Grünwald-Giemsa) and observed under an optical microscope, exhibit common characteristics (Figure 17, Figure 18). These include :

- **Cytoplasm is** :
  - Blue for trophozoites, schizonts, and female gametocytes.
  - Lilac-purple for male gametocytes.
- **Nucleus** : Appears as a mass of chromatin stained red.
- **Vacuole** : A white vacuole is present in trophozoites.

- **Black Hemozoin Granules** : These appear as the parasite matures (a product of hemoglobin degradation).

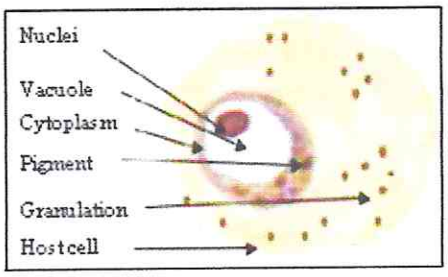


Figure 17. Plasmodium (Trophozoite Form) in an Infected Erythrocyte

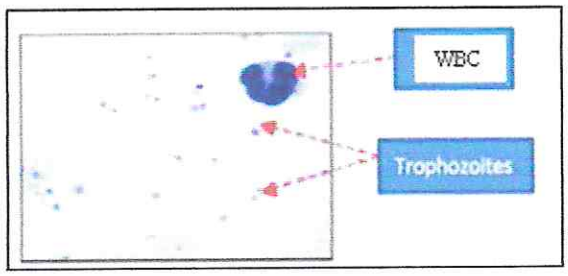


Figure 18. Trophozoites and White Blood Cells (WBC) in the Thick Blood Smear

The examination of a thin smear for the presence of *Plasmodium* involves studying the infected red blood cell, where the following can be observed :

- **Size** : It may be decreased, normal, or increased.
- **Shape** : The red blood cell may be regular in shape or have "ragged" edges.
- **Content** :
  - Presence or absence of granulations, such as :
    - **Schüffner's dots** (small, fine, brown-red granules of equal size and shape).
    - **Maurer's clefts** (masses of red-brown color with variable size and shape).
- The parasitic element may be :
  - A young or old trophozoite.
  - A young or old schizont (often referred to as "rosette bodies").
  - A gametocyte.

In the host cell (the erythrocyte), the parasite goes through three stages of development :

Stage 1 : Trophozoite (Figure 19)

This is the most frequently observed stage. Often referred to as the ring stage, its size varies from small to fairly large within the host cell.

Typically, there is one chromatin dot, but two are commonly observed with *P. falciparum*. As the parasite grows, pigment begins to appear.

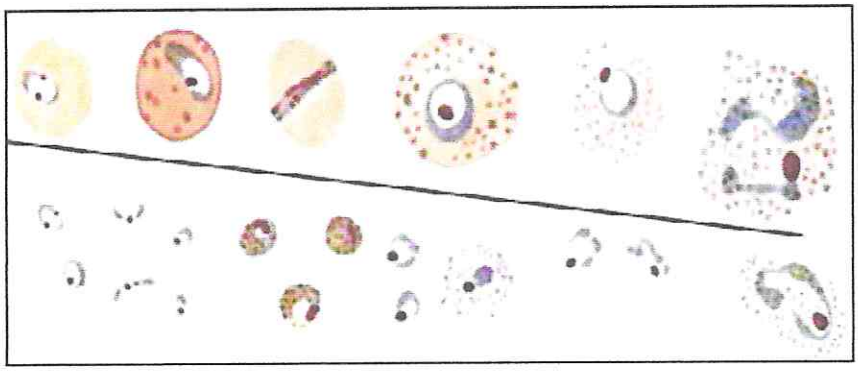


Figure 19. Top Row : Trophozoites on Thin Smear / Bottom Row: Trophozoites on Thick Smear

Stage 2 : Schizont (Figure 20)

The schizont stage begins when the trophozoite has reached full development and the chromatin divides into two. The parasite begins to reproduce asexually, giving rise to cells called **merozoites**. Several successive divisions of the chromatin occur, indicating the growth of the schizont, until numerous chromatin masses are formed, each with its own cytoplasm. The number of merozoites helps identify the species. The newly formed parasites, now clearly defined, are ready to leave the host cell and invade new red blood cells.

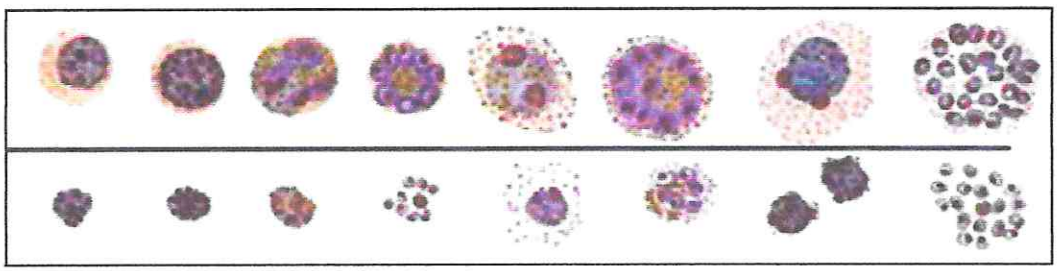


Figure 20. Top Row: Schizonts on Thin Smear / Bottom Row: Schizonts on Thick Smear

Stage 3 : Gametocyte (Figure 21)

The parasite then develops into male and/or female gametocytes in preparation for the sexual phase, which takes place in the body of the female Anopheles mosquito. Depending on the species, the gametocytes are either round or banana-shaped.

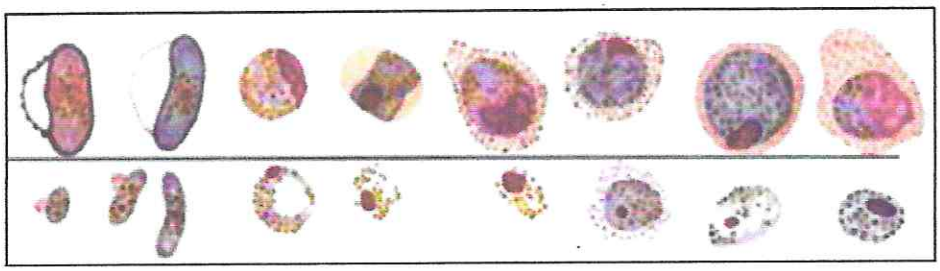
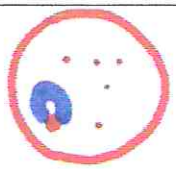
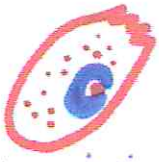
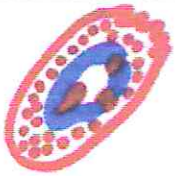
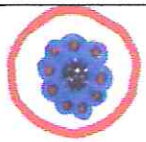
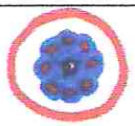
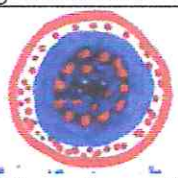
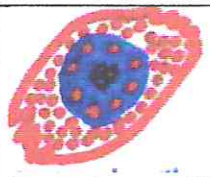
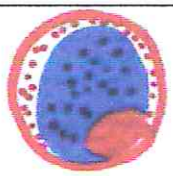
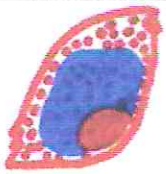


Figure 21 : Top Row: Male and Female Gametocytes on Thin Smear / Bottom Row: Male and Female Gametocytes on Thick Smear

Table 4 presents the distinguishing characteristics between the different *Plasmodium* species as observed from a thin smear.

Table 4 : Distinctive characteristics between the four *Plasmodium* species

Species	*P. falciparum*	*P. malariae*	*P. vivax*	*P. ovale*
Appearance of the Infected Erythrocyte	 It remains unchanged.	 Retracted	 Enlarged	 Enlarged, Oval, with Jagged Edges
Trophozoite	 Bi or Triparasitism Common (1/5 of diameter)	 1/4 to 1/3 of the diameter	 1/4 to 2/3 of the diameter	 1/4 to 2/3 du diameter
young schizont	 Presence of Maurer granulations		Fine  Fine Schüffner granulations	 Schüffner granulations
mature schizont (rosette body)	 8 to 16 merozoites	 6 to 8 merozoites	 12 to 18 merozoites	 8 to 10 merozoites
Gametocytes				
	Female gametocytes			
Stages encountered	Trophozoites or gametocytes or both forms together	All forms are encountered on the same slide	All forms are encountered on the same slide	All forms are encountered on the same slide

Modified table (Personal)

III. Other methods

III.1. Vaginal and Urethral Samples for the Detection of *Trichomonas vaginalis* -Method

In Women

1. The cervical mucus should be collected before any intimate hygiene and treatment.
2. The patient should avoid all sexual relations 24 to 48 hours before the sample collection.
3. The sample is taken at multiple levels of the vaginal mucosa using a sterile swab moistened with saline under a speculum.
4. Rapid transport to the laboratory.
5. If the sample is taken outside the laboratory, a swab with a transport medium (Stuart's medium) should be used to preserve the parasites for 24 hours at room temperature.

In Men

1. The sample is collected before any morning urination.
2. Collect the first morning discharge from the meatus using a swab or directly on a slide.
3. Prostate massage increases the sensitivity of the sample.

III.2. Microscopic Examination

III.2.1. Direct Fresh Examination

This is performed quickly using the swab moistened with saline, by placing a drop between a slide and a coverslip, which will be observed under an optical microscope at magnifications of x10 and x40 to search for *Trichomonas vaginalis*, which is easily recognizable by its mobility (Figure 22).

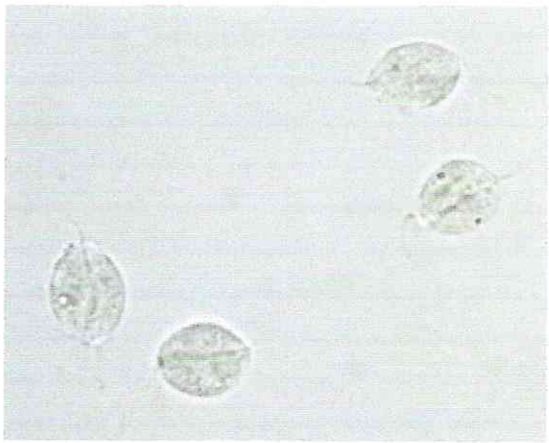


Figure 22. Trophozoites of *Trichomonas vaginalis* (Fresh Examination)

III.2.2. Direct Examination after Staining with MGG

A drop of the sample is spread into a thin smear, dried, fixed with May-Grünwald, and stained with 10% Giemsa to search for trophozoites of *Trichomonas vaginalis* (Figure 23) (See staining of blood smears for the detection of plasmodial species).

Figure 23. Trophozoites of *Trichomonas vaginalis*
(Examination after MGG Staining)

IV. Culture

It is a good diagnostic method when the vegetative forms are rare in the stools. The culture works well with vegetative forms when they are still viable, so it should be done with freshly passed stools.

There are three types of culture media:

- **Xenic or polyxenic medium**: A medium in which the parasite develops in the presence of microbial flora.
- **Monoxenic medium**: A medium in which the parasite is associated with only one bacterial species.
- **Axenic medium**: A medium in which the parasite develops in the absence of any metabolizing cells.

IV.1. Polyxenic Diphasic Medium of DOBELL and LAIDLAW (an interesting medium for amoebas and flagellates).

This medium consists of a solid phase (coagulated horse serum on an inclined plane) and a liquid phase containing Ringer's solution, horse serum, and rice starch (Figure 24). Incubation occurs at 37°C, with readings taken after 24 to 48 hours, and subcultures performed every 2 to 3 days.

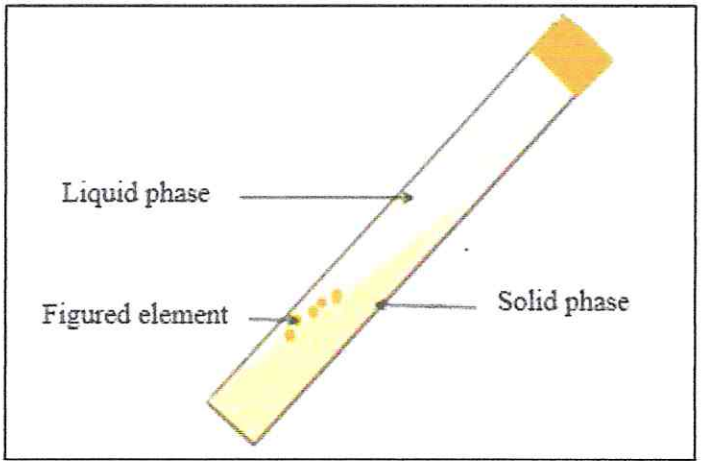


Figure 24. Diphasic Medium

The medium includes :

- **Solid Phase** (Base : Coagulated Horse Serum)
    1. Thaw the horse serum.
    2. Sterile distribute 3 to 4 ml of serum into 10 cm long glass tubes capped with autoclavable plastic caps.
    3. Allow to coagulate in an oven at 90°C, with the tubes inclined at 30° from the horizontal, for 1 to 2 hours.

- **Liquid Phase :** 7 volumes of Ringer + 1 volume of Horse Serum

Ringer Solution :

Solution 1

NaCl.....6.5 g

KCl.....0.25 g

$CaCl_2$.....0.30 g

Distilled Water.....900 ml

Solution 2

$NaHCO_3$.....0.20 g

Distilled Water.....100 ml

1. Autoclave the two solutions separately, along with the materials (tubes, test tubes, etc.).
2. Mix the two cooled solutions.

Horse Serum :

1. Add 140 ml of horse serum per liter of Ringer's solution.
2. Sterile distribute into screw-cap tubes.
3. If preparing Ringer with antibiotics, add per liter before distribution :
    - Penicillin G.....1 million IU
    - Streptomycin.....1 g (or Gentamicin 400 mg)

Ringer media with and without antibiotics should be renewed every 15 days.

IV.2. Rice sarch-based medium

- Distribute about 1 to 2 g into sterile hemolysis tubes capped with cotton.
- Tyndallize for 1 hour in a Pasteur oven at 60°C, three times over three days.

Tyndallization consists of a series of 3 pasteurizations of 1 hour at 70-80°C, separated by an interval of 24 hours at room temperature, which allows for germination and destruction of spores without using excessive temperatures. This method is used for delicate media containing serum, egg, or any thermosensitive substance of high viscosity that cannot be sterilized by filtration.

- Method

To reconstitute the medium, use Ringer with antibiotics for stool samples and Ringer without antibiotics for pus from abscesses, biopsies, etc.

1. Pour a small amount of starch into the tube containing the base.
2. Cover the base with Ringer solution (± 5 ml).
3. Inoculate with a fragment of stool, mucus, or pus.
4. Scrape against the wall of the tube.
5. Place the tube vertically in the incubator.
6. At 24 h, 48 h, and 72 h, collect with a Pasteur pipette from the interface between the starch and the sample.
7. Observe between a slide and a coverslip on the heated stage at 37°C.

V. Molecular identification

Contamination by parasites is necessarily indicated by the presence of their genetic material in the infected organism. PCR (Polymerase Chain Reaction) is therefore a particularly effective tool for detecting the presence of a pathogen in a biological sample, as it has high sensitivity and specificity.

Several techniques are used: standard PCR (qualitative PCR), real-time PCR (quantitative PCR) used alone (utilizing two primers targeting a specific parasite species) or combined in multiplex for the detection of other protozoa from the same sample.

All these techniques share the same principle, consisting of a series of cycles that repeat in a loop, each comprising three temperature phases. On average, a PCR consists of 20 to 40 cycles.

V.1. Actors of PCR

V.1.1. DNA (Deoxyribonucleic Acid)

Before the PCR reaction, DNA is extracted from the sample to be analyzed (stool, blood, bone marrow, etc.). The chemical components of DNA are (Figure 25) :

a) A C5 sugar : deoxyribose

b) Nitrogenous bases

- Adenine, Guanine → **Purine bases**
- Cytosine, Thymine → **Pyrimidine bases**

c) phosphate group

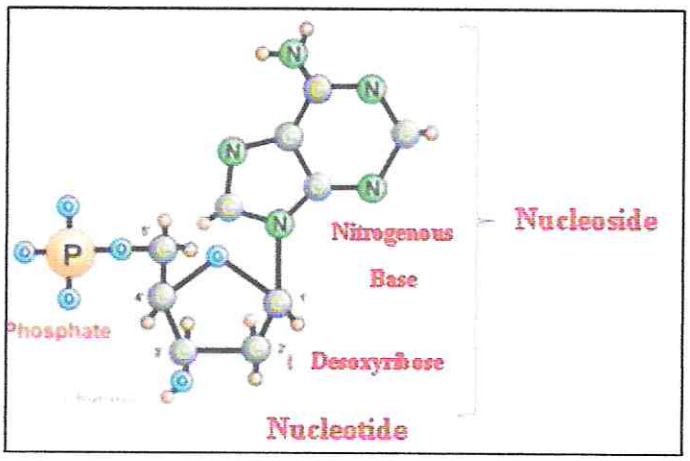


Figure 25. Chemical Components of DNA

The length of DNA molecules is measured in base pairs.

V.1.2. The two primers

These are short fragments of DNA capable of specifically hybridizing, due to base complementarity, to one of the two strands of DNA (Figure 26).

The primers (forward and reverse) are selected to flank the DNA sequence to be amplified (example of primers for *Entamoeba* sp : The primers used were FOR2 (CAC-GGG-AAA-ACT-TAC-CAA-GAC-C) and REV (CCA-AGA-TGT-CTA-AGG-GCA-TCA).

The length of these primers is typically around twenty deoxyribonucleotides.

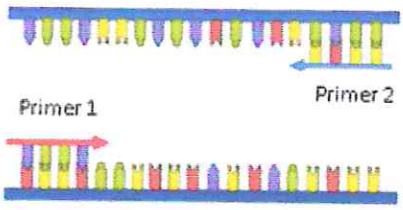


Figure 26. Hybridization of Primers

V.1.3. Deoxyribonucleoside Triphosphates (dATP, dCTP, dGTP, dTTP)

dNTPs (deoxyribonucleoside triphosphates) are the basic molecules that make up DNA and are used by Taq polymerase for the synthesis of the complementary DNA strand.

V.1.4. The enzyme, Taq Polymerase

The enzyme used is a polymerase, meaning it can synthesize a new DNA strand from the template DNA strand after binding to a primer.

V.1.5. The reaction medium

The PCR reaction medium includes the DNA to be amplified, the dNTPs, the two primers, Taq polymerase, a buffer, and magnesium ions ($MgCl_2$). These last two components define a medium with an optimal pH and salt concentration for the proper functioning of the enzyme.

V.2. PCR Reaction

The PCR reaction takes place in a thermocycler, a device that contains a heating block where the samples (reaction medium) are inserted and where the temperature can vary very quickly and precisely from 0°C to 100°C.

The thermocycler is programmed to perform the different cycles of the PCR. Each cycle consists of a succession of predetermined temperature phases, each with a defined duration. These two parameters, temperature and time, depend on the size of the sequence to be amplified and the composition of the deoxyribonucleotide primers.

Each cycle is thus composed of three different steps :

- **Denaturation** : The temperature in the tube is set to 95°C. At this point, the DNA denatures. The DNA loses its characteristic double helix structure, as the hydrogen bonds linking the bases of each DNA strand are unstable at this temperature. The double-stranded DNA (2 strands) is denatured into single-stranded DNA (1 strand).
- **Hybridization** : The temperature is generally between 50°C and 60°C. This depends on the composition of the deoxyribonucleotides (dATP, dTTP, dGTP, and dCTP) in the primers.

  The primers recognize and bind to their complementary sequences, reforming hydrogen bonds. It is said that the primers hybridize to the DNA strand.
- **Elongation** : The temperature is set to 72°C, which is the ideal temperature for Taq polymerase activity.

  At 72°C, Taq polymerase binds to the annealed single-stranded DNA and catalyzes replication using the deoxyribonucleoside triphosphates present in the reaction mixture. The regions of the template DNA downstream of the primers are selectively synthesized. In the next cycle, the fragments synthesized in the previous cycle serve as templates, and after a few cycles, the predominant species corresponds to the DNA sequence between the regions where the primers hybridize. Typically, 20 to 40 cycles are needed to synthesize an analyzable amount of DNA. Each cycle theoretically doubles the amount of DNA present during the previous cycle.

In the case of standard PCR, the DNA is revealed by staining with ethidium bromide. Thus, the products are instantly visible by transillumination under ultraviolet light (Figure 27).

Figure 27. Result of the amplification of the ITS2 region (400 bp) of *Leishmania* by standard PCR (Personal Photo)

Positive Samples (17, 28, 29, 31, 36, 37, 39, 40, 43, 44).
M : Size Marker

In the case of real-time PCR, the principle is based on visualizing the exponential phase of the reaction using a DNA-specific fluorochrome or a fluorescent probe specific to a sequence. Monitoring this fluorescence allows observation of its increase and, therefore, the number of PCR fragments in three distinct phases (Figure 28) :

- **Background Noise Phase** : The amount of amplified fragment is insufficient to generate a fluorescent signal above the detection threshold of the device.
- **Exponential Phase** : The quantity of amplified fragment generates a fluorescent signal above the detection threshold of the device, and the number of amplified products doubles with each cycle. Taq polymerase degrades the probe on its path, releasing the reporter from the quencher (Figure 29). The fluorescence associated with the reporter fluorochrome is proportional to the amount of product generated.
- **Plateau (or Saturation) Phase** : Certain components of the reaction (especially the number of available Taq molecules) become limiting. The system no longer allows for exponential amplification.

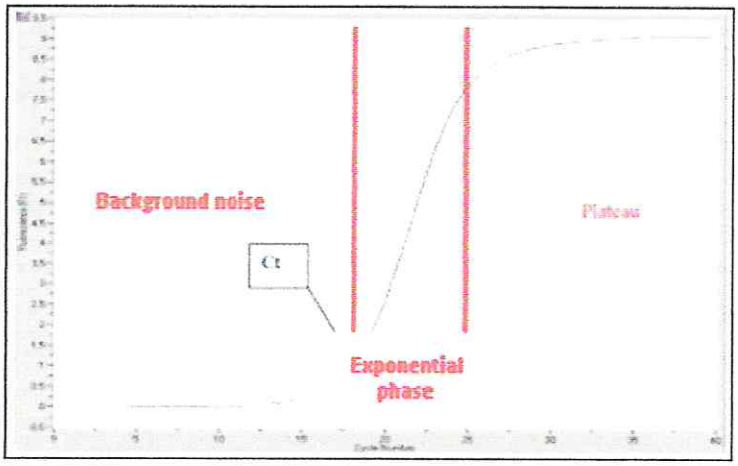


Figure 28. Graphical model of Real-Time PCR

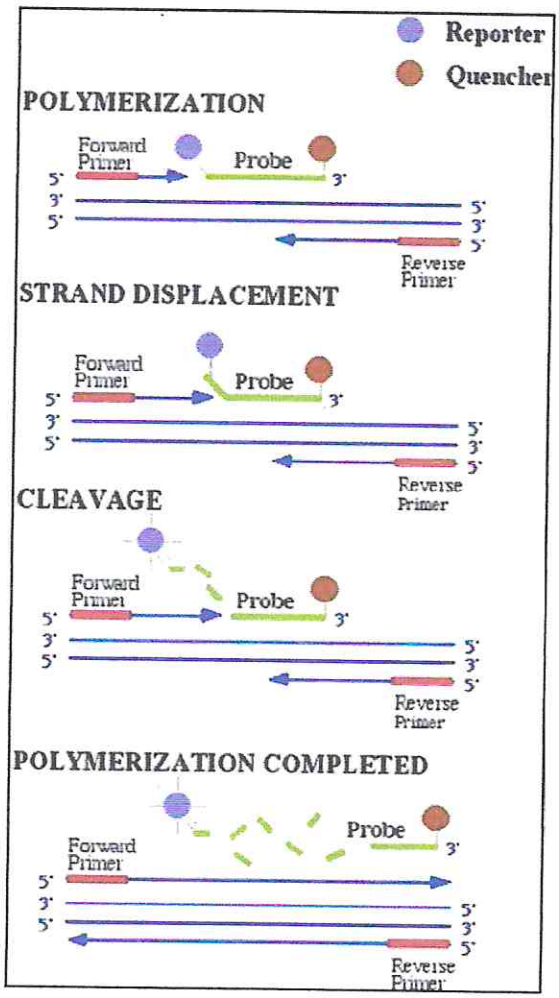


Figure 29 : Principle of Real-Time PCR

Conclusion

Protozoal infections pose a significant public health problem for many developing countries and can jeopardize vital prognosis. Intestinal amoebiasis caused by *Entamoeba histolytica* is the third leading cause of mortality from parasitic diseases worldwide, following malaria and schistosomiasis. It affects approximately 180 million people, with 40000 to 110000 deaths each year. Additionally, giardiasis, caused by *Giardia intestinalis*, is a common cause of diarrhea that can negatively impact the growth and development of children.

As a result, patient welfare must be at the heart of health system concerns, which includes biologists and laboratory technicians. Their expertise during sampling and parasite detection is crucial to ensuring the quality of the results obtained.

This document, with its procedural guidelines, serves as a laboratory manual to assist undergraduate students specializing in Parasitology in the detection and identification of parasitic protozoa from various samples.

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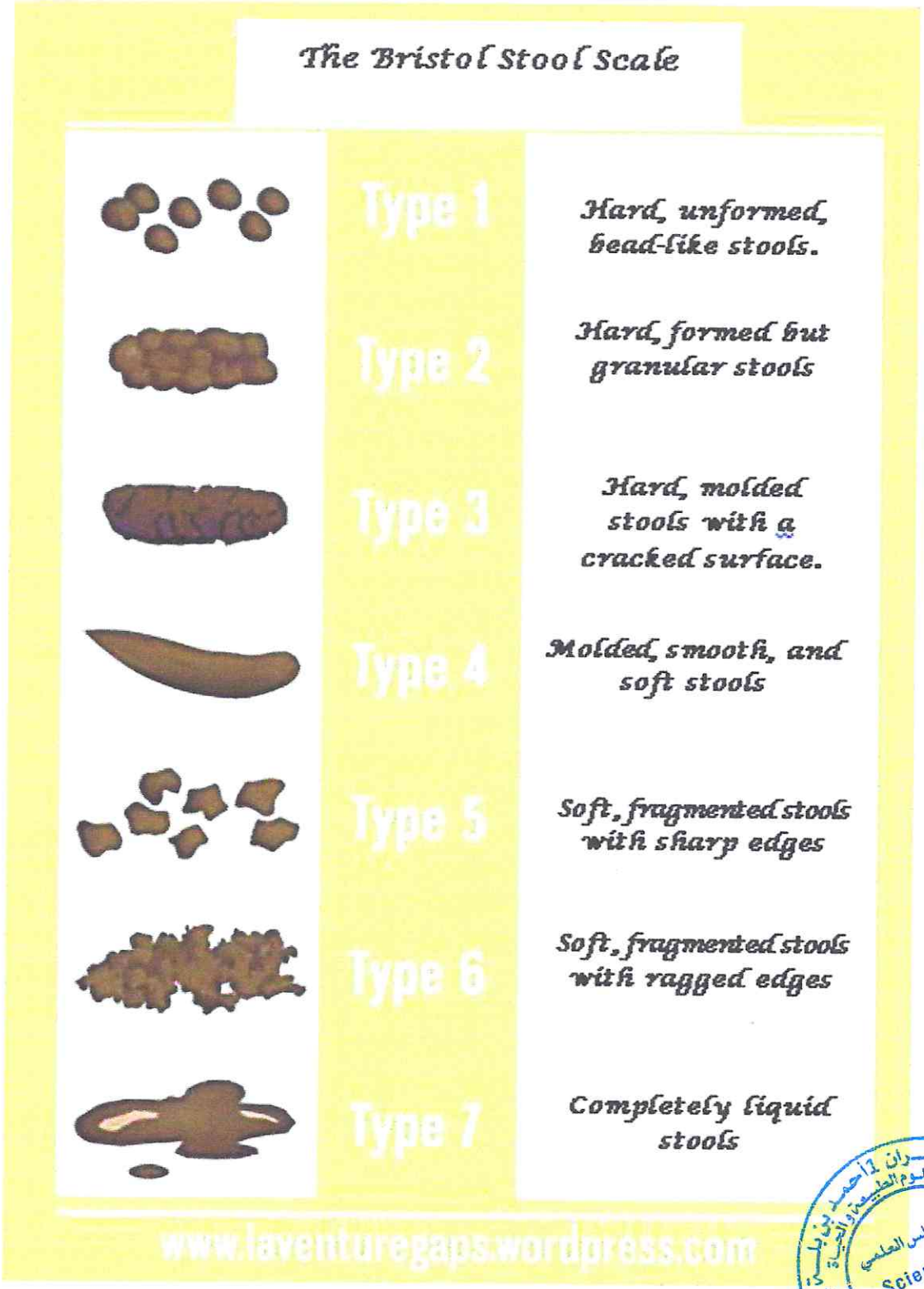
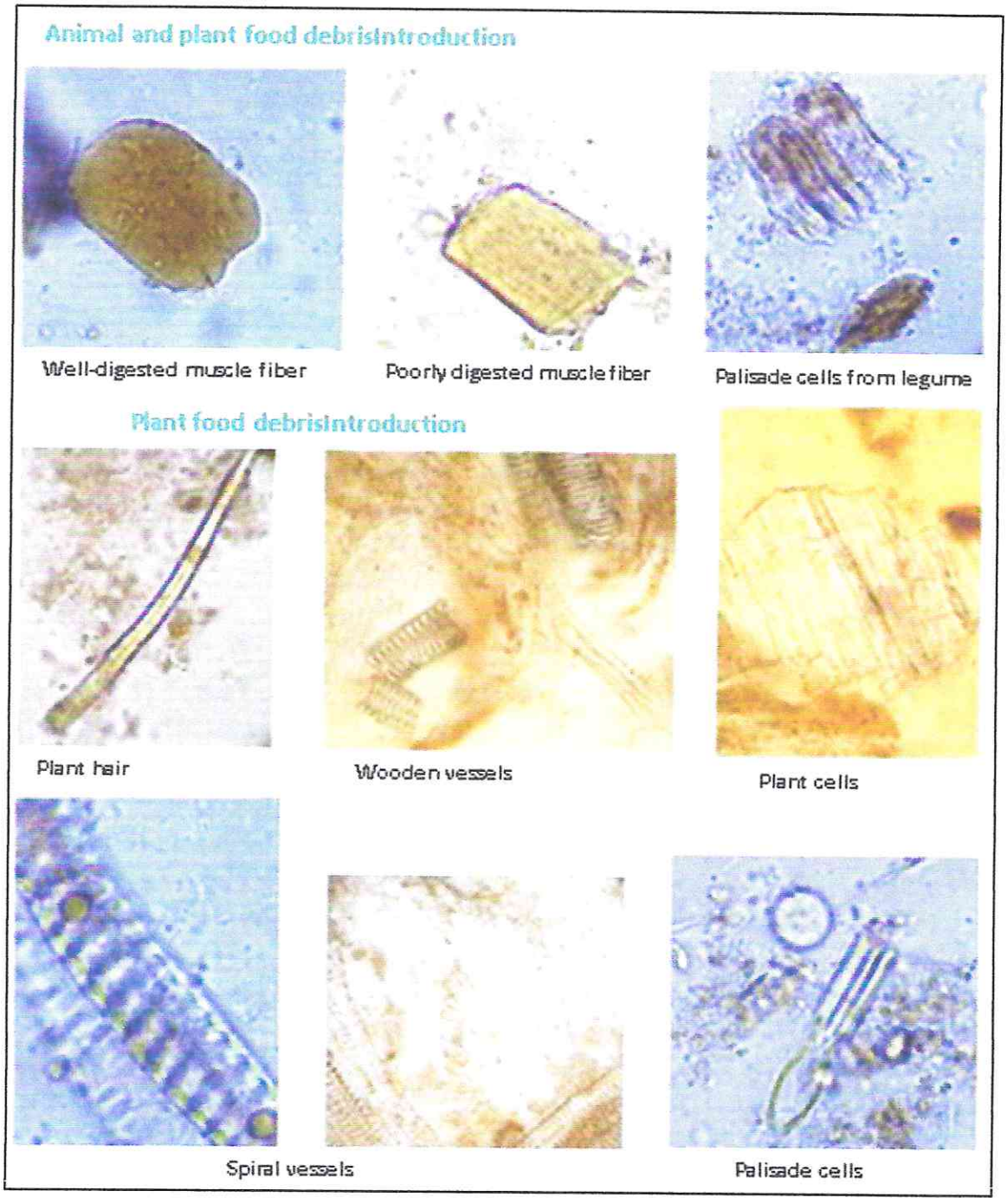
Annexes

Annex 1

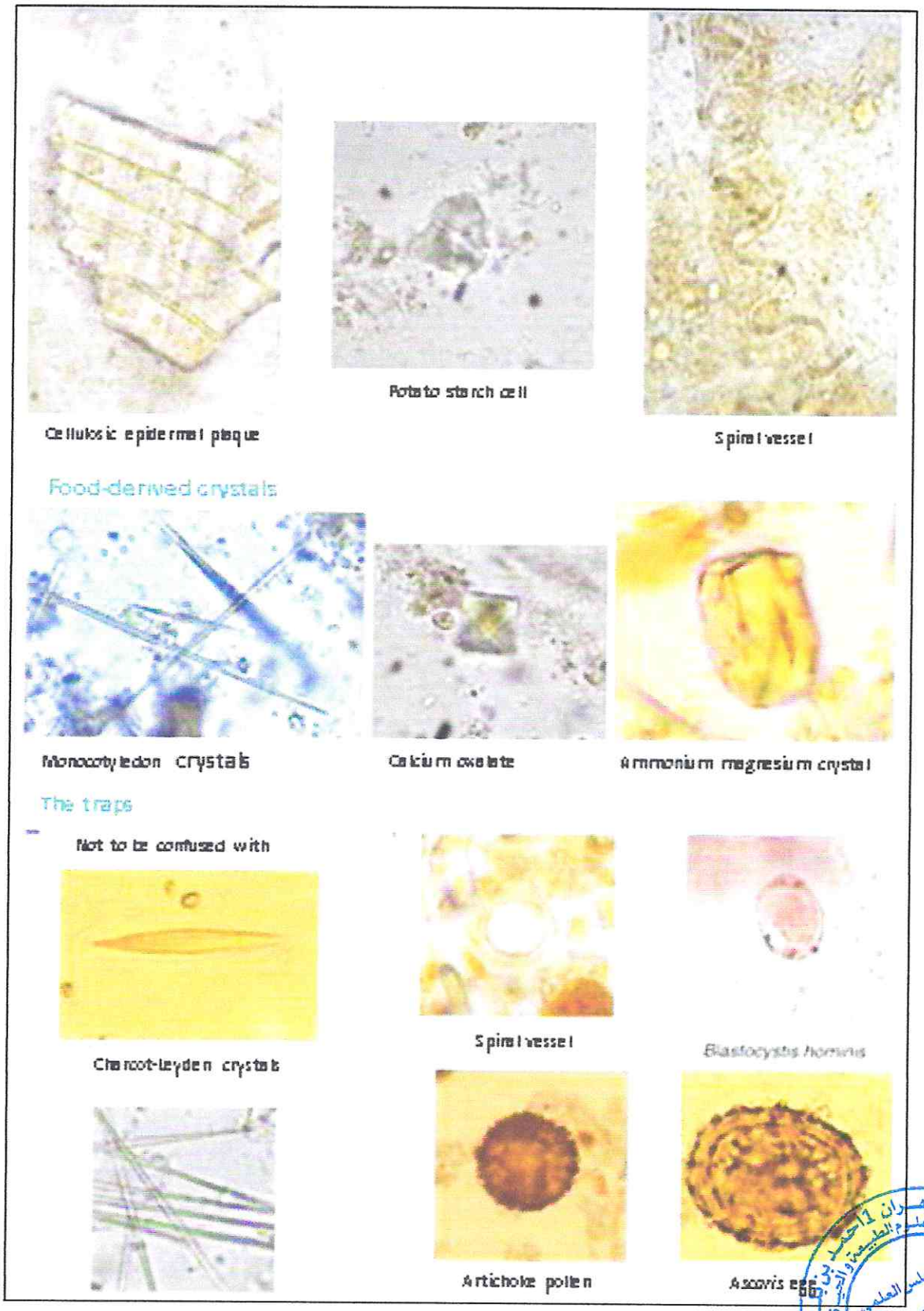
Stool polymorphism according to the Bristol stool scale



Annex 2. Non-parasitic elements



Annex 2. Non-parasitic elements (continued).



Annex 3. Composition of the 'Wheatley Trichome' Stain

Preparation of the stain : Work under a fume hood with gloves, a mask, and protective goggles.

- Chromotrope 2R 6g
- Fast Green (or Light Green) 3g
- Phosphotungstic Acid 7g
- Acetic Acid 10ml
- Distilled Water 1000ml

Chromotrope 2R : Acidic red dye used as a counterstain.

Annex 4. Fixatives Used in Ferric Hematoxylin Staining

1. Ordinary Bouin Fixative :
   - Saturated aqueous solution of picric acid : 15 ml
   - Commercial formaldehyde : 5 ml
   - Crystallizable acetic acid : 1 ml
2. Duboscq-Brasil Fixative (Alcoholic Bouin) :
   - 80% Ethyl alcohol : 150 ml
   - Commercial formaldehyde : 60 ml
   - Crystallizable acetic acid : 15 ml
   - Picric acid : 1 g
3. Acetic Schaudinn Solution :
   - Saturated aqueous solution of mercuric chloride : 200 ml
   - 90% Ethyl alcohol : 100 ml"

Chapter III. Other methods

Chapter IV. Culture

Chapter V. Molecular identification