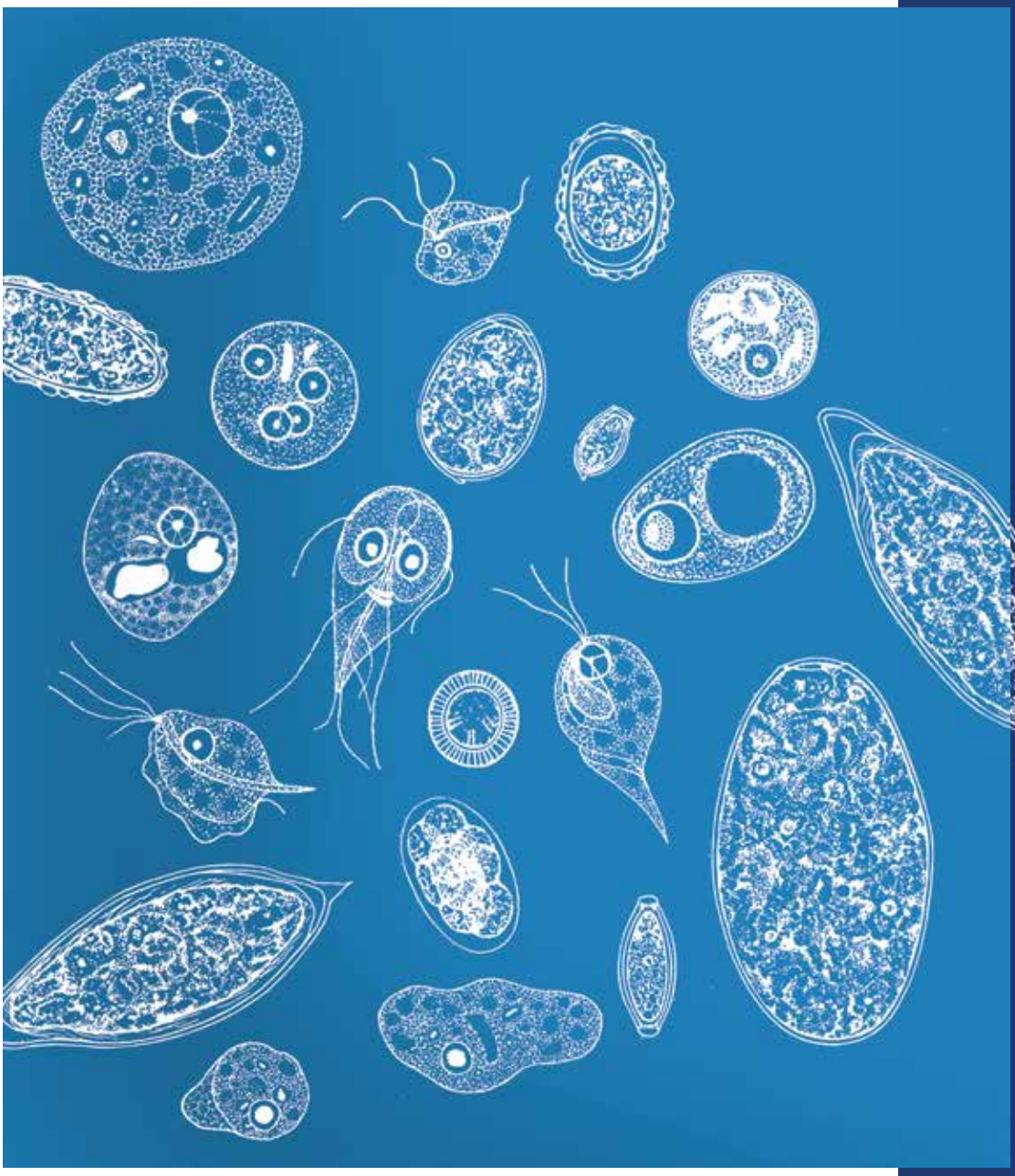
Bench aids

for the diagnosis of intestinal parasites



second edition

World Health Organization

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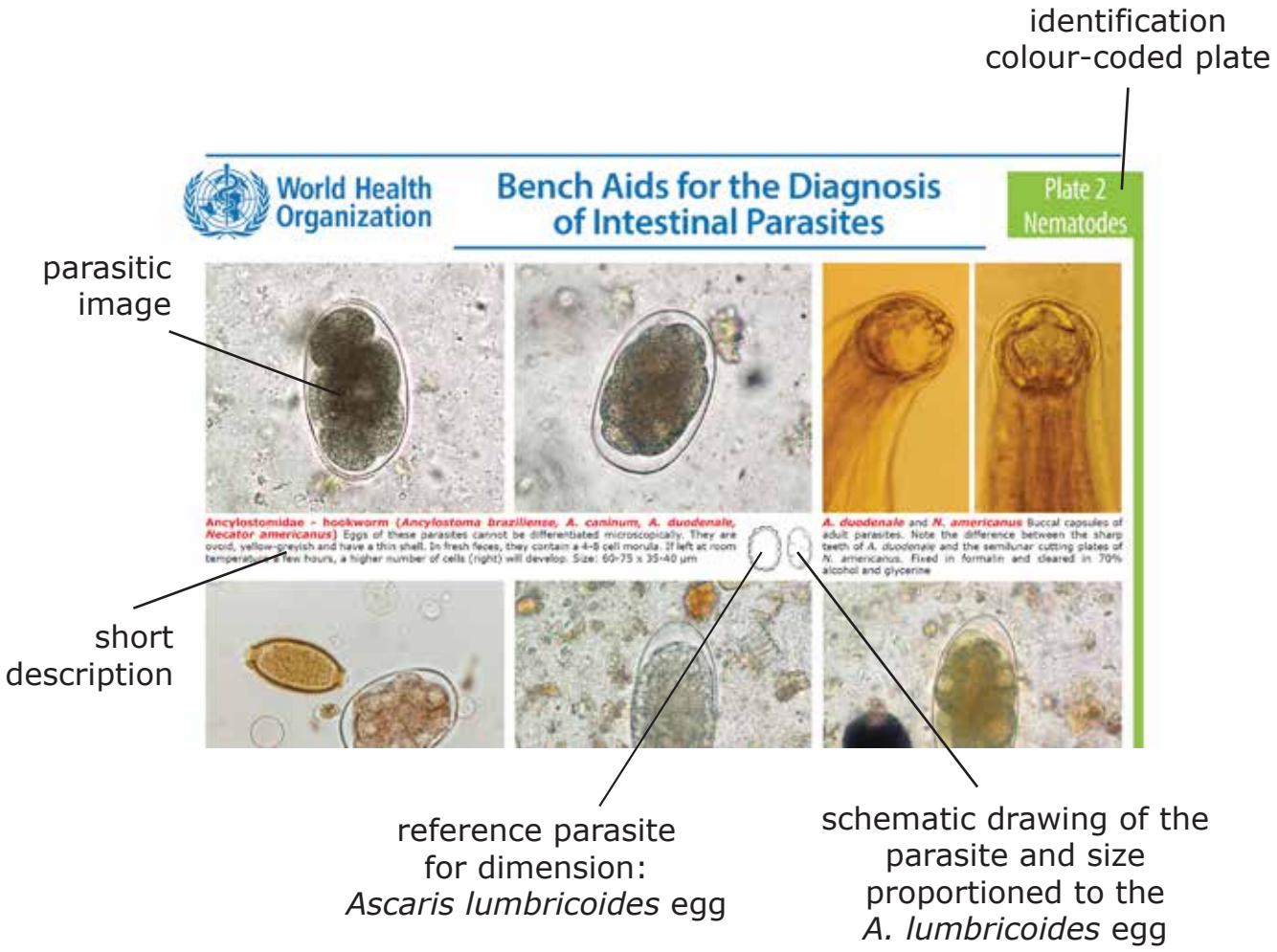
Introduction

This second edition of *Bench aids for the diagnosis of intestinal parasites* is intended both as a practical tool for the diagnosis of intestinal parasitic infections for laboratory and field workers and as a teaching aid for students and trainees. The plates are arranged on two sides: the recto with microphotographs for the identification of eggs, larvae, trophozoites, cysts and oocysts occurring in faeces, and the verso dedicated to the different copromicroscopical methods (procedures) and main staining techniques used in parasitology.

Special attention has been devoted to all graphical and pictorial contents. The decision to include the outline of an *Ascaris lumbricoides* egg in its relative size next to each parasitic structure fulfils the intention of visualizing the actual dimensions that the eye needs to be looking for when examining the specimens with a microscope. For each image, the size of the parasite and a short description are provided to assist in the microscopical identification.

Two summary plates, one for helminths and the other for protozoa, are also included to provide a visual overview of the different presentations of parasitic elements.

The bench aids have been produced in a weatherproof plastic-sealed format that is robust and easy to use at the bench. They are recommended for use by all health workers engaged in the routine diagnosis of intestinal parasitic infections.



Good laboratory practices and biosafety

Some basic practices and biosafety principles that must be followed in the laboratory are presented below. For more detailed information, refer to:

https://apps.who.int/iris/handle/10665/42981

A laboratory of parasitology is generally classified as a basic biosafety 2 laboratory (BSL-2) (see http://www.hse.gov.uk/pubns/misc208.pdf). This requires the application of good laboratory practices, the use of personal protective equipment and the display of an international biological hazards sign. The laboratory must also be equipped with safety hoods and specific disinfection and treatment procedures for biological materials, to be used also in case of accidental spillage. It should have regulated access and there should be enough space for laboratory benches and equipment, which must be arranged to allow for adequate cleaning. Facilities for storing personal clothing and items must be provided for all personnel, and storage areas for specimens, reagents and equipment should be available. Moreover, it is crucial that "clean" and "dirty" areas are clearly distinguished, adequately lit and ventilated; that barriers against arthropods are in place if the windows can be opened; that an easily accessible water source is available; and that the benches, walls and floors are smooth, water-repellent, and easy to clean and disinfect. Finally, the laboratory must be separated from any changing rooms and recreational areas provided to the staff.

The disinfectants normally used are sodium hypochlorite (bleach), 70% ethanol or isopropanol, and quaternary ammonium compounds. Bleach is easily available and inexpensive. When diluted at 5–10%, bleach is suitable for disinfecting benches and work areas. Alcohols are effective in decontaminating stainless steel surfaces and removing bleach residues from metals to minimize corrosion. Quaternary ammonium compounds must be used after removal of organic matter, which reduce their effectiveness.

For waste disposal, under ideal conditions all infected or potentially infected material should be decontaminated, autoclaved or incinerated in the laboratory. Contaminated waste containers, including those provided for sharps waste disposal, must be easily identifiable and fit for purpose.

Basic laboratory rules can be summarized as follows:

1. Keep the work areas uncluttered (e.g. never place backpacks, bags, books, etc. on the laboratory bench).
2. Always wash your hands with soap and water when you enter and leave the laboratory.
3. Always wear your laboratory coat when in the laboratory and remove it when leaving; laboratory coats and personal clothing should not be stored in the same locker.
4. Always wear gloves when handling potentially dangerous biological or chemical substances.
5. Wear safety glasses for protection against splashes, sprays and UV radiation.
6. Use proper shoes (no sandals).
7. Handle toxic substances (e.g. formalin) under a safety hood.
8. Unequivocally label all preparations and samples to be analysed.
9. Dispose of all waste appropriately and safely.
10. Clean and disinfect the work area at the beginning and end of each laboratory session.
11. Do not take out of the laboratory the clipboard/notepad/pen/pencil used because they are potentially contaminated.
12. Do not store food and/or drinks in the laboratory.
13. Do not eat and/or drink in the laboratory, nor bring hands or other objects (e.g. pencils, make-up, contact lenses) to your mouth or eyes.

Basic laboratory supplies in medical parasitology

Equipment

- Microscope, objective 4x, 10x, 40x, 100x
- Supplementary objective 20x, 60x
- Ocular scale and stage micrometer for microscope calibration
- Centrifuge (if possible, with rotor for microtiter plates)
- Laboratory scale
- Hot plate with magnetic stirrer
- Fridge
- Stereomicroscope (if possible)

Materials

- Adhesive tape (transparent) and paper: 2 cm wide
- Beakers (plastic and glass): 250 mL, 500 mL, 1000 mL
- Conical glasses
- Bottles (plastic or glass): 25 mL, 30 mL, 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL, with stoppers or dropper-tops and screw-caps
- Centrifuge tubes, conical, flat-top, graduated: 15 mL, 50 mL
- Centrifuge tubes, conical, plastic disposable: 12 mL
- Graduated cylinder: 10 mL, 25 mL, 50 mL, 100 mL, 250 mL, 1000 mL
- Detergents and disinfectants
- Dishes for staining, Coplin jars
- Dropper bottle for saline, iodine, etc.
- Forceps and scissors
- Funnel (plastic and glass)
- Gauze
- Hot plate
- Hydrometer (specific gravity 1.10–1.40)
- Immersion oil, low viscosity
- Permanent markers, pens, pencils
- Mortar and pestle (laboratory porcelain)
- Gloves (latex or nitrile), disposable
- Membrane filter (12 µm or 15 µm) and filter holder
- Microscope slide and coverslips
- Paper towels
- Pasteur pipettes and rubber bulbs, electronic pipette
- Petri dishes (plastic and glass)
- Pipettes (plastic droppers) disposable: full pipette capacity 7 mL
- "Squeeze" plastic: 100 ml, 250 mL, 500 mL
- Rod (plastic)
- Slide tray (plastic)
- Stirring rods
- Rack for centrifuge tubes
- Record forms and notepaper
- Self-adhesive labels
- Strainer, metal (tea strainer), 7.5 cm diameter
- Timer
- Wooden applicator sticks, cotton swabs and tongue depressors

Reagents

- Chromotrope 2R
- Glacial acetic acid
- Distilled water
- Ethanol: 70%, 95%, 100%
- Ethyl acetate
- Formaldehyde (37–40%)
- Glycerol
- Hydrochloric acid (HCl)
- Iodine crystals (I_2)
- Light green SF
- Malachite green
- Potassium iodide (KI)
- Saline solution
- Sodium acetate
- Methylene blue

Solutions

- Carbol fuchsin: liquefy 5 g of phenol crystals with a small amount of distilled water, using a warm water bath at 95 °C. Dissolve 1 g of basic fuchsin in the liquefied phenol. Add 10 mL of 95% ethanol and mix. Add 100 mL of distilled water. Filter and store in a dark flask, well labelled. The solution is ready for use.
- Formalin 5%: 50 mL formaldehyde + 950 mL distilled water or saline (recommended for all-purpose use and for preservation of protozoan cysts).
- Formalin 10%: 100 mL formaldehyde + 900 mL distilled water or saline (recommended for helminth eggs and larvae).
- Lugol's solution: 2 g potassium iodide (KI) + 1.5 g powered iodine crystals (add after KI dissolves) + 100 mL distilled water. Store in a brown, glass-stoppered bottle at room temperature and in the dark; the expiration date is 1 year. The solution is ready to use. For routine use, put 20 mL in a brown dropper bottle for 10–14 days.
- SAF (sodium acetate-acetic acid-formalin fixative): sodium acetate 1.5 g + acetic acid, glacial 2.0 mL + formalin 4 mL + distilled water 92.0 mL).

Flotation solutions

Saturated sodium chloride (NaCl), specific gravity – 1.20: warm water 1000 mL + NaCl 500 g, dissolve overnight by magnetic stirrer. Check the specific gravity with a hydrometer.

Saturated sodium chloride (NaCl), sg 1.20

Hookworms
(*Ascaris lumbricoides*)
(*Trichuris* spp.)
Trichostrongylus spp.
Strongyloides stercoralis
Enterobius vermicularis
Hymenolepis spp.
Taenia spp.

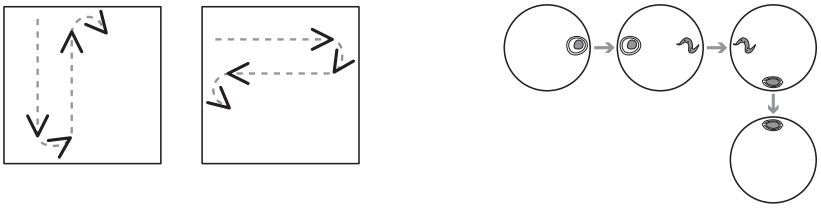
Zinc sulfate ($ZnSO_4$ $7H_2O$), specific gravity 1.35: water 685 mL + zinc sulfate 685 g, dissolve overnight by magnetic stirrer. Check the specific gravity with a hydrometer.

Zinc sulfate ($ZnSO_4$ $7H_2O$), sg - 1.35

Ascaris lumbricoides
Trichuris spp.
Fasciola hepatica
Schistosoma mansoni
Dicrocoelium dendriticum
Balantidium coli
Dientamoeba fragilis
Blastocystis hominis
Entamoeba spp.
Endolimax nana
Giardia duodenalis
Enteromonas hominis

Microscopic examination

The search for eggs and larvae of helminths (and of ciliates) is classically done using the 10x objective. The entire preparation is examined. To accomplish this, one should work systematically. Always start at a corner of the cover slip and work in a straight line from the chosen corner towards the opposite side. Once there, move one row aside and work back until the entire preparation has been examined. Always proceed by looking at the next microscopic field with a small overlap: when a field has been examined, an object in this field is chosen, and is brought towards the opposite side of the field. This second field is then examined. When parasitic structures are found, details are examined at 40x objective.



For searching most of the protozoans, the 40x objective is used. In the same way as described above, a few overlapping rows (3 or 4) should be examined. For morphological identification, oil immersion can be used with the Lugol's solution smear. For differentiation of species, trophozoites and/or cysts must be measured.

Calibration of ocular micrometer

In order to measure structures in the microscopic field, it is necessary to have a measuring scale in the eye-piece of the microscope. Before it can be used, the scale must be calibrated.

Instructions

1. Remove the eyepiece (10x or other) from the micro-scope and place the ocular scale on the diaphragm within the eyepiece. Screw back the lens and re-insert the eyepiece into the microscope.

2. Place the stage micrometer on the microscope stage and focus on the scale.

3. Adjust the stage micrometer by moving the stage so that the 0 line of the ocular micrometer is exactly super-imposed on the 0 line of the stage micrometer.

4. Without moving the stage micrometer, find another point at the extreme right where two other lines are exactly superimposed. This second set of superimposed lines should be as far to the right as possible from the 0 lines.

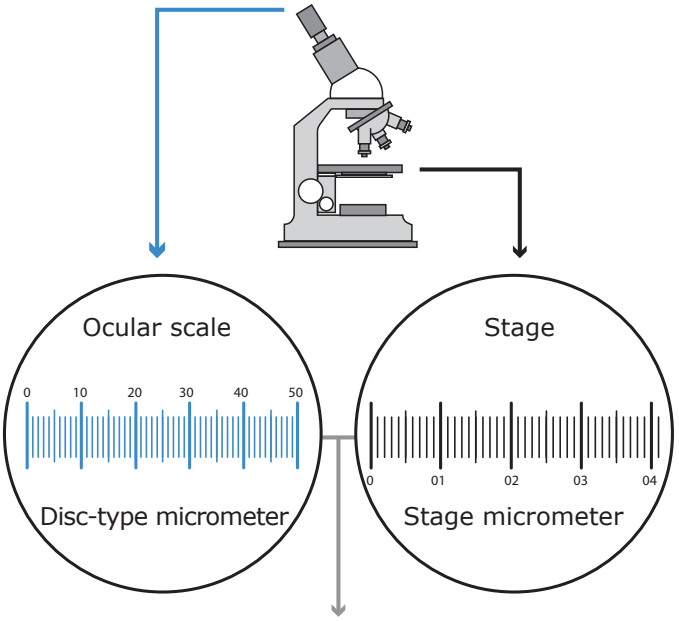
5. Count the number of division lines on the ocular micrometer between the 0 line and the point where the second set of lines is superimposed. In the example provided in the figure, this number, indicated by the black arrows, equal 27 ocular units.

6. Then count the number of 0.1 mm division lines between the 0 line and the second superimposed line on the stage micrometer; in the figure, this number, indicated by the red arrow, equals 0.2 mm.

7. To calculate the length represented by one ocular unit: 1 ocular unit = (0.2 mm/27) x 1000 = 7.4 µm.

8. Thus, 1 ocular unit = 7.4 µm for this specific objec-tive. Each objective on the microscope must be calibrated separately.

9. When all objectives have been calibrated, prepare a simple chart that displays the calibration factor for each objective.



Adjust the stage micrometer to align 0 lines

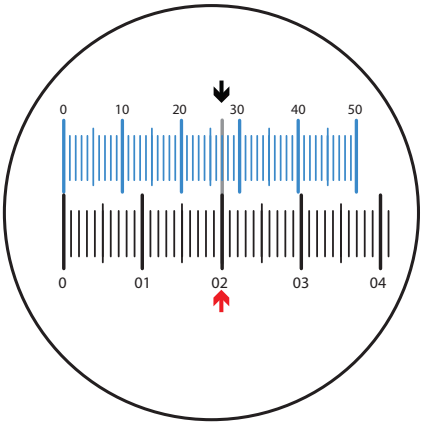


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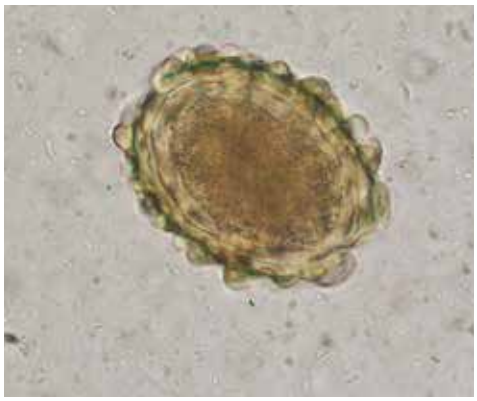
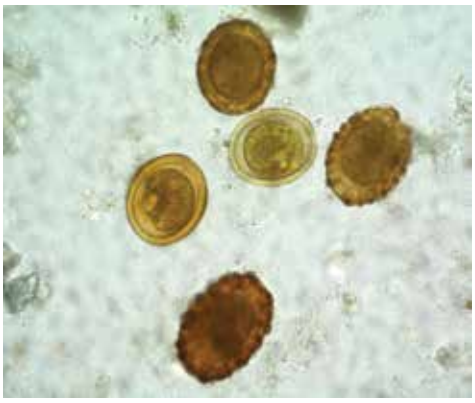
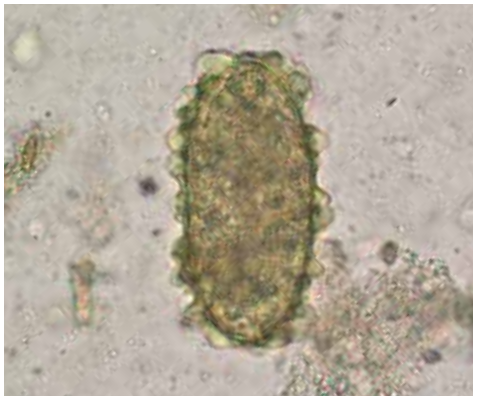
Plate 1
Nematodes



Ascaris lumbricoides Fertile egg (left) as seen in fresh stools; infective egg (right), containing larva after a period of embryonation of 2–4 weeks. Roundish-shaped, yellow to brown in colour with a thick shell and mamillated layer. Size: 45-75 x 35-40 µm



A. lumbricoides Fertile eggs, either with or without the mamillated layer ("decorticated" eggs). Note the lighter colour of decorticated eggs



A. lumbricoides Infertile eggs are elongated and larger in size than fertile eggs, their shell is thinner and the size of the mamillated layer is more variable (left). The content of the egg consists of unorganized material, composed of an amorphous mass of refractile granules. Sometimes these eggs can be decorticated (right). Size: 85-95 x 38-45 µm

A. lumbricoides Infective larva from a broken egg

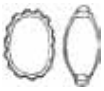
***Trichuris trichiura* - whipworm** Lemon-shaped egg, yellow to brown in colour with smooth shell and typical bipolar prominences (plugs). On the right, an infective egg containing the larva can be seen. Size: 50-55 x 20-25 µm

T. trichiura Egg, atypical form

***Capillaria* spp.** Note the asymmetrical shape of the egg

C. philippinensis Lemon-shaped yellowish egg with striated shell and two poorly evident polar plugs. Size: 35-45 x 20-25 µm

C. aerophila Lemon-shaped greyish egg with thin shell and poorly evident bipolar plugs. Size: 59-80 x 30-40 µm



Ascaris (left), ***Trichuris*** (upper) and ***Capillaria*** (right) eggs

Concentration (sedimentation and flotation)

These procedures allow for the detection of parasitic elements (eggs, larvae, oocysts and cysts) that may be missed when examining only a direct wet smear.

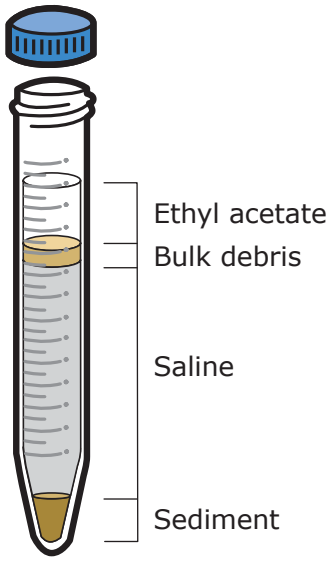
Formalin-ethyl acetate sedimentation concentration

This procedure leads to recovery of all protozoan cysts and oocysts, helminth eggs and larvae present in the stool specimen; it is recommended as being the easiest to perform and the least subject to technical error, allowing recovery of the broadest range of parasitic elements. The specimen can be fresh or fixed stool. The preparation will often contain more debris than that obtained with the flotation and otherprocedures.

***Note**: This technique is not recommended for eggs of* Fasciola *spp. and larvae of* Strongyloides stercoralis.

Procedure

1. Mix about 1 g of faeces (size of a hazelnut) with 10 mL of fixative (SAF or formalin 5–10%), and leave for at least 30 minutes.
2. Strain the suspension into a 15 ml conical tube through a sieve or double layer of gauze allocated into a small funnel and centrifuge at 500 *g* for 10 minutes.
3. Remove the supernatant and break the sediment with a wooden toothpick.
4. Add 7 mL of saline to the sediment, seal the tube with a stopper and mix.
5. Add 3 mL of ethyl acetate (or gasoline or ether. Caution: these reagents should be handled with special care as they are very volatile and may cause explode), seal the tube with a rubber stopper (check that it is tightly closed) and shake vigorously for 30 seconds.
6. Wait 15–30 seconds and carefully remove the stopper.
7. Centrifuge at 500 *g* for 3 minutes.
8. The contents in the tube will separate in four layers, starting from the bottom: sediment (containing the parasitic elements), saline, plug of faecal debris, and top layer of ethyl acetate (or ether or gasoline).
9. Detach the plug of debris from the tube wall with the help of an applicator stick. Pour off the top three layers by inverting the tube with a brisk movement.
10. Mix the sediment with the remaining liquid (if needed, add a few drops of saline).
11. Place a drop of the sediment on a slide and cover with a coverslip. A Lugol-stained preparation can be placed on the same slide.
12. Examine using a microscope.



Concentration by flotation

The flotation technique allows separation of parasitic elements from the coarsest organic debris, using a high specific density flotation solution. Eggs, cysts and oocysts, with a specific density lower than the flotation solution, will rise to the