Basic laboratory methods in medical parasitology

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Contents

Preface

This manual is intended as a practical guide for use by workers in laboratories in health centres and first-referral hospitals. Diagnostic methods are restricted to microscopy, though reference specimens may need to be sent for serodiagnosis.

The content of this manual is based on information contained in training material and manuals produced by the Hospital for Tropical Diseases, London, England; the Parasitology Training Section, Division of Laboratory Training and Consultation, Laboratory Program Office, Centers for Disease Control, Atlanta, Georgia, USA; the World Health Organization, Geneva, Switzerland; and the Pan American Health Organization, Washington, DC, USA. In particular, a number of illustrations were taken from the following publications: Brooke, M. M. & Melvin, D. M., *Morphology of diagnostic stages of intestinal parasites of humans*, 2nd ed. Atlanta, GA, US Department of Health and Human Services, 1984 (HHS Publication No. (CDC) 84-8116); Melvin, D. M. & Brooke, M. M., *Laboratory procedures for the diagnosis of intestinal parasites*, 3rd ed. Atlanta, GA, US Department of Health and Human Services, 1982 (HHS Publication No. (CDC) 82-8282).

List of contributors

The following have contributed to the preparation of this publication:

Dr P. L. Chiodini, Consultant Parasitologist, Hospital for Tropical Diseases, London, England;

Dr K. Engbaek, Department of Clinical Microbiology, Copenhagen County Hospital, Herlev, Denmark;

Dr C. C. Heuck, Health Laboratory Technology and Blood Safety, WHO, Geneva, Switzerland;

Dr L. Houang, Annemasse, France;

Dr R. C. Mahajan, Department of Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India;

Dr M. A. Melvin, Atlanta, Georgia, USA;

Dr L. Monjour, Parasitology and Mycology, Tropical and Parasitological Diseases, Groupe Hospitalier Pitié-Salpétrière, Pavillon Laverean, Paris, France;

Dr J. C. Petithory, National Quality Control in Parasitology, Department of Medical Biology, Centre Hospitalier, Gonesse, France;

Dr J. Vandepitte, Department of Clinical Microbiology, University Hospital St Raphael, Leuven, Belgium.

Introduction

Parasitic diseases are responsible for considerable morbidity and mortality throughout the world, and often present with nonspecific symptoms and signs. Most parasitic diseases cannot be diagnosed by physical examination alone, and laboratory investigation is necessary to decide whether or not the patient is infected with a parasite and, if so, what species of parasite is present. Thus the laboratory plays an important role in establishing the diagnosis of parasitic diseases and is therefore the key to the selection of the appropriate drug for treatment. Laboratory tests must be accurate and reliable if the results are to help the physician and benefit the patient.

This manual is a guide for the laboratory worker. Section 1 presents the techniques to be used when examining faeces, blood, urine, and other materials for the presence of parasites. Pitfalls and possible errors are pointed out and methods for avoiding these indicated. Quality control measures are also discussed. The laboratory worker must understand that only careful performance of the techniques required to recover and demonstrate parasites will make it possible for them to be seen clearly on microscopic examination.

Section 2 of the manual describes the morphological criteria used to identify parasites. Artefacts and problems of identification are also discussed.

Information about the equipment and reagents required is contained in four annexes. Annex 1 lists the materials and equipment needed in health centres and hospital laboratories at the primary health care level, Annex 2 gives the formulae and directions for preparing reagents, Annex 3 gives the formulae and directions for preparing culture media, and Annex 4 describes the procedure for cleaning and storing slides to be used for preparing blood films.

Laboratory safety

General principles

1. Each laboratory should have a written manual of safe laboratory methods, to be followed at all times.
2. The laboratory should have a first aid box and a designated first aider on the staff.
3. Non-laboratory staff must not be allowed to enter the working area of the laboratory.
4. Eating, drinking, smoking, and applying cosmetics should not be permitted in the laboratory.
5. Laboratory personnel should wear protective clothing, which should be removed on leaving the laboratory area.
6. Laboratory personnel should clean the benches with a detergent solution (soap) and disinfect the working surfaces after every working day, or after having spilt infectious material. The most commonly used disinfectants are:
   - 96% ethanol or isopropanol (irritant to skin),
   - 1% phenol solution (corrosive, caustic),
   - 0.5–1% hypochlorite solution (caustic, corrosive) (alkaline hypochlorite solution is more aggressive than neutral hypochlorite solution),
   - 1% formaldehyde or 2% glutaraldehyde solution (toxic and irritant to skin).

   Aldehyde and phenol solutions are active over a longer period. It is advisable to wipe the working areas with tissue soaked with a disinfectant solution rather than using a spray.
7. Laboratory personnel must always wash their hands before leaving the laboratory.

Handling specimens

Great care is needed in handling all laboratory specimens and rubber gloves should always be worn.

Blood samples. All blood samples must be regarded as potentially infectious. As very serious pathogens can be transmitted by blood (e.g., human immunodeficiency virus (HIV), hepatitis B virus) great care is required when collecting and processing samples. Particular risks are:

(*a*) Stabbing or cutting injuries—dispose of used needles or lancets in a container which can then be incinerated or buried in a disposable specimen container after soaking in disinfectant solution. Do not reuse lancets. Do not leave used lancets lying around the laboratory. Do not use chipped or cracked glassware.

(*b*) Contamination of damaged skin or of mucous membranes—cover any cuts with impervious dressings. Avoid spilling blood on to the skin or mucous membranes. Pipetting by mouth should be absolutely forbidden! If blood is spilt on to the skin, immediately wash the affected area with soap and water; if blood gets into the eyes, they should be irrigated with large amounts of water. Any blood spilt on to laboratory surfaces should be soaked with hypochlorite solution and then wiped up with a cloth impregnated with hypochlorite solution.

Stool samples. Skin contact must be avoided. When finished with, samples should be either (*a*) incinerated or (*b*) soaked in disinfectant solution and then buried in disposable specimen containers.

Urine samples. Skin contact must be avoided. Samples can be discarded via the sewage system.

Disposal of microscope slides

Slides should be discarded into a pot containing 1% hypochlorite solution and buried in disposable specimen containers, if they are not to be cleaned for reuse.

Section 1

Techniques of collection, preparation, and examination of samples

Care of the microscope

Do's

1. Do keep the microscope covered with a clean plastic or cloth cover when it is not in use.
2. Do take special care to protect the microscope from dust in hot dry periods.
3. Do take special care to protect the microscope lenses and prisms from fungal growth in hot humid periods. This can be done by:
   - keeping the microscope in an air-conditioned room,
     OR
   - storing the microscope in a special dehumidified room—an electric dehumidifier is about half the price of an air-conditioner,
     OR
   - connecting a number of 15 or 25 watt bulbs inside a cupboard with tightly fitting doors,
     OR
   - placing a 15 watt bulb in the individual microscope box which then acts as a warm cupboard,
     OR
   - in areas without electricity, placing a shelf to hold the microscope box about 30 cm over the chimney of the gas- or kerosene-operated refrigerator or freezer; an airtight bag and silica gel in its dry state (as indicated by its blue colour) will keep a microscope sufficiently dry to protect lenses from fungi.
4. Do clean the immersion oil from the immersion objective every day; use a soft cloth dampened with ethanol/ether (3 ml/7 ml) or benzine/ethanol/ether (2 ml/2 ml/1 ml) and polish with a clean, lint-free cloth.
5. Do clean the oculars with a soft, lint-free cloth; as an alternative, use lens tissue or facial tissue, if available.
6. Do use the microscope retaining screw fitted at the base of the microscope box to prevent damage to the instrument while in transit.
7. Do quote the model number and, if possible, the instrument and part number when ordering replacement parts.

Dont's

1. Don't use the tissue or cloth used for the oil immersion objective to clean the oculars.
2. Don't use alcohol to clean painted surfaces of the microscope.
3. Don't dismantle or try to clean parts of the microscope that are difficult to reach unless you have been trained to do so.
4. Don't leave the lens ports empty; use the appropriate cover or some sticking plaster to cover the empty port.
5. Don't exchange lenses from microscopes of different manufacture—even some models by the same manufacturer have different specifications.

Calibrating the microscope for measurement

Size is an important criterion for the identification of many parasites, particularly cysts and ova. Size can be determined using a blood cell counting chamber (Neubauer), or alternatively an eyepiece micrometer. Using an eyepiece micrometer, the procedure is as follows:

1. The eyepiece scale is divided into 100 small divisions.
2. The stage micrometer scale consists of 1 mm divided into 0.1 mm divisions and each 0.1 mm is divided in 0.01 mm.
3. Insert the eyepiece scale (a round glass disc) into the eyepiece by removing the uppermost lens and placing the scale on the field stop.
4. Insert the eyepiece into the microscope.
5. Place the stage micrometer on the microscope stage.
6. Focus the low-power objective on the stage scale.
7. Adjust the stage and eyepiece scales until the eyepiece scale and the stage scale are parallel.
8. Note the number of eyepiece divisions and its appropriate stage measurement, e.g., 50 eyepiece divisions = 0.75 mm; 10 eyepiece divisions = 0.15 mm.
9. From this reading, work out the value for one eyepiece division, as follows:

   50 eyepiece divisions = 0.75 mm
   1 eyepiece division = 0.75/50 = 0.015 mm
   OR
   10 eyepiece divisions = 0.15 mm
   1 eyepiece division = 0.15/10 = 0.015 mm.

10. Change the measurement value from mm to μm (1 mm = 1000 μm), e.g., 0.015 mm = 15 μm.
11. Repeat for all objectives and note the reading for each.
12. Calibration need be done only once for each microscope used.

Faecal specimens

Faecal specimens are examined for the presence of protozoa and helminth larvae or eggs.

The stages of protozoa found in stools are trophozoites and cysts. The stages of helminths usually found in stools are eggs and larvae, though whole adult worms or segments of worms may also be seen. Adult worms and segments of tapeworms are usually visible to the naked eye, but eggs, larvae, trophozoites, and cysts can be seen only with the microscope. In order to see these structures, the faecal material must be properly prepared and examined.

Collection of faecal specimens

Because of the fragile nature of many intestinal parasites, and the need to maintain their morphology for accurate identification, reliable microscopic diagnosis cannot be made unless the stool is collected properly.

1. Give the patient the following:

   – a waxed cardboard box with an overlapping lid, or a plastic cup or box with a tight-fitting lid, and
   – 2 applicator sticks.

   If waxed boxes or plastic cups are not available, tin boxes or glass jars can be used. Banana leaves and match boxes are not satisfactory containers for the collection and storage of stool specimens.
   In control programmes, it is often sufficient to examine a single specimen, but for patients, three specimens are usually required, at 3-day intervals, to detect all parasitic infections. A variety of substances may interfere with the examination of stool specimens for parasites (e.g., laxatives, antacids, ingested contrast media, certain antibiotics).
2. Tell the patient to pass the stool specimen directly into the container, or to pass the stool on to a piece of paper and use the applicator sticks to transfer it to the container. If paper is not available, the faeces can be passed on to a large, clean leaf, such as a banana leaf. However, the stool *must be transferred immediately to the specimen container*. It should not remain on the leaf, or be brought to the laboratory on the leaf.
3. Some organisms, especially amoebic trophozoites, will begin to disintegrate or change within a short time after passage and become unrecognizable. Warm temperatures will hasten these changes. Therefore, specimens must reach the laboratory very soon (i.e., within half an hour) after passage. If this is not possible, the specimen must be treated with preservatives (see pp. 28–29).
4. The container with the specimen should be labelled clearly with the following information:

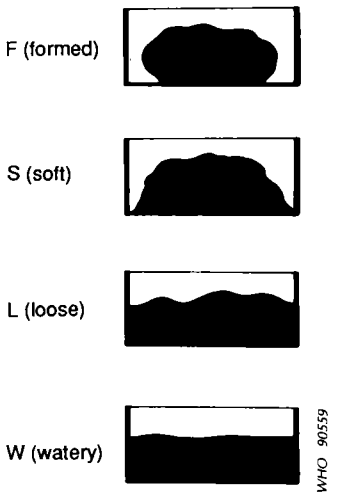
   – patient's name or number
   – date of collection
   – time the patient passed the stool (ask the patient when he/she passed the stool).

5. The stool specimen must be large enough for satisfactory examination. The smallest quantity that should be accepted is about the size of a pigeon's egg. Urine and dirt should be excluded. Urine will destroy any amoebic trophozoites and dirt will interfere with the examination.
   If the specimen is too small, or if it is mixed with urine or dirt, it should not be accepted. Ask the patient to pass another specimen.

6. Keep the carton containing the specimen in a refrigerator, or if this is not possible in the coolest, shadiest area in the laboratory. Do not keep the specimen artificially warm and do not leave it in the sun.

Examination

Macroscopic examination of stool



1. As soon as the specimen is received in the laboratory, check the consistency (degree of moisture) and write one of the following letters on the container: F (formed), S (soft), L (loose), or W (watery).
   If mucus is present write M, and if blood is present write B. For example, a loose stool with blood and mucus would be recorded as L, B, M. The consistency, or degree of moisture, will be a guide as to whether the trophozoite stage or the cyst stage of protozoa is likely to be present. The various categories of stool and the appropriate techniques to be used are shown in Table 1.
2. If several specimens are received at the same time, those containing blood and mucus should be examined first, followed by liquid specimens. These specimens are the most likely to contain amoebic trophozoites (which die soon after being passed) and must be examined within 1 hour after passage. Formed specimens may be examined at any time during the first day, but should not be left overnight (cysts may disintegrate).

Microscopic examination of wet mounts

Wet mounting is the simplest and easiest technique for the examination of faeces, and this method should be performed in all laboratories at the peripheral level.

A wet mount can be prepared directly from faecal material or from concentrated specimens (see p. 16). The basic types of wet mount that should be used for each faecal examination are saline, iodine, and buffered methylene blue:

– The *saline wet mount* is used for the initial microscopic examination of stools. It is employed primarily to demonstrate worm eggs, larvae, protozoan trophozoites, and cysts. This type of mount can also reveal the presence of red blood cells and white blood cells.

Table 1. Categories of stool and appropriate techniques to be used

Consistency	Protozoan stage most likely to be found[a]	Technique to use		
		Saline	Iodine	Buffered methylene blue (if trophozoites are seen)
Formed	Cysts	+	+	
Soft	Cysts (occasionally trophozoites)	+	+	+
Loose	Trophozoites	+		+
Watery	Trophozoites	+		+

[a] Worm eggs and larvae may be found in stools of any consistency.

- The *iodine wet mount* is used mainly to stain glycogen and the nuclei of cysts, if present. Cysts can usually be specifically identified in this mount.
- The *buffered methylene blue (BMB) wet mount* should be prepared each time amoebic trophozoites are seen in a saline wet mount, or when their presence is suspected. BMB stains amoebic trophozoites, but does not stain amoebic cysts, flagellate trophozoites, or flagellate cysts. BMB stain is appropriate only for fresh unpreserved specimens. It is not used on preserved specimens in which the organisms have been killed.

Materials and reagents

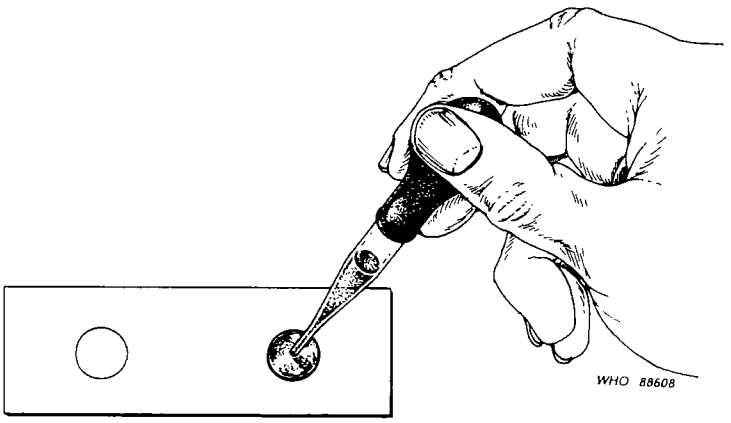
1. Coverslips
2. Dropping-bottles containing: saline solution, isotonic (reagent no. 24)[1]
   Lugol's iodine (1% solution) (reagent no. 18)
   buffered methylene blue (reagent no. 2)
3. Microscope slides
4. Pens or markers for labelling
5. Wire loop (or applicator sticks, matchsticks, or toothpicks).

Direct saline and iodine mounts

1. With a wax pencil write the patient's name or number and the date at the left-hand end of the slide.

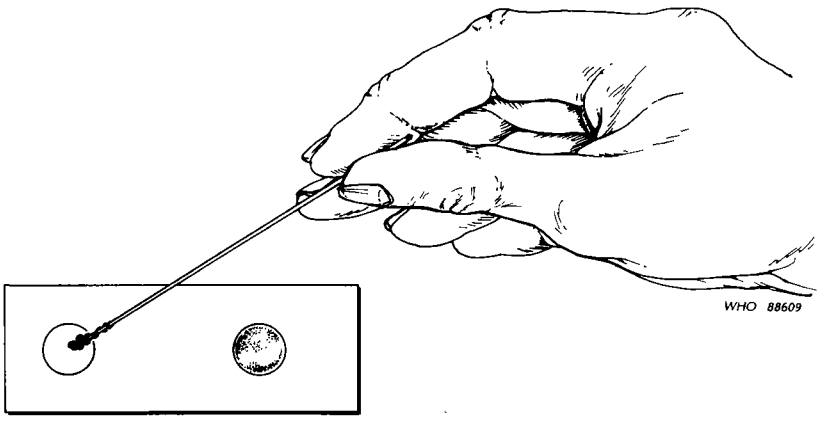
2. Place a drop of saline in the centre of the left half of the slide and place a drop of iodine solution in the centre of the right half of the slide.

   NOTE
   If the presence of amoebic trophozoites is suspected, warm saline (37 °C) should be used.



[1] Throughout this manual the "reagent no." refers to the number assigned to the reagent in Annex 2.

3. With an applicator stick (match or toothpick), pick up a small portion of the specimen (size of a match head) and mix with the drop of saline.



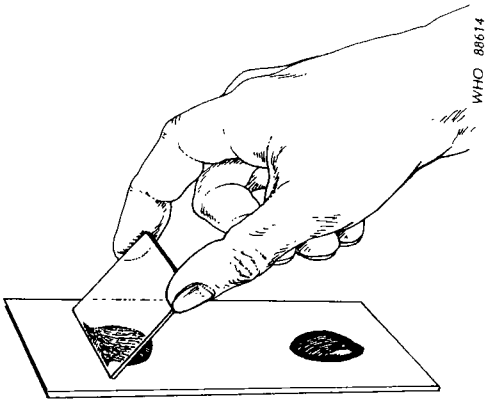
NOTE

Formed stool: take the portion of stool from an area to include inside and outside parts of the specimen.

Stool with mucus: if mucus is present, label a second slide with the patient's name or number. Put a drop of saline on the slide, pick up a small portion of mucus and mix with the saline. Trophozoites, if present, are sometimes more readily found in mucus than in the solid parts of the stool.

Loose watery stool: if mucus is not present, pick up a small portion of the stool (any part) and mix with the saline.

4. Similarly, pick up a small amount of the stool and mix with the drop of iodine, to prepare an iodine mount. If a wire loop is used, flame it after making the mount. If applicator sticks are used, discard them.
5. Cover the drop of saline and the drop of iodine with a coverslip. Hold the coverslip at an angle, touch the edge of the drop, and lower gently on to the slide. This will reduce the chance of including air bubbles in the mount.



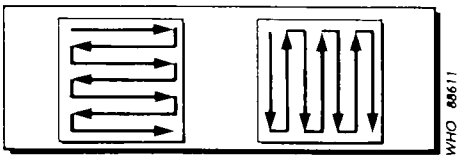
Buffered methylene blue (BMB) mount (to be prepared if amoebic trophozoites are seen in the saline mount)

Proceed as in steps 1 to 5 for "Direct saline and iodine mounts", but place a large drop of BMB on the slide instead of saline or iodine. Wait 5–10 minutes before

examining, to allow the stain to penetrate the trophozoites. BMB will overstain the trophozoites in about 30 minutes. Therefore the slide must be examined within 30 minutes after preparation.

Examination

1. Put the slide with the mounts on the microscope stage and focus on the mount with the ×10 or low-power objective.
2. Regulate the light in the microscope field with the substage diaphragm. You should be able to see objects in the field distinctly. Too much or too little light is not good.
3. Examine the entire coverslip area with the ×10 objective; focus the objective on the top left-hand corner and move the slide systematically backwards and forwards, or up and down.



4. When organisms or suspicious material are seen, switch to the high-dry objective, and increase the light by opening the substage diaphragm to observe the detailed morphology.

This is a systematic examination. If mounts are examined in this way, any parasites present will usually be found. If the mount is not examined systematically, parasites may be missed. Examine each microscopic field carefully, focusing up and down, before moving to the next field.

REMEMBER
EXAMINE MOUNTS SYSTEMATICALLY

Fig. 1 indicates which examinations should be carried out at laboratories at the different levels.

Identification of parasites

Worm eggs and larvae in saline mounts

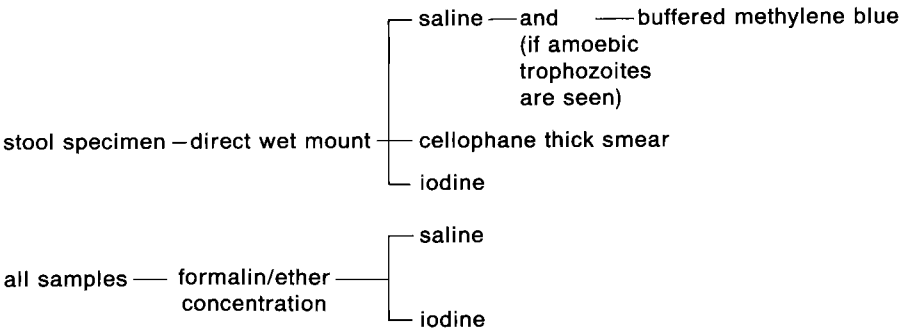
Eggs may be easily detected and identified in saline mounts. They should not be stained (stains may interfere with identification). Most of the eggs are large enough to be recognized with the low-power (×10) objective, but a few small eggs will require a high-power, dry lens.

In saline mounts, larvae of *Strongyloides stercoralis* may be seen. Hookworm larvae are not usually present if the sample is fresh, but it may be necessary to distinguish between these two species if an old sample is examined.

The characteristics and features used to identify species of eggs and larvae are described in Section 2, pp. 67–70. The diagrams and keys in Section 2 can be used to help identify eggs and larvae.

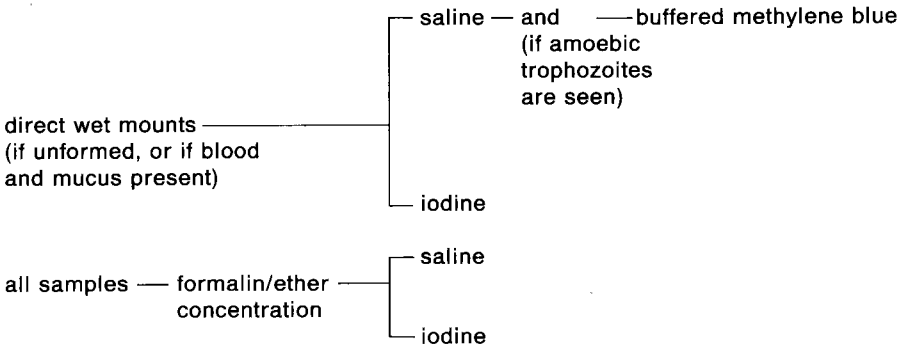
Fig. 1. Techniques of stool examination according to level of laboratory

Health centre laboratory

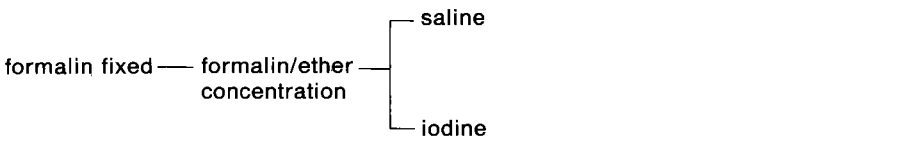


District hospital

Unpreserved stool specimens



Preserved stool specimens



Not all worms are found in every area of the world; some have a restricted geographical distribution. You should make a list of the species that are found in your area.

Protozoa in wet mounts

Saline wet mounts

In saline mounts, trophozoites and cysts of amoebae and flagellates may be seen. Cysts will appear as round or oval, refractile structures; the trophozoites of amoebae may be round or irregular; the trophozoites of flagellates are usually pyriform (elongated, pear-shaped). In freshly passed faeces (the stool must not be more than 1 hour old), motile trophozoites may be seen. Motility can be very helpful in identifying species, especially in the case of flagellates. The characteristic motility of each species is described in Section 2, pp. 71–79.

Organisms may be detected with the low-power ($\times 10$) objective, but a high-power, dry objective will be necessary to identify reliably the structure as a cyst or

trophozoite. With the high-power, dry objective, you can see motility, inclusions like erythrocytes and yeasts in amoebic trophozoites, chromatoid bodies in amoebic cysts, and the shape and structural details (e.g., sucking discs, spiral grooves, or filaments) of flagellate trophozoites and cysts. You will not be able to see any detail in the nucleus in saline mounts. However, it is necessary to regulate carefully the microscope illumination so that the objects appear clearly. Too much or too little light will interfere with your observations. It is also necessary to focus up and down to see all the layers (levels) of the specimen. Remember to examine the whole coverslip area in a systematic manner to reduce the chances of overlooking organisms.

Buffered methylene blue wet mount

If you see amoebic trophozoites, or structures that resemble trophozoites, you should prepare and examine a BMB mount. After 5–10 minutes of staining, the trophozoites sometimes remain motile, but often they curl up in BMB preparations. (Do not confuse curled trophozoites with cysts; cysts do not stain with BMB solution.) In the trophozoites, the nucleus and inclusions (erythrocytes, yeasts) will stain dark blue; the cytoplasm will stain light blue. Occasionally, some trophozoites will not stain, so you should look for well-stained organisms. Look for peripheral nuclear granules (granules in the membrane around the nucleus); if these are present, the trophozoite is an *Entamoeba* species, and you must identify the species. (Characteristics for identifying amoebic trophozoites will be found in Section 2, pp. 71–74.) If there are no peripheral nuclear granules, the trophozoite is not an *Entamoeba* species.

Iodine wet mount

Iodine mounts are examined for amoebic and flagellate cysts. They can be detected with the ×10 objective, but they are not as refractile as in saline mounts. High-power, dry magnification must be used to see the characteristics of the cysts and they must be measured to ensure correct identification.

In the iodine mount, cytoplasm of the cysts will stain yellow or light brown and nuclei will stain dark brown. In iodine-stained cysts of *Entamoeba*, the arrangement of the peripheral chromatin and the position of the karyosome can be seen. (If the peripheral chromatin is not present, the cyst is not an *Entamoeba* species.) These peripheral chromatoid bodies stain light yellow and may not be very clear. Sometimes, young cysts contain glycogen; this stains dark brown with iodine.

In iodine-stained flagellate cysts, the fibrils (filaments) can be seen.

Specific identification of amoebic and flagellate cysts can usually be made from iodine wet mounts. However, occasionally a definite identification cannot be made, and it may be necessary to use permanent stains.

Characteristics for identifying cysts will be found in Section 2, pp. 73–79.

Supplementary techniques

In addition to direct wet mounts, supplementary procedures are available for the diagnosis of intestinal parasites. The most commonly used procedures are concentration techniques for recovering eggs, larvae, and cysts, and permanent staining techniques for demonstrating trophozoites and cysts.

Concentration technique

If the number of organisms in the stool specimen is low, examination of a direct wet mount may not detect parasites. Thus, whenever possible, the stool should be concentrated. Worm eggs, larvae, and protozoan cysts may be recovered by concentration but protozoan trophozoites will NOT be seen as they are usually destroyed during the concentration procedure. This makes direct wet mount examination obligatory as the initial phase of microscopic examination.

The concentration procedure is indicated when the initial wet mount examination is negative despite the clinical symptoms indicating parasitic infection of a patient, and for the detection of *Schistosoma* and *Taenia*.

The concentration procedure recommended is the formalin–ether (or formalin–ethyl acetate) method.[1] All types of worm eggs (roundworms, tapeworms, schistosomes, and other fluke eggs), larvae, and protozoan cysts may be recovered by this method.

Materials and reagents

1. Applicator sticks, wooden
2. Bottles, dispensing or plastic "squeeze", 250 ml or 500 ml. These bottles are convenient for adding formalin to the centrifuge tubes. However, any small bottles or flasks may be used.
3. Centrifuge, with head and cups to hold 15-ml conical tubes. Sealed buckets must be used.
4. Centrifuge tubes, 15 ml, conical (make a graduation at 7 ml and 10 ml with a grease pencil)
5. Cotton swabs
6. Coverslips
7. Funnel
8. Surgical gauze
9. Microscope slides
10. Pipettes, Pasteur, with rubber bulbs
11. Rack or support for tubes
12. Formalin, 10% (reagent no. 10). For everyday use, pour some of the solution into a "squeeze" bottle. Label the bottle.
13. Ether or ethyl acetate.[1]
14. Lugol's iodine, 1% solution—in a dispensing bottle with a pipette (reagent no. 18).
15. Saline solution, isotonic (reagent no. 24).

CAUTION

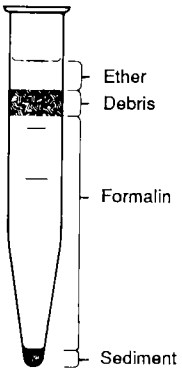
Ether is a highly flammable compound and will ignite and explode quickly if there is a flame or spark nearby. Store opened cans or bottles on an open shelf in the coolest part of the laboratory. Be sure the cans or bottles are stoppered. Do NOT put an opened container of ether in a refrigerator: fumes build up inside the refrigerator, even if the container is closed, and may explode when the door is opened. Do not put opened containers in a cabinet. It is better to leave the container on an open shelf so that the fumes can disperse readily.

Technique

1. Add 10 ml of 10% formalin to approximately 1 g of faeces and stir using an applicator stick, until you get a slightly cloudy suspension.

[1] If ether or ethyl acetate is not available, use ordinary petrol (gasoline) in exactly the same quantities as ether. Ethyl acetate is not as flammable or as explosive as ether, therefore it is less hazardous to use in the laboratory.

2. Fit a gauze filter into a funnel and place the funnel on top of the centrifuge tube.
3. Pass the faecal suspension through the filter into the centrifuge tube until the 7 ml mark is reached.
4. Remove the filter and discard the filter with the lumpy residue.
5. Add 3 ml of ether or ethyl acetate and mix well for one minute.
6. Transfer back to the centrifuge tube and centrifuge for 1 minute. The tube should then look like the diagram below.
7. Loosen the fatty plug (debris) with an applicator stick, and pour away the supernatant by quickly inverting the tube.
8. Replace the tube in its rack and allow the fluid on the sides of the tube to drain down to the sediment. Mix well and transfer a drop to a slide for examination under a coverslip. Also make an iodine-stained preparation.
9. Use the ×10 and ×40 objectives to examine the whole area under the coverslip for ova, cysts, and larvae.



Four layers after final centrifugation

WHO 90560

Examination of sediment

Mounts of concentrated material should be examined in the same way as described for direct wet mounts (see p. 13). The saline (or unstained) mount should be examined systematically, looking for eggs, larvae, and cysts. If cysts, or structures resembling cysts, are seen, you should examine the iodine mount to see more details.

Organisms will look the same as described for direct wet mounts. In saline mounts of formalin–ether (or ethyl acetate) concentrate, the nuclei of cysts are fixed and may be visible. However, iodine wet mounts should still be examined for more reliable identification.

Permanent staining techniques

Permanently stained slides are not made routinely in diagnostic practice and are not required for the identification of worm eggs or larvae. However, permanently stained preparations are occasionally required for the following purposes:

- identification of oocysts of *Cryptosporidium*;
- identification of protozoan trophozoites, if doubt exists;
- confirmation of the identity of protozoan cysts, where doubt exists;
- keeping a permanent record;
- sending to a reference laboratory for an expert opinion.

Staining for oocysts of *Cryptosporidium*

Oocysts of *Cryptosporidium* passed in faeces are spherical, measuring 4–6 µm in diameter. They may be concentrated using a modified formalin–ether technique, but must be identified by staining methods. The recommended method is the modified Ziehl–Neelsen technique. An alternative is the safranin–methylene blue technique.

Materials

Applicator sticks, wooden
Coverslips
Forceps
Microscope slides
Pen or marker for labelling
Rod, glass

Slide holder, for finished slides
Small bottle of mounting medium
Staining dishes
Paper towel or sponge.

Modified Ziehl–Neelsen technique

Reagents

Carbol-fuchsin (reagent no. 4)
Formalin (formaldehyde) (reagent no. 10)
Hydrochloric acid–ethanol solution (reagent no. 13)
Glycerol–malachite green (or methylene blue) solution (reagent no. 12)
Hydrochloric acid–methanol solution (reagent no. 14)
Water.

Preparation

1. Make a thin faecal smear, leave it to air-dry and fix it in methanol for 2–3 minutes. Further fixation in formalin vapour should be performed if possible, to reduce infectivity. (The sediment from formalin–ether extraction cannot be used.)
2. Stain the smear with cold carbol–fuchsin for 5–10 minutes.
3. Differentiate in 1% hydrochloric acid–ethanol until colour ceases to flood out.
4. Rinse in tap-water.
5. Counterstain with 0.25% malachite green (or methylene blue) for 30 seconds.
6. Rinse in tap-water.
7. Blot or drain dry.
8. Examine using the high-power, dry objective and confirm the morphology using oil immersion. Measure the cysts. *Cryptosporidium* cysts measure 4–6 µm.

When stained by this technique, *Cryptosporidium* oocysts appear as bright rose-pink spherules on a pale green background. Different degrees of internal staining are seen, depending on the age and condition of the oocyst.

Safranin–methylene blue technique

Reagents

Hydrochloric acid–methanol solution (reagent no. 14)
Methylene blue–phosphate solution (reagent no. 19)
Safranin solution (reagent no. 23)
Water.

Preparation

1. Prepare a thin smear of faeces.
2. Air-dry the smear. Pass the slide once through the flame of a spirit lamp.
3. Fix the smear in hydrochloric acid–methanol solution for 4 minutes.
4. Wash with clean tap-water.
5. Stain the smear with 1% aqueous safranin solution for 1 minute. Heat the stain by holding a lighted spirit swab under the slide. Do not let the stain dry on the smear.
6. Wash off the stain with clean tap-water.
7. Counterstain with 1% (w/v) methylene blue solution for 30 seconds.
8. Wash with clean tap-water and dry the slide.
9. Scan the smear for oocysts with the ×40 objective and identify oocysts with a × 100 objective. *Cryptosporidium* oocysts are round to oval orange–pink bodies (4–6 µm diameter). The sporozoites within the oocysts stain slightly darker.

The trichrome stain technique for protozoa

The trichrome stain procedure is of value for staining fresh faecal specimens, as well as material fixed with polyvinyl alcohol (PVA). However, it is important to ensure that the shelf-life of the reagents is not exceeded and that the method is followed exactly, if reliable results are to be obtained.

Reagents

Acetic acid–alcohol destain solution (reagent no. 1)
70% ethanol (reagent no. 7)
95% ethanol[1]
Carbol–xylene solution (reagent no. 5) or absolute ethanol
Iodine–alcohol solution (reagent no. 16)
Schaudinn's fixative (reagent no. 25)
Trichrome stain solution (reagent no. 27)
Xylene[1]

Preparing the staining dishes, and replacement of the stains

(i) Label the staining dishes necessary for the procedure and arrange in a row in the following order:

1. Schaudinn's fixative
2. Iodine–alcohol solution
3. 70% ethanol (1)
4. 70% ethanol (2)
5. Trichrome stain solution
6. Acetic acid–alcohol destain solution
7. 95% ethanol (1)
8. 95% ethanol (2)
9. Carbol–xylene or absolute ethanol
10. Xylene

Put several layers of paper towel in front of the dishes. If paper towel is not available, use sponges or newspaper.

(ii) Fill each dish with the appropriate solution. Be sure to use the Schaudinn's fixative with acetic acid added.

(iii) Pour a small amount of mounting medium into a small bottle with a lid or stopper. (Keep the big stock-bottle tightly closed to prevent drying as a result of evaporation.)

The set of staining dishes is arranged in a convenient place and left, ready for use as needed. If the dish tops have ground glass rims, put a thin layer of petroleum jelly on the ground surface to make a good seal with the cover.

The solutions will need to be changed at intervals as follows:

1. Schaudinn's fixative—change every month. Pour out the used fixative into a waste bottle for organic solutions and replace with fresh solution.
2. Iodine–alcohol solution—change every 3 weeks. If the colour fades, or becomes too pale, replace immediately.
3. 70% ethanol (1). This solution will become yellow with iodine from the iodine–alcohol. Change it every 3 weeks or after staining 30 smears, whichever is sooner.
4. 70% ethanol (2)—change every 3 weeks.
5. Trichrome stain solution—change only when solution becomes greenish. Gently rock the jar back and forth. If the stain on the walls of the jar looks green rather than purple, discard the stain in the jar and replace it with fresh stain.

[1] This reagent requires no preparation. It is used directly from the container in which it is purchased.

6. Acetic acid–alcohol destain solution—change after destaining 20 smears, or each week, whichever comes first.
7. 95% ethanol (1)—change every week.
8. 95% ethanol (2)—change every 2 weeks.
9. Absolute ethanol—change every week; carbol–xylene—change every month.
10. Xylene—change every month.

Keep a record of each solution and the date it was poured into the jar, for example:

Name of solution	*Starting date*	*Changing date*
Schaudinn's	13 January 1990	12 February 1990
Iodine–alcohol	7 January 1990	28 January 1990

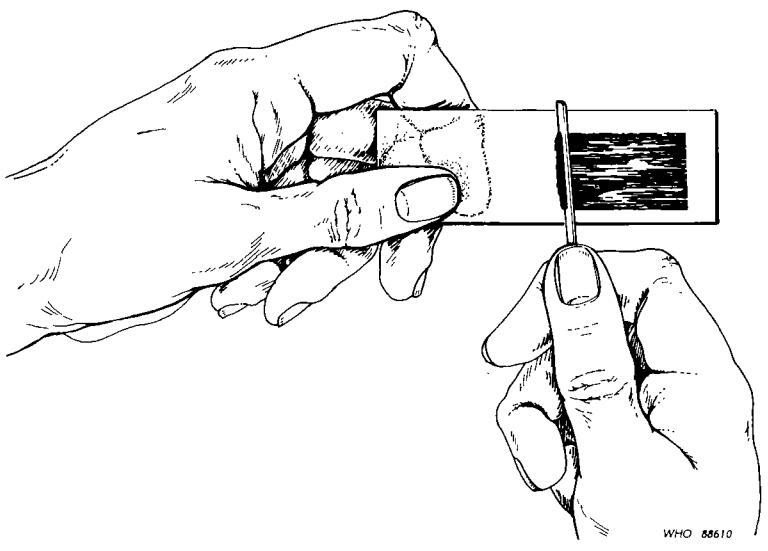
Check this record each week. In this way, the stain solutions will always be satisfactory for staining faecal smears.

NOTE
Keep covers on the stain jars at all times except when slides are being put in or taken out. Do not leave the covers off while staining. The solutions will absorb moisture from the air if not kept covered, and will become unsatisfactory for staining.

The carbol–xylene solution and xylene are dehydrating and clearing solutions; they remove water from the smear and make the material translucent so it can be examined. If moisture gets into these solutions, they will not work properly. Sometimes beads of moisture will collect and can be seen in the jars. If this happens, discard the solution and replace with fresh solution.

Technique

1. Label a microscope slide with the patient's name or number, and the date.
2. With an applicator stick, pick up a small amount of the stool and spread it in a thin smear by rubbing the material back and forth with the applicator stick over the middle part of the slide. The layer of stool must be as uniform and even as possible and of the correct density.



This must be done quickly to prevent drying of the specimen. Immediately put the smear into Schaudinn's fixative.

NOTE
If the stool is hard, a small portion can be mixed with saline to soften it and the smear made from this.

THE SMEAR MUST NOT DRY
FROM THE TIME IT IS MADE
UNTIL THE TIME IT IS MOUNTED

3. Fix with Schaudinn's fixative for 1 hour at room temperature. (The smear may be left in the fixative for as long as 2 days, if necessary.) Put the slide in the dish, so that the end with the patient's name is at the top.
4. Use forceps to pick up the slide from the dish. Drain the excess fluid by touching the end of the slide with a paper towel or sponge. After draining, put the slide into the next staining dish.
5. Leave in iodine–alcohol solution for 1 minute (not more or less). Remove the smear and drain as directed in step 4. Transfer into next dish.
6. Leave in 70% ethanol (1) for 1 minute. Remove slide and drain.
7. Leave in 70% ethanol (2)[1] for 1 minute. Remove slide and drain.
8. Stain with trichrome stain solution for 8 minutes. Remove slide and drain.
9. Destain with acetic acid–alcohol solution—hold the slide with forceps and dip it in the solution twice (total time: 5 seconds). The acid–alcohol will continue to destain the specimen as long as it is in contact with the smear, and 5 seconds is therefore enough time for dipping the slide in the dish of acetic acid–alcohol. (Do NOT put the slide in the dish and count to 5.)
   Drain the slide on a paper towel or sponge. Immediately rinse the smear in the 95% ethanol (1) to remove the acid.
10. Dip the slide once in the 95% ethanol (1) for 1–2 seconds. Drain the smear before putting it in the next dish.
11. Dip the slide twice in the 95% ethanol (2) for 2–3 seconds. Drain the smear before putting it in the next dish.
12. Dip the slide into the carbol–xylene or absolute ethanol for 1 minute. Drain the smear before putting it in the next dish.
13. Leave in xylene for 2–3 minutes.
14. Remove the slide and drain. DO NOT LET THE SLIDE DRY BEFORE MOUNTING WITH THE COVERSLIP. If it starts to dry, dip it in the xylene again—but drain the excess xylene before putting the mounting medium on the smear.
15. Lay the slide flat on a paper towel, or a piece of newspaper, and with a glass rod put 3 or 4 drops of mounting medium on the smear. Hold a coverslip at an angle and touch it to the edge of the smear. Lower it gently on to the smear so the medium spreads out under the coverslip and air bubbles are not trapped between the coverslip and smear.

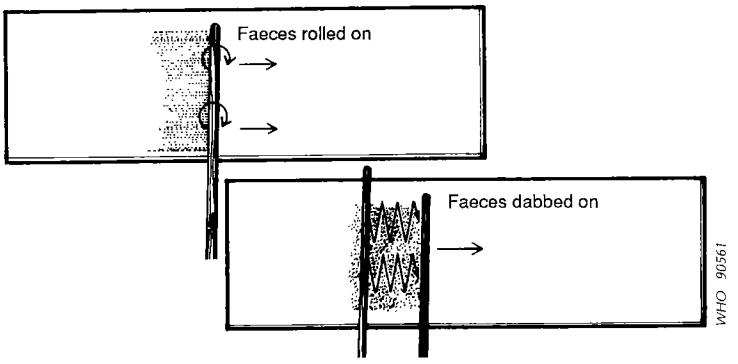
NOTE
If more than one smear is being stained at the same time, destain each one separately. Take one smear from the stain, drain it, destain it, rinse in the 95% ethanol, drain, and put it in carbol–xylene or absolute ethanol (steps 9–12). Then remove another slide from the stain and treat it the same way. Continue until all of the smears have been destained.

[1] If the staining procedure must be interrupted, the slide may be left in the 70% ethanol (2). This is the only stage at which the process may be interrupted. If the staining procedure has gone past the second 70% ethanol (step 7), it must be completed; it must not be stopped or interrupted after step 7.

If more than 1 slide is being stained at the same time, remove from the xylene one at a time, drain and mount. If some of the smears remain in the xylene for 4 or 5 minutes, they will not be harmed. Leave the mounted preparation on a table or counter-top in a flat, horizontal position on a paper towel or a piece of newspaper; it should remain in a flat position until it dries. This may take overnight or longer. However, although not completely dry, the preparations may be examined after 30 minutes. After examination, do not attempt to remove the immersion oil until the mount is completely dry (about 24 hours) as wiping off the oil may remove the coverslip. If this should happen, immediately rinse the smear in xylene for 5 seconds and remount.

Technique for PVA-fixed stool specimens

1. Stir the PVA-fixed material (see pp. 28–29) gently but thoroughly, to mix the stool evenly with the fixative.
2. Label a microscope slide as indicated in step 1 for fresh stools.
3. Dip an applicator stick into the PVA-fixed specimen to pick up some of the material. Spread the material by rolling it on to the slide with the stick. The material can also be spread by dabbing it on—moving the stick up and down as you spread the specimen. The layer of the specimen should be as uniform and even as possible. Fine print should be just visible through the smear, but not clear enough to read. Preparations that are too thick do not stain well and are difficult to examine. If the preparation is too thin, there is not enough material for a reliable examination.



4. Smears MUST DRY before they can be stained. Leave the smears on a flat surface or in a slide holder to dry. They can be left at room temperature or in a 37 °C incubator. Drying takes 8–10 hours, usually overnight. It is better to make the smears one day, let them dry overnight, and stain them the next day. If necessary, dry unstained smears may be kept 3–4 weeks before staining.
5. Put the dried PVA-fixed smear into iodine–alcohol solution.

   (i) Iodine–alcohol solution—15 minutes. Drain the smear as indicated previously for smears made from fresh faeces.
   (ii) 70% ethanol (1)—5 minutes. Drain.
   (iii) 70% ethanol (2)—5 minutes. Drain.
   (iv) Trichrome stain solution—8 minutes. Take one slide out of stain and drain it. If more than one slide is being stained, leave the others in the stain. Destain only one slide at a time.
   (v) Acetic acid–alcohol destain solution—hold the slide with the forceps and dip in and out of the destain 2 or 3 times (total time—5 seconds). Drain slide for 1–2 seconds. (See step 9 for fresh faeces, p. 21.)
   (vi) 95% ethanol (1)—dip the slide in the solution twice to rinse off the acid. Drain slide for 2 seconds.
   (vii) 95% ethanol (2)—5 minutes. Drain smear.
   (viii) Carbol–xylene solution or absolute alcohol—7 minutes. Drain smear.

(ix) Xylene—10 minutes.
(x) Take one slide from the xylene, drain it for 2 seconds, then lay it flat on a paper towel or piece of paper. For mounting, proceed as indicated for fresh faecal specimens (p. 21).

Microscopic appearance of the stained smear

The appearance of smears from unpreserved and PVA-fixed stools is the same, but there is variation from smear to smear because:

- the thickness of the smears will not be exactly the same,
- the destaining time may be slightly different, and
- stool specimens vary from person to person.

In general, the background material stains green or blue-green. Occasionally a specimen will stain red, but this does not interfere with recognizing and identifying organisms. The different inclusions in the stool stain as follows:

GREEN (or blue-green)

- cytoplasm of trophozoites and cysts (well-fixed and well-stained),
- cytoplasm of pus cells and tissue cells,
- yeasts and moulds (usually),
- degenerate protozoa,
- organisms that have been destained too much or too little,
- *Blastocystis hominis* (a protozoan often seen in stools)—contains red granules around the outer edge of the cell.

PURPLE (or blue-purple or red-purple)

- cytoplasm of trophozoites and cysts (sometimes),
- *Entamoeba coli* cysts (sometimes),
- ingested red blood cells and bacteria inside trophozoites.

RED (or purple-red)

- ingested red blood cells and bacteria inside trophozoites,
- yeasts and moulds (sometimes),
- cysts that have not been properly fixed,
- nuclei of pus cells and tissue cells,
- nuclear chromatin of trophozoites and cysts,
- chromatoid bodies of amoebic cysts.

Examination of the stained smear

1. Place the mounted, completely dry smear on the stage of the microscope and focus on it with the low-power objective (×10). Select an area that looks neither too thin nor too thick. If the smear appears uniform (all areas are about equally thick), focus on any area with the ×10 objective. Some of the smear may be too thick to see through easily; other areas may be too thin.
   If the whole smear is heavy or thick, look for the thinnest part. Better stained organisms will usually be seen in the thinner areas.
   If the whole smear looks light or thin, look for the heavier or thicker areas. Organisms will probably be better stained in thicker areas.
2. Put a drop of immersion oil on the selected area and change to the oil-immersion objective. Stained smears of stool specimens must be examined with the oil-immersion objective. Do not use the high-power, dry objective.
3. Focus carefully with the oil-immersion objective. Regulate the microscope illumination with the substage iris diaphragm so that there is adequate light, and cells, bacteria, and other things in the field may be seen distinctly. Move the

slide across the microscope stage while focusing carefully on each new field to see things at different levels in the smear. You should examine about half of the smear; you do not need to examine the entire coverslip area as directed for saline mounts.

4. You are looking for *Entamoeba histolytica* trophozoites and cysts and *Giardia* trophozoites and cysts. If there are many organisms in the specimen, you may find them within a few minutes. If you do not, continue to examine the smear. If you do *not* find *E. histolytica* or *Giardia* organisms report the smear as "No pathogens found".

Identification of parasites in stained smears

In stained smears, both trophozoites and cysts of amoebae and flagellates will be seen. The cytoplasm will stain greenish-blue or green; the nuclei, inclusions like red blood cells and bacteria, chromatoid bodies in amoebic cysts, and fibrils (filaments) in flagellates will usually stain red or purple. Glycogen is dissolved during the staining process and is not visible in stained preparations. You will see a clear or white area where the glycogen has been removed.

Characteristics used to identify protozoa in stained smears are presented in Section 2, pp. 71–79.

Anal swabs for pinworm

Anal swabs are used to detect the presence of pinworms (*Enterobius vermicularis*). Pinworms are more common in children than adults. Often, however, if one child in a family has pinworms other members of the family will be infected. Therefore, if a child is found positive, it is desirable to examine swabs from all members of the family group, especially the children. Pinworm eggs are usually found in the folds of skin around the anus. They rarely appear in the stool.

Collection of specimens

Materials for Method A

Centrifuge
Cotton swabs
Microscope slides
Pipettes, Pasteur, with rubber bulbs
Saline solution to moisten cotton swabs
Test tubes, 100 × 13 mm.

Technique—Method A

Spread buttocks apart, and rub cotton swab over the area around the anus, but do not insert into the anus. Place the cotton swab in the tube.

Materials for Method B

Transparent adhesive tape
Tongue depressor or plastic spoon with handle 10 cm long
Microscope slide.

Technique—Method B

1. Fold a strip of transparent adhesive tape over the end of a spoon handle or tongue depressor.

2. Separate the patient's buttocks with the other hand. Press the end of the spoon covered with tape against the skin around the anus in several places.
3. Place the tape with the sticky side down on a microscope slide. Before examining the slide, lift the tape up and place a drop of immersion oil under the middle of the tape and replace the tape. This will improve the transparency of the tape.

Wash your hands after sample collection; otherwise, eggs that may have contaminated your hands could get into your mouth and lead to infection.

In order to increase the chances of picking up eggs, the swab should be taken between 22h 00 and midnight, or early in the morning before the patient urinates, defecates, or bathes. It may be necessary to collect several swabs before a positive diagnosis can be made.

Examination procedure

Tape-swab slides may be examined directly. For cotton swabs, proceed as follows:

1. Into the tube containing the swab, pour enough saline to cover the cotton swab—about 5 ml.
2. Let stand for 4–5 minutes.
3. Remove the swab from the saline. Roll it against the side of the tube to squeeze out the saline.
4. Discard the swab.
5. Concentrate by centrifugation for 1 minute.
6. With a pipette, remove the supernatant fluid *carefully* so as not to disturb the small quantity of sediment present.
7. With a pipette, transfer the sediment to a slide for examination. Reduce the microscope illumination and focus up and down to see the *Enterobius* eggs (for description see Section 2, pp. 67–69).

Cellophane faecal thick-smear for diagnosis of intestinal schistosomiasis (Kato-Katz technique)

The cellophane faecal thick-smear examination technique has proved to be an efficient means of diagnosis of intestinal schistosomiasis and intestinal helminths. Cellophane thick-smear slides can be prepared in the field, stored in microscopic slide boxes, and shipped great distances, for examination at a central laboratory if required. The technique is not suitable for examining larvae, cysts, or eggs from certain intestinal parasites.

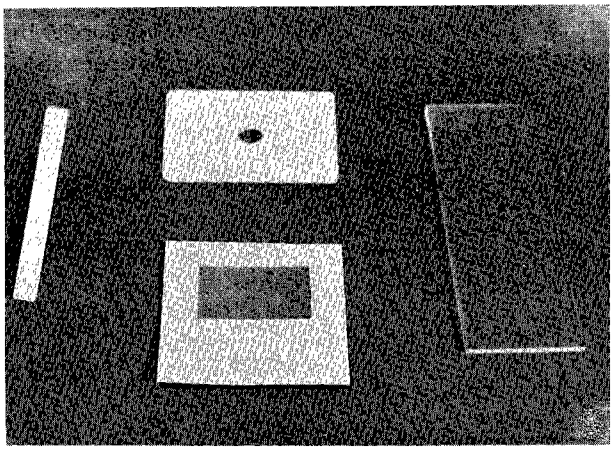
Materials and reagent

Applicator sticks, wooden
Screen, stainless steel, nylon or plastic—60–105 mesh
Template, stainless steel, plastic or cardboard
Microscope slides
Cellophane, 40–50 µm thick, strips 25 × 30 or 25 × 35 mm
Flat-bottomed jar
Forceps
Toilet paper or absorbent tissue
Newspaper
Glycerol–malachite green (or methylene blue) solution (reagent no. 12).

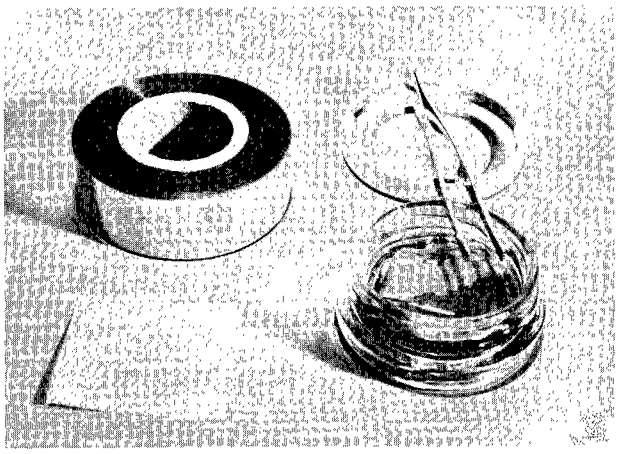
Technique

Care must be taken during collection of stool specimens. Always wear gloves to avoid contamination of the fingers.

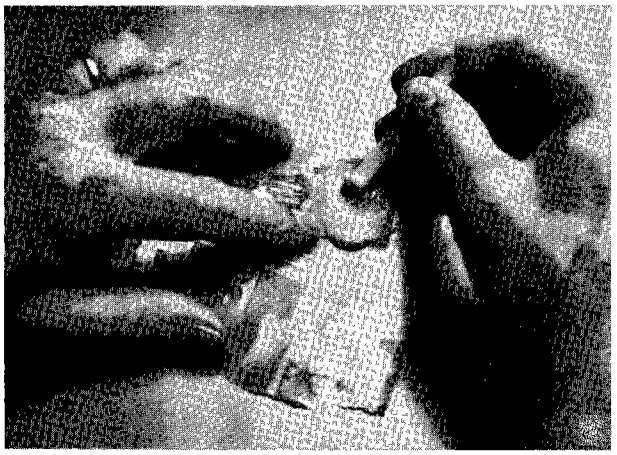
Fig. 2. Cellophane faecal thick-smear examination technique (Kato) for diagnosis of intestinal schistosomiasis and gastrointestinal helminth infections



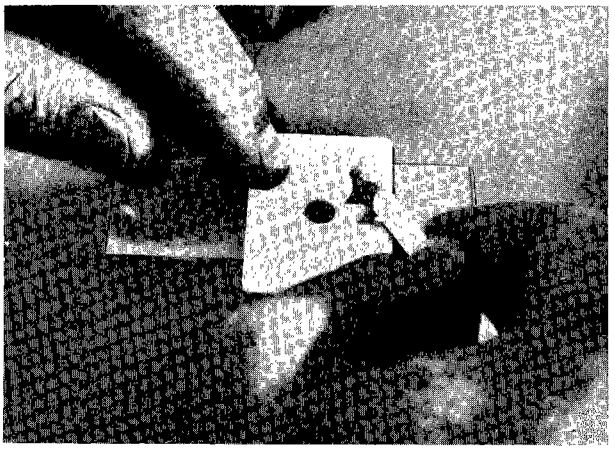
1. Different materials are available. This slide shows the plastic spatula, plastic template and nylon screen in a commercially available Kato-Katz kit. The first two items and the microscope slides may be reused. The nylon screen is disposable. A few kits may be ordered to provide standard reusable templates.



2. The nylon screen and the cellophane required for the thick smear technique may be purchased in bulk. From the roll, cellophane is cut into 25–30 mm sections and placed in a wide-mouth, flat-bottom jar containing a 50% (or greater) glycerol solution with malachite-green or methylene blue stain (100 ml water, 100 ml glycerol, 1 ml 3% aqueous malachite green or methylene blue).



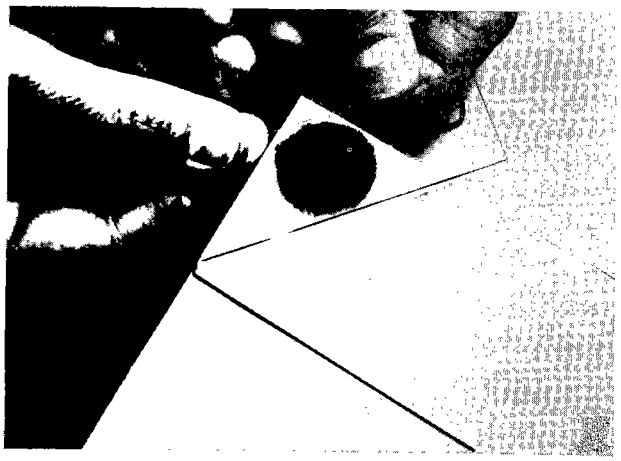
3. The procedure for this technique is the same no matter which material is used. The faecal specimen is forced through the screen by a spatula to separate faecal material from the large debris.



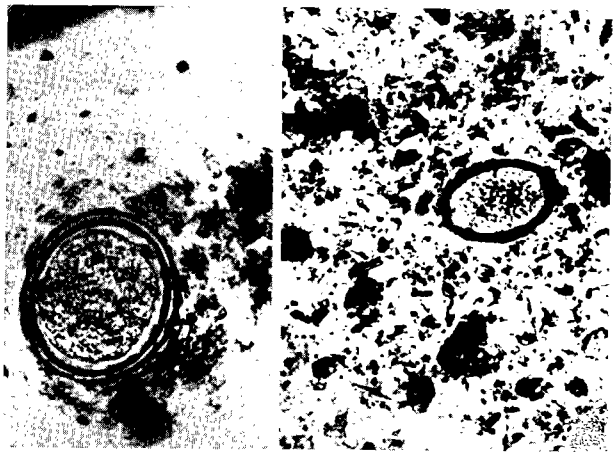
4. The screened faecal material is transferred to the template which is laid flat centrally on a microscope slide. The template hole is completely filled with screened faecal material and levelled to the surface of the template. The Kato-Katz template shown delivers 41.7 mg of faeces. The number of eggs observed is multiplied by 24 to obtain the number of eggs per gram of faeces.

Fig. 2. (continued)

5. The cellophane square soaked in glycerol for at least 24 hours is placed over the faecal specimen.



6. The slide is inverted against a piece of glass or another glass slide and the faecal specimen spread evenly under the cellophane as shown here. After the slide is prepared an additional drop of glycerol may be placed on the cellophane and the edges of the cellophane pressed smooth to ensure conservation of the slide. If air bubbles form under the cellophane during storage, a couple of drops of glycerol on the cellophane allowed to stand overnight will eliminate the bubbles. Cellophane thick-smear slides can be prepared in the field, stored in microscope slide boxes and shipped great distances, which permits examination at a central laboratory if required within days or weeks after preparation.



7. *Ascaris* (left) and *Trichuris* eggs (right) are visible at any time. Hookworm eggs (not shown) are visible for up to 30 minutes after preparation.

8. The ideal time for observing *S. mansoni, S. intercalatum* or *S. japonicum* eggs is 24 hours after preparation. In bright sunlight the slides clear rapidly and a 24-hour delay may not be necessary.

1. Soak the cellophane strips in the 50% glycerol–malachite green (or methylene blue) solution for at least 24 hours before use.
2. Transfer a small amount of faeces on to a piece of scrap paper (newspaper is ideal).
3. Press the screen on top of the faecal sample.
4. Using a flat-sided applicator stick, scrape across the upper surface of the screen to sieve the faecal sample.
5. Place a template on a clean microscope slide.
6. Transfer a small amount of sieved faecal material into the hole of the template and carefully fill the hole. Level with the applicator stick.
7. Remove the template carefully so that all the faecal material is left on the slide and none is left sticking to the template.
8. Cover the faecal sample on the slide with a glycerol-soaked cellophane strip.
9. If an excess of glycerol is present on the upper surface of the cellophane, wipe off the excess with a small piece of toilet paper or absorbent tissue.
10. Invert the microscope slide and press the faecal sample against the cellophane on a smooth surface (a piece of tile or flat stone is ideal) to spread the sample evenly.
11. Do not lift the slide straight up. The cellophane may separate. Gently slide the microscope slide sideways holding the cellophane.

Preparation of the slide is now complete. It may be necessary to wipe off excess glycerol with a piece of toilet paper to ensure that the cellophane stays fixed. After practice you can obtain perfect preparations. The various stages of preparation of the thick smear are shown in Fig. 2.

Thick or hard stool specimens

The main problem with the thick-smear technique has been that it is impossible to see the helminth eggs in some hard (constipated) stool specimens. In such cases:

- after preparation by the standard method, be sure to wait 24–48 hours before counting eggs on these slides—the slide may clear slowly;
- make another pair of samples on a large (5 × 7.6 cm) microscope slide and use a slightly larger piece of cellophane (35 × 35 mm), then press very hard to flatten the specimen as much as possible;
- when the large slide is used, the stool may be softened with saline or glycerol before sieving.

Proper reading of slides

At ambient temperature the slide should be kept for at least 24 hours before microscopic examination (see below regarding hookworm eggs). If the slide is placed in an incubator (40 °C) or under an intense fluorescent or incandescent light in the laboratory, or in sunlight in the field, it may be read within minutes.

To facilitate the microscopic reading, one or two drops of eosin in saline (1:100) may be placed on the upper surface of the cellophane, left for 3–5 minutes, then wiped off with a piece of toilet paper or absorbent paper. This makes the *Schistosoma* eggs easier to see.

Sending specimens to a reference laboratory

If specimens are to be sent to another laboratory for examination, they must be preserved to keep the parasites in good condition. Two preservatives are used:

- 10% formalin—preserves eggs, larvae and cysts for wet mount examinations;
- PVA-fixative—preserves trophozoites and cysts so that permanent stained smears can be made.

Materials and reagents

Adhesive tape
Applicator sticks, wooden
Bottles, 1000 ml
Labels
Pen or marker for labelling
Vials, 20 ml, with tight-fitting screw-caps
Formalin (formaldehyde), 10% (reagent no. 10)
PVA-fixative[1] (reagent no. 22).

Preservation of specimens

1. Label two 20-ml vials with the patient's name or number. Write F in the upper right-hand corner of the label on one vial; write PVA in the upper right-hand corner of the label on the other vial.
2. Fill the "F" vial about half full with 10% formalin. Fill the "PVA" vial about half full with PVA-fixative.
3. With an applicator stick, pick up a portion of the stool to include areas from the inside and edges of the sample and mix with the 10% formalin. Be sure to mix *very* well; break up lumps. Use enough, but not too much, stool so that the mixture will occupy about 2/3 to 3/4 of the vial.
4. With an applicator stick, pick up a portion of the softest part of the stool and mix with the PVA-fixative as described for formalin. The total amount of stool–PVA mixture should not occupy more than 3/4 of the vial. Be *very* sure that the stool is thoroughly mixed with the fixative. Break up lumps by crushing them against the side of the vial.
5. Screw the caps of the vials securely. Wrap a piece of adhesive tape around the top of each vial to prevent leaking.
6. Pack the vials carefully in a box or shipping container and send to the reference laboratory. Be sure that the vials are surrounded by absorbent material (e.g., cotton wool, newspaper) and are packed so they will not break.
7. Be sure to include the necessary information: patient's name or number, date of shipping, organisms you found.

Disposal of specimens

1. If stools are collected in paper boxes, the best way to dispose of them is by burning the entire container. If they cannot be burned, or if the stool was collected in a metal or glass container, add enough 10% formalin to cover the stool left in the container. This will kill any parasites that might be present. Allow to stand for 1 hour or more before discarding or washing (if the container is glass).
2. Slides used for wet mounts should be put in a pan of disinfectant (e.g., sodium hypochlorite) for at least 1 hour before washing. Use an applicator stick to push the coverslip off into a beaker or small pan of disinfectant and then put the slide into another pan of disinfectant.
   Coverslips break easily, and if put in with the slides, they may break and cut the hands of the person washing them.

[1] Preparation of PVA-fixative is complicated, and involves poisonous and corrosive solutions. The technique is presented in Annex 2 (reagent no. 22), but preparation is perhaps best done in a higher level laboratory. Alternatively, the fixative may be purchased already prepared.

3. Funnels, stoppers, and centrifuge tubes should also be put into disinfectant for 1 hour before washing.
4. Applicator sticks and gauze squares should be burned. If burning is not possible, they can be discarded after soaking in disinfectant.

Quality control for faecal examination

To ensure accurate and reliable results, quality control must be applied to laboratory procedures for diagnosing parasitic infections. Controls must apply to collection of specimens, preparation of reagents, performance of the techniques and examination of the final preparations.

Collection of specimens

If faecal specimens are not properly collected and taken care of before examination, they will be of little or no value for accurate diagnosis. This is especially true of protozoa. Amoebic trophozoites will begin to degenerate 1–2 hours after passage and alterations in appearance may result in erroneous identification. Flagellate trophozoites may also undergo changes that would make differentiation difficult. Cysts will deteriorate if faecal specimens are left standing for many hours or overnight, especially if the temperature is high.

Helminth eggs and larvae are less affected by the age of the specimen than are protozoa. Nevertheless, changes may occur that would affect identification. Hookworm eggs, for example, may become embryonated and larvae may hatch from the eggs. Even *Ascaris* eggs may develop to multicellular stages. In addition, larvae may degenerate in old stools making it impossible to identify the species.

To ensure that good specimens are provided for examination, pay attention to the following points:

1. Use clean, dry containers for collecting faeces. (Dirt will interfere with examinations and may introduce free-living organisms from the soil that would cause problems in identifying the species. Urine and water will destroy trophozoites, if present).
2. Have the specimen brought to the laboratory as soon as it is passed to prevent deterioration of protozoa and alterations in the morphology of protozoa and helminths. Note the patient's name and the date and time of passage on the specimen.
3. Accept only freshly passed specimens for examination. Do not attempt to examine old specimens or those contaminated with dirt, water, or urine. Instead, ask the patient to pass another specimen.
4. If specimens cannot be examined as soon as they arrive, put them in a refrigerator (4–5 °C) or in the coolest, shadiest area in the laboratory. Do not leave them in the sun.
5. Examine diarrhoeal specimens and those containing blood and mucus immediately upon their receipt in the laboratory.

Preparation of reagents

Reagents should be prepared exactly according to the formula and directions. Do not alter the ingredients, or their quantity, or the method of preparation in any way. Store reagents as recommended in the preparation procedure.

Some reagents will last indefinitely if kept properly stoppered and out of direct sunlight. Examples are formalin solutions, isotonic saline, fixatives, and alcohol solutions (unless evaporation occurs). Other reagents may last for only a short time and are ineffective if too old. The "life" of each solution is indicated in the directions for preparing it.

1. Label all reagents with the date of preparation. Keep records for each solution. Review these every week and discard outdated solutions.
2. Many of the solutions used in the method for trichrome stain need to be changed at regular intervals. The intervals are stated in the directions for that technique and must be observed. Failure to do so will result in poor preparations that are of no value for diagnosis.

Performance of techniques

No procedure used for examining faecal specimens is 100% effective—that is, the procedures will not always recover all the species present and, if a particular species is present in only very low numbers, they may fail to demonstrate them when used on a single specimen. Because the techniques are not perfect, you should perform them as carefully as possible for optimum results. Also, be sure to use techniques that are appropriate for the material you are examining.

Direct wet mounts

1. Be sure the density of the mount is correct. You should be able to read small print through it (but not too clearly). If it is too thick or too thin, observation of the elements in the mount may be difficult.
2. Be sure to prepare fresh iodine solutions every 10–14 days. Old iodine will not stain cysts properly. The iodine must not be too strong or the faecal material may clump and trap organisms in the clumps so that they cannot be seen. If the iodine is too weak, it will not stain cysts properly.

Concentration procedure

1. Select a fully representative sample of the stool for concentration.
2. Prepare well mixed suspensions of faeces and water or saline.
3. Use the appropriate quantities of materials.
4. Use the correct centrifuge speed and time.
5. Prepare and examine mounts carefully as described for direct wet mounts.
6. Do not discard the tube containing the concentrated material until you have completed your examination. You may need to make another mount.

Staining procedure

1. Select soft portions of the stool, and portions of mucus, if present, for making smears for staining.
2. Be sure that solutions are in good condition. Change them as described in the procedures for staining. Keep the bottles of stock solutions tightly closed and store away from direct sunlight.
3. Be sure to keep dishes covered. Keep the covers on except when putting slides into, or taking them out of, the dish. If the staining dishes are left uncovered, the solutions may evaporate or debris may get into them and adhere to the preparation.

4. Use the correct quantity of mounting medium. Too much medium may result in a thick layer through which it will be difficult to focus, and you will not be able to see the smear clearly. Too little medium will leave gaps under the coverslip. If the smear was prepared from fresh, unpreserved faeces, areas where there are gaps may become unsatisfactory for diagnosis. Gaps in the medium also make the mount difficult to examine.
5. Always examine stained smears with the oil-immersion objective. Use the ×10 objective to focus on the smear. If the smear is not uniform, locate an area for examination where the smear is not too thin or too thick and the staining is good. Then change to the oil-immersion lens and examine for protozoan trophozoites and/or cysts.

REMEMBER

RELIABLE AND ACCURATE PARASITE IDENTIFICATION DEPENDS ON:

- **COLLECTING SATISFACTORY SPECIMENS**
- **PREPARING AND MAINTAINING REAGENTS CORRECTLY**
- **CAREFUL PERFORMANCE OF APPROPRIATE TECHNIQUES AND THOROUGH EXAMINATION OF FINISHED PREPARATIONS**

Urine specimens

Urine specimens are usually examined for *Schistosoma haematobium* eggs. *Trichomonas vaginalis* trophozoites may also be seen. Microfilariae of *Wuchereria bancrofti* and *Onchocerca volvulus* may be found in the centrifuged sediment of sometimes milky urine from patients in countries where filariasis is endemic.

In areas where schistosomiasis is endemic, the first indirect evidence of infection is haematuria and/or proteinuria, detectable using a reagent strip. Gross haematuria indicates heavy infection.

Collection of urine for diagnosis of Schistosoma infection

The number of ova in the urine varies throughout the day, being highest in urine obtained between 10h 00 and 14h 00. The specimen should be collected between these times and consist of a single, terminal urine of at least 10 ml. Alternatively a 24-hour collection of terminal urine can be made. The whole specimen must be examined as ova may be very scanty. Ask the patient to pass the urine into a clean flask or bottle, and examine the urine at once.

If the urine must stand for an hour or longer, add 1 ml of undiluted formalin (37% formaldehyde solution) to each 100 ml of urine. This will preserve any eggs that might be present.

NOTE
If formalin is not available, 2 ml of ordinary household bleach can be added to each 100 ml of urine.

WARNING
Formalin and bleach are corrosive, and dangerous if swallowed.

Examination of urine

The two methods used for detection of *Schistosoma haematobium* ova are sedimentation and filtration. The sedimentation method is less sensitive but cheaper and simpler to perform. The filtration technique is used in public health care mainly when quantitative information is required.

Sedimentation method for 24-hour terminal urine collection

Materials

Centrifuge, with head and cups to hold 15-ml tubes
Centrifuge tubes, conical, 15 ml
Coverslips
Flask, conical for urine collection
Microscope slides
Pen or marker for labelling
Pipettes, Pasteur, with rubber bulbs.

Technique

1. Shake the urine specimen well and pour into a conical urine flask.
2. Allow the urine to sediment for 1 hour. Withdraw the supernatant, transfer the sediment into a centrifuge tube, and centrifuge at 2000 *g* for 2 minutes.
3. Examine the deposit of the centrifuged sample for the presence of ova, using the × 10 objective to screen the whole of the deposit.

Do not increase the centrifugation time and do not exceed 2000 *g* as this may rupture the ova and release miracidia.

- **PROCESS AS SOON AS POSSIBLE**
- **SHAKE CONTAINER BEFORE POURING**
- **LABEL SLIDES/TUBES/PAPERS CAREFULLY**

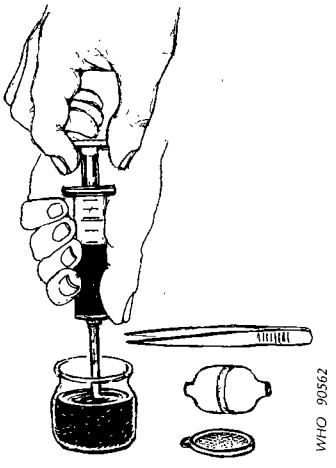
Syringe filtration method

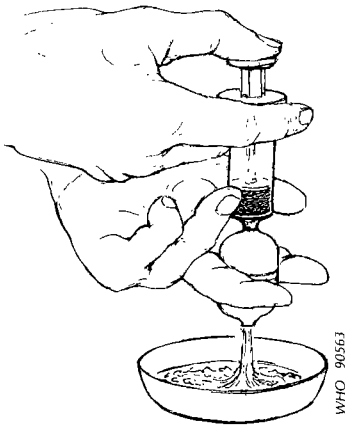
Materials and reagents

Coverslips
Filter holder, diameter 13 mm or 16 mm
Forceps
Syringe, plastic, 10 ml
Membrane filter,[1] 12 µm or 20 µm (polycarbonate), nylon filter, or paper filter
Microscope slides
Lugol's iodine (stock 5% solution) (reagent no. 17).

Technique

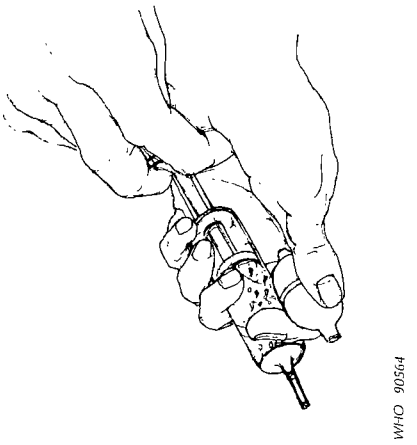
1. Place a polycarbonate or nylon filter (pore size 12–20 µm) in the filter holder. Alternatively, paper filters (Whatman No. 541 or No. 1) can be used. Agitate the urine sample by shaking it gently or by filling and emptying the syringe twice.
2. Draw 10 ml of the urine into the syringe and attach the filter-holder to the bottom of the syringe. (If less than 10 ml is available record in notebook.)
3. Keeping the unit level, expel the urine from the syringe into the filter holder over a bucket or sink.

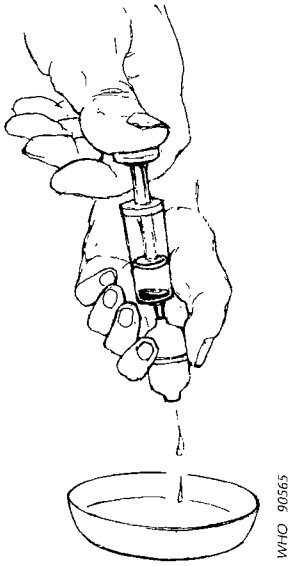




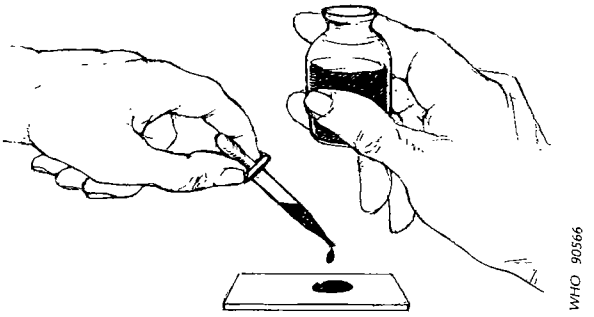
[1] Polycarbonate filters and filter holders are available from: Sartorius GmbH, PO Box 19, D-3400 Göttingen, Germany; Millipore Intertech Inc., Ashby Road, PO Box 255, Bedford, Massachusetts 31730, USA. Nylon filters (Nytrel TI HD 20) in rolls or packages of 500 are available from l'Union Gazes à Bluter, B.P. 2, F-42360 Panissières, France.

4. Carefully unscrew the filter holder, draw air into the syringe, reattach the syringe to the holder, and expel the air. (This is important as it helps to remove excess urine and also makes sure the eggs, if present, are attached to the filter.)





5. Unscrew the filter holder, remove the filter with the forceps and place it (top side up) on a microscope slide. Add one drop of Lugol's iodine and wait for 15 seconds for the stain to penetrate the eggs.



6. Examine the whole filter under the microscope immediately at low power (×40). Schistosome eggs stain orange and can be seen clearly. Infection loads are recorded as the number of eggs per 10 ml of urine. Therefore it is important to note the amount of urine examined, if it is less than 10 ml. To estimate the intensity of infection of the sample, divide the number of eggs counted by 10. If less than 10 ml was examined use the following equation:

$$\text{number of eggs per 10 ml sample} = \frac{\text{number of eggs counted}}{x} \times 10$$

where x = no. of ml of filtered urine examined.

Reuse of filters

Remove the plastic filter immediately after use and soak it overnight in a 1% hypochlorite solution (domestic bleach). Wash the filter thoroughly with detergent solution and then several times with clean water. Check the filter microscopically to ensure that it is free of parasites before being reused.

Identification

Schistosoma haematobium eggs are large, about 120–150 µm long, and have a terminal spine at one end. An embryo (the miracidium) can be seen inside the egg.

Sometimes, it is necessary to determine whether the eggs are viable. This can be done if the specimen is fresh and no preservatives have been added.

Look carefully at the eggs to see if the embryos are moving. This is the best indication of viability. If no movement is seen, look for the "flame cells". There are 4 flame cells, one at each corner of the embryo. Use high-power, dry magnification, reduce the illumination slightly and look for the rapid movement of cilia (short hairs) in the cells.

Quantitative urine examination

Quantitative data from urine examinations, by the syringe filtration technique for detection of *S. haematobium* infection, may be reported according to egg count categories:

light infection—1–49 eggs per 10 ml of urine
heavy infection—>50 eggs per 10 ml of urine.

A third category, such as over 500 or over 1000 *S. haematobium* eggs per 10 ml of urine, may be appropriate in areas where the intensity of infection frequently reaches this level (e.g., in more than 10% of cases).

Vaginal and urethral material

Vaginal and urethral materials are examined for the presence of *Trichomonas vaginalis*, a flagellate parasite of the urogenital system. It parasitizes both men and women, but men are usually asymptomatic. *Trichomonas vaginalis* is usually identified in wet mounts of vaginal and urethral material. (In stained preparations these organisms are badly distorted and may not be recognizable.)

Collection of specimens

Materials

Centrifuge with head and cups to hold 100 × 13-mm tubes (the same cups will hold both 15-ml conical tubes and 100 × 13-mm tubes)
Coverslips
Cotton swabs, sterile
Microscope slides
Pipettes, Pasteur, with rubber bulbs
Pen or marker for labelling
Test tubes, small, 100 × 13 mm, with cotton plugs or screw-caps and 3 ml of sterile saline in each.

Technique

1. With a sterile cotton swab, collect the vaginal or urethral discharge.
2. Put the swab immediately into a sterile tube containing about 3 ml of sterile saline. The top of the stick can be broken off if it is too long for the tube.
3. Smears for staining can be made if desired. For these, collect more material with a second sterile swab and smear on the slide. Allow to dry.
4. Label tubes and slides with patient's name or number, and the date of collection.

NOTE
If the patient can come to the laboratory, wet mounts can be examined directly; tubes are not needed.

Direct examination of vaginal and urethral smears

1. If the patient can come to the laboratory, obtain some of the vaginal or urethral discharge with a sterile swab and put into a drop of saline on a microscope slide.
2. Cover with a coverslip and examine with the ×10 and ×40 objectives for motile flagellates.

Centrifuged or sedimented material

1. If a swab in saline is received, remove the excess fluid from the swab by squeezing it against the side of the tube. Discard the swab.
2. Centrifuge the tube for 2 minutes. If a centrifuge is not available, let the tube stand for 10 minutes to allow any sediment to settle on the bottom.
3. With a pipette remove the supernatant fluid. Do not disturb the sediment.
4. Take a drop of the sediment and put on a microscope slide.
5. Cover with a coverslip and examine with ×10 and ×40 objectives for motile flagellates.

In wet mounts, flagellates can be identified by their pattern of movement. *Trichomonas* trophozoites move with a nervous, jerky or jumpy movement. Since *T. vaginalis* is the only species of *Trichomonas* that inhabits the urogenital system, there is no need to study the morphological features or to differentiate it from *T. hominis*, which lives in the intestine. On rare occasions, ciliated bodies from epithelial cells of the genital tract may be mistakenly identified as some sort of parasitic organism.

Blood and other specimens

Blood is examined for the following parasites:

- *Plasmodium*
- microfilariae
- *Trypanosoma*
- *Leishmania*

The most commonly used technique for blood examination is stained blood films. Giemsa stain (one of the Romanowsky stains) is usually used to stain the films. Field's stain is an alternative where rapid diagnosis is required. Delafield's haematoxylin stain is used for microfilariae. Either thick films or thin films may be used depending on the circumstances. The thick film is more sensitive in detecting parasites and also saves time in examination. However, the thin film technique causes very little distortion of the parasite, and permits species identification when it may not be possible in thick films, but many fields must be examined to detect parasites when they are few in number. Therefore, both thick and thin films must always be prepared when searching for plasmodia and trypanosomes; if a precise identification cannot be made from the thick film, the thin film will be available. Thick films should be used when searching for microfilariae.

The most economical use of slides is achieved by making a combination thick and thin slide, i.e., a thick film and a thin film on the same slide. However, combination films must dry thoroughly (8–10 hours or overnight) before they can be satisfactorily stained. Slides for malaria should be stained the same day. Sometimes, the physician may need a diagnosis quickly. In these cases, make thin films and thick films on separate slides.

The thin films will dry quickly and can be stained as soon as they are dry. Use the rapid Field's stain method and examine while the Giemsa stain is working. Examine for malaria parasites. If parasites are seen, a diagnosis of malaria can be made and, using the Giemsa stain, the species can be identified.

If parasites are not seen in the thin film, stain the thick film using Field's stain. Examine the thick film for malaria parasites.

Sometimes, species cannot be readily identified in thick films and it may be necessary to send to another laboratory for an expert opinion.

Direct wet mounts of fresh whole blood (or centrifuged blood) are usually used for detection of microfilariae and trypanosomes. This only gives evidence of infection and stained films are necessary for confirmation of the species present.

In areas where malaria, trypanosomes, and/or microfilariae may all be present, both wet and stained films should be prepared and examined. If neither trypanosomes nor microfilariae occur in the region, only stained films need to be made for detection of plasmodia.

Stained blood films

Collection of specimens

Careful attention to technique is necessary in the collection of blood and the preparation of blood films. One should always be aware that a number of viral, bacterial, and parasitological diseases may be transmitted in blood.

Materials and reagents

Block, wooden with grooves (to hold slides)
Bottle, small (30–100 ml capacity) with a dropper-top and screw-cap, or small glass bottle (30–100 ml capacity) with a screw-cap and a separate dropper (with rubber bulb)
Cylinders, graduated, 10 ml, 25 ml, and 50 ml
Forceps
Gauze pads
Glass rod
Lancets, sterile
Pen or marker for labelling
Microscope slides
Register or record form
Staining dishes
Towels (paper), or sponge
Alcohol, 70% ethanol or isopropanol
Phosphate buffer (reagent no. 3)
Methanol in a dropping bottle.
Stain dilution (see "Staining blood films with Giemsa stain" pp. 42–43). The directions for preparing the stain dilution needed for each type of film are given with details of the technique.[1] Giemsa stain dilutions are good for only 8 hours. The dilution should be prepared at the time it is needed, not before. Diluted stains should be discarded at the end of the day.

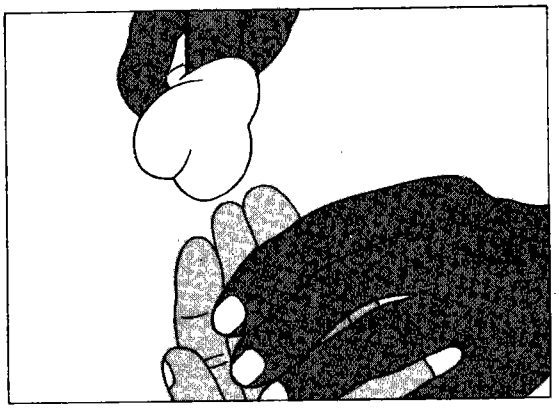
Preparation of a thick and thin blood film on the same slide

For routine malaria microscopy, a thin and a thick film are made on the same slide. The thin film is used as a label but, if well prepared, is also available for species confirmation. The thick film should be used for examination.

Technique

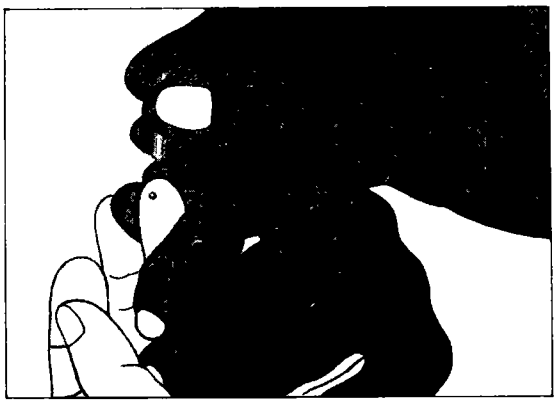
After patient information has been recorded in the appropriate form or register, the blood films are made as follows:

1. With the patient's left hand, palm upwards, select the third finger. (The big toe can be used with infants. The thumb should never be used for adults or children.) Use cotton wool lightly soaked in alcohol to clean the finger—using firm strokes to remove dirt and grease from the ball of the finger.
   With a clean cotton towel dry the finger, using firm strokes to stimulate blood circulation.

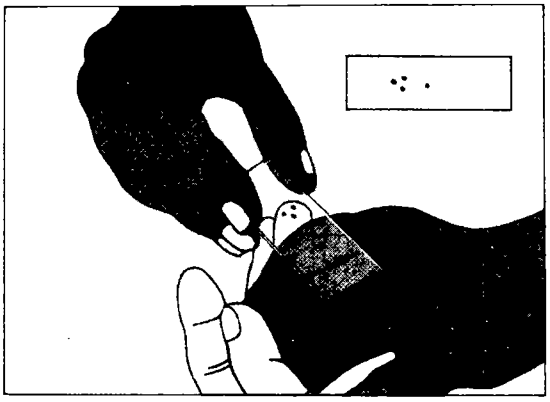


[1] Stock Giemsa stain is usually purchased as a prepared solution.

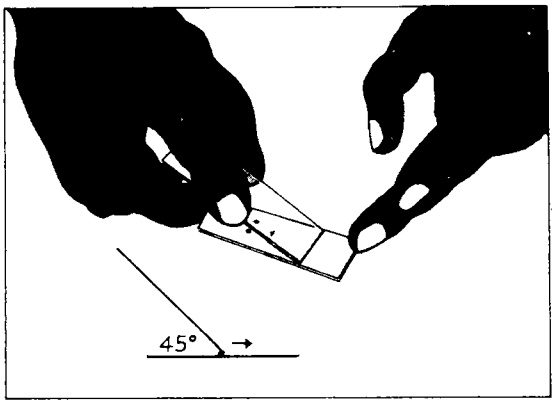
2. With a sterile lancet puncture the ball of the finger using a quick rolling action. By applying gentle pressure to the finger, express the first drop of blood and wipe it away with dry cotton wool. Make sure no strands of cotton remain on the finger.



3. Working quickly and handling clean slides only by the edges, collect the blood as follows:
   Apply gentle pressure to the finger and collect a single small drop of blood, about this size ●, on to the middle of the slide. This is for the thin film.
   Apply further pressure to express more blood and collect two or three larger drops, about this size ●, on to the slide about 1 cm from the drop intended for the thin film as illustrated.
   Wipe the remaining blood away from the finger with cotton wool.

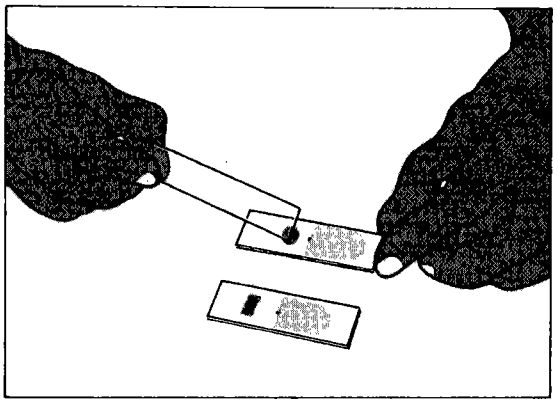


4. *Thin film.* Using another clean slide as a "spreader", and with the slide with the blood drops resting on a flat, firm surface, touch the small drop with the spreader and allow the blood to run along its edge. Firmly push the spreader along the

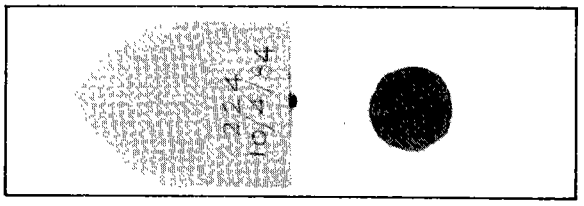


slide, away from the largest drops, keeping the spreader at an angle of 45°. Make sure the spreader is in even contact with the surface of the slide all the time the blood is being spread. The blood film should not extend to the edges of the slide in order to prevent infection of the investigator.

5. *Thick film.* Always handle slides by the edges, or by a corner, to make the thick film as follows:
   Using the corner of the spreader, quickly join the larger drops of blood and spread them to make an even, thick film. The blood should not be excessively stirred but can be spread in a circular or rectangular form with 3–6 movements.



6. Allow the thick film to dry in a flat, level position protected from flies, dust, and extreme heat. Label the dry film with a pen or marker pencil by writing across the thicker portion of the thin film the patient's name or number and date (as shown below). Do not use a ball pen to label the slide.



7. Wrap the dry slide in clean paper, and dispatch with the patient's record form to the laboratory as soon as possible.
8. The slide used for spreading the blood films must be disinfected and could then be used for the next patient, another clean slide from the pack being used as a spreader.

Staining blood films with Giemsa stain

Regular method for staining thick and thin blood films on the same slide

For optimum staining, the thick and thin films should be made on separate slides and different concentrations and times used for staining. This is often not possible and the thick and thin films are generally made on the same slide. When this is done, good-quality staining of the thick film is of primary importance. Best results are obtained if the blood films have dried overnight.

1. Fix the thin film by adding 3 drops of methanol, or by dipping it in a container of methanol for a few seconds. With prolonged fixation it may be difficult to demonstrate Schüffner's dots and Maurer's dots. To permit dehaemoglobinization, the thick film should not be fixed; therefore avoid exposure of the film to methanol or methanol vapour.

2. Place the slides back to back in a staining dish.
3. Prepare a 3% Giemsa solution in buffered, distilled or deionized water, pH 7.2, in sufficient quantity to fill the number of dishes being used. Mix the stain well.
4. Pour the stain gently into the dish, until the slides are totally covered.
5. Allow to stain for 30–45 minutes out of sunlight.
6. Pour clean water gently into the dish to float off the iridescent scum on the surface of the stain. Alternatively, gently immerse the whole dish in a vessel filled with clean water.
7. Gently pour off the remaining stain, and rinse again in clean water for a few seconds. Pour the water off.
8. Remove the slides one by one and place them in a slide rack to drain and dry, film side downwards, making sure that the film does not touch the slide rack.

Rapid method for staining thick and thin blood films on the same slide

This method is suitable for rapid staining of thick films in a busy laboratory when urgent results are required, but it uses much more stain.

1. Allow the thick film to dry thoroughly; if results are required urgently, drying may be hastened by fanning, or briefly exposing the slide to gentle heat such as that from the microscope lamp. Care should be taken to avoid overheating, otherwise the thick film will be heat-fixed.
2. Fix the thin film by gently dabbing with cotton wool dampened with methanol, or by dipping it in a container of methanol for a few seconds. To permit dehaemoglobinization, the thick film should not be fixed; therefore avoid exposure of the film to methanol or methanol vapour.
3. Prepare a 10% Giemsa solution in buffered, distilled or deionized water, pH 7.2; if a small quantity is being used, 3 drops of stain per ml of buffered water will give the correct concentration of Giemsa solution. One slide requires about 3 ml of made-up stain.
4. Gently pour the stain on the slide; a pipette can be used for this purpose. Alternatively, slides can be placed face down on a concave staining-plate and the stain introduced underneath the slide.
5. Stain for 5–10 minutes.
6. Gently flush the stain off the slide by adding drops of clean water; do not tip off the stain and then wash, as this will leave a deposit of scum over the smear.
7. Place the slide in the rack, film side downwards, to drain and dry, making sure that the film does not touch the slide rack.

Staining blood films with Field's stain

Staining with Field's stain allows rapid detection of malaria parasites (but it does not always stain Schüffner's dots).

Method for staining thick films

Materials

One staining dish filled with Field's stain A
One staining dish filled with Field's stain B
Two dishes filled with clean water.

Technique

1. Dip the slide into Field's stain A for 3 seconds.
2. Wash gently by dipping (once) into clean water.

3. Dip into Field's stain B for 3 seconds.
4. Wash gently as in step 2.
5. Place slide upright in a draining rack to air-dry.

Method for staining thin films

Reagents

Field's stain A.
Field's stain B diluted—1 part by volume of stain plus 4 volumes of buffered water (pH 7.2).
Buffered water, pH 7.2.

Technique

1. Fix film in methanol for one minute.
2. Wash off methanol with water.
3. Using a pipette, cover the film with diluted Field's stain B.
4. Immediately add an equal volume of Field's stain A and mix well by tilting the slide.
5. Leave to stain for one minute.
6. Wash off stain with clean water.
7. Place slide upright in a draining rack to air-dry.

Staining blood films with Delafield's haematoxylin stain for microfilariae

Materials and reagents

Five staining dishes
Ether–ethanol fixative (reagent no. 8)
Hydrochloric acid–water destain (reagent no. 15)
Delafield's haematoxylin stain (reagent no. 6). This stain may be purchased as a prepared solution.

Technique

1. Prepare thick films[1] of blood obtained by finger-prick. Allow to dry for 8–10 hours or overnight.
2. Prepare staining dishes as follows:

   dish 1—tap-water
   dish 2—ether–ethanol
   dish 3—Delafield's haematoxylin stain
   dish 4—hydrochloric acid–water (0.05% HCl)
   dish 5—tap-water.
3. Put the dry film in tap-water (dish 1) for 5–10 minutes. The red blood cells lyse and the film will be clear, or white, when lysis is complete.
4. Let film dry.
5. Put the film in ether–ethanol for 10 minutes (dish 2).
6. Let dry.
7. Put the film in Delafield's haematoxylin stain for 15 minutes (dish 3).
8. Destain with hydrochloric acid–water (dish 4). Dip slide into hydrochloric acid–water twice. Do this QUICKLY. The film will turn red.
9. *Immediately* put slide in tap-water (dish 5) to wash off acid. Put dish under a stream of running water until the film turns blue. Put a piece of rubber tubing

[1] If films of sediment from concentrated blood are used, start with step 5.

on the tap long enough to reach the top of the dish and let the water run gently; if the flow of water is too strong, the film will be washed off.
If running water cannot be used, change the water in the dish several times until the film turns blue. Put your finger over the top of the dish to keep the slide from falling out, pour the water out, and refill the dish.

10. Let the film dry and examine with the ×10 and oil-immersion objectives for microfilariae.

Examination

Thick films

1. Focus on the film with the ×10 objective and search for microfilariae. They are easily detected with the ×10 objective.
2. If microfilariae are present, switch to the oil-immersion objective and identify the species. Also look for malaria parasites with the oil immersion objective. At least 100 fields should be examined.

Microscopy of thick films should reveal the following features:

- The background should be clean, free from debris, with a pale mottled-grey colour derived from the lysed erythrocytes.
- Leukocyte nuclei are stained a deep, rich purple.
- Malaria parasites are well defined with deep-red chromatin and pale purplish-blue cytoplasm. In *Plasmodium vivax* and *P. ovale* infections, the presence of Schüffner's stippling in the "ghost" of the host erythrocyte can sometimes be seen at the edge of the film.

Thin films

1. Focus with the ×10 objective on the thin terminal end of the film where the red blood cells are in one layer.
2. Put immersion oil on the slide and switch to the oil-immersion objective.

When examining for malaria parasites and trypanosomes, at least 200 fields should be examined. Microscopy should reveal the following features:

- The background should be clean and free from debris; erythrocytes are stained a pale greyish-pink.
- Neutrophil leukocytes have deep purple nuclei and well defined granules.
- The chromatin of malaria parasites is stained a deep purplish-red and cytoplasm a clear purplish-blue.
- Schüffner's dots should be seen as stippling in erythrocytes containing *P. vivax* or *P. ovale*, and Maurer's dots show as stippling in erythrocytes containing the larger ring forms of *P. falciparum*.

Quality control for blood examination

To ensure reliable examination for blood parasites, the laboratory worker must pay attention to the following points:

1. *Equipment must be clean.* Slides must be free from dust, grease, soap, fingerprints, and debris, otherwise the blood may not adhere to the slide or may not stain properly. The lancet used for puncturing the finger (or ear or toe) must be sterile to avoid transferring disease from one patient to another. The puncture should be deep enough to provide sufficient blood for the films to be made. Gauze, rather than cotton, should be used to clean the finger. (Cotton may leave fibres

that will get into the blood film.) The finger should be cleaned thoroughly before puncturing to remove dirt, mould, or other contaminants. Blood obtained by venepuncture can be used if the films are made immediately after the blood is collected. Anticoagulants will affect adhesion of the blood to the slide and staining.

2. *Films must be of the correct density.* The thin film should have a terminal edge where the blood cells are in a single layer so the morphology of the red blood cells can be seen. If the film is too thick the red blood cells may be "piled-up" in layers and the morphology may not be clear. If it is too thin, the blood cells may be grossly distorted and thin layers of blood often do not stain well. In addition, in thin areas the parasites are usually distorted.
   It should just be possible to read small print through a thick film. If the film is too thick, the blood may flake or peel off during staining and portions of the film be lost. If the film is too thin, the advantage of the thick film containing a larger sample may be lost.

3. *Films must be allowed to dry in a horizontal position and for the correct time* to ensure good results. If thick films are tilted or slanted the blood may run to one edge of the slide producing an uneven smear. The thick area may flake off. If films do not dry thoroughly, they will not stain properly.
   While drying, films must be protected from dust, mould, or other debris that may fall on to the blood and cause problems in diagnosis. They must also be protected from flies or other insects that may damage the film.
   Thin films must be fixed with methanol before staining to prevent the red blood cells from lysing. The methanol must be absolute and moisture-free. Otherwise the blood cells will be damaged, and the staining will be poor. **Never fix thick films**. Be careful not to let methanol get on the thick film.

4. The stain dilutions and the buffered water used for staining must be accurately prepared and the stock stain must be of good quality. The bottle of stock Giemsa stain must be kept tightly closed and out of sunlight. If moisture gets into the stain it will be ruined. It is recommended that a portion of the stock stain be poured into a clean, dry bottle for use. This will protect the remainder of the stock from accidental contamination with moisture or debris. **Never put a wet pipette into the bottle of stock stain**.
   Diluted Giemsa stain is good for about 8 hours. Therefore, stain dilutions must be prepared fresh on the day they are required.
   The pH of the stain is a very important factor in obtaining well stained films. The pH is controlled by using water buffered to pH 7.2 which gives the best staining results. Buffered water can be kept for some time if the bottle is kept tightly closed. However, the pH should be checked occasionally to be sure it is still neutral.

5. *The staining procedure should be followed very carefully.* Be sure to use the procedure developed for the type of film you are staining. Wash the stain exactly as indicated in the procedure. If thin films are washed too much, the colour will wash out. If the thick films are not washed enough, a lot of cellular and stain residue may remain on the slide causing problems in examination. Be sure combination thick and thin films are dried in a vertical position with the thick film at the bottom. Otherwise, water will run on to the thin film and wash out the colour.

Special techniques for plasmodia

Identification of malaria parasites

Three components of malaria parasites may be seen. These are blue-staining cytoplasm, red or purple chromatin, and brown or black pigment granules or rods.

Except for the early (young) ring stages, you should be able to see all three components. (Early rings usually do not have pigment.) Observation of the three components is important in order to distinguish malaria parasites from host cells, like white blood cells, and artefacts that may appear on the slide during preparation.

In thin films, look at the appearance of the parasite and the appearance of the red blood cells containing the parasites. Observe the following:

1. The appearance of the red blood cell containing the parasite.
   - *Size.* Is the parasitized cell the same size as the blood cells without parasites (that is, normal size) or is it bigger (enlarged)?
   - *Stippling.* Is the red blood cell filled with pink- or red-staining dots? These are Schüffner's dots and occur only in *P. vivax* and *P. ovale* infections. (They will *not* be present in blood cells without parasites.) Cells containing late stage trophozoites of *P. falciparum* often include irregular red-mauve dots. These are Maurer's dots.
2. The appearance of the parasite.
   - Are the growing trophozoite stages irregular in outline?
   - Are they regular or smooth?
   - What colour is the pigment in the older trophozoites, schizonts, and gametocytes?
   - How many merozoites are there (if any) in the mature schizont?
   - What is the shape of the gametocytes, if seen?
   - What stages (rings, growing trophozoites, schizonts, gametocytes) are present?

Let the films dry completely before examining. If you are in an area where filariasis occurs, you should scan the thick film with the ×10 objective for microfilariae (see pp. 57–58). Use of the oil-immersion objective is necessary to observe the morphology of the blood parasites.

Examine the centre portion of the thick film first. Organisms are more likely to be detected in this thicker area. If the morphology is not distinct, go to the thinner, outer edges to find parasites with a more characteristic appearance.

In thick films, the red blood cells are lysed and no longer present. The blood layer is much thicker than in a thin film and parasites may be located at different levels. Focus up and down carefully to see the organisms. In thick films, the parasites often appear smaller than in thin films, but the same parasite characteristics are used to separate species. Sometimes, parasites in the thinner, outer edges of the thick film are more like those in thin films than are those in the centre. Occasionally, the outlines of the red blood cells can be seen in the thin edges.

Specific characteristics used to identify species of malaria in thick and thin films are presented and problems in diagnosis discussed in Section 2, pp. 80–88.

Examination of chloroquine resistance in falciparum malaria

A drug-resistant parasite is defined as a parasite that will survive and multiply in a patient despite treatment with the drug in a dosage that normally cures the infection. The commonly used standard field test requires the examination of a thick blood smear daily for the first 7 days of treatment. If asexual forms have disappeared in the peripheral blood on day 7, the examination has to be extended to 28 days to exclude a case of RI resistance with late recrudescence. It is simpler to examine a thick blood film on day 2 in severely ill patients, or on day 4 in less sick

patients. If the parasite count on either of these days is 20–25% above the level before treatment, it indicates a resistant strain and a need for a change in treatment.

Technique—the standard field test

1. Make a total white blood cell count and calculate the number of white blood cells per µl of blood (WBC).
2. Make a thick blood film (pretreatment parasite level) and count the number of parasites (*P*) and leukocytes until 300 leukocytes have been enumerated. The number of parasites per µl of blood is then WBC × *P*/300.
3. Give the patient 10 mg of chloroquine (base) per kg of body weight orally once a day for the first 2 days and 5 mg/kg of body weight on the third day (a total of 25 mg of chloroquine base per kg of body weight during the 3 days).
4. Make a thick blood film each day for the first 7 days, and on days 14 and 28, if no sign of remission has appeared during the first 1–2 weeks.

Interpretation of the standard field test

1. If no asexual forms are found by day 6 and no parasites (ring forms and gametocytes) are present on day 7, the infection may be either sensitive (S) or resistant at the RI level. To differentiate between these two, the observation should be extended to 28 days. If ring forms do not reappear by day 28, the strain is sensitive; if the ring forms reappear, the strain is resistant at the RI level.
2. If ring forms disappear for at least 2 consecutive days, but return and are present on day 7, the parasites are resistant at the RI level.
3. If ring forms do not disappear, but are reduced to 25% or less of the pretreatment level during the first 48 hours of treatment, the parasites are resistant at the RII level.
4. If the ring forms are reduced by less than 75% during the first 48 hours, if they remain at the same level, or if they continue to increase, the parasites are resistant to the standard dose of the drug at the RIII level.

Special techniques for Trypanosoma[1]

Trypanosomes can be identified in:

- blood films
- cerebrospinal fluid (CSF)
- lymph node aspirates.

Detection of trypanosomes in blood

Of the *Trypanosoma* species occurring in Africa, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cannot be distinguished morphologically. They may be identified in either thick or thin blood films. However, in the thick film, they may be distorted and difficult to differentiate from cellular debris. *Trypanosoma cruzi*, found in the Americas, is badly distorted in thick films and is more readily identified in the thicker areas of the thin film.

Like plasmodia, the cytoplasm of trypanosomes stains blue. The nucleus and kinetoplast stain red or purple. Look for an elongated organism with a prominent nucleus located near the centre of the body and a smaller dot, the kinetoplast, located near one end. The flagellum orginates from the posterior part of the trypanosome close to the kinetoplast. The flagellum is attached to the cell wall except at the anterior tip, where it terminates with a free end. Since the flagellum is

[1] Adapted from the *Trypanosomiasis control manual*, unpublished WHO document, 1983.

constantly moving it pulls the cell wall into irregular extensions, this feature being known as the undulating membrane. The trypanosome may be undulated (with 2 or 3 curves) or it may be in a C or U shape. The shape, the position of the nucleus, and the size and location of the kinetoplast are features used in identifying species. More detailed characteristics for species identification are described in Section 2, pp. 88–89.

The wet blood film

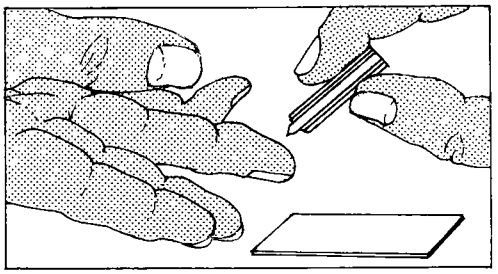
This method is the easiest and least expensive way to show the presence of parasites in blood, but it is the least sensitive test.

Materials

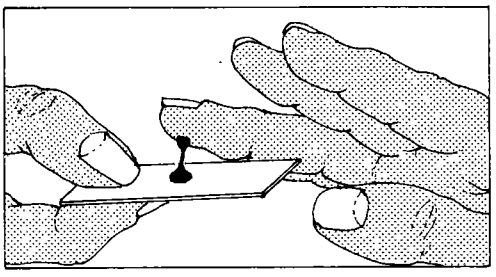
Blood lancet
Cotton wool swabs
Coverslip
Microscope slide
Saline solution (reagent no. 24)
Ethanol.

Method

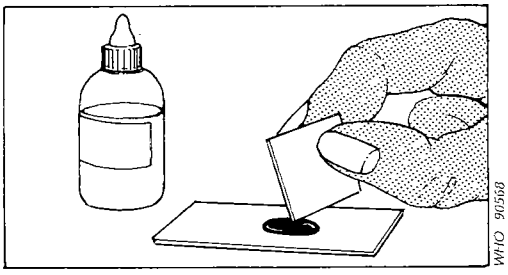
1. Select the third finger from the thumb. Clean the finger with a cotton wool swab lightly soaked in ethanol. Dry well. Prick with the lancet.



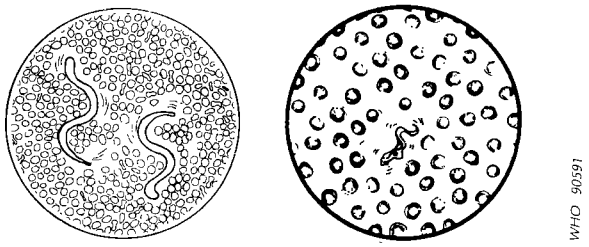
2. Collect the *first* drop of blood that appears directly on to the middle of the slide.



3. Add an equal drop of saline. Mix the blood and saline solution using the corner of a coverslip. Cover the preparation with the coverslip.



4. Prepare two thick films on another slide using 2 more drops of blood. Examine the fresh smear systematically under the microscope (×10 objective with reduced condenser aperture). The first sign of the presence of live trypanosomes or microfilariae is rapid movement among the red cells.



(The illustration on the right shows a view (×40) of a trypanosome and red blood cells, that on the left, microfilariae (×10).)

5. Scan the whole preparation systematically. The trypanosomes are refractile and difficult to see. They are easier to see with slightly reduced lighting.

The thick blood film

This is more sensitive than the wet blood film because more blood is observed per microscope field.

1. Place a drop of blood on to a clean dry microscope slide.
2. Prepare and stain a thick blood film in the same way as for malaria parasites (see pp. 40–43).
3. Examine the whole film using the oil-immersion objective.
4. Trypanosomes are seen among the mass of lysed red blood cells and can be recognized by their typical shape and light bluish colour, with a dark nucleus and kinetoplast.

The microhaematocrit method

Materials and reagents

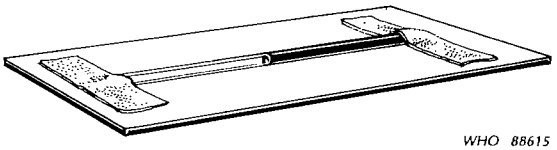
Adhesive tape
Capillary tube
Microhaematocrit centrifuge
Plasticine
Sodium citrate solution (reagent no. 26).

Only a small quantity of blood is needed, so blood obtained by finger-prick can be used if venepuncture cannot be performed. However, a special centrifuge that will accommodate microhaematocrit tubes is needed.

Method

1. Prick the finger and obtain 2 drops of blood on a slide. Add 1 drop of 2% sodium citrate solution and mix. Fill the capillary tube 3/4 full. If venous blood has been collected, fill the capillary tube 3/4 full with blood from the bottle or flask.
2. Seal the open end of the tube by heating or with plasticine.
3. Centrifuge in a microhaematocrit centrifuge for 2 minutes for microfilariae or 4 minutes for trypanosomes.

4. Lay the capillary tube on a microscope slide and secure the ends with adhesive tape to keep the tube from rolling around.



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5. With the ×10 objective, examine the area between the red blood cells and the plasma. Motile microfilariae or trypanosomes may be seen. Switch to the high-power, dry lens for better observation.

The sterile mini-anion exchange centrifugation technique (m-AECT)

This method is the most sensitive test so far developed for the detection of trypanosomes in human blood. Prepared columns in kits with sterile reagents require no refrigeration for storage. Once open, the column and the buffer must be used immediately. Care should be taken to dispose of every item in disinfectant solution (e.g., water with 2% bleach).

Materials and reagents

The kit must include the materials and reagents required for one test, which are:

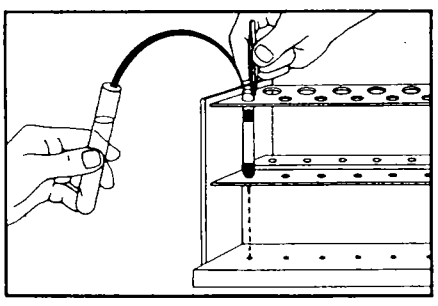
Sterile column in a tube container kept in phosphate-buffered saline
Tube with pre-weighed glucose or glucose solution
Heparinized capillary tube (Caraway tube) for blood collection
Lancet
Reservoir and centrifuge tube
Plastic pipette to handle the buffer.

The additional equipment required is:

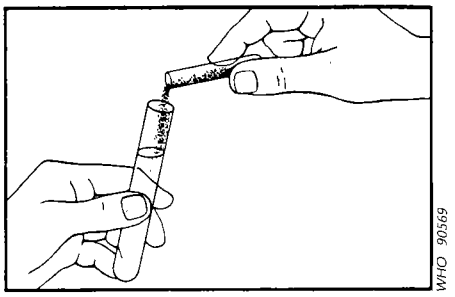
Manual centrifuge
Column rack
Good microscope, allowing magnification up to ×150
Standard microscope glass slides
Coverslips
Masking tape
Modelling clay for the fabrication of the viewing chamber.

Technique

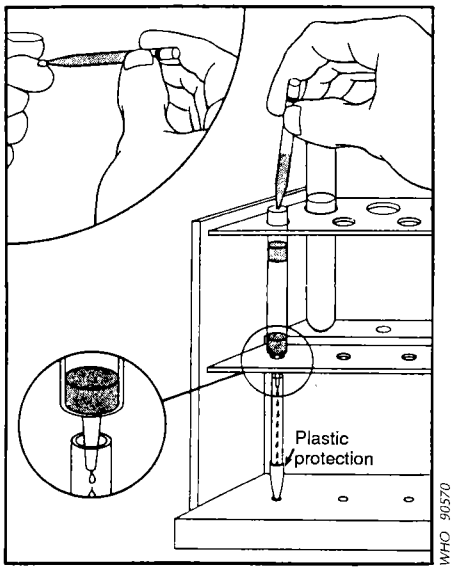
1. Remove the column from its tube container and place it in the front row of the rack and let it drain.



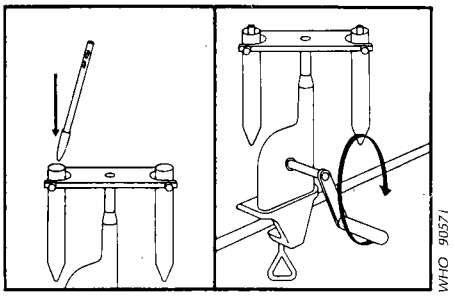
2. Add the pre-weighed glucose or glucose solution to the remaining buffer in the container and mix thoroughly. Store the tube behind the column in the back row of the rack. With the pipette, add a small amount of the buffered saline–glucose solution to the column and let the column drain out. Repeat this step. You will use the rest of the buffer later.



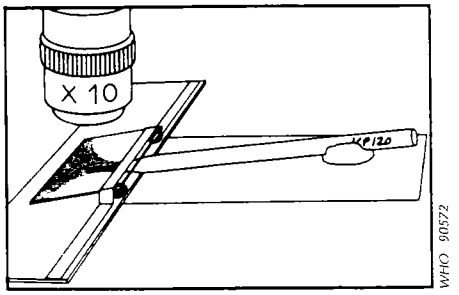
3. Following the finger-prick, fill the capillary tube (Caraway tube) with blood up to the red mark, and empty it on to the column immediately. Let the blood soak in the upper filter until the column stops running. Add a few drops of buffer with the pipette and immediately place the reservoir on the column and fill it up with buffer. The column will start draining drop by drop. Count 6–7 drops and place the collecting tube under the column, making sure that no air lock forms. Only the very tip of the column should enter the centrifuge tube (the column height is adjustable)—this avoids the formation of an air lock and any loss of eluate out of the centrifuge tube. Top up the reservoir with buffer and let the column run slowly by itself.



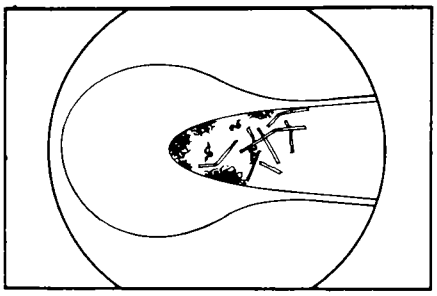
4. When the centrifuge tube is full, remove it and place it in the swinging bucket of the manual centrifuge. Balance the centrifuge by placing an equivalent weight in the opposite bucket. Centrifuge for 5 minutes.



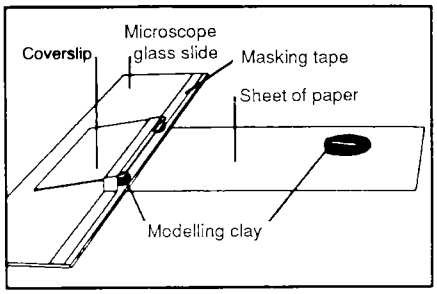
5. Take the collecting tube from the centrifuge and remove the plastic protection. Place the tip of the centrifuge tube in a viewing chamber and add water between the slide and the coverslip. Examine the very tip of the m-AECT centrifuge tube under the microscope using the ×10 objective.



6. You will easily see trypanosomes as small wiggling organisms at the very tip of the tube. Occasionally cellulose particles may have passed through the bottom filter of the column and may complicate the examination. In that case rotate the tube.



7. This is a viewing chamber:



WARNINGS

REMEMBER TO IDENTIFY the collecting tube with a label or a special felt pen.

REMEMBER TO BALANCE the centrifuge rotor if an uneven number of tubes are centrifuged.

REMEMBER TO DISCARD all contaminated items in a bucket with disinfectant solution.

Detection of trypanosomes in lymph node aspirates

The standard method of diagnosis of sleeping sickness, in the early stage, is to search for trypanosomes in aspirates from enlarged cervical lymph nodes. The

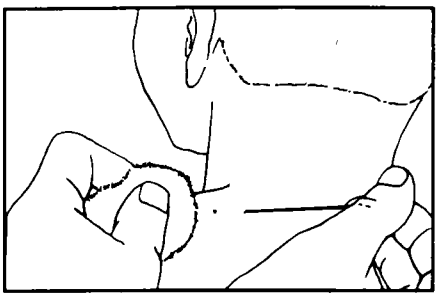
sample is examined under a coverslip and the trypanosomes are identified by their movements.

Materials

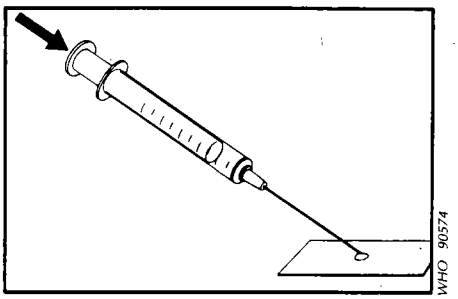
Cotton swabs
Cotton wool
Coverslips
Microscope slides
Needle (for subcutaneous injection), 25 gauge, 0.5 × 16 mm
Syringe, 5 or 10 ml
70% ethanol.

Technique

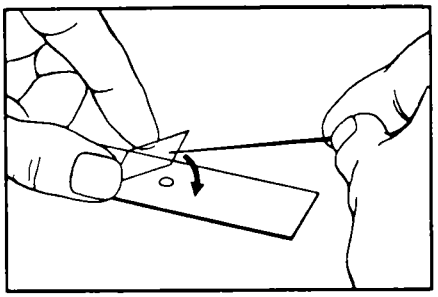
1. Prepare the syringe; pull the piston as far back as possible.
2. Wash your hands with soap.
3. Ask the person to sit down. Disinfect the chosen site on the neck with 70% ethanol.
4. With your left hand, take the gland between the thumb and index finger and make it stand out. Hold your hand steady.
5. Holding the needle between your thumb and finger, introduce it at right angles into the centre of the gland. First pierce the skin, then penetrate the centre of the gland. Make sure that you avoid the jugular veins and arteries.



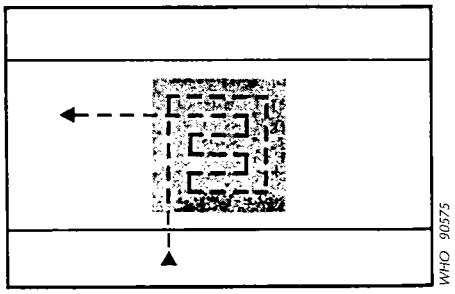
6. With your left hand, gently knead the gland. With your right hand, revolve the needle in both directions.
7. The glandular fluid will ooze into the needle. The operation should last about one minute.
8. Withdraw the needle in one rapid movement, holding your index finger over the hub. Apply a swab dipped in disinfectant to the point of entry. Never apply the disinfectant swab before you withdraw the needle, as some disinfectant may get on to the tip of the needle and make the trypanosomes non-motile.
9. Attach the needle to the syringe, with the piston pulled back. Push the piston gently half way down the barrel to discharge the glandular fluid in the needle on to the slide.



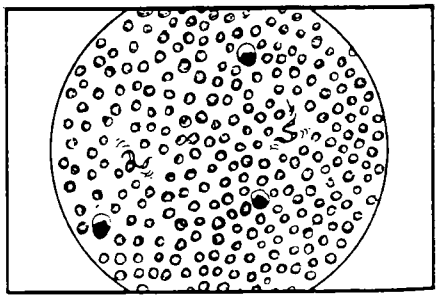
10. Cover the preparation with a coverslip. Examine at once under the microscope at a magnification of approximately ×400, using the ×40 objective.



11. Wait until the convection currents stop. It is impossible to see the movement of trypanosomes among moving cells. Begin by examining the periphery of the preparation, near the edges of the coverslip, as the trypanosomes tend to make their way to the edges. Then examine the rest of the preparation.



12. The preparation will contain red blood cells and leukocytes. Trypanosomes are about 20 µm long and are often hidden by the cellular elements, which are disturbed by the flagella as they move. Any movement is suspicious. Trypanosomes occasionally disappear, hiding under masses of cells. Look very carefully!



Detection of trypanosomes in cerebrospinal fluid (CSF)

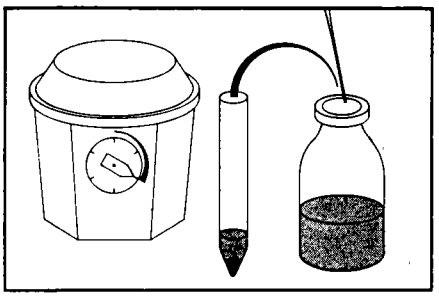
When the trypanosomes have passed the blood–brain barrier and invade the central nervous system, the patient is in the second stage of the disease. The only way to diagnose trypanosomes in the nervous system is to examine the cerebrospinal fluid. Three tests are commonly done:

- white cell counts in the CSF;
- measurement of protein concentration in CSF;
- detection of trypanosomes in CSF.

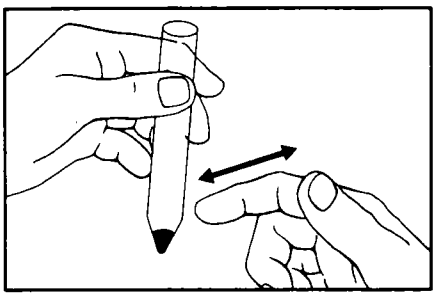
Cells and trypanosomes are rapidly lysed. Therefore the cell count must be carried out soon after collection of the CSF. Trypanosomes are examined after concentration in the CSF by single or double centrifugation. The more convenient technique of single centrifugation will be described. The number of parasites found in the CSF of patients in the advanced stage of disease is highly variable.

Single centrifugation technique

1. After making a white cell count, centrifuge the CSF collected by lumbar puncture for 10 minutes at 900 *g*. Decant the supernatant and save it for other tests.

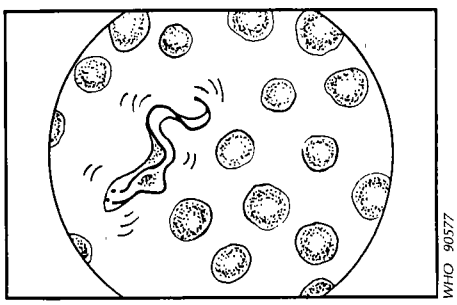


2. Resuspend the sediment in the small amount of CSF left at the bottom of the tube by tapping it with the finger.



3. Place one drop of this resuspended CSF pellet on a clean dry microscope slide. Cover the drop with a coverslip immediately. Wait a few minutes until convection stops.
4. Place the preparation on the stage of the microscope and examine with the ×10 objective first. Use higher magnification to confirm the presence of trypanosomes. Scan the whole preparation starting with the edges since trypanosomes are often found at the very edge of the preparation.

If motile trypanosomes are found in the CSF it means that the patient has reached the later stage of the disease and that the central nervous system is involved.



Indirect detection

Inoculation of biological material from man, host animals, or vectors into susceptible animals has been used to detect trypanosomes. This method is more sensitive for *T. b. rhodesiense* than for *T. b. gambiense.*

Several culture systems have been developed that can support trypanosome growth, but these *in vitro* systems are not commonly used for primary isolation of trypanosomes from patients with sleeping sickness.

Special techniques for microfilariae

Collection of blood for microfilariae

In cases of suspected filariasis, the time of day is important in taking blood samples for examination. Some species have a "periodicity"—that is, microfilariae are present in the blood only at certain times of the day and to detect their presence the blood must be collected at the appropriate time (see Table 2).

Table 2. Blood collection times for suspected filariasis patients

Species	When to take a specimen[a]	Part of world
Wuchereria bancrofti	At night (between 22 h and 24 h)	Tropical Africa, Asia, Central and South America, Indian Ocean
Wuchereria bancrofti (var. pacifica)	Any time	Pacific Ocean
Brugia malayi	Mainly at night (between 22 h and 24 h)	Southern China, Southern India, South-East Asia
Loa loa	During the day (between 10 h and 12 h)	West and Central Africa
Doubtfully pathogenic filariae:		
– *Mansonella perstans*	Any time	Tropical Africa
– *Mansonella ozzardi*	Any time	Tropical Africa

[a] These periods are not invariable.

Detection of microfilariae in peripheral blood

Three microscopic methods are commonly used for detection of microfilariae in peripheral blood:

- the thick blood film
- capillary blood examination
- examination of haemolysed venous blood.

Microfilariae must be stained to confirm the species present.

The thick blood film

Prepare and stain as described on pp. 40–43.

Capillary blood examination

Mix a fresh smear of capillary blood from the finger with saline, place between a slide and coverslip, and examine for motile microfilariae under the microscope. The microfilariae can also be concentrated using venous blood. The examination should be carried out as described for trypanosomes (see pp. 49–50) at the appropriate time of day.

Examination of haemolysed venous blood

Materials and reagents

Bottle, 10 ml
Centrifuge
Centrifuge tubes, conical, 15 ml
Microscope slides
Needles for venepuncture
Rack for centrifuge tubes
Syringe, 5 ml
Anticoagulant—2% sodium citrate solution (reagent no. 26)
2% formalin solution (reagent no. 10)
Giemsa stain (reagent no. 11)
Ether
Ethanol.

Method

1. Add 1 ml of venous blood to 9 ml of 2% formalin; wait for 5 minutes for the red cells to haemolyse and then centrifuge at high speed.
2. Pour off the supernatant fluid. Tap the tube to mix the deposit.
3. Place 1 drop of the deposit on a slide. Spread the drop to form a thin smear. Leave to dry in the air. Fix the smear using a mixture of equal parts of ether and ethanol. Leave to dry for 2 minutes. Stain immediately with Giemsa stain. The microfilariae stain well.

Other filtration techniques have been developed for the isolation of microfilariae, but as these techniques are restricted to specialized laboratories, a description of the methods would be beyond the scope of this manual. They are described by Melvin & Brooke.[1]

Special techniques for Leishmania

Leishmaniasis is caused by infection with flagellate protozoan parasites of the genus *Leishmania*. Depending on the species concerned, the disease may take a cutaneous, mucocutaneous, or visceral form. Promastigote forms of the parasite are transmitted by the bite of infected sandflies. They are taken up by mononuclear phagocytes in the skin or viscera, where they develop intracellularly into amastigote forms. Although amastigotes can occasionally be detected in mononuclear leukocytes in peripheral blood, microscopic examination of bone marrow, lymph node, and splenic aspirates is more sensitive. For the examination, blood leukocytes can be concentrated in a buffy coat layer by the microhaematocrit method as described for the detection of trypanosomes (see p. 50).

On the basis of clinical signs and symptoms, it is impossible to differentiate visceral leishmaniasis from other causes of febrile splenomegaly. Parasites may be

[1] MELVIN, D. M. & BROOKE, M. M., ed. *Laboratory procedures for the diagnosis of intestinal parasites.* Atlanta, US Department of Health and Human Services, 1982 (HHS Publication No. (CDC) 82–8282).

demonstrated in aspirates of spleen (98% positivity), bone marrow (54–86%), or enlarged lymph nodes (64%). Splenic aspiration can be a high-risk technique and there is no consensus on its use, although it is preferred by some because of its superior sensitivity. It requires no special equipment, is less painful than bone-marrow aspiration, and is relatively easy to perform in experienced hands and when proper precautions are taken. In acute visceral leishmaniasis, when the spleen is small and soft or impalpable, bone-marrow or lymph-node aspiration is recommended.

Splenic aspiration should be performed in patients only after a measurement of prothrombin time and platelet count. It should not be done if the prothrombin time is more than 5 seconds longer than the control, or if the platelet count is below 40×10^9 per litre (40 000/mm^3).

The two important precautions to take, if the procedure is to be safe, are:

- be quick, so that the needle remains within the spleen less than 1 second; and
- be sure that the entry and exit axes of the aspirating needle are identical, to avoid tearing the splenic capsule.

Materials

Cotton swabs
Microscope slides
Needle, 21 gauge (32 × 0.8 mm)
Pen or marker for labelling
Syringe, 5 ml
Tubes for culture media
Novy Nicolle-McNeal (NNN) culture medium[1]
Schneider's enriched medium.[1]

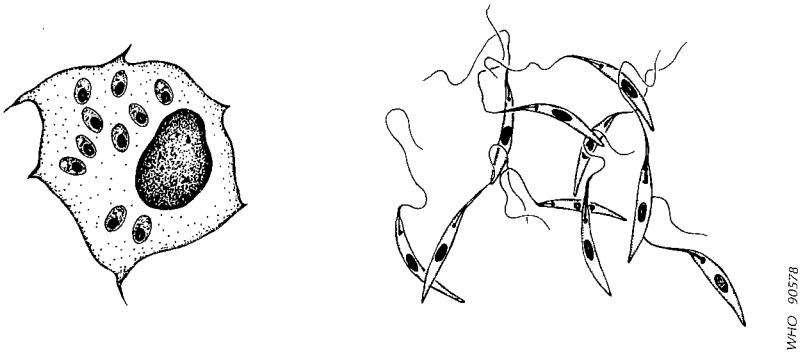
Technique

1. Clean 3 glass slides and label with patient's name, date, and "splenic aspirate". Have culture media ready (one tube each of NNN and Schneider's) and labelled in the same way as the slides. Allow the culture media to reach room temperature. Attach a 21 gauge (32 × 0.8 mm) needle to a 5 ml syringe. Place all items on a table at the bedside.
2. Obtain the patient's informed consent to the procedure. Palpate the spleen and outline its margins on the patient's abdomen with a pen. For safety reasons, the spleen should be palpable at least 3 cm below the costal margin on expiration. Use an alcohol swab to clean the skin at the site of aspiration and allow the alcohol to dry.
3. With the 21 gauge (0.8 mm) needle attached to the 5 ml syringe, just penetrate the skin, midway between the edges of the spleen, 2–4 cm below the costal margin. Aim the needle cranially at an angle of 45° to the abdominal wall. The actual aspiration is done as follows: pull the syringe plunger back to approximately the 1 ml mark to apply suction, and with a quick in and out movement push the needle into the spleen to the full needle depth and then withdraw it completely, maintaining suction throughout.
4. In young restless children, arrange for two assistants to hold the child (arms folded across chest, with shirt raised to obstruct line of vision, and pelvis held firmly). Carry out the aspiration as a single-stage procedure, using the same landmarks, angles and suction as in step 3, all in one quick motion. The insertion should be timed with the patient's breathing so that the diaphragm is not moving; this should be during fixed expiration if the child is crying. Only a

[1] See Annex 3 for the preparation of culture media.

minute amount of splenic material is obtained but this is sufficient for culture and smear.

5. Slowly pull the plunger back to the 2–3 ml mark and, using sterile techniques, insert the needle into a tube of culture medium and briskly push the plunger to expel the contents of the needle on to the side walls of the tube. If necessary, repeat once or twice until splenic material is visible in the tube. Replace the cap on the tube and invert to wash splenic material off the side of the tube. Repeat the expulsion procedure for the second tube of culture medium. Sterile techniques are essential throughout.
6. Expel additional material gently on to glass slides holding the needle tip on the surface of the slide. Immediately spread evenly with the needle using a linear (not circular) motion. The smear should not be quite as thick as a thick blood film for malaria. Remove the needle and use the end of it to obtain additional material from the tip of the syringe and spread on slides. Further material may be found on the end of the plunger and dabbed directly on to a slide and spread. Allow the slides to dry.
7. Write the time of aspiration on the patient's chart and the instructions: "Record pulse and blood pressure every 30 min for 4 hours, then hourly for 6 hours. The patient must remain in bed for 12 hours." See that the patient understands the instructions. Enter the procedure in the notes and sign.
8. Take the slides and media to the laboratory. Cultures are incubated at 25 °C and examined regularly for up to two weeks. Slides are stained with Giemsa or Field's stain and examined under oil-immersion (see illustration below). The pH of the buffered saline used in the Giemsa stain should be 6.8 for *Leishmania* (not 7.2 as used for malaria). The parasite density is graded according to Table 3.



WHO 90578

A. *Leishmania* amastigotes B. *Leishmania* promastigotes

For visceral leishmaniasis, serodiagnostic methods provide useful support for the diagnosis. Serodiagnosis is of less value for mucocutaneous leishmaniasis and of no value for cutaneous leishmaniasis.

Table 3. Grading of *Leishmania* parasites

Grade	Average parasite density
6+	>100 parasites/field[a]
5+	10–100 parasites/field
4+	1–10 parasites/field
3+	1–10 parasites/10 fields
2+	1–10 parasites/100 fields
1+	1–10 parasites/1000 fields
0	0 parasites/1000 fields

[a] Using ×10 eyepiece and ×100 oil-immersion lens.

A detailed description of the numerous immunological techniques available is beyond the scope of this manual. The enzyme-linked immunosorbent assay (ELISA) and the indirect fluorescent antibody test (IFAT) are the most suitable. The test may be carried out on serum or on a measured volume of blood collected by finger-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted in the laboratory and tested at a single dilution that has previously been determined as giving an acceptable sensitivity and specificity in that area. Cross-reactions may occur with *Trypanosoma cruzi* infections. These can usually be eliminated by dilution, or identified by testing in parallel against *T. cruzi* antigens.

Skin specimens

Small pieces of skin are examined for onchocerciasis (river blindness), a filarial infection of human beings in Africa, Central and South America, and parts of the eastern Mediterranean region. The worms live in nodules in the subcutaneous tissues. Corneoscleral punches are most commonly used to take bloodless skin snips.

Materials and reagents

Coverslips
Gauze pads
Microscope slides, or microtitration plates
Needle, 22 gauge
Scalpel, razor blade, or 2 mm punch[1]
Ethanol, 95%
Saline solution, isotonic (reagent no. 24) or distilled water.

Collection of specimens

Patients with nodules

Look for nodules:

- on the chest (over the ribs),
- on the hips,
- on the legs (calves),
- on the back (shoulder-blades).

The nodules are round and hard, 1–5 cm in diameter; when pushed with the fingertips they slide about under the skin. Take the specimen from the skin in the centre of the nodule.

Patients without nodules

Take the skin specimen from:

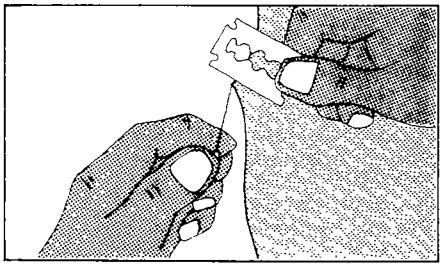
- the top of the buttocks (the upper outer part where intramuscular injections are given),
- the calf (upper outer part),
- the back (centre of shoulder-blade).

It is recommended that 6 specimens (2 from buttocks, 2 from calves, 2 from shoulder-blades) be examined.

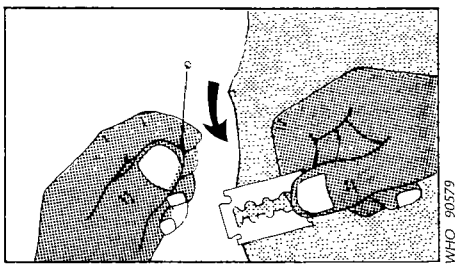
1. If a corneoscleral punch is not available, use a sterile, disposable scalpel, and needles or punches. If disposable equipment is not available, the apparatus must be sterilized before use on each patient. This can be done by placing the scalpel, razor blade, punch or needle in a little alcohol and setting fire to it.
2. Disinfect the skin area with a gauze pad dipped in alcohol.

[1] The punch is supplied by: Karl Storz, Tuttlingen, Germany.

3. Push the point of the needle 2–3 mm into the skin and lift up.



4. Place the cutting edge of the scalpel or razor blade on the stretched skin above the point of the needle. Cut with a quick stroke the piece of skin pulled up by the point of the needle, as close to the needle as possible. The specimen should be about 2–3 mm wide. It should remain attached to the tip of the needle.



The specimen should not be blood-stained. The biopsy must be bloodless to avoid possible contamination with blood parasites.

Examination of specimens

1. Put a drop of distilled water or saline solution on a microscope slide.
2. Put the small piece of skin in the drop and place a coverslip over it. *Do not press on the skin or coverslip.* If there is not enough saline to cover the skin, add some at the edge of the coverslip, so that it will run under.
3. Let the mount stand for 30 minutes, then examine under the microscope with the ×10 objective. If microfilariae are present, they can usually be seen wriggling around in the saline. If they are not present, skin snips should be allowed to stand in saline solution for 4 hours at room temperature, then re-examined.

Quantitative examination can be achieved as follows:

1. Weigh a skin specimen with a balance (1–10 mg).
2. Transfer the specimen into one hole of a microtitration plate.
3. Add 0.1 ml of saline solution.
4. Cover the plate to avoid evaporation of water and leave to incubate at ambient temperature for 24 hours.
5. Count the number of microfilariae in the saline by microscopy using the ×10 objective, and express the results per wet weight of the specimen.

Collagenase digestion of the skin over more than 24 hours may increase the sensitivity of examination in specimens from patients with low parasite loads. The digestion can also be performed on ethanol-fixed material stored at ambient temperature.

Section 2

Identification of parasite species

Intestinal parasites

Helminths

There are three groups of medically important helminths—nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes). The usual diagnostic stages are eggs and larvae. Less frequently, adult worms like *Ascaris* and *Enterobius* may be seen and segments or proglottids are used for diagnosing certain of the tapeworms. However, for the majority of the worm infections, eggs are used for identification.

Key to identification of eggs

The characteristics used to identify species of eggs are as follows:

1. *Size.* The length and width are measured and are generally within a specific range.
2. *Shape.* Each species has its own particular shape.
3. *Stage of development when passed.* In some species, the eggs consist of a single cell; in some, there may be several cells; and some species are usually embryonated (i.e., they contain a larva) when passed in the faeces.
   Occasionally, if the stool specimens are several hours or 1–2 days old, eggs may develop to more advanced stages. *Ascaris* eggs usually have only 1 cell when passed in the faeces; however, the single cell may divide and, in old specimens, eggs with 2 or 4 cells may be seen. Hookworm eggs in specimens that are several hours old may contain 16, 32, or more cells. In 12–24 hours, the egg may be embryonated and later still the larvae may hatch. Therefore, when observing the stage of development of helminth eggs, be sure that the stool specimen is freshly passed. If it is several hours or a day old, expect to see changes in the stage of development of some species. Ideally only fresh samples should be accepted for diagnosis.
4. *Thickness of the egg shell.* Some species, like *Ascaris*, have thick egg shells; others, like hookworm, have thin shells.
5. *Colour.* Some eggs are colourless (e.g., hookworm, *Enterobius*), others are yellow or brown (*Ascaris, Trichuris*).
6. Presence of characteristics like opercula (lids), spines, plugs, hooklets, or mammillated outer coats.

If an egg, or an object that looks like an egg, is found, these features should be carefully observed in order to make a specific identification. Occasionally, atypical or distorted eggs will be seen. In such cases, it will be necessary to look for more typical forms in order to make a reliable diagnosis. Remember that more than one species of helminth may be present in an individual patient.

The following key (Fig. 3) and the diagrams of helminth eggs shown in Fig. 4, will be helpful in identifying species.

REMEMBER

Look at the size, shape, stage of development, thickness of the egg shell, and colour, and for the presence of special structures like opercula, spines, plugs, hooklets, and bumpy outer coat to identify species of eggs.

Fig. 3 Key to the identification of helminth eggs

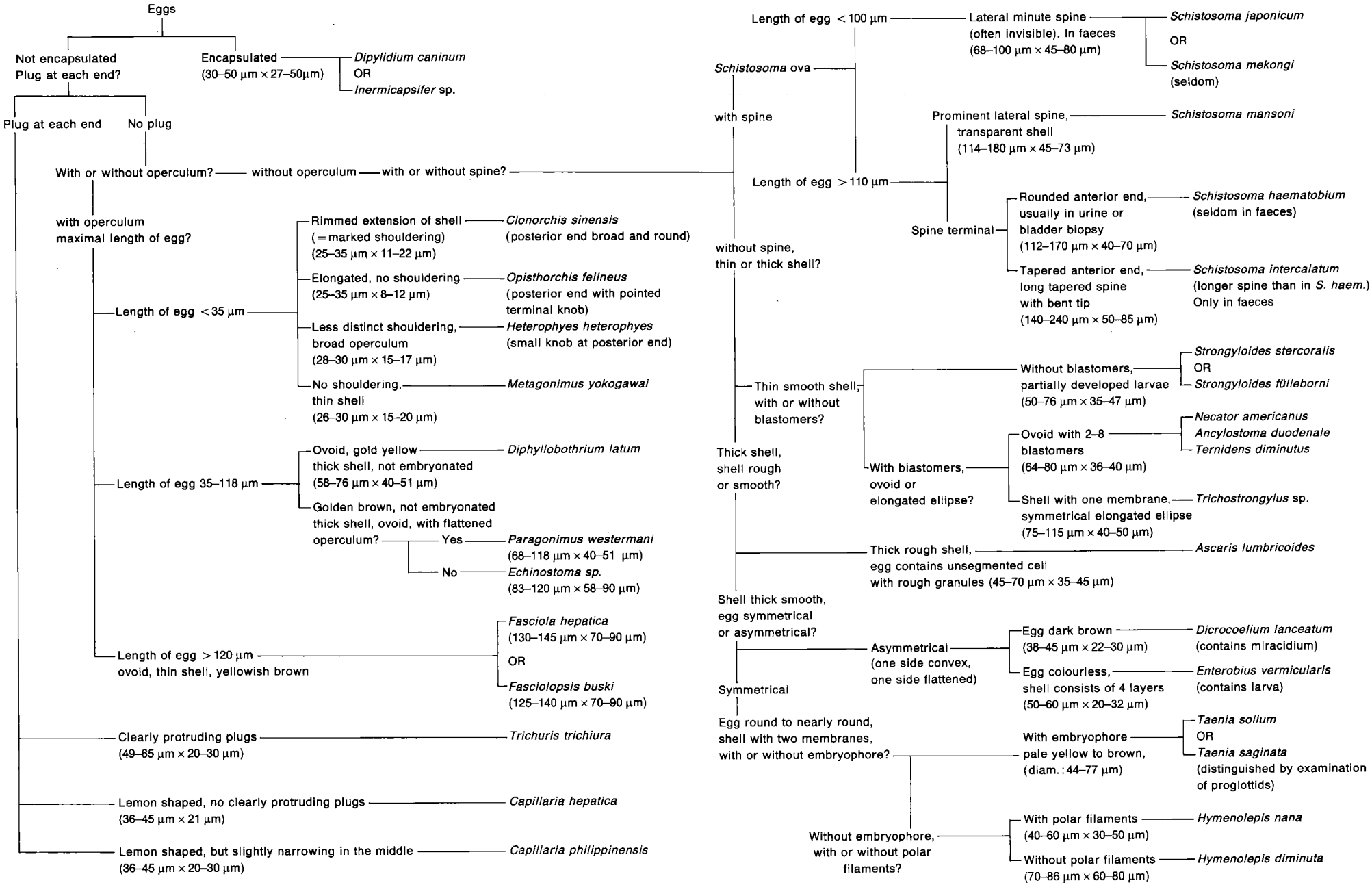
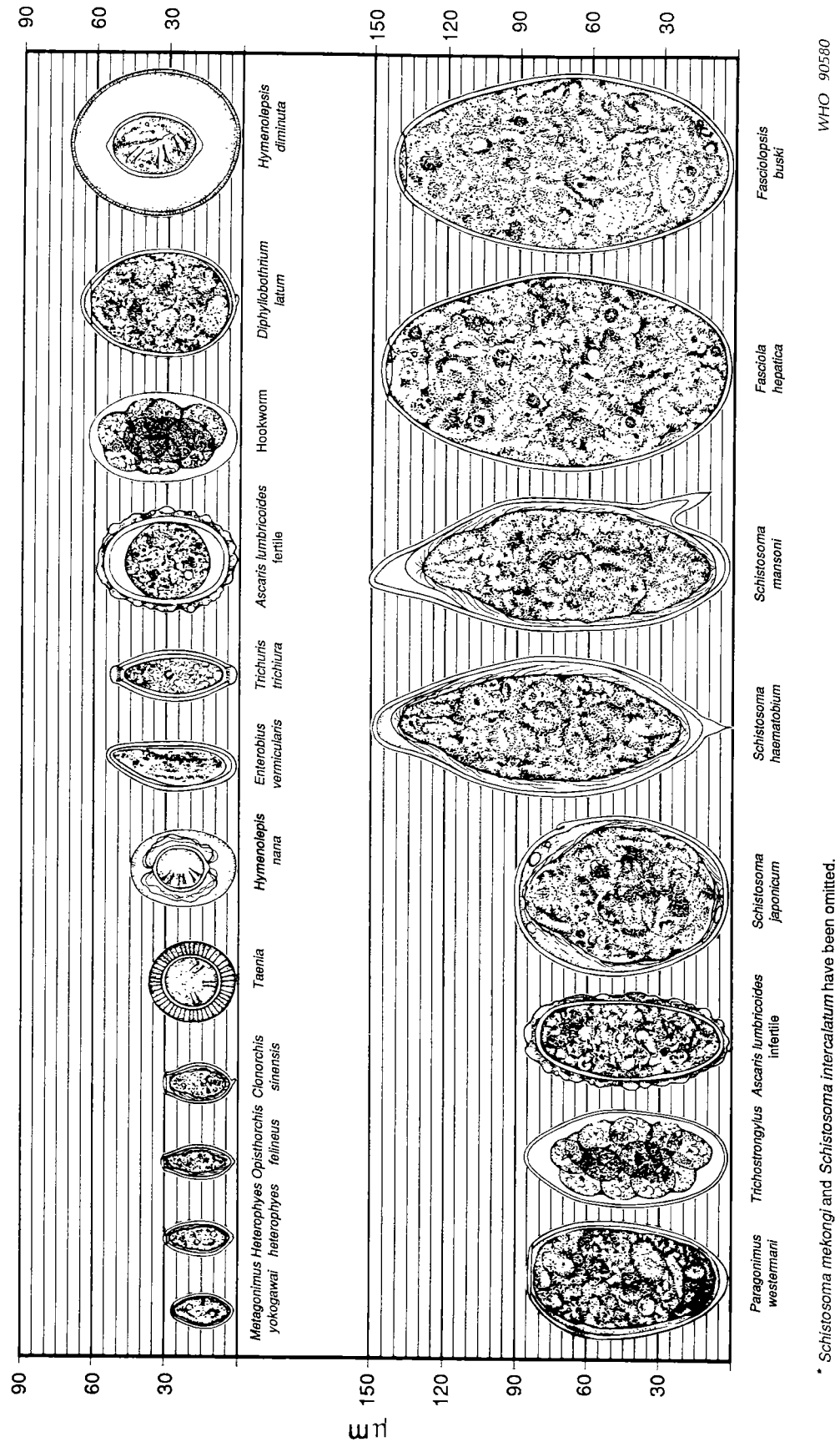


Fig. 4. Identification of intestinal parasites—helminths



* *Schistosoma mekongi* and *Schistosoma intercalatum* have been omitted.

Worm larvae

In fresh stool specimens, the larvae seen are usually rhabditiform (= first stage) larvae from *Strongyloides stercoralis*. However, if the stool has been passed for more than 12 hours, the larvae may hatch into filariform larvae (infective stage); these must be differentiated from hookworm larvae, which may hatch in stool within 12–24 hours. The appearance of filariform larvae of *Strongyloides stercoralis* may indicate a systemic hyperinfection.

The characteristics used to separate the species are shown in Fig. 5 and in Table 4.

In iodine preparations, the genital primordium will be more visible. Iodine will kill the larvae and you will be able to see the features better. You will need to use high-power, dry magnification to see these structures.

- If you see a larva with a short mouth opening and a prominent (clearly visible) genital primordium, it is *Strongyloides*.
- If you see a larva with a long mouth opening and do not see a genital primordium, it is *hookworm*.

Fig. 5. Helminth larvae

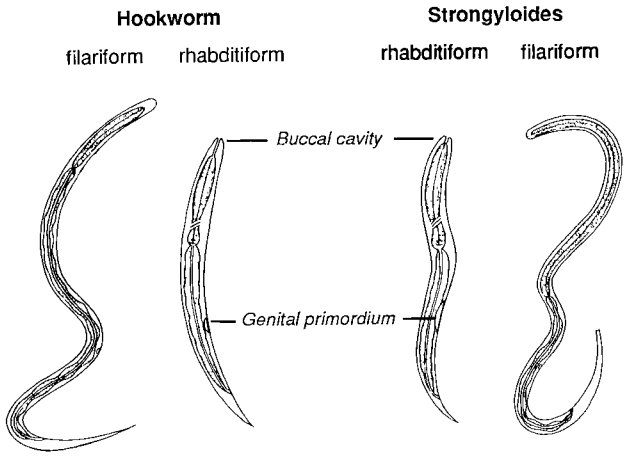


Table 4. Characteristics of helminth larvae

Hookworm	*Strongyloides*
Filariform larvae	*Filariform larvae*
Size 500 × 14–20 µm	Size 500 × 14–20 µm
Sheathed	Unsheathed
Tail tapered	Tail forked or blunt
Oesophagus one-third of body length with no swelling	Oesophagus half of body length with no swelling
Rhabditiform larvae	*Rhabditiform larvae*
Size 100–150 × 15–17 µm	Size 200–300 × 15–18 µm
Buccal cavity long (15 µm)	Buccal cavity short (4 µm)
Oesophagus one-third of body length with two swellings	Oesophagus one-third of body length with two swellings
Genital primordium small (7 µm)	Genital primordium large (22 µm)
Anal pore 80 µm from posterior end	Anal pore 50 µm from posterior end

Protozoa

Intestinal protozoa include amoebae and flagellates. Two diagnostic stages are recognized: the vegetative or trophozoite stage and the dormant cyst stage. Both stages may be passed in the faeces. Trophozoites are usually found in diarrhoeal or loose stools; cysts are usually found in formed stools. However, both stages may be present in the same stool specimen.

Trophozoites and cysts can be seen in saline mounts of fresh faeces. On occasions, species identification may require stained preparations. The types of preparation usually used for detection and identification of protozoa and the characteristics that can be seen in each type of mount are shown in Table 5.

Amoebic trophozoites

Saline wet mount

Motile amoebic trophozoites may be seen in saline mounts of freshly passed faeces. These will be slightly greenish and refractile (shiny). With high-power, dry

Table 5. Characteristics of protozoa seen in different types of preparation

		Temporary stains		
	Saline	BMB[a]	Iodine[b]	Permanent stain (Trichrome)[c]
Amoebae				
Trophozoites				
Motility	+	−		−
Cytoplasm	+	+		+
Inclusions	+	+		+
Nucleus	−	+		+
Cysts				
Nuclei	−		+	+
Chromatoid bodies	+		+[d]	+
Glycogen	−		+	−
Flagellates				
Trophozoites				
Motility	+		−	−
Shape	+		+	+
Nucleus	−		+	+
Cysts				
Shape	+		+	+
Nuclei	−		+	+
Fibrils	+[f]		+	+

[a] BMB stain is used *only* to stain living amoebic trophozoites.
[b] Iodine is used to stain cysts, although flagellate trophozoites may also stain.
[c] Permanent staining techniques are not routinely used in diagnostic laboratories, but are reserved for special circumstances as detailed in the text (e.g., diagnosis of cryptosporidiosis).
[d] Chromatoid bodies in amoebic cysts are better seen in saline mounts than in iodine mounts. They are best seen in permanent stained smears.
[e] Glycogen is dissolved during the staining process, and in permanently stained smears only a clear space (vacuole) will be seen.
[f] Fibrils (filaments) can sometimes be seen in saline mounts.

magnification you can see the type of motility and inclusions like red blood cells and ingested yeasts. *You will not be able to see the nucleus.* (Macrophages may also contain red blood cells, and may move.)

If a trophozoite moves quickly in one direction and forms pseudopods rapidly, it may be *Entamoeba histolytica.* Other species of amoebae do not usually move like this. If the trophozoite moves as described and if red blood cells are present in the cytoplasm, it can be assumed that it is *E. histolytica.* On occasions it may be necessary to use BMB to stain the nucleus for confirmation.

Saline mount:

DEFINITE DIRECTIONAL MOTILITY

+

INGESTED RED BLOOD CELLS

= *E. histolytica*

BMB wet mount

If you suspect the presence of amoebic trophozoites, examine a BMB mount (see Table 5). The trophozoites may curl up and no longer be motile *but the nucleus and inclusions will stain dark blue,* while the cytoplasm will stain light blue. Look for peripheral nuclear chromatin granules (granules in the membrane around the nucleus); if present, it is an *Entamoeba* species. If there is no peripheral chromatin, it is not *Entamoeba.* When peripheral nuclear chromatin is seen, you must identify the species. Use the "Key for the identification of amoebic trophozoites in stained smears" (Fig. 6).

E. histolytica trophozoites are about 12–40 μm long. The nucleus will appear as a dark blue circle with a dark dot (the karyosome) towards the centre. If the peripheral nuclear chromatin granules are fine and even and there is a small central karyosome, it is probably *E. histolytica,* but you must look at several organisms to be sure. If red blood cells are seen in the cytoplasm, this will help confirm the diagnosis. The blood cells will be dark blue in BMB stained mounts.

Entamoeba coli is about the same size as *E. histolytica* and also has a nucleus with peripheral chromatin. It has rough, coarse cytoplasm, often containing bacteria or moulds. The peripheral nuclear chromatin is irregular and the karyosome is not in the centre.

Trichrome stained smears

In stained smears, trophozoites may be round, elongated, or irregular in shape. Amoebic trophozoites usually stain green or blue-green with purple or red nuclei. The nuclei of *Entamoeba* species have peripheral chromatin granules (as described for BMB mounts) which may appear as a circle, or as a beaded circle. The karyosome may be seen as a red or purple dot within the nucleus. In *E. histolytica,* the karyosome is usually in the centre of the nucleus, but may occasionally be eccentric and you must look at several organisms to decide whether the species is *E. histolytica* or *E. coli.*

In addition to amoebic trophozoites, other cells resembling trophozoites may be seen. These must be differentiated from the amoebae. Compare the objects you see in the stained smears with the figures in the key and on p. 79, "Problems of

identification", to decide whether they are amoebic trophozoites, or tissue cells, or some other structure.

Trophozoites may be identified as *E. histolytica* if you see:

- **peripheral nuclear chromatin**
  and
- **red blood cells in cytoplasm**

OR

- **uniform peripheral nuclear chromatin**
  and
- **small central karyosome in nucleus**
  and
- **finely granular, smooth cytoplasm**
  and
- **length of trophozoite = 2–6 × red blood cell diameter**

Amoebic cysts

Accurate measurement of cysts is *essential* for correct identification.

Saline wet mount

When examining with the ×40 objective, focus up and down and look for shiny round objects with a diameter roughly equal to 1–3 red blood cells. Look also for chromatoid bodies (rod-shaped structures), which are best seen with the high-power objective. They are more distinct in the saline mounts than in the iodine mounts. These bodies are characteristic in appearance, and occur in *E. histolytica* and *E. coli* cysts. In *E. histolytica*, the rod-shaped bodies have blunt rounded ends, in contrast to the pointed ends of the chromatoid bodies in *E. coli*. These structures are seen less frequently in cysts of *E. coli* than in those of *E. histolytica*.

Nuclei are not easily visible in saline, but are well seen in iodine mounts. The appearance of the nucleus is important in differentiating species of amoebae. Therefore, if cysts (or things that look like cysts) are seen in the saline mount, examine the iodine mount.

Measure any cysts found.

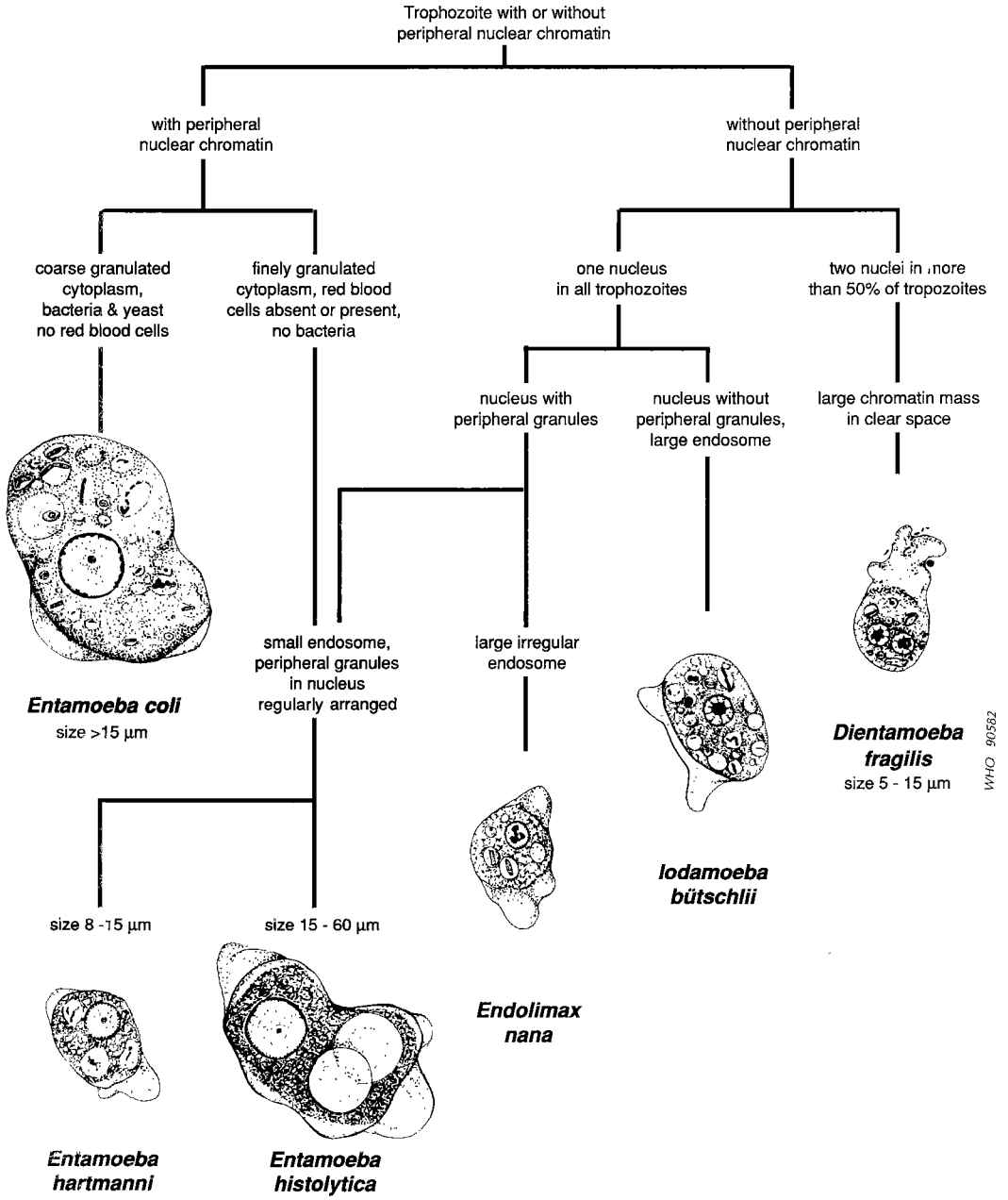
Iodine wet mount

Focus on the mount with the ×40 objective and search for cysts. When you find cysts, or objects that look like cysts, switch to the high-power dry objective to see the details necessary to identify the species. Measure the cysts.

Use the "Key to identification of amoebic and flagellate cysts" in iodine mounts and stained smears (Fig. 7) and the following description to identify the species of cysts found.

E. histolytica cysts measure 10–15 µm in diameter. Mature *E. histolytica* cysts have four nuclei that have uniform peripheral nuclear chromatin and a central karyosome. Usually the nuclei are not in the same plane, so it is necessary to focus up and down to see all four. Carefully move the objective up until the cyst is just out of focus.

Fig. 6. Key for the identification of amoebic trophozoites in stained smears

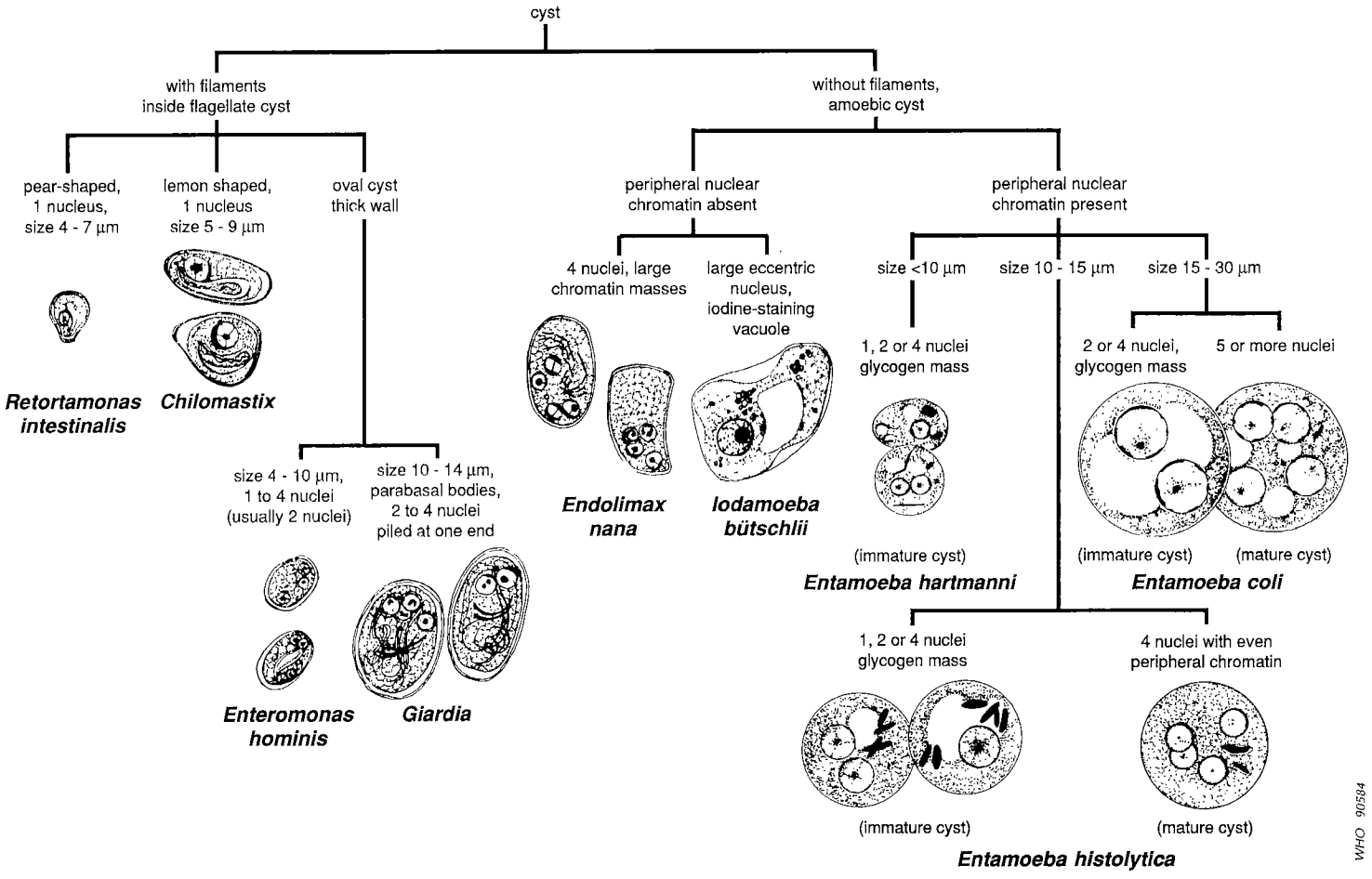


Focus carefully and continue to move the objective *slowly* downward to see all levels of the cyst. Count the nuclei as they become visible.

Sometimes immature *E. histolytica* cysts are seen. These may have 1, 2, or 4 nuclei. If only one nucleus is present, it is usually quite large. Often cysts with one nucleus have large glycogen masses and several small chromatoid masses. (The chromatoid masses have not yet formed into the typical rod-shaped bodies.) Measure the cyst.

E. coli cysts are usually larger than those of *E. histolytica* (15–30 µm). *E. coli* cysts have eight nuclei that have irregular peripheral chromatin and karyosomes that are not central. They are usually on different planes, so you must focus up and down carefully to see and count them.

Fig. 7. Key to identification of amoebic and flagellate cysts



Immature *E. coli* cysts may be seen and should not be confused with *E. histolytica.* The most commonly seen immature form is a cyst with two nuclei (*E. coli* cysts with one nucleus are rarely seen), a large glycogen mass and several small chromatoid masses. As with *E. histolytica,* the masses have not yet formed into typical chromatoid bodies. Accurate measurement will help distinguish cysts of *E. coli* from those of *E. histolytica.*

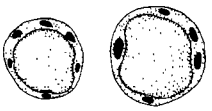
Cysts from *E. hartmanni* are similar to those from *E. histolytica.* However, they are smaller (7–9 μm).

In iodine preparations, cysts of *Iodamoeba bütschlii* show a compact glycogen body, and this differentiates them from *E. histolytica.* Cysts of *I. bütschlii* have no chromatoid bodies and contain a single nucleus.

If at first you see immature cysts, look for mature forms. Mature cysts can usually be found and the species identified. If you see cysts with five or more nuclei, they are *E. coli.*

In recent years, several cases of primary amoebic meningoencephalitis caused by free-living amoebae, *Acanthamoeba* spp and *Naegleria* spp, have been reported throughout the world. Most infections have been traced to polluted water. Laboratory diagnosis is made by microscopical examination of the purulent cerebrospinal fluid containing polymorphonuclear cells but no bacteria. The amoebae can be seen in the Giemsa stained smear.

Blastocystis hominis, a protozoan that is also found in stools, can be distinguished from amoebic cysts because the centre stains green (or sometimes clear) with refractile granules around the edge.



Blastocystis hominis

WHO 90583

Flagellates

Saline wet mount for detection and identification of trophozoites

Flagellate trophozoites (Fig. 8) are best identified by the way they move in saline mounts. The flagella will usually not be seen and nuclei will not be visible.

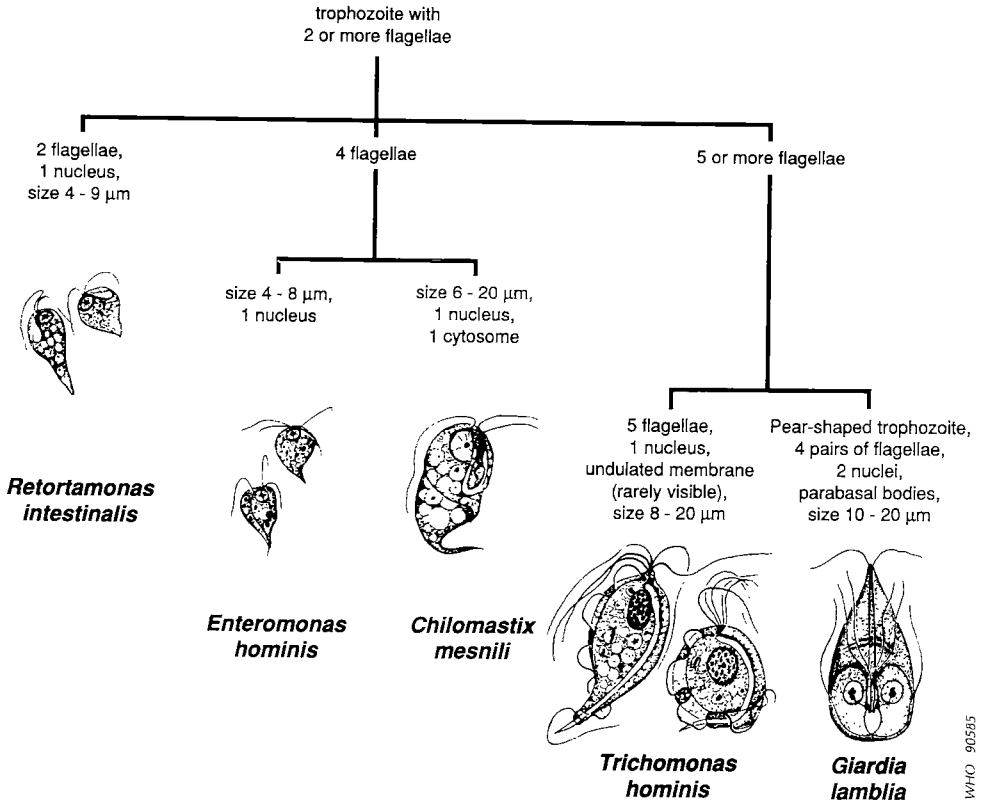
- *Giardia intestinalis* trophozoites (pathogenic) move like a "falling leaf"; they flip back and forth.
- *Chilomastix mesnili* trophozoites (nonpathogenic) rotate as they move.
- *Trichomonas hominis* trophozoites (rarely pathogenic) have a jerky movement.

NOTE
BMB does not stain flagellate trophozoites, so a BMB mount is not of value in their diagnosis.

Iodine wet mount for detection and identification of cysts

Iodine solutions are used primarily to stain cysts and make it possible to see the structure of the nuclei. Use the "Key to identification of amoebic and flagellate cysts" (Fig. 7) for identification.

Fig. 8. Key to identification of flagellate trophozoites in stained smears



NOTE
Trichomonas hominis does not have a cyst stage.

Giardia intestinalis trophozoites and cysts in stained smears

Giardia organisms are characteristic and usually match the illustrations in manuals and text books. They are not hard to identify in stained smears.

The other species of intestinal flagellates are rarely pathogenic.

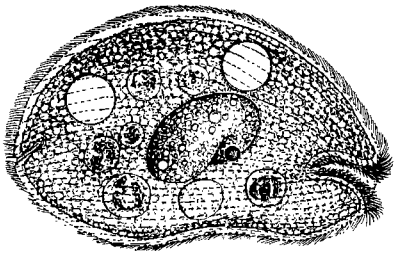
Balantidium coli

This is the only ciliate parasite of human beings and infections are not common. It has not been discussed elsewhere in this manual because human cases are rare. (It is primarily a parasite of swine and monkeys.) However, it may occasionally be seen in human stool specimens, so a brief description is included here.

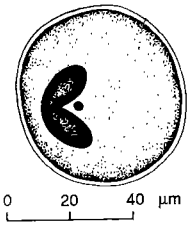
Balantidium coli has a trophozoite and a cyst stage. The trophozoites are large (50–200 µm long by 40–70 µm wide) and very active, and can easily be seen in saline mounts using the low-power objective (see p. 78). They are covered with short hairs (cilia) that beat rapidly and cause the organisms to move quickly. They die very quickly outside the body, so stool specimens must be examined within 1 hour after passage.

The cysts are 45–75 µm in diameter. Neither the trophozoites nor the cysts stain

well with iodine or permanent stains. The best technique, therefore, is the saline wet mount.



Balantidium coli trophozoite



Balantidium coli cyst

WHO 90586

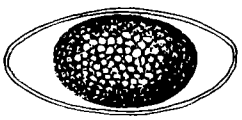
Isospora belli and *Cryptosporidium* spp

Isospora belli and *Cryptosporidium* are intestinal coccidian parasites. Human infection with *Isospora belli* is uncommon, rarely serious and often asymptomatic. Infection with *Cryptosporidium* is a significant cause of diarrhoea in children below the age of 5 years and is particularly severe in immunocompromised patients.

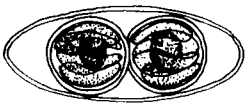
Laboratory diagnosis

Oocysts of *Isospora belli* can be detected in direct faecal smears. If required the oocysts can be concentrated by the formalin–ether technique (see Section 1, pp. 16–17).

Mix a small amount of faeces in saline at the end of a slide with a small amount of iodine solution at the other end. Oocysts from *Isospora belli* are oval (about 32 × 16 µm) and contain a central individual mass of protoplasm (see diagrams below).



Immature oocyst of *Isospora belli*



Mature oocyst of *Isospora belli*

WHO 90587

NOTE
Prepare iodine solution by mixing 10 ml of Lugol's iodine (reagent no. 16) with 10 ml of 25% v/v acetic acid. This solution gives good staining of nuclei.

Cryptosporidium oocysts are examined in stained faecal smears (see Section 1, pp. 17–18).

Toxoplasma gondii

Toxoplasma gondii is an animal parasite that causes toxoplasmosis. Human toxoplasmosis is often asymptomatic. It can cause fever, a rash, enlargement of lymph glands, and lymphocytosis. The most serious form of human infection is congenital toxoplasmosis, which often causes severe cerebral damage in the fetus. Infection occurring in early pregnancy may result in abortion, while infection in late pregnancy may cause symptoms of infection in the infant 2–3 months after birth. Clinical manifestations of *Toxoplasma* infection often occur in patients with acquired immunodeficiency syndrome (AIDS).

Laboratory diagnosis of toxoplasmosis is by:

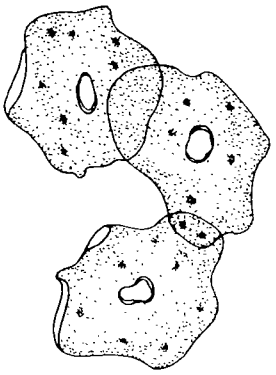
1. Serological tests, which include the Sabin–Feldman dye test, the indirect fluorescent antibody test (IFAT), the indirect haemagglutination test (IHA), a complement-fixation test (CFT), and more recently the enzyme-linked immunosorbent assay (ELISA), which is claimed to be more specific. Further discussion is outside the scope of this manual.
2. Microscopical investigation is occasionally useful for diagnosis of an acute infection, using Giemsa or Field's stained preparations of lymph node aspirates, bone marrow aspirates, cerebrospinal fluid, peritoneal, or pleural fluids.

The parasites are crescent-shaped and small (3 × 7 μm). One end is rounded, the other end is pointed. The cytoplasm stains blue, the dark red-stained nucleus is at the rounded end. Occasionally the organisms may appear round and resemble *Leishmania* amastigotes in tissue sections.

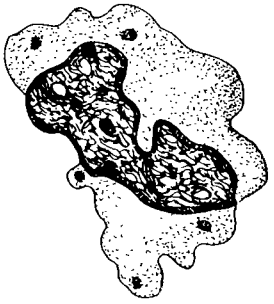
Problems of identification

Many things in stool specimens look like parasites but are not.

Epithelial cells and macrophages can be confused with amoebic trophozoites, especially macrophages that show slight amoeboid movement and may contain red blood cells. The nuclei, which can be seen in BMB stained mounts, appear much larger than nuclei of amoebae and usually contain several granules or particles of chromatin (see below).



Epithelial cells



Macrophage

Pus cells can be confused with amoebic cysts. The nuclei appear as 3 or 4 rings and usually stain heavily. The cytoplasm is ragged and the cell membrane is often not seen. Amoebic cysts have a distinct cell wall.

Hairs and fibres may be confused with larvae. However, hairs and fibres do not have the same internal structure as larvae.

Plant cells (e.g., moulds or yeasts) can be confused with cysts or eggs. Plant cells usually have a thick wall; cysts have a thin wall. Yeasts and moulds are usually smaller than amoebic cysts and do not have nuclei such as are seen in amoebic cysts.

NOTE
E. histolytica trophozoites and cysts are often not typical, and several organisms should be examined to be certain of the species.

IDENTIFICATION MAY NEED CAREFUL STUDY OF SEVERAL CYSTS OR TROPHOZOITES. MEASUREMENT IS ESSENTIAL

Blood parasites

Malaria

Identification of malaria parasites in thin blood films

In examining thin blood films for malaria, you must look at the infected red blood cells and the parasites inside the cells.

Infected red cells

– Look at the size of the infected red cells.
– Is Schüffner's stippling present or not present?

Infected red cells

- Enlarged: Schüffner's stippling present[1] → Suggests *P. vivax* or *P. ovale.* Look at parasites.
- Normal: Schüffner's stippling not present (infected cells may be smaller than normal in *P. malariae* infections) → *P. malariae* or *P. falciparum*

Parasites

Rings of the four main species may look alike. If you see rings, look for older stages. In patients with *P. falciparum* only rings are usually seen; older stages are present only in severe infections.

NOTE
If *Plasmodium falciparum* is seen, the percentage parasitaemia (number of infected red cells per hundred) *must* be reported.

See Tables 6 and 7, and Fig. 9, 10, 11, and 12, for the diagnostic features to look for in thin and thick blood films.[2]

Identification of malaria parasites in thick blood films

In stained thick blood films, the red blood cells are lysed, so diagnosis is based on the appearance of the parasite. In thick films, organisms tend to be more compact and denser than in thin films.

[1] In poorly stained slides, Schüffner's dots may not be visible, so it is essential that correct staining methods are used. Also Schüffner's dots may not be present in the early rings of *P. vivax* or *P. ovale.*

[2] Table 7 and Fig. 9–13 are taken from *Bench aids for the diagnosis of malaria*, Plates No. 1–8, Geneva, World Health Organization, 1988.

Table 6. Morphological features of malaria parasites in thin blood films

Stage	*P. vivax*	*P. ovale*	*P. malariae*	*P. falciparum*
Infected red cell	Enlarged; Schüffner's dots present	Enlarged; may be oval with fimbriae; Schüffner's dots present	Size normal or smaller than normal	Size normal; Maurer's clefts may be seen
Ring stage (early trophozoite)	Quite large; one or two chromatin dots; may be two rings per rbc	Compact; two rings per rbc; rare	Compact; two rings per rbc; rare	Small and delicate; often two chromatin dots; often two or more rings per rbc; accolé forms common
Late trophozoite	Large; amoeboid; pigment seen as fine rods	Small; not amoeboid; pigment coarse	Small; compact; often band-shaped; pigment coarse	Moderate size; usually compact; pigment granular
Mature schizont	Large; merozoites large (12–24 in number); coalescent pigment	Smaller than *P. vivax*; 6–12 merozoites; pigment darker than in *P. vivax*	Small but merozoites (6–12) large; "daisy head" appearance characteristic; pigment coarse	Rare in peripheral blood; merozoites (8–26) small; single pigment mass
Gametocytes	Spherical; compact; single nucleus; pigment diffuse and coarse	Similar to, but smaller than, *P. vivax*	Resemble *P. vivax* but smaller, less numerous and Schüffner's dots absent	Crescent shaped; single nucleus

Methods of counting malaria parasites in thick blood films

Parasites per microlitre

The following is a practical method of adequate accuracy. It is based on the number of parasites per µl of blood in a thick film, these being counted in relation to a predetermined number of leukocytes. An average of 8000 leukocytes per µl is taken as the standard. Despite inaccuracies due to variations in the number of leukocytes between individuals in normal health, and greater variations in ill health, this standard allows for reasonable comparisons. Before counting begins, the equivalent of 0.25 µl of blood (about 100 fields, using a ×7 ocular and a ×100 oil-immersion objective) should be examined in the thick film to determine the parasite species and the stages that are present. When this has been done, the following counting method should be employed for positive blood films.

1. Two tally counters are required to count parasites and leukocytes separately.
2. (a) If, after 200 leukocytes have been counted, 10 or more parasites have been identified, record the results in the record form, showing the number of parasites per 200 leukocytes.
   (b) If, after 200 leukocytes have been counted, 9 or fewer parasites have been counted, continue counting until 500 leukocytes have been counted and record the number of parasites per 500 leukocytes.
3. In each case, the parasite count in relation to the leukocyte count can be converted to parasites per µl by the simple mathematical formula:

$$\frac{\text{No. of parasites} \times 8000}{\text{No. of leukocytes}} = \text{No. of parasites per } \mu\text{l}$$

This means that if 200 leukocytes are counted, the number of parasites counted is multiplied by 40, and if 500 leukocytes are counted the number of parasites is multiplied by 16.

(continued on p. 88)

Table 7. Species identification of malaria parasites in Giemsa-stained thick blood films

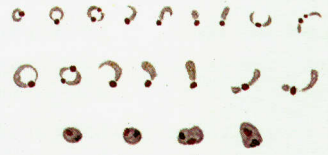
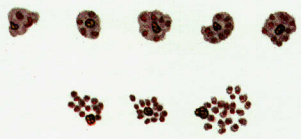
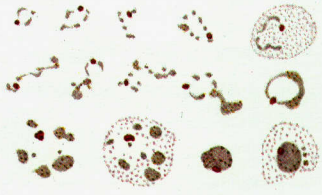
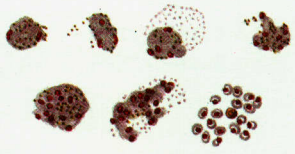
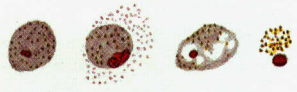
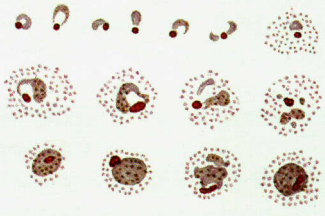
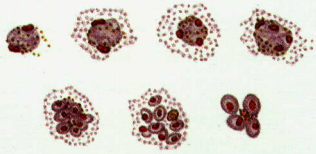
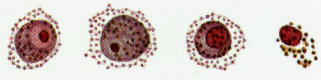
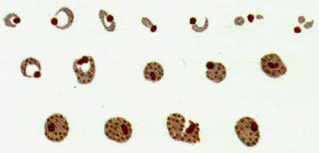
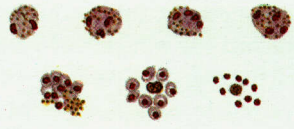
Species		Stage of parasite in peripheral blood		
		Trophozoite	Schizont	Gametocyte
Plasmodium falciparum	Young, growing trophozoites and/or mature gametocytes usually seen.	 *Size:* small to medium; *number:* often numerous; *shape:* ring and comma forms common; *chromatin:* often two dots; *cytoplasm:* regular, fine to fleshy; *mature forms:* sometimes present in severe malaria, compact with pigment as few coarse grains or a mass.	 Usually associated with many young ring forms. *Size:* small, compact; *number:* few, uncommon, usually in severe malaria; *mature forms:* 12–30 or more merozoites in compact cluster; *pigment:* single dark mass.	 Immature pointed-end forms uncommon. *Mature forms:* banana-shaped or rounded; *chromatin:* single, well defined; *pigment:* scattered, coarse, rice-grain like; pink extrusion body sometimes present. Eroded forms with only chromatin and pigment often seen.
P. vivax	All stages seen; Schüffner's stippling in 'ghost' of host red cells, especially at film edge.	 *Size:* small to large; *number:* few to moderate; *shape:* broken ring to irregular forms common; *chromatin:* single, occasionally two; *cytoplasm:* irregular or fragmented; *mature forms:* compact, dense; *pigment:* scattered, fine.	 *Size:* large; *number:* few to moderate; *mature forms:* 12–24 merozoites, usually 16, in irregular cluster; *pigment:* loose mass.	 Immature forms difficult to distinguish from mature trophozoites. *Mature forms:* round, large; *chromatin:* single, well defined; *pigment:* scattered, fine. Eroded forms with scanty or no cytoplasm and only chromatin and pigment present.
P. ovale	All stages seen; prominent Schüffner's stippling in 'ghost' of host red cells, especially at film edge.	 *Size:* may be smaller than *P. vivax*; *number:* usually few; *shape:* ring to rounded, compact forms; *chromatin:* single, prominent; *cytoplasm:* fairly regular, fleshy; *pigment:* scattered, coarse.	 *Size:* rather like *P. malariae*; *number:* few; *mature forms:* 4–12 merozoites, usually 8, in loose cluster; *pigment:* concentrated mass.	 Immature forms difficult to distinguish from mature trophozoites. *Mature forms:* round, may be smaller than *P. vivax; chromatin:* single, well defined; *pigment:* scattered, coarse. Eroded forms with only chromatin and pigment present.
P. malariae	All stages seen.	 *Size:* small; *number:* usually few; *shape:* ring to rounded, compact forms; *chromatin:* single, large; *cytoplasm:* regular, dense; *pigment:* scattered, abundant, with yellow tinge in older forms.	 *Size:* small, compact; *number:* usually few; *mature forms:* 6–12 merozoites, usually 8, in loose cluster; some apparently without cytoplasm; *pigment:* concentrated.	 Immature and certain mature forms difficult to distinguish from mature trophozoites. *Mature forms:* round, compact; *chromatin:* single, well defined; *pigment:* scattered, coarse, may be peripherally distributed. Eroded forms with only chromatin and pigment present.

Fig. 9. ***Plasmodium falciparum***

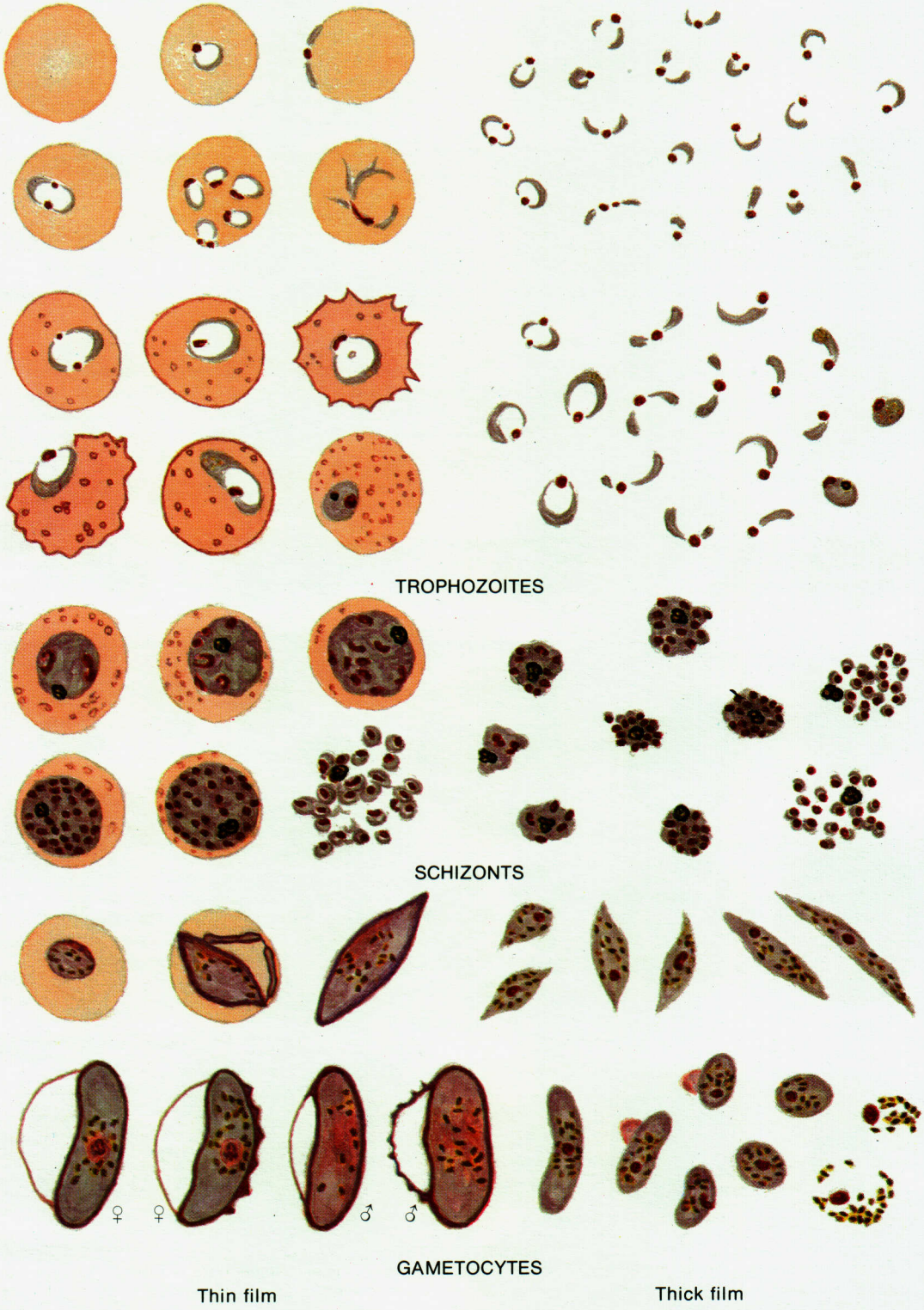


Fig. 10. ***Plasmodium vivax***

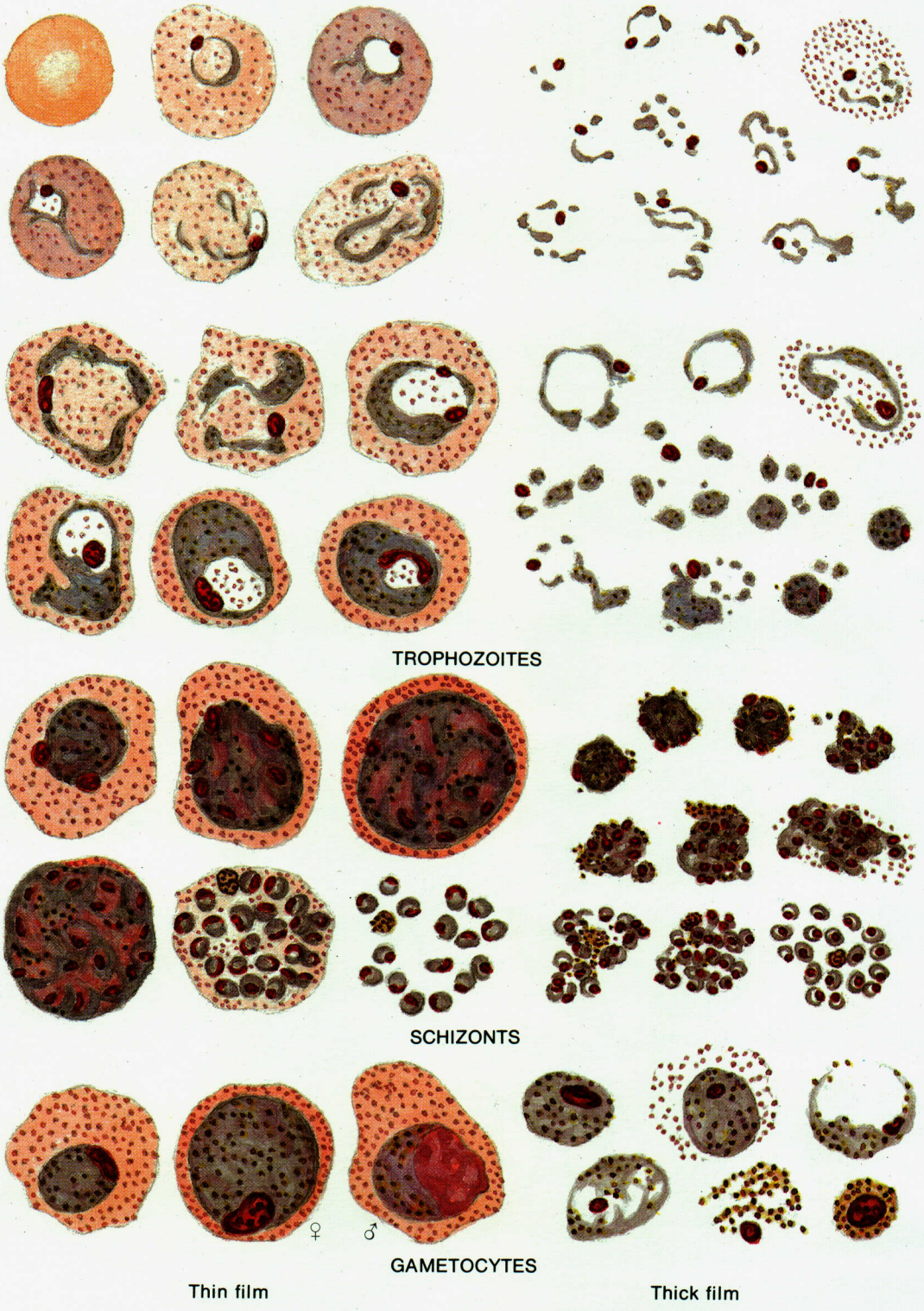


Fig. 11. ***Plasmodium malariae***

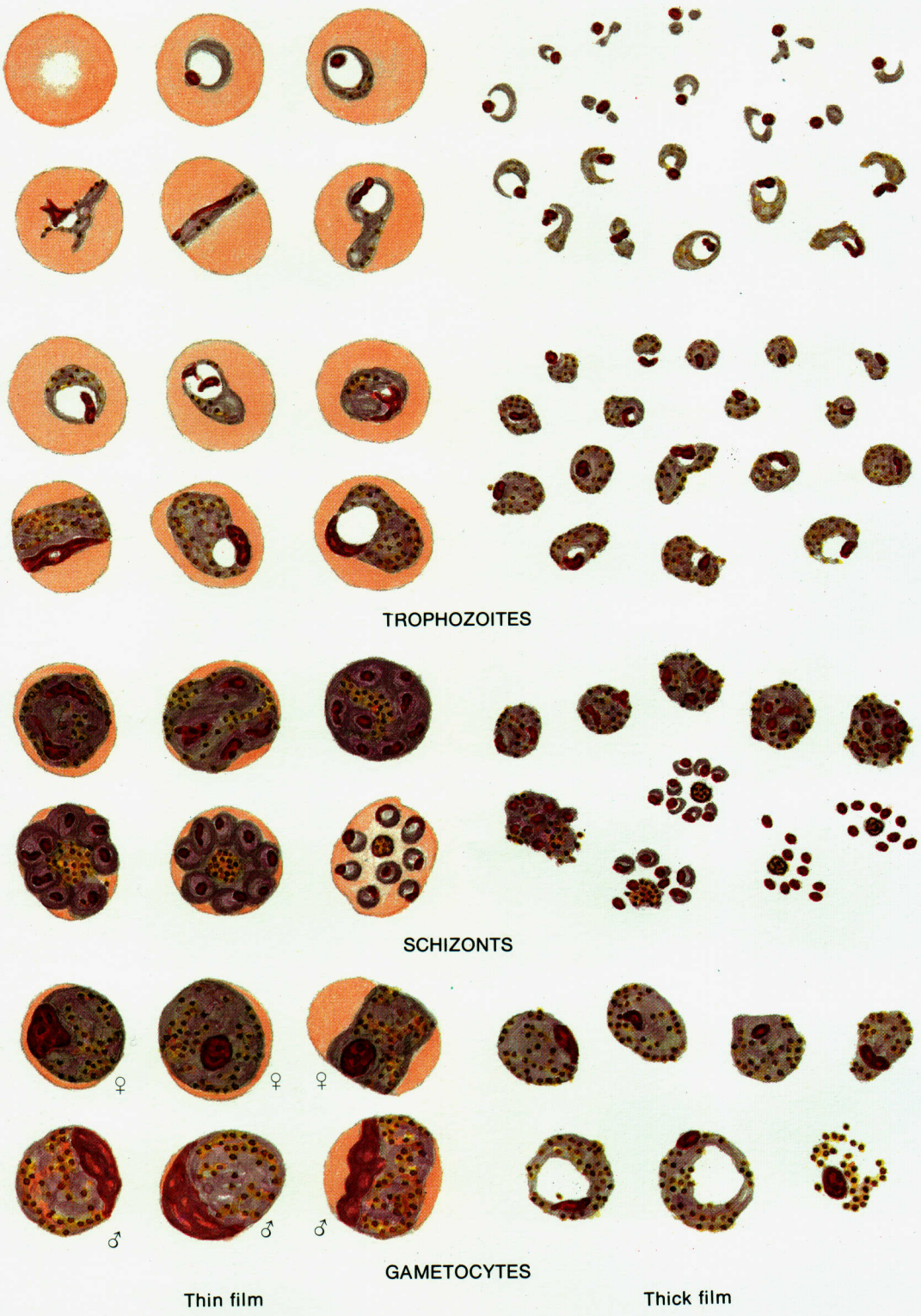


Fig. 12. ***Plasmodium ovale***

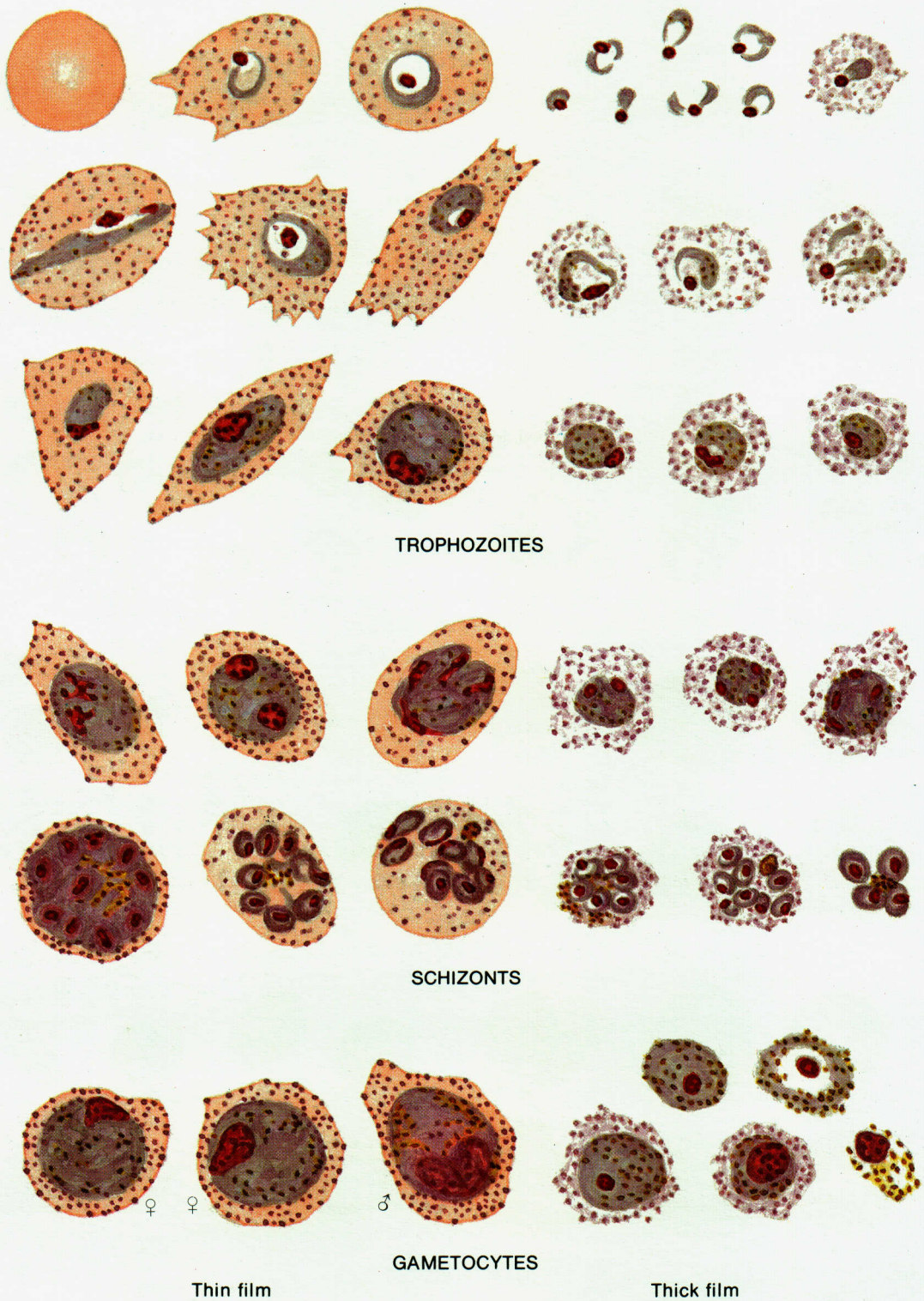
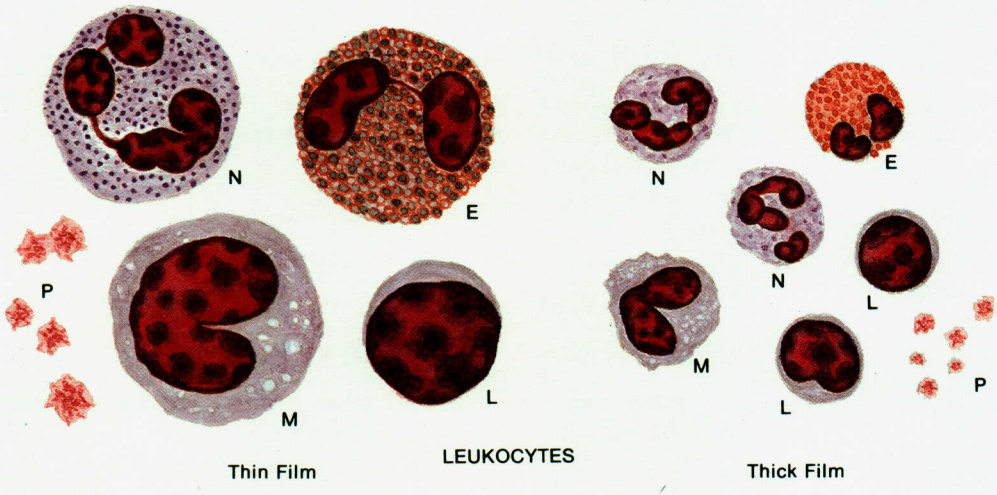
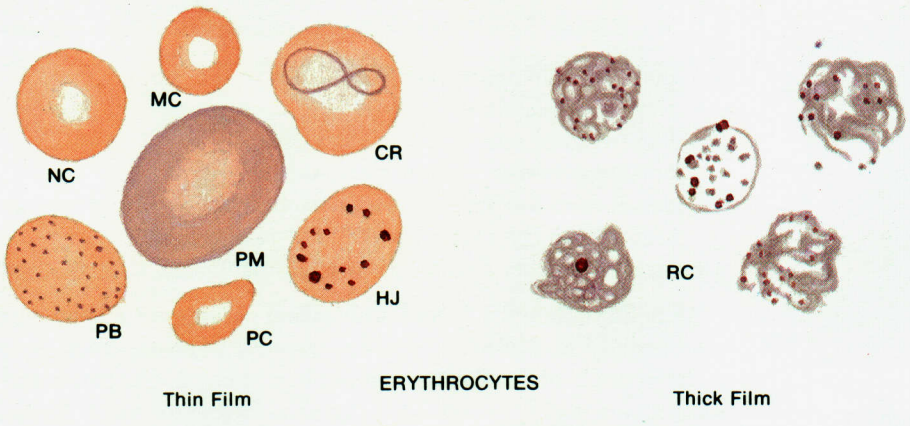


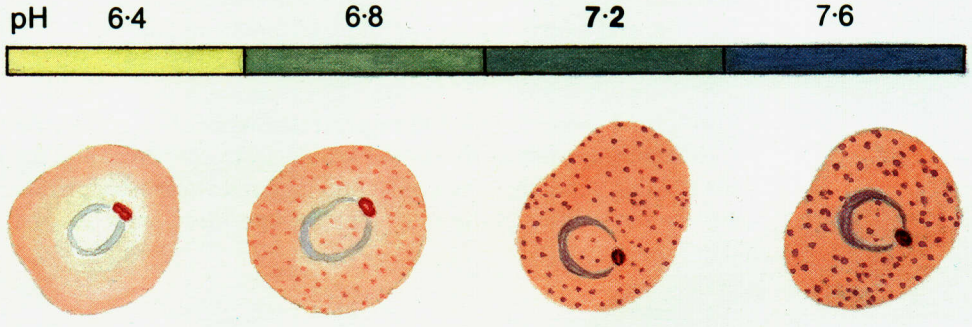
Fig. 13. Appearance of cellular elements in Giemsa-stained thin and thick blood films: effect of pH on Giemsa staining of malaria parasites



N = Neutrophil, E = Eosinophil, M = Monocyte, L = Lymphocyte, P = Platelets



NC = Normocyte, MC = Microcyte, PM = Polychromatic macrocyte, PC = Poikilocyte, PB = Punctate basophilia, CR = Cabot's ring, HJ = Howell-Jolly bodies, RC = Reticular 'clouds' and chromatoid bodies in severe anaemia



MALARIA STAINING AND pH

4. It is normal practice to count all the species present and to include both sexual and asexual parasites together. Occasionally, a separate count is made of the gametocytes of *Plasmodium falciparum*, but when this is done, they should still be included in the general parasite count. It is rarely possible to separate the gametocytes of *P. vivax* or *P. malariae* from the asexual parasites with sufficient accuracy to justify a gametocyte count.

The plus system

A simpler method of enumerating parasites in thick blood films is to use the plus system. This indicates the relative parasite count and entails using a code of 1–4 pluses, as follows:

\+ = 1–10 parasites per 100 thick-film fields

\+ + = 11–100 parasites per 100 thick-film fields

\+ + + = 1–10 parasites per thick-film field

\+ + + + = more than 10 parasites per thick-film field

This system should be used only when it is not possible to undertake the more acceptable parasite count per µl of blood.

Things that may be confused with malaria parasites

In blood films, the following things may look like malaria parasites (see also Fig. 13):

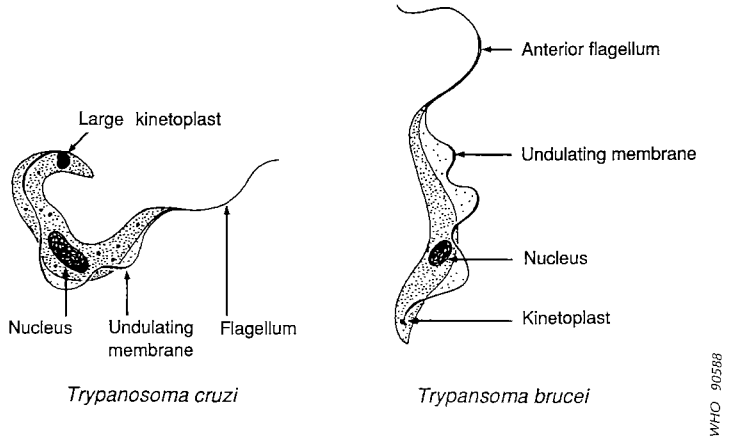
- platelets adhering to red blood cells in thin films,
- clumps of platelets,
- fragments of white cells in thick films,
- precipitated stain on red blood cells,
- debris from the patient's skin, dust, bacteria, yeasts, spores, and other organisms that get on the blood film while it is drying,
- algae and other organisms from contaminated staining solutions.

All of these things may look like malaria parasites in stained blood films and can be mistakenly identified as malaria. Usually, however, they do not have all three components of a malaria organism: blue staining cytoplasm, red or purple staining chromatin, and brown or black pigment. Except for rings (which lack pigment), you should see all three characteristics before you identify any structure as a parasite. If you are uncertain whether a particular object or structure is a malaria parasite, look for more typical organisms. If you do not find things that are clearly malaria parasites, report the film as "No malaria parasites found," preferably after making, staining, and examining another film. Diagrams of some of the things that can be confused with malaria parasites are included in Fig. 13.

You should keep the blood films protected while they are drying to prevent dust and organisms, such as spores or bacteria, from contaminating the film. Also, keep the staining solutions (stock stain and buffered water) stoppered or closed so that dust and organisms in the air do not get into them, and then on to the film when you stain it.

Trypanosoma

Trypanosomes may be distorted in thick films. If organisms cannot be recognized in thick films, look for them in the thicker areas of the thin film. They are between the red blood cells. Look at the length, the shape, and the size of the kinetoplast of the parasites.



Trypanosoma cruzi *Trypansoma brucei*

Remember

1. In Africa, the two *Trypanosoma* subspecies that infect man are identical. You cannot determine the subspecies from the appearance in stained films.
2. In South and Central America, *T. cruzi* and *T. rangeli* must be distinguished from each other.
3. *T. rangeli* is longer than *T. cruzi.*
4. *T. cruzi* has a very large, prominent kinetoplast and is often seen in C, U, or S shapes in stained films.

Microfilariae

The following characteristics are used for the identification of microfilariae (see Fig. 14):

- presence or absence of a sheath,
- presence or absence of nuclei in the tip of the tail,
- innerbody—can or cannot be demonstrated,
- size of the microfilaria.

Sometimes other characteristics have to be used to identify species. Unfortunately, not all the diagnostic features can be seen in Giemsa stained preparations, so occasionally special stains like Delafield's haematoxylin must be used to demonstrate them.

Remember

1. Use a ×10 objective to locate microfilariae.
2. Search the blood film systematically.
3. Use high-power, dry (×40) or oil-immersion objectives to examine microfilariae for specific identification.

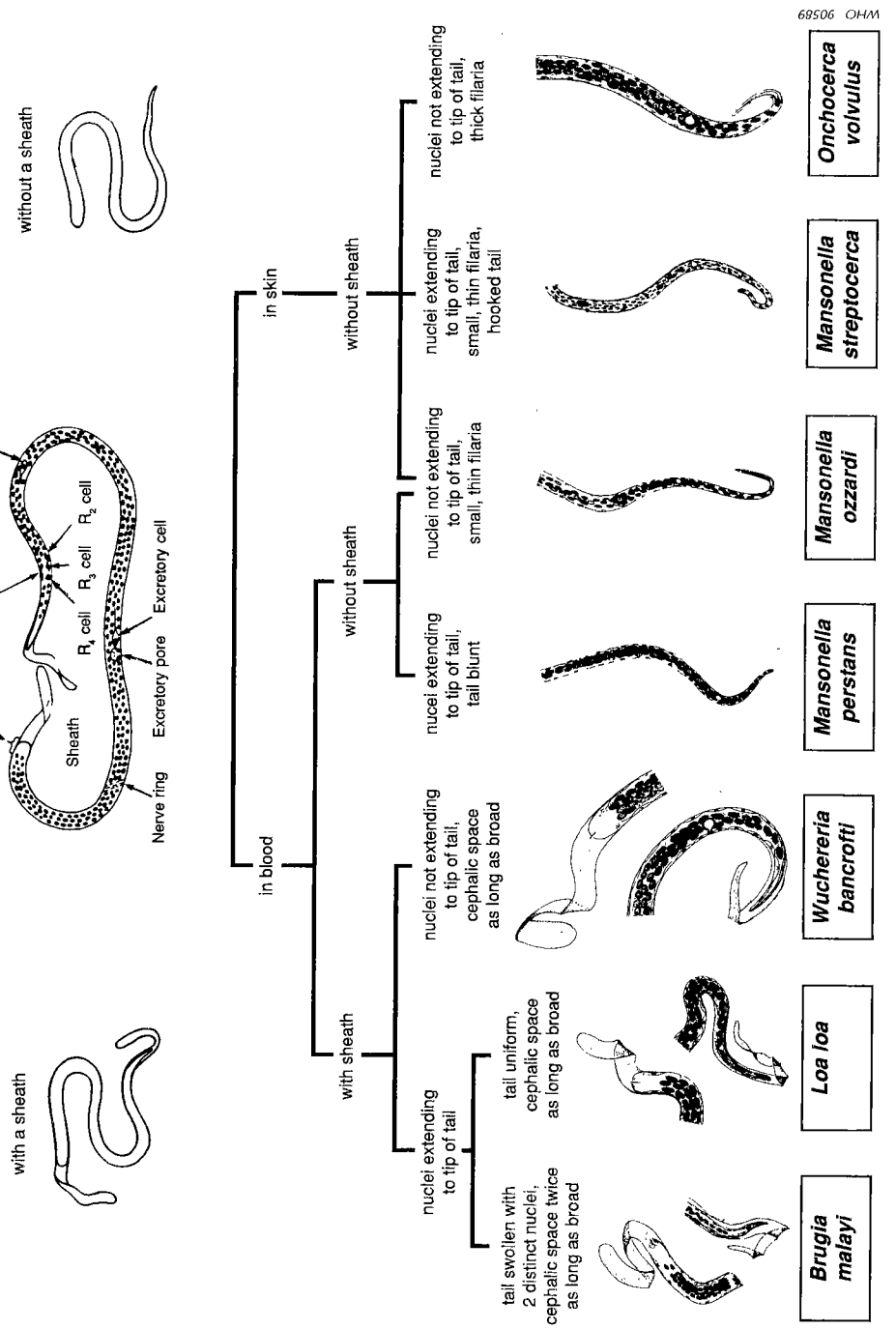
A. *Wuchereria bancrofti* (Asia, Africa, Central and South America, West Indies)

Sheath may or may not stain with Giemsa; does stain with haematoxylin stains.
Discrete nuclei. Empty space between the nuclei and the body wall.
No nuclei in tip of tail.
Innerbody rarely visible in Giemsa. Does not stain with haematoxylin. Cephalic space as long as it is broad.
Tip of tail may be bent back underneath the body. Found in blood.

B. *Brugia malayi* (South-East Asia, India)

Kinked microfilaria.
Sheath stains deep pink with Giemsa stain. Does stain with haematoxylin stains.

Fig. 14. Microfilariae found in human beings



Nuclei crowded and fill the whole body. Empty space between nuclei and body wall.
Cephalic space twice as long as it is broad.
Innerbody may or may not stain; when it does, it is prominent. Found in blood.

C. *Loa loa* (only in Africa)

Kinked and sheathed microfilaria.
Sheath does not stain with Giemsa stain; does stain with haematoxylin stains.
Nuclei crowded extending to tip of tail; tip of tail tapers. Cephalic space as long as it is broad.
Innerbody does not usually stain. Found in blood.

D. *Mansonella perstans* (Africa and South America)

Small, thin microfilaria.
Does not have a sheath.
Nuclei extend to end of tail; last nucleus bigger; tip of tail is blunt.
Nuclei stain deeply and "run together". Found in blood.

E. *Mansonella ozzardi* (Central and South America)

Small thin microfilaria.
Does not have a sheath.
Nuclei do not extend to end of tail; tip of tail tapers.
Stains very lightly; tip of tail difficult to see. Found in blood and skin.

F. *Mansonella streptocerca* (West and Central Africa)

Small, thin, microfilaria.
Does not have a sheath.
Nuclei extend to end of tail.
Tail is hooked; its tip is rounded or forked. Found only in skin.

G. *Onchocerca volvulus*

Thick microfilaria. Does not have a sheath.
Head often spatulate.
Nuclei do not extend to tip of tail.
Found only in skin.

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[1] Available on request from Division of Control of Tropical Diseases, World Health Organization, 1211 Geneva 27, Switzerland.

[2] Available on request from Diarrhoeal Diseases Control, World Health Organization, 1211 Geneva 27, Switzerland.

ANNEX 1

Equipment and materials for diagnostic parasitology in health centres and district hospital laboratories

Equipment

Microscopes with adequate illumination, preferably built-in light sources. Microscopes with adjustable mirrors will be needed if the light source is separate, e.g., sunlight or lamp. Microscopes should have an adjustable iris diaphragm and substage condenser; ×10 oculars, and ×10, ×40, and oil-immersion objectives.

Centrifuge—either table model or floor model—with head and cups to hold 15-ml centrifuge tubes. Sealed buckets are preferred.

Microhaematocrit centrifuge.

Refrigerator, 4–5 °C.

Materials

Adhesive tape, transparent, 2 cm wide, for anal swabs

Applicator sticks, wooden

Block, wooden, with grooves to hold slides

Bottles, 1000 ml

Bottles, small, 25 ml, 30 ml, 50 ml, and 100 ml, with rubber stoppers or dropper-top and screw-cap

Bottles, dispensing or plastic "squeeze", 100 ml, 250 ml, and 500 ml

Bottles, glass, with glass stoppers, 250 ml, 1000 ml

Centrifuge tubes, conical, graduated, 15 ml

Cotton swabs

Cellophane, wettable, 40–50 μm thick, 25 × 30–35 mm strips

Coverslips, 20–22 mm square

Cylinders, graduated, 10 ml, 25 ml, 50 ml, 100 ml, and 1000 ml

Dishes for staining

Dropping bottles for saline, iodine, buffered methylene blue, and methanol (for blood staining).

Filter, brass wire, 40 mesh (425 μm) 7.5 cm diameter

Flasks, 100 ml, 250 ml, 500 ml, 1000 ml, and 5000 ml

Flask, conical, for urine collection

Forceps

Funnel

Gauze pads, sterile, for cleaning fingers

Glass rod

Hot plate (metal plate with spirit lamp beneath)

Immersion oil, low viscosity

Labelling pens or markers

Labels

Lancets, disposable, individual

Membrane filter, 12 µm or 15 µm and filter holder

Microscope slides—25 × 75 mm, 50 × 75 mm (optional)

Needle for splenic aspiration

Needle, 25 gauge, 0.5 × 16 mm, for subcutaenous injection

Needle for venepuncture

Petroleum jelly

Pipettes, capillary, about 14 cm long with rubber bulbs (for concentration procedure and general use)

Pipettes, serological, 1 ml, 5 ml, 10 ml capacity with rubber bulbs

Pipettes, Pasteur, with rubber bulbs

Pipettes, Sahli

Rack to hold centrifuge tubes

Rack for staining, glass rods or small jars

Rack, wooden, for drying blood smears

Reagent tubes

Register or record forms

Scalpel or razor blades

Screen, stainless steel, nylon or plastic, 60–105 mesh

Stoppers, rubber, to fit 15-ml centrifuge tubes (usually size 0 or size 1)

Syringe, plastic, 5 or 10 ml

Template, stainless steel, plastic or cardboard, size 20–50 mm

Test tubes, small, 100 × 13 mm, with cotton plugs or screw-caps

Test tubes, large, 150 × 13 mm, for boiling

Timer (clock)

Toilet paper, facial tissue or lens paper

Tongue depressor (or plastic spoons)

Towel, cotton, lint-free

Towels, paper or sponges

Tubes, capillary

Vials, 20 ml, with tight-fitting screw-caps

Chemicals and solutions for the preparation of reagents (preparation is described in Annex 2)

Disodium hydrogen phosphate (Na_2HPO_4) (for buffered water)

Distilled water

Dyes (stain powder):

- Azur 1—for Field's stain A
- Chromotrope 2R—for trichrome stain
- Eosin—for Field's stain B
- Light Green SF—for trichrome stain

- Fast Green FCF—for trichrome stain
- Malachite green
- Methylene blue

Ethanol (ethyl alcohol), 70%, 95%, 100% (absolute)

Ether, anaesthesia or technical grade, or ethyl acetate

Formalin (formaldehyde)

Glacial acetic acid

Glycerol

Hydrochloric acid, concentrated (HCl)

Iodine crystals (I_2)

Isopropanol (isopropyl alcohol)

Mercuric chloride crystals ($HgCl_2$)

Methanol (methyl alcohol)

Phenol crystals (carbolic acid)

Phosphotungstic acid crystals ($H_3[PO_4(W_{12}O_{36})] . 5H_2O$)

Polyvinyl alcohol (PVA)

Potassium iodide crystals (KI)

Potassium dihydrogen phosphate (KH_2PO_4) (for buffered water)

Sodium acetate powder (CH_3COONa) or sodium acetate crystals ($CH_3COONa . 3H_2O$)

Sodium chloride (NaCl)

Sodium citrate crystals ($C_6H_5O_7Na_3 . 2H_2O$)

Stains:

- Buffered methylene blue
- Field's stain A
- Field's stain B
- Giemsa, stock
- Glycerol–malachite green; glycerol–methylene blue
- Delafield's haematoxylin, stock
- Safranin solution
- Trichrome, stock

Xylene

If Delafield's haematoxylin is prepared in the laboratory the following will be needed:

- aluminium ammonium sulfate to prepare a saturated aluminium ammonium sulfate solution
- haematoxylin powder or crystals.

ANNEX 2

Reagents and solutions and their preparation

Acetic acid alcohol destain solution (No. 1)

Ethanol, 95%	600 ml
Glacial acetic acid (CH_3COOH)	4 ml
Distilled water	350 ml

Pour the 95% ethanol into a 1000-ml graduated cylinder. Add the distilled water making a total quantity of 950 ml. Pour into a 1000 ml bottle. Add the glacial acetic acid and mix.

Label the bottle: ACID ALCOHOL DESTAIN and write the date. Store on a shelf or in a cabinet. This solution will remain good for a year or more.

Warning: Glacial acetic acid is highly corrosive.

Buffered methylene blue solution (No. 2)

Solution A (acetic acid solution)

Glacial acetic acid (CH_3COOH)	1.2 ml
Distilled water	98.8 ml

Measure the 98.8 ml of distilled water into a clean flask or bottle, add the 1.2 ml of glacial acetic acid and mix well. Store in a clean bottle.

Warning: Glacial acetic acid is highly corrosive.

Solution B (sodium acetate solution)

Sodium acetate (CH_3COONa)	1.6 g
(If you are using crystalline sodium acetate ($CH_3COONa.3H_2O$)	2.6 g)
Distilled water	100 ml

Measure the 100 ml of distilled water into a small flask. Weigh out 1.6 g of sodium acetate (or 2.6 g of crystalline sodium acetate) and dissolve in water; mix thoroughly. Store in a clean bottle.

Working solution (for stain)

Solution A (acetic acid solution)	46.3 ml
Solution B (sodium acetate solution)	3.7 ml
Methylene blue dye	0.5 g
Distilled water	50 ml

Pour the 50 ml of distilled water into a flask or beaker of about 150 or 200 ml capacity. Add solutions A and B and mix well. Add about 0.5 g of methylene blue dye and shake or stir until the dye has dissolved. If all the dye does not dissolve in about 15 minutes, the solution should be filtered. Pour or filter into a clean stoppered bottle and label: BUFFERED METHYLENE BLUE SOLUTION. Write

the date on the label. This solution will keep indefinitely. If stain particles settle in the bottom of the bottle, the solution should be filtered. Pour a small amount (20 ml) into a dispensing or dropping-bottle for ready use. The dropping-bottle should have a pipette with a rubber bulb.

Note: Acetate buffers with a pH of 3.6 are most satisfactory for this stain.

Buffer solutions for malaria staining (No. 3)

A phosphate buffer solution, balanced to pH 7.2, is essential for Giemsa staining of malaria parasites.

Preparation of a solution for daily use

1. Dissolve 1.0 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) and 0.7 g of potassium dihydrogen phosphate (KH_2PO_4) in 1 litre of distilled or deionized water. Filtered rainwater or even tap-water may be used if no other is available.
2. Check the pH with a pH meter or a colour-based indicator, such as the Lovibond comparator.
3. If the pH is below 7.2, add small quantities of a 2% Na_2HPO_4 solution; if it is above 7.2, add small quantities of a 2% solution of KH_2PO_4.
4. When balanced to pH 7.2, store in a tightly stoppered bottle, preferably of dark glass, in a cool place away from direct sunlight.

This solution will remain good for some weeks, but needs to be regularly checked to ensure that growths or moulds do not become established. This may be done by shaking the solution; if cloudy, discard.

Preparation of a concentrated stock solution (useful for field trips or dispatch to distant stations)

1. Dissolve 3.0 g of anhydrous Na_2HPO_4 and 2.1 g of KH_2PO_4 in 25 ml of distilled or deionized water.
2. Adjust the pH to 7.2 in the way described in point 3 above.
3. Store in a dark bottle away from direct sunlight; this will remain good for several weeks.
4. To make up a working solution, dilute 1 ml of the concentrate with 20 ml of distilled or deionized water.

Preparation of preweighed packs

The two phosphate salts can be preweighed and placed together in a clearly labelled, tightly stoppered tube, bottle, or well sealed plastic bag, and stored in a screw-capped jar. To make the solution, add the contents of the packet to 1 litre of water, and adjust the pH to 7.2.

Carbol–fuchsin solution (No. 4)

Basic fuchsin	10 g
Ethanol, absolute, technical grade	100 ml
Phenol (carbol)	50 g
Distilled water	1 litre

Preparation

1. Weigh the basic fuchsin powder and trasnfer it into a 1.5-litre bottle.
2. Add 100 ml of absolute ethanol and dissolve the dye completely.
3. Weigh the phenol in a beaker and dissolve in a small volume of distilled water.
4. Add the aqueous phenol solution to the dye solution and mix well.
5. Add the rest of the water, mix well, and label the bottle. The dye solution will be stable indefinitely

Note: Ethanol is flammable. Phenol is toxic and corrosive.

Carbol–xylene solution (No. 5)

Note: This should be prepared at an intermediate level laboratory because of the dangerous reagent involved. Loosen the top of a jar of phenol[1] (carbolic acid) crystals and put the jar in a water bath. Heat the water to liquefy the crystals. DO NOT HEAT PHENOL CRYSTALS DIRECTLY OVER A FLAME. ALWAYS PUT THE JAR INTO A WATER BATH.

Liquid phenol	200 ml
Xylene	600 ml

Measure the liquid phenol and pour into a 1000-ml glass-stoppered bottle. Add the xylene. Use an unopened bottle of xylene, if possible. Mix by shaking the bottle.

Label the bottle: CARBOL–XYLENE and write the date. Store in a cabinet or on a shelf away from direct light. The solution is good for a year or more, but the bottle must be kept tightly closed. Adhesive tape can be wrapped around the stopper to keep moisture out. If moisture gets into the solution, it will be unsatisfactory.

Warning: Phenol is highly corrosive and poisonous.

Delafield's haematoxylin stain (No. 6)

Haematoxylin crystals	1 g
Ethanol, absolute	10 ml
Saturated solution of aluminium ammonium sulfate ($NH_4Al(SO_4)_2$) in distilled water	100 ml
Glycerol ($C_3H_5(OH)_3$)	25 ml
Methanol, absolute	25 ml

Dissolve the haematoxylin crystals in the absolute ethanol. Add a few drops at a time to the saturated aluminium ammonium sulfate solution. Leave this solution unstoppered in direct sunlight or in a 37 °C incubator for 3–4 months to oxidize the haematoxylin to haematein.[2]

Stopper and label the bottle: DELAFIELD'S HAEMATOXYLIN and write the date. When reopened, filter and add the glycerol and methyl alcohol, and the stain is then ready for use. Keep stoppered to prevent evaporation. The stain will remain good for at least 18 months.

[1] Liquid phenol available from commercial sources must **not** be used. It contains water which makes it unsatisfactory for staining.
[2] This is not the same as the blood product haematin.

Ethanol, 70% (No. 7)

Ethanol, 95% . . . . . 726 ml
Distilled water . . . . . 274 ml

Pour the ethanol into a 1000-ml graduated cylinder. Add the distilled water making a total amount of 1000 ml. Pour into a 1000-ml glass-stoppered bottle.

Label the bottle: 70% ETHANOL and write the date. This solution will remain good indefinitely but keep the bottle tightly closed. Store on a shelf or in a cabinet.

Warning: Ethanol is inflammable.

Ether–alcohol fixative (No. 8)

Ether . . . . . 20 ml
Ethanol, 95% . . . . . 20 ml

Add the ether to the alcohol in a graduated cylinder and mix. Pour into a jar with a screw-cap lid or a lid with a ground-glass rim.

Warning: Ether is highly inflammable and potentially explosive.

Field's stain (No. 9)

Field's stain A

Preparation from prepared powders:

Field's stain A powder . . . . . 5.9 g
Hot distilled water . . . . . 600 ml

Mix until dissolved. Filter when cool.

Preparation from original stains and chemicals:

Methylene blue (medicinal) . . . . . 1.6 g
Azur I . . . . . 1.0 g
Disodium hydrogen phosphate (Na_2HPO_4), anhydrous . . . . . 10.0 g
Potassium dihydrogen phosphate (KH_2PO_4) . . . . . 12.5 g
Distilled water . . . . . 1000 ml

Dissolve the two phosphates in the water. Pour about half of the phosphate solution into a 1-litre bottle containing a few glass beads. Add the stain powders and mix well. Add the remainder of the phosphate solution. Mix well and filter.

Field's stain B

Preparation from prepared powders:

Field's stain B powder . . . . . 4.8 g
Hot distilled water . . . . . 600 ml

Mix until dissolved. Filter when cool.

Preparation from original stain and chemicals:

Eosin (yellow, water-soluble) . . . . . 2.0 g
Disodium hydrogen phosphate (Na_2HPO_4), anhydrous . . . . . 10.0 g
Potassium dihydrogen phosphate (KH_2PO_4) . . . . . 12.5 g
Distilled water . . . . . 1000 ml

Dissolve the two phosphates in the water. Pour into a 1-litre bottle. Add the eosin. Mix until dissolved. Filter.

Formalin solutions (No. 10)

10% Formalin solution for preserving faeces

Formalin (neutral formaldehyde, at least 37%) . . . . . 100 ml
Distilled water . . . . . 900 ml

Measure the aqueous formalin solution into a graduated measuring cylinder and pour it into a 1000-ml bottle with a glass stopper or screw-cap. Add the distilled water to the bottle and mix.

Label the bottle: 10% FORMALIN and write the date. Store on a shelf or in a cabinet. The solution will remain good for two years or more.

2% Formalin (concentration method for microfilariae)

Formalin (neutral formaldehyde, at least 37%) . . . . . 20 ml
Distilled water . . . . . 980 ml

Measure the aqueous formalin solution into a graduated measuring cylinder and pour it into a 1000-ml bottle with a glass stopper or screw-cap. Add the distilled water to the bottle and mix.

Label the bottle: 2% FORMALIN and write the date. Store on a shelf or in a cabinet. The solution will remain good for two years or more.

Warning: Formalin is corrosive and poisonous.

Giemsa stain (stock solution) (No. 11)

Giemsa is the standard stain for reliability in the routine staining of blood films for malaria diagnosis. However, the quality of this stain, in ready-made solution or powder form, varies according to its source of supply and it is advisable to obtain it from a reputable manufacturer. Even so, you will need to establish stain quality by testing each batch after it has been made up and prior to routine staining of large numbers of blood films.

Giemsa powder . . . . . 3.8 g
Methanol . . . . . 250 ml
Glycerol . . . . . 250 ml

A dark bottle is preferred, but if one is not available, use a chemically clean and dry, clear, hard glass or polyethylene bottle of suitable size. You will also need about 50 solid glass beads of about 5 mm in diameter.

1. Put the glass beads in the bottle; pour in the measured amount of methanol and add the stain powder.
2. Tightly stopper the bottle. Allow the stain powder to sink slowly through the methanol until it settles to the bottom. Shake the bottle in a circular motion for 2–3 minutes.
3. Add the measured amount of glycerol and repeat the shaking process. Continue to shake for 2–3 minutes at half-hourly intervals, for at least six times.
4. Leave the bottle for 2–3 days, shaking it 3–4 times each day until the stain is thoroughly mixed. Keep some of this stock solution in a small bottle for routine use to avoid contamination of the stock solution.

Each newly prepared batch of stain should be properly labelled, including date of preparation, and should be tested for optimum stain dilution and staining time. Always keep the bottle tightly stoppered, in a cool place, away from direct sunlight. Clear glass stock bottles can be covered with a thick dark paper jacket to keep out the light.

Glycerol–malachite green solution or glycerol–methylene blue solution (No. 12)

Glycerol . . . . . 100 ml
3% aqueous malachite green, or 3% aqueous methylene blue . . . . . 1 ml
Distilled water . . . . . 100 ml

Grind some malachite green or methylene blue powder with a pestle in a clean, dry mortar. Weigh out 3 g of the powder, pour it into a bottle and add distilled water to give 100 ml. Seal and label the bottle: 3% AQUEOUS MALACHITE GREEN or 3% AQUEOUS METHYLENE BLUE. Store in a cabinet away from light.

To prepare the solution: pour 1 ml of the 3% aqueous solution into a 250-ml bottle. Add 100 ml of glycerol and 100 ml of distilled water and seal the bottle; mix thoroughly before use.

Hydrochloric acid–ethanol solution (No. 13)

Hydrochloric acid (concentrated) . . . . . 1 ml
Ethanol, 95% . . . . . 100 ml

Put 100 ml of 95% ethanol into a clean 250-ml bottle with glass stopper. Add 1 ml of concentrated hydrochloric acid and mix.

Warning: Hydrochloric acid is highly corrosive. Ethanol is flammable.

Hydrochloric acid–methanol solution (No. 14)

Hydrochloric acid (concentrated) . . . . . 3 ml
Methanol, absolute . . . . . 100 ml

Measure 100 ml of absolute methanol and pour into a clean 250-ml bottle with glass stopper. Add 3 ml of concentrated hydrochloric acid and mix.

Warning: Hydrochloric acid is highly corrosive. Methanol is flammable.

Hydrochloric acid–water destain (No. 15)

Hydrochloric acid (HCl), concentrated	0.5 ml
Distilled water	100 ml

Measure the distilled water and pour into a 1000-ml glass-stoppered bottle. Add the hydrochloric acid. Mix thoroughly.

Label the bottle: 0.5% HCl and write the date.

Warning: Hydrochloric acid is highly corrosive.

Iodine–alcohol solution (No. 16)

Ethanol, 70%	40 ml
Iodine crystals	a few

Pour the 70% alcohol into a small flask. Using two applicator sticks held together, pick up some iodine crystals and add to the alcohol. Shake or stir the mixture and add more iodine if necessary until the solution has the colour of strong tea. The colour is important.

The purpose of iodine–alcohol is to remove mercuric chloride left by the fixative and it must be the correct strength to do this. If the iodine–alcohol is not strong enough, it will not remove the mercuric chloride residue on the preparation, which will interfere with examination. If the iodine–alcohol is too strong, the iodine will penetrate the protozoa and prevent staining by the trichrome solution.

Label the bottle: IODINE–ALCOHOL SOLUTION and write the date. Iodine–alcohol solutions must be prepared fresh every three weeks.

Lugol's iodine (stock 5% solution) (No. 17)

Iodine	5 g
Potassium iodide (KI)	10 g
Distilled water	up to 100 ml

Weigh the iodine in a porcelain dish or a watch glass. Grind the dry iodine and potassium iodide in a mortar. Add water, a few millilitres at a time, and grind thoroughly after each addition until the iodine and iodide dissolve. Put the solution into an amber glass bottle with the remainder of the distilled water.

Alternatively: Dissolve the potassium iodide in about 30 ml of the water. Add the iodine and mix until dissolved. Add a further 70 ml of water and mix well. Store in a brown bottle.

Lugol's iodine (1% solution for wet mounts) (No. 18)

Lugol's iodine stock solution is too strong for wet mounts of stool. It will cause the faecal material to clump and organisms may get trapped and not be seen. Therefore, the stock Lugol's iodine solution should be diluted.

Lugol's iodine (stock, 5% solution) (No. 17)	5 ml
Saline solution, isotonic (No. 24)	20 ml

Measure the isotonic saline into a dispensing or dropping bottle. Add the 5% Lugol's iodine stock solution. Mix thoroughly. This will give a 1% iodine solution which will satisfactorily stain cysts.

Label the bottle: LUGOL'S IODINE 1% and write the date. The 1% solution must be prepared fresh every 14 days.

Methylene blue–phosphate solution (No. 19)

Methylene blue powder[1] . . . . . 1 g
Disodium hydrogen phosphate (Na_2HPO_4) . . . . . 3 g
Potassium dihydrogen phosphate (KH_2PO_4) . . . . . 1 g
Distilled water . . . . . 300 ml

Weigh out the methylene blue powder and put in a clean dry mortar. Add the disodium hydrogen phosphate and potassium dihydrogen phosphate. With a pestle, grind the dye and phosphate powders together and mix thoroughly. Weigh 1 g portions of the mixture and put in small well-stoppered vials.

Label the vials: METHYLENE BLUE–PHOSPHATE and write the date. The dry mixture will keep for a long time, if the vials are kept tightly closed. Put a piece of adhesive tape around the stopper to seal the vial and keep out moisture.

To prepare the solution

Put 1 g of the mixture in a 500-ml flask. Add the distilled water and shake the flask or stir to dissolve the dye mixture. Filter through filter paper into a 500-ml clean, dry, glass-stoppered bottle.

Label the bottle: METHYLENE BLUE–PHOSPHATE SOLUTION and write the date. Store in a cabinet away from the light. The solution will remain good for two years or more.

Methylene blue–saline (No. 20)

Methylene blue . . . . . 0.1 g
Isotonic saline . . . . . 100 ml

Weigh the methylene blue and transfer it to a clean bottle. Add the saline and mix until the dye crystals are completely dissolved.

For use: Filter a small amount of the stain solution into a dropper bottle.

Potassium iodide solution, 10% (No. 21)

Potassium iodide (KI) . . . . . 100 g
Distilled water . . . . . 1000 ml

Weigh out the potassium iodide. Measure the distilled water into a clean glass-stoppered bottle. Dissolve the potassium iodide in the water.

[1] Medicinal methylene blue powder is preferred, but any good quality methylene blue can be used.

Label the bottle: 10% POTASSIUM IODIDE and write the date. (Put a piece of paper or string in the neck of the bottle to prevent the stopper sticking.) Store in a cabinet or on a shelf out of the light. On standing, the solution may become slightly yellow, but this does not interfere with its use.

PVA-fixative preparation (No. 22)

Note: This should be prepared at an intermediate level laboratory, because of the dangerous reagents involved.

Modified Schaudinn's fixative

Mercuric chloride crystals ($HgCl_2$)	1.5 g
Ethanol, 95%	31.0 ml
Glacial acetic acid	5.0 ml

Dissolve the mercuric chloride in the ethanol in a stoppered flask (50 or 125 ml) by swirling at intervals. Add the acetic acid, stopper, and mix by swirling.

Warning: Mercuric chloride is highly poisonous. Glacial acetic acid is highly corrosive.

PVA mixture

Glycerol	1.5 ml
Polyvinyl alcohol (PVA) powder (low viscosity)	5.0 g
Distilled water	62.5 ml

In a small beaker, add the glycerol to the PVA powder and mix thoroughly with a glass rod until all particles appear coated with the glycerol. Scrape the mixture into a 125-ml flask. Add the distilled water, stopper, and leave at room temperature for 3 hours or overnight. Swirl mixture occasionally to mix.

PVA powder and PVA-fixative solutions are available from several commercial sources. There are many grades of PVA powder on the market, but the grades with high hydrolysis and low or medium viscosity are most satisfactory for preparing PVA-fixative for protozoa.

PVA-fixative working solution

1. Heat a water-bath (or large beaker of water) to 70–75 °C. Adjust the heat to maintain this temperature range.
2. Place the loosely stoppered flask containing the PVA mixture in the bath for about 10 minutes, swirling frequently.
3. When the PVA powder appears to be mostly dissolved, pour in the modified Schaudinn's fixative solution, restopper and swirl to mix.
4. Continue to swirl mixture in the bath for 2–3 minutes to dissolve the remainder of the PVA, to allow bubbles to escape, and to clear the solution.
5. Remove the flask from the water-bath and let cool. Store the PVA-fixative in a screw-cap or glass-stoppered bottle. Label: PVA-FIXATIVE and write the date. Fixative will keep for 6–12 months.

For mixing, a high-speed agitator that will produce a whirlpool in the fixative solution is highly desirable.

Safranin solution (No. 23)

Stock solution

Safranin O . . . . . 2.5 g
Ethanol, 95% . . . . . 100 ml

Weigh the dye powder and dissolve in 100 ml of ethanol. Store in a labelled bottle.

Working solution

Stock solution . . . . . 10 ml
Distilled water . . . . . 90 ml

Saline solution, isotonic (No. 24)

Sodium chloride (NaCl) . . . . . 8.5 g
Distilled water . . . . . 1000 ml

Weigh out the sodium chloride. Measure the distilled water into a clean, glass-stoppered bottle. Dissolve the sodium chloride in the water and mix thoroughly. Put a piece of string or a narrow strip of paper between the glass stopper and the neck of the bottle, to keep the stopper from sticking.

Label the bottle: ISOTONIC SALINE and write the date. Store on a shelf or in a cabinet. Pour some saline into a dispensing or dropping-bottle for daily use. Write the date on the label. The dispensing bottle should have a pipette with a rubber bulb.

Schaudinn's fixative (No. 25)

Note: This should be prepared at an intermediate level laboratory, because of the dangerous reagents involved.

Stock solution

Mercuric chloride, saturated, aqueous ($HgCl_2$)[1] . . . . . 600 ml
Ethanol, 95% . . . . . 300 ml

Measure out the saturated aqueous mercuric chloride and pour into a 1-litre glass-stoppered bottle. Add the 95% ethanol and mix by shaking the bottle.

Label the bottle: SCHAUDINN'S FIXATIVE–STOCK and write the date. The stock solution will remain good indefinitely (a year or more).

Working solution for staining

Schaudinn's fixative stock solution . . . . . 100 ml
Glacial acetic acid (CH_3COOH) . . . . . 5 ml

[1] Add about 80 g of mercuric chloride crystals to 1000 ml of distilled water and heat to dissolve the crystals. Allow to cool. Some crystals should form in the bottom of the flask if the solution is saturated.

Measure the Schaudinn's fixative stock solution and pour into a 250-ml glass-stoppered bottle. Add the glacial acetic acid and mix by shaking the bottle.

Label the bottle: SCHAUDINN'S FIXATIVE WITH ACETIC ACID, and write the date. This solution will remain good for 2–3 months.

Warning: Mercuric chloride is highly poisonous. Glacial acetic acid is highly corrosive. Ethyl alcohol is inflammable.

Sodium citrate solution, 2% (No. 26)

Sodium citrate crystals ($C_6H_5O_7Na_3 . 2H_2O$) . . . . . 20 g
Distilled water . . . . . 1000 ml

Measure the distilled water and pour into a clean bottle (glass-stoppered or screw-cap). Weigh out the sodium citrate crystals and add to the water. Stir until dissolved.

Label the bottle: SODIUM CITRATE SOLUTION, 2% and write the date. Store on a shelf or in a cabinet. The solution will remain good for one year or more.

Trichrome stain solution (No. 27)

Chromotrope 2R . . . . . 6.0 g
Light green SF . . . . . 1.5 g
Fast green FCF . . . . . 1.5 g
Phosphotungstic acid crystals ($H_3[PO_4(W_{12}O_{36})] . 5H_2O$ . . . . . 7 g
Glacial acetic acid (CH_3COOH) . . . . . 10 ml
Distilled water . . . . . 1000 ml

Weigh each dye powder separately. Put the dyes into a 1-litre flask.

Weigh out the phosphotungstic acid crystals and add to the flask with the dyes. Measure the glacial acetic acid and pour into the flask. Swirl the flask so that the acetic acid wets the dyes. Let stand for 30 minutes. Add the distilled water and mix. Pour the stain into a 1-litre, clean, glass-stoppered bottle.

Label the bottle: TRICHROME STAIN and write the date. Store on a shelf or in a cabinet away from the light. Good trichrome stain is a deep purple-black colour. The stain will remain good for a year or more.

Warning: Glacial acetic acid is highly corrosive.

ANNEX 3

Preparation of culture media

Schneider's enriched medium for the in vitro culture of Leishmania

Materials

Schneider's *Drosophila* medium[1]	80 ml
Fetal calf serum[1]	20 ml
Antibiotic-antimycotic solution[1]	1.2 ml

The antibiotic-antimycotic solution contains penicillin, streptomycin, and amphotericin.

Preparation

1. Inactivate the fetal calf serum for 30 minutes at 56 °C and allow to cool.
2. Mix 20 ml of inactivated fetal calf serum with 80 ml of Schneider's medium.
3. Add 1.2 ml of the antibiotic-antimycotic solution, mix, aseptically dispense the medium in 3-ml amounts into sterile 16 × 100 mm tubes, and stopper.
4. Label and freeze at −20 °C. The medium can be kept for up to 1 year at −20 °C. It can be kept for up to 6 weeks at 4–6 °C.

Note: The medium should be prepared in aseptic working conditions.

Use

1. Warm 2 tubes of medium to room temperature.
2. Inoculate each tube with 0.1 ml of the specimen.
3. Incubate the cultures at 24 °C (±2 °C) in the dark for up to 14 days. Room temperature is usually suitable.
4. Examine daily for promastigotes. Transfer a drop of the culture to a slide for examination using a wire loop. Cover with a coverslip and look for motile, flagellated promastigotes.

Note: Negative cultures must be subcultured after 4 days into fresh medium and examined daily for a further 10 days.

Novy Nicolle–McNeal (NNN) culture medium for the in vitro culture of Leishmania

Materials

Difco blood agar base[2]	8 g
Distilled water	200 ml
Defibrinated rabbit blood	0.6 ml in each 5 ml of medium

[1] All the ingredients are obtainable from the Institut Pasteur, 3 boulevard Raymond Poincaré, BP 3, F-92430 Marnes-la-Coquette, France.

[2] Difco Blood Agar Base is obtainable from Difco Laboratories, PO Box 10587, Detroit, Michigan 48233, USA.

Preparation of defibrinated rabbit blood

Collect 20 ml of rabbit blood into a sterile flask containing about 100 glass beads of 4 mm diameter. Defibrinate the blood by rotating the flask for 5 minutes. Add 200 IU of penicillin, 200 mg of gentamicin, and 2 mg of streptomycin per ml of defibrinated blood.

Preparation

1. Pour 200 ml of water into a flask, add the agar to the water, mix, and warm the flask in boiling water until the agar is completely dissolved.
2. Dispense the medium in 5 ml amounts into screw-cap bottles (20 ml capacity). Sterilize by autoclaving (with caps loosened) at 121 °C for 15 minutes and allow the agar to cool to 45–50 °C.
3. Add 0.6 ml of sterile defibrinated rabbit blood to each bottle and mix *gently*. Allow the medium to solidify with the bottles in a sloped position.
4. Leave the bottles in an upright position at room temperature for 24 hours to allow fluid of condensation to form. The bottles should be stored at 4–6 °C until required.

Note: The medium should be prepared in aseptic working conditions.

Use

1. Inoculate about 0.1 ml of specimen aseptically *into the fluid of condensation* of each of 2 bottles at room temperature.
2. Incubate the cultures at 24 °C (± 2 °C) in the dark.
3. Examine every 4 days. Transfer a drop of the culture using a sterile wire loop to a slide for examination for promastigotes.

Note: Negative cultures must be subcultured after 8 days into fresh medium and examined every 4 days for a further 20 days.

ANNEX 4

Cleaning and storage of microscope slides

Cleaning

The availability of clean, good quality glass slides for the preparation of blood specimens for microscopic examination needs to be emphasized. All slides must be scrupulously clean and free from grease or moisture. This will prevent most of the artefacts which confuse malaria diagnosis and will avoid the detachment and washing away of thick blood films during the staining process. Reject defective materials such as:

- slides with an iridescent bloom or frosted appearance;
- imperfectly cleaned slides, whether new or old;
- old slides with surface scratches or notched edges.

New slides

It is prudent to clean all new slides (including commercially pre-cleaned slides) by soaking in water with a reliable detergent[1] and then placing them in running tap-water, or several changes of clean water, for some hours. Each slide should be wiped dry and polished with a dry, clean, lint-free cloth. Always handle the cleaned slides by the edges to avoid finger marks.

Used slides

Used slides must be soaked for at least 60 minutes in hypochlorite solution before washing. They should be washed in hot soapy water and both sides scrubbed with a brush. Wash only a few at a time to avoid scratching or chipping. The slides should then be cleaned one by one with gauze or cotton wool. Then the slides should be transferred to a fresh solution of detergent and later to running water, or several changes of clean water, before drying with a clean cotton cloth. Slightly scratched slides that are considered unsuitable for blood films may still be usefully passed on to the entomology section for routine laboratory use.

Storage of slides

In the humid tropics, glass slides should not be kept in the ambient climate for more than a few weeks. Otherwise they will adhere to each other because of entrapped moisture and there will be a loss of transparency due to "frosting". After cleaning, slides are best stored in a dry place or a warm air cabinet.

It is recommended that cleaned slides be stored in packages of 10, which have been wrapped in thin paper and secured with adhesive tape or rubber bands, so that they are ready for use. Packages of slides can be put in the original cardboard boxes or other suitable boxes for mailing or transportation, but should be protected with corrugated cardboard, expanded polystyrene, or cotton wool.

[1] The use of acid-bichromate as a cleaning solution is not recommended.

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